APPROVED BY

Decision of the Board of
the Eurasian Economic Commission
No. 100 dated August 11, 2020

PHARMACOPOEIA

of the Eurasian Economic Union

1. GENERAL NOTICES

1.1. General Provisions

The provisions of Section *1. General Notices* apply to all the texts of the Pharmacopoeia of the Eurasian Economic Union.

The Pharmacopoeia of the Eurasian Economic Union is a set of regional requirements and regulations that establish the maximum allowable quality level of medicinal products in the pharmaceutical market of the Eurasian Economic Union. The Pharmacopoeia of the Union is the abbreviated name of the Pharmacopoeia given in the texts. In some cases, the word "Pharmacopoeia" may be used to indicate the Pharmacopoeia of the Union.

The official texts of the Pharmacopoeia of the Union include general notices, general chapters, monographs, and annexes, which are published in Russian.

Monograph is a document that sets out the requirements and statements of the Pharmacopoeia for drugs, excipients and other articles as well as tests and their methods.

Monographs can be general or individual.

General monograph is a monograph that establishes general requirements and regulations for the quality and packaging of medicinal products, excipients and other articles, as well as tests, their methods, and reagents used.

Individual monograph (monograph) is a pharmacopoeial monograph that sets special requirements for the quality of specific medicinal products, excipients and other articles.

The reference to a monograph and/or its section in the texts of the Pharmacopoeia means that the drugs, excipients and other articles meet the requirements of this monograph. References to monographs in the texts of the Pharmacopoeia are shown using the monograph title and reference number in italics.

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The requirements of the Pharmacopoeia for medicinal products must be met throughout their valid shelf life. Pharmacopoeial monographs do not regulate shelf life of medicinal products in opened packages and/or their quality specifications, which must be approved by the competent authority. Any other materials (active pharmaceutical ingredients, excipients, etc.) must meet the requirements of the pharmacopoeial monograph throughout their period of use. The shelf life and the time which the shelf life is calculated from must be approved by the competent authority based on experimental results of stability tests.

The requirements of pharmacopoeial monographs may be mandatory, advisory, and informative. The requirements of monographs are mandatory unless otherwise specified in *1. General Notices* or general monographs. General chapters become mandatory if they are referenced in the monograph, except in cases where the reference is advisory or given for information.

Active pharmaceutical substances, excipients, drug products and other auxiliary materials described in monographs are intended for human use and veterinary use, unless a monograph gives instructions for use in only one of these fields.

Quality Systems. The quality standards established in the monographs apply to drug products, excipients and auxiliary materials only if they are produced in terms of the relevant quality system. The quality system must ensure that drugs, excipients and auxiliary materials always meet the requirements of the standards of the Pharmacopoeia.

Alternative Procedures. All tests and procedures described are the official methods upon which the quality standards of the Pharmacopoeia are based.

Subject to approval by the competent authority, alternative procedures may be used for quality control of medicinal products.

Alternative methods included in the manufacturer's quality specifications and/or quality regulatory documents must ensure that an unequivocal decision should be made as to whether drug compliance with the standards of the monograph would be achieved if the official procedures were used. The alternativeness of proposed procedures is confirmed by validation tests using the same validation characteristics as in the case of pharmacopoeial procedures. Analytical procedures should be validated in accordance with the requirements stated in General Chapter *Analytical Procedures Validation*. In the event of doubt or dispute, the pharmacopoeial analytical procedures are alone authoritative.

Confirmation of compliance with requirements of the Pharmacopoeia. (1) Drug, excipient or auxiliary material is considered to be of pharmacopoeial grade only if it meets all the requirements of the corresponding monograph. This requirement does not mean that the manufacturer must perform all the tests described in the monograph when evaluating compliance with the Pharmacopoeia before the product is released to the market. The manufacturer may obtain assurance that a medicinal product is of Pharmacopoeia quality on the basis of its design, together with its control strategy and data derived, for example, from validation studies of the manufacturing process.

(2) An advanced approach to quality control could utilise process analytical technology and/or real-time release testing (including parametric release) strategies as alternatives to end-product testing alone. Real-time release testing in circumstances deemed appropriate by the competent authority is thus not precluded by the need to meet with the Pharmacopoeia requirements.

(3) Reduction of animal testing: the Pharmacopoeia is dedicated to phasing out the use of animals for test purposes by replacing, reducing and refining tests in accordance with the provisions set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

In proving compliance with the Pharmacopoeia requirements as indicated above (1), manufacturers may establish additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia requirements when animal tests are prescribed, is established in such a way that animal usage is minimised as much as possible.

Qualification of Materials. Some materials, requirements to which are described in monographs may be produced with different quality depending on their intended use. Unless otherwise specified in the monograph, its requirements apply to all qualifications (categories, forms, brands, classes) of materials. In some monographs, for example, on excipients, a list of functional characteristics of the material that are important for its use may be provided as additional information. In addition, the monograph may provide procedures for determining one or more of these characteristics for information. The informative or advisory nature of data on excipients is indicated in the general chapter *Functional Characteristics of Excipients*.

Validation of Pharmacopoeial Procedures. The test methods given in general and individual monographs are validated in accordance with good scientific practice and modern recommendations for the validation of analytical procedures. Unless otherwise specified in the general and individual monographs, validation of an analytical procedure is not required.

Implementation of Pharmacopoeial Procedures. When implementing a pharmacopoeial procedures, the user must assess (verify) whether and to what extent the suitability of the procedure under the actual conditions of use needs to be demonstrated according to the relevant monographs, general chapters and quality systems. The assessment of the pharmacopoeial procedure suitability and the extent thereof should be carried out in accordance with the requirements stated in General Chapter *Verification of Pharmacopeia Procedures*.

Conventional Terms. The term "competent authority (organisation)" means the body (organisation) vested with the authority for making decisions concerning the issue of drug circulation.

The expression "unless otherwise justified and authorised" means that the monograph requirements have to be met, unless the competent authority authorises a modification to or an exemption from these requirements where justified in a particular case.

In certain monographs or other texts, the term "suitable" and "appropriate" are used to describe a reagent, micro-organism, test procedure, etc. If criteria for suitability are not described in the monograph, suitability is demonstrated to the satisfaction of the competent authority.

The following key terms and definitions are used in the Pharmacopoeia.

Medicinal product (drug) is any substance or combination of substances that come into contact with the human body and may be used in or administered to human beings with a view either to treating, preventing, or restoring, correcting, or modifying physiological functions by exerting a pharmacological, immunological, or metabolic action or to making a medical diagnosis.

Drug preparation means a drug in a dosage form.

Dosage form is the state of the medicinal product corresponding to the methods of its administration and application and ensuring the achievement of the desired effect.

Substance for pharmaceutical use means a substance intended for the production and manufacture of drug preparations.

Active pharmaceutical ingredient is a substance for pharmaceutical use containing active ingredient(s) of chemical, plant, animal, or human origin.

Excipient (auxiliary substance) is any substance for pharmaceutical use that is not an active substance for the given drug preparation and intended to create a dosage form with specific properties.

References to regulatory documents. Monographs and general chapters may contain references to documents issued by regulatory authorities of the Union. These references are provided for information for Pharmacopoeia users. Inclusion of such a reference does not modify the status of the documents referred to, which may be mandatory or for guidance.

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1.2. Other Provisions Applying to General Chapters and Monographs

Substance quantity. In tests with numerical limits and quantitation procedures, the quantity stated to be taken for examination is approximate. The amount of substance actually used, which may deviate by not greater than 10% from that stated in the monograph, is accurately weighed or measured and the result is calculated from this exact quantity. In tests where the limit is not numerical, but usually depends upon comparison with the behavior of a reference substance in the same conditions, the quantity stated in the monograph is taken for examination. Reagents are used in the prescribed amounts.

Substance quantities are weighed or measured with accuracy according to the indicated degree of precision. For weighings, the precision should correspond to ±5 units after the last figure stated (for example, the weighed amount of 0.25 g is to be interpreted as 0.245 g to 0.255 g). The measurement of volumes is carried out as follows. If the figure after the decimal point is 0 or ends in 0 (for example, 10.0 mL or 0.50 mL), the required volume is measured using a pipette, a volumetric flask, or a burette. Otherwise, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

However, in certain cases, the precision with which substance quantities are stated does not meet to the number of significant figures stated in a specified numerical limit. The weighings and measurements are then carried out with a sufficiently improved accuracy.

Apparatus and Procedures. Volumetric glassware should comply with Class A requirements of the appropriate International Standard issued by the International Organisation for Standardisation (ISO). It is allowed to use the volumetric apparatus of accuracy Class 1 according to the appropriate standard of a member state of the Union, provided that the replacement of Class A volumetric glassware with that of Class 1 does not increase the value of the extended uncertainty of the test result.

Unless otherwise prescribed in the monograph, analytical procedures are carried out at a temperature between 15 °C and 25 °C.

Unless otherwise prescribed in the monograph, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base; the volumes of liquid prescribed are for use with tubes having an internal diameter of 16 mm; tubes with a larger internal diameter may be used provided the volume of liquid used is adjusted (see chapter *2.1.1.5*). Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background, or if necessary against a black background. The examination is carried out in diffuse light.

Any solvent required in a test or quantitation in which an indicator is to be used is previously neutralised to the indicator unless a blank test is prescribed.

Water Bath. The term "water bath" means a bath of boiling water unless water at another temperature is indicated in the monograph.

Other methods of heating may be substituted provided the temperature is near to but not higher than 100 °C or the indicated temperature.

Drying and ignition to constant mass. The terms "dried to constant mass" and "ignited to constant mass" mean that two consecutive weighings do not differ by greater than 0.5 mg, the second weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue to be dried or ignited.

Where drying is prescribed using a desiccator or in vacuo, it is carried out using the conditions described in General Chapter. *Loss on drying.*

Reagents. The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, particularly, upon the quality of the reagents used. The specifications of reagents are given in General Chapter *Reagents*. It is assumed that reagents of analytical grade are used, for some reagents, tests to determine suitability are included in the specifications.

Solvents. Where the name of the solvent is not stated, the term "solution" implies an aqueous solution.

The term "water" means purified water. Where the use of water is specified or implied in the analytical procedures described in the Pharmacopoeia or for the preparation of reagents, water complying with the requirements of the monograph *Purified water* is used, except that for many purposes the requirements for bacterial endotoxins (*Purified water in bulk*) and microbial contamination (*Purified water in containers*) are not relevant. The term "distilled water" indicates purified water prepared by distillation.

The term "ethanol" without qualification means anhydrous ethanol. The term "alcohol" without qualification means 96% ethanol (*V/V*). Other dilutions of ethanol are indicated by the term "ethanol" or "alcohol" followed by a statement of the percentage by volume of ethanol (C_2H_6O) required.

Expression of content. In defining content, the expression "percent" is used according to circumstances with one of three meanings:

- mass percent (*m/m*, mass in mass) expresses the quantity of grams of substance in 100 grams of the final product;

- volume percent (*V/V*, volume in volume) expresses the number of millilitres of substance in 100 millilitres of the final product;

- mass/volume percent (*m/V*, mass in volume) expresses the number of grams of substance in 100 millilitres of the final product.

The expression "parts per million" (or ppm) and "parts per billion" (or ppb) refers to mass in mass unless otherwise specified in the monograph.

Quantities of liquid substances and solutions used in the preparation of mixtures can be expressed as volume in volume (*V/V*).

Temperature. Where an analytical procedure describes temperature without a figure, the general terms used have the following meaning:

In a deep-freeze	below -15 °C
In a refrigerator	between 2 °C and 8 °C
In a cold/cool place	between 8 °C and 15 °C
Warm	between 40 °C and 50 °C
Hot	between 80 °C and 90 °C
Water bath temperature	from 98 °C to 100 °C
Ice bath temperature	0 °C

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1.3. General Chapters and General Monographs

The names of general chapters and general monographs are given in Russian. In some cases (for example, in general monographs on drug dosage forms), additional names in Latin are allowed.

General Chapters. General chapters of the Pharmacopoeia may include several general monographs grouped by types of objects of the standardisation of the Pharmacopoeia, types of tests and their methods, the nature of requirements, etc. For example, general chapters may contain general monographs on physical and chemical test methods, biological test methods, packaging and packaging materials, reagents, etc.

General monographs. Substances for pharmaceutical use, drug preparations, and other articles that are the subject of an individual monograph are also required to comply with relevant general monographs.

General monographs apply to all substances and preparations within the scope of the Definition section of the general monograph, except where a preamble limits the application, for example to substances and preparations that are the subject of an individual monograph of the Pharmacopoeia.

General monographs on dosage forms apply to all preparations of the type defined. The requirements are not necessarily comprehensive for a given specific preparation and requirements additional to those prescribed in the general monograph may be imposed by the competent authority.

General monographs and individual monographs are complementary. If the requirements of a general monograph do not apply to a particular product, this is expressly stated in the individual monograph.

Packaging (containers). Materials used for containers are described in General Chapter *Pharmacopoeia of the Union.* General names used for packaging materials, particularly plastic materials, each cover a range of products varying not only in the properties of the principal constituent but also in the additives used. The test procedures and limits for materials depend on the formulation and are therefore applicable only for materials whose formulation is covered by the preamble to the specification. The use of materials with different formulations, and the test procedures and limits applied to them, are subject to agreement by the competent authority.

The specifications for containers in General Chapter *Pharmacopoeia of the Union have been developed for general application to containers of the stated category. However, in view of the wide variety of containers available and possible new developments, the publication of a specification does not exclude the use, in justified circumstances, of containers that comply with other specifications, subject to agreement by the competent authority*.

Reference may be made within the monographs of the Pharmacopoeia to the definitions and specifications for containers provided in chapter *Packaging*. The general monographs for pharmaceutical dosage forms may, under the heading *Definition* and *Production*, require the use of certain types of container. Certain other monographs may, under the heading *Storage*, indicate the type of container that is recommended for use.

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1.4. Monographs

A monograph may include the following sections.

TITLES

Monograph titles are in Russian and there are Latin and English subtitles.

In the titles of monographs on substances for pharmaceutical use, the international nonproprietary name (INN) is indicated, and in its absence, the generally accepted name of the active substance. If necessary, it is supplemented with the name of the anion or cation and the degree of hydration.

In the names of monographs on drug products, the dosage form is additionally indicated.

RELATIVE ATOMIC AND MOLECULAR MASSES

Relative atomic masses and relative molecular masses are indicated in monographs on substances for pharmaceutical use.

The relative atomic mass (A_r) or the relative molecular mass (M_r) is shown, as and where appropriate, at the beginning of each monograph. The relative atomic and molecular masses and the molecular and graphic formulae are given for information.

CHEMICAL ABSTRACTS SERVICE (CAS) REGISTRY NUMBERS

Chemical abstracts service (CAS) registry numbers are included for information in individual monographs, where applicable, to provide convenient access to useful information for users. CAS Registry Number is a registered trademark of the American Chemical Society.

DEFINITION

Statements under the heading *Definition* constitute an official definition of the substance, preparation, or another article that is the subject of the monograph.

Limits of content. Where limits of content are prescribed in monographs, they are those determined by the procedure described in *Quantitation* section.

Herbal drugs. In monographs on herbal drugs, the *Definition* indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form. Where a monograph applies to the drug in several states, for example, both to the whole drug and the drug in powdered form, the definition states this.

PRODUCTION

Statements under the heading *Production* draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute mandatory requirements for manufacturers unless otherwise stated.

They may relate, for example, to source materials; to the manufacturing process itself and its validation and control, to in-process testing, or to testing that is to be carried out by the manufacturer on the final product, either on selected batches or on each batch prior to release. These requirements cannot necessarily be verified on a sample of the final product by an independent analyst. The competent authority may establish that the requirements have been followed, for example, by examination of data received from the manufacturer, by inspection of manufacture, or by testing appropriate samples.

The absence of a *Production* section in a monograph does not imply that attention to features such as those referred to above is not required.

Choice of vaccine strain. Choice of vaccine composition. The *Production* section of a monograph may define the characteristics of a vaccine strain or vaccine composition. Unless otherwise stated, test methods given for verification of these characteristics are provided for information as examples of suitable procedures. Subject to approval by the competent authority, other test procedures may be used without validation against the procedures shown in the monograph.

POTENTIAL ADULTERATION

Due to the increasing number of fraudulent activities and cases of adulteration, information may be made available to users of the Pharmacopoeia to help detect adulterated materials (i.e. active pharmaceutical ingredients, excipients, intermediate products, bulk products, and finished products).

To this purpose, a procedure for the detection of potential adulterants and relevant limits, together with a reminder that all stages of production and sourcing are subjected to a suitable quality system, may be included in this section of monographs on substances for which an incident has occurred or that present a risk of deliberate contamination. The frequency of testing by manufacturers or by users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant) depends on a risk assessment, taking into account the level of knowledge of the whole supply chain and national requirements.

This section constitutes requirements for the whole supply chain, from manufacturers to users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant). The absence of this section in a monograph does not imply that attention to features such as those referred to above is not required.

CHARACTERS

The *Characters* section is given in monographs on substances for pharmaceutical use.

The statements in this section of a monograph are not to be interpreted as strict requirements and are given for information only.

IDENTIFICATION

The *Identification* section is given in monographs on both substances for pharmaceutical use and drug preparations.

Scope. The tests given in the Identification section are not designed to give full confirmation of the chemical structure or composition of the medicinal product or excipient. They are intended to give confirmation, with an acceptable degree of assurance, that the medicinal product, excipient, or material conforms to the description on the label.

First and second identifications. Certain monographs have subdivisions entitled *First identification* and *Second identification.* The test or tests that constitute the *First identification subsection may be used in all circumstances.* The test or tests that constitute the *Second identification* subsection may be used in pharmacies provided it can be demonstrated that the substance for pharmaceutical use or drug preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the *Tests* section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

Powdered herbal drugs. Individual monographs

Where a quantitation of a residual solvent is carried out and a test for loss on drying is not carried out, the content of the residual solvent is taken into account for the calculation of the assay content of the substance, the specific optical rotation and the specific absorbance.

Limits. The limits prescribed in a monograph are based on data obtained in normal analytical practice when they take account of normal analytical errors, of acceptable variations in manufacture and compounding, and of deterioration to an extent considered acceptable when storing. No further tolerances are to be applied to the limits prescribed to determine whether the excipient or another article being examined complies with the

on herbal drugs may contain schematic illustrations (drawings, micrographs) of the powdered drug and its diagnostic anatomical features. These drawings complement the description given in the relevant identification test.

TESTS AND QUANTITATION

The sections *Tests* and *Quantitation* are given in monographs on both substances for pharmaceutical use and drug products.

Scope. The requirements in monographs are not framed to take account of all possible impurities. It is not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated if common sense and good pharmaceutical practice require that it be absent (see also below under *Impurities*).

Calculations. Where the result of a test or quantitation is required to be calculated with reference to the dried or anhydrous substance or on some other specified basis, the determination of loss on drying, water content, or other property is carried out by the test procedure prescribed in the relevant test in the monograph.

In determining compliance with a numerical limit, the calculated result of a test or quantitation is first rounded to the number of significant figures stated, unless otherwise prescribed in the monograph. The limits, regardless of whether the values are expressed as percentages or as absolute values, are considered significant to the last digit shown (for example 140 indicates 3 significant figures). The last figure of the result is rounded up when the part rejected is equal to or exceeds five; whereas it is not modified when the part rejected is less than five.

Indication of permitted limit of impurities. The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values. For comparative tests, the approximate content of impurity tolerated, or the sum of impurities may be indicated in brackets for information only. Acceptance or rejection is determined on the basis of compliance or non-compliance with the test stated in the monograph. If the use of a reference standard for the impurity is not prescribed, this content may be expressed as a nominal concentration of the substance used to prepare the reference solution specified in the monograph, unless otherwise directed.

Herbal drugs. For herbal drugs, the sulfated ash, total ash, water-soluble matter, alcohol-soluble matter, water content, the content of essential oil, and content of active substances are calculated with reference to the drug that has not been specially dried, unless otherwise prescribed in the monograph.

Equivalents. Where an equivalent is given, for the purposes of the Pharmacopoeia only the figures shown are to be used in applying the requirements of the monograph.

Culture media. The culture media described in monographs and general chapters have been found to be satisfactory for the intended purpose. However, the components of media, particularly those of biological origin, are of variable quality, and it may be necessary for optimal performance to modulate the concentration of some ingredients, notably:

- peptones and meat or yeast extracts, with respect to their nutritive properties;
- buffering substances;
- bile salts, bile extract, deoxycholate, and colouring matter, depending on their selective properties;
- antibiotics, with respect to their activity.

STORAGE

The information and recommendations given under the heading *Storage* do not constitute a pharmacopoeial requirement but the competent authority may specify particular storage conditions that must be met.

The medicinal products, excipients and materials described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Where special conditions of storage are recommended, including the type of container (see *General Chapters and General Monographs*) and limits of temperature, they are stated in the individual monograph.

The following expressions are used in monographs under *Storage* with the meaning shown.

In an airtight container means that the drug products, excipients, and materials are stored in an airtight container. Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

Protected from light means one of the three requirements:

- the medicinal product, excipient and other article should be stored in a container made of a material that absorbs actinic light sufficiently;
- the packaging with the medicinal product, excipient and other article should be enclosed in an outer cover that provides protection from actinic light;
- the medicinal product, excipient and other article should be stored in a place from which actinic light is excluded.

LABELLING

The requirements under the heading Labelling are not comprehensive and, moreover, for the purposes of the Pharmacopoeia, only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are given as recommendations. In cases where the term “label” is used in the Pharmacopoeia, by the decision of the competent authority, the relevant labelling information and statements may be provided on the container, the package, a leaflet accompanying the package, or a certificate of analysis accompanying the drug, as decided by the competent authority.

WARNINGS

Materials described in monographs and reagents specified for use in the Pharmacopoeia may be injurious to health unless adequate precautions are taken.

The principles of good quality control laboratory practice and the provisions of any appropriate regulations are to be observed at all times. Attention is drawn to particular hazards in certain monographs by means of a warning statement. Absence of such a statement is not to be taken to mean that no hazard exists.

IMPURITIES

The *Impurities* section is given in the monographs on substances for pharmaceutical use.

A list of all known and potential impurities that have been shown to be detected by the tests in the monograph may be given (see also chapter *Control of Impurities in Substances for Pharmaceutical Use*). The impurities are designated by a letter or letters of the Latin alphabet. Where a letter appears to be missing, the impurity designated by this letter has been deleted from the list during monograph development prior to publication or during monograph revision.

FUNCTIONALITY-RELATED CHARACTERISTICS OF EXCIPIENTS

Monographs on excipients may have a section on functionality-related characteristics. The characteristics, and test procedures for determination, and any tolerances described in this chapter are not mandatory requirements. They may nevertheless be relevant for use of the excipient and are given for information (see also section *1.1. General Provisions*).

REFERENCE STANDARDS

Certain monographs require the use of reference standards that include chemical reference substances, herbal reference standards, biological reference preparations, reference spectra (see also chapter *Reference Standards*).

Reference standards of the Member States of the Union and the main pharmacopeias, with which the Pharmacopoeia of the Union is harmonised, are accepted as reference standards of the Pharmacopoeia of the Union.

1.5. Abbreviations and symbols

A	Absorption (optical density)
$A_{1\text{ см}}^{1\%}$	Specific absorbance
A_r	Relative atomic mass
$[\alpha]_D^{20}$	Specific optical rotation
BRP	Biological reference standard
CRS	Chemical reference substance
d_{20}^{20}	Relative Density
λ	Wavelength
HRS	Herbal reference standard
M	Molarity
IU	International Unit
M_r	Relative molecular mass
n_D^{20}	Refractive index
Ph. Eur. U.	European Pharmacopoeia Unit
ppb	Parts per billion (micrograms per kilogram)
ppm	Parts per million (milligrams per kilogram)
R	Substance or solution defined under section *Reagents*
R_F	Retardation factor (see chapter *2.1.2.36*)
R_{st}	Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by a reference standard
RV	Substance used as a primary standard in volumetric analysis (chapter *2.2.2.1*)
T_{bp}	Boiling Point
T_{mp}	Melting point

ABBREVIATIONS USED IN THE MONOGRAPHS ON IMMUNOGLOBULINS, IMMUNOSERA AND VACCINES

CFU LD_{50}	Colony-forming units. The statistically determined amount of substance that, when administered by the specified route, may be expected to cause the death of 50% of the test animals within a given period
MLD	Minimum lethal dose
L+/10 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
L+ dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
Ir/100 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period
Lp/10 dose	The smallest quantity of toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period

Lo/10 dose	The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given period
Lf dose	The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin
$CCID_{50}$	The statistically determined quantity of virus that may be expected to infect 50% of the cell cultures to which it is added
EID_{50}	The statistically determined quantity of virus that may be expected to infect 50% of the fertilised eggs into which it is inoculated
ID_{50}	The statistically determined quantity of a virus that may be expected to infect 50% of the animals into which it is inoculated
PD_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to protect 50% of the animals against a challenge dose of the micro-organisms or toxins against which it is active
ED_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to induce specific antibodies in 50% of the animals for the relevant vaccine antigens
PFU	Pock-forming units or plaque-forming units
SPF	Specified-pathogen-free

COLLECTIONS AND DEPOSITORIES OF MICROORGANISMS

ATCC	American Type Culture Collection - 10801 University Boulevard Manassas, Virginia 20110 - 2209, USA
C. I. P.	Collection de Bactéries de l'Institut Pasteur - B. P. 52, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France
IMI	International Mycological Institute - Bakeham Lane Surrey TW20 9TY, Great Britain
I. P.	Collection Nationale de Culture de Microorganismes (C. N. C. M.) Institut Pasteur 25, rue du Docteur Roux 75724 Paris Cedex 15, France
NCIMB	National Collection of Industrial and Marine Bacteria Ltd - 23 St Machar Drive Aberdeen AB2 1 RY, Great Britain
NCPF	National Collection of Pathogenic Fungi - London School of Hygiene and Tropical Medicine Keppel Street London WC1E 7HT, Great Britain
NCTC	National Collection of Type Cultures - Central Public Health Laboratory, Colindale Avenue London NW9 5HT, Great Britain

NCYC	National Collection of Yeast Cultures - AFRC Food Research Institute Colney Lane Norwich NR4 7UA, Great Britain
NITE	Biological Resource Center Department of Biotechnology National Institute of Technology and Evaluation - - - 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba, 292-0818, Japan
S. S. I.	Statens Serum Institut - 80 Amager Boulevard, Copenhagen, Denmark

REPUBLIC OF BELARUS

SCVB	Specialised collection of viruses and bacteria pathogenic to humans: State Institution "Republican Research and Practical Center for Epidemiology and Microbiology" of the Ministry of Health of the Republic of Belarus
BIM	Belarusian collection of non-pathogenic microorganisms: State Research Institution "Institute of Microbiology, National Academy of Sciences, the Republic of Belarus"

REPUBLIC OF KAZAKHSTAN

KVOOI	Collection of pathogens of particularly dangerous infections: RGP on PKhV Kazakh Scientific Center of Quarantine and Zoonotic Infections named after M. AikimBaev, Ministry of Health of the Republic of Kazakhstan; RGP on PKhV National Reference Center for Veterinary Medicine, Ministry of Agriculture of the Republic of Kazakhstan
KPM	Collection of industrial microorganisms: RGP on PKhV National Center of Biotechnology, Ministry of Education and Science of the Republic of Kazakhstan.
DVOOI	Depository of pathogens of particularly dangerous infections: RGP on PKhV Kazakh Scientific Center of Quarantine and Zoonotic Infections named after M. AikimBaev, Ministry of Health of the Republic of Kazakhstan; RGP on PKhV Research Institute of biological safety problem, Ministry of Education and Science of the Republic of Kazakhstan; RGP on PKhV National Reference Center for Veterinary Medicine, Ministry of Agriculture of the Republic of Kazakhstan

RUSSIAN FEDERATION

GKPM	State collection of pathogenic microorganisms: Federal State Budgetary Institution "Scientific Center for Examination of Medical Devices", Ministry of Health of the Russian Federation (FGBU NCESMP); Russian Research Anti-Plague Institute "Microb".
VKPM	All-Russian collection of industrial microorganisms: State Research Institute of Genetics and Selection of Industrial Microorganisms.
VKNPM	All-Russian collection of non-pathogenic microorganisms: Institute of Biochemistry and Physiology of Microorganisms named after K. Skryabin, RAS; State Scientific Center of Applied Microbiology and Biotechnology (GNTs PMB).

RKPG	Russian collection of pathogenic fungi: Saint Petersburg Medical Academy of Postgraduate Education; Research Institute of Medical Mycology named after P. N. Kashkin.
GKMOBZh	State collection of microorganisms that cause dangerous, very dangerous, including zooanthroponotic and non-native animal diseases: Federal State Budgetary Scientific Institution Federal Research Center of Virology and Microbiology (FGBNU FICViM)
VKPiVSM	All-Russian collection of pathogenic and vaccine strains of microorganisms that cause infectious diseases of animals: FGBNU All-Russian Research Institute of Experimental Veterinary Medicine named after Ya. R. Kovalenko.
KNPMSN	A collection of nonpathogenic microorganisms of agricultural purpose: All-Russian Research Institute for Agricultural Microbiology (VNIISHM).
VGKShMVZh	All-Russian state collection of microorganism strains used in veterinary medicine and animal husbandry: Federal State Budgetary Institution "All-Russian State Center for Quality and Standardisation of Drugs for Animals and Feed" (FGBU VGNKI).
GKV	State collection of viruses: Division of the Institute of Virology named after D.I. Ivanovsky FGBU NICEM named after N.F. Gamalei, Ministry of Health of the Russian Federation. National Museum of Brucella Cultures; Collection of Legionella and Listeria bacterial cultures; International collection of reference strains of pathogenic and saprophytic Leptospira; Mycoplasma Collection; Collection of Borrelia bacteria; Collection of Francisellatularensis cultures; All-Russian Museum of Rickettsia cultures: Federal State Budgetary Institution "Research Center of Epidemiology and Microbiology named after N.F. Gamalei, Ministry of Health of the Russian Federation (FGBU NICEM).
CC	Collection center: Volgograd Research Anti-Plague Institute (VolgNIPChI).
MLC	Museum of Live Cultures (working collection of bacteria of pathogenicity groups I-IV – vibrions and Yersinia of different types): Rostov Research Anti-Plague Institute.

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1.6. Units of the international system (SI) used in the Pharmacopoeia of the Union and equivalence with other units

INTERNATIONAL SYSTEM OF UNITS

The International System of Units (SI) comprises three classes of units: base units, derived units, and supplementary units. The base units and their definitions are set out in Table 1.6-1.

The derived units may be formed by combining the base units according to the algebraic relationships linking the corresponding quantities. Some of these derived units have special names and symbols. The SI units used in the Pharmacopoeia of the Union are shown in Table 1.6-2.

Table 1.6.-1. – *SI base units*

Quantity		Unit		Definition
Name	Symbol	Name	Symbol	
Length	*l*	metre	m	The metre is the length of the path travelled by light in a vacuum during a time interval of 1/299 792 458 of a second
Weight	*m*	kilogram	kg	The kilogram is equal to the mass of the international prototype of the kilogram
Time	*t*	second	s	The second is the duration of 9 192 631 770 periods of the radiation corresponding to the transition between the two hyperfine levels of the ground state of the cesium-133 atom
Electric current	*I*	ampere	A	The ampere is that constant current which, maintained in two straight parallel conductors of infinite length, of negligible circular cross-section and placed 1 metre apart in vacuum would produce between these conductors a force equal to $2 \cdot 10^{-7}$ newton per metre of length
Thermodynamic temperature	*T*	kelvin	K	The kelvin is the fraction 1/273.16 of the thermodynamic temperature of the triple point of water
Substance quantity	*n*	mol	mol	The mole is the amount of substance of a system containing as many elementary entities as there are atoms in 0.012 kilograms of carbon-12.
Luminous intensity	I_v	candela	cd	The candela is the luminous intensity in a given direction of a source emitting monochromatic radiation with a frequency of $540 \cdot 10^{12}$ hertz and whose energy intensity in that direction is 1/683 watt per steradian

Table 1.6.-2. – – *SI units used in the Pharmacopoeia of the Union and equivalence with other units*

Quantity		Unit				
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	Conversion of other units into SI units
Wavenumber	*v*	one per metre	1/m	m^{-1}		
Wavelength	*λ*	micrometre nanometre	µm nm	10^{-6} m 10^{-9} m		
Area	*A, S*	square metre	m^2	m^2		
Volume	*V*	cubic metre	m^3	m^3		1 mL = 1 cm^3 = 10^{-6} m^3
Frequency	*v*	hertz	Hz	s^{-1}		
Density	*s*	kilogram per cubic metre	kg/m^3	$kg \cdot m^{-3}$		1 g/mL = 1 g/cm^3 = 10^3 kg· m^3
Rate	*v*	metre per second	m/s	$m \cdot s^{-1}$		
Force	*F*	newton	N	m· $kg \cdot s^{-2}$		1 dyne = 1 g· $cm \cdot s^{-2}$ = 10^{-5} N 1 kp = 9.806 65 N
Pressure	*p*	pascal	Pa	m_{-1}· $kg \cdot s_{-}^{2}$	$N \cdot m^{-2}$	1 dyne/cm^2 = 10^{-1} Pa = 10^{-1} $N \cdot m^{-2}$ 1 ATM = 101,325 Pa = = 101.325 kPa 1 bar = 10^5 PA = 0.1 MPa 1 mm Hg = 133,322,387 Pa 1 Torr = 133,322,368 Pa 1 psi = 6.894 757 kPa

Table 1.6.-2. – (*cont.*)

Quantity		Unit				
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	Conversion of other units into SI units
Dynamic viscosity	η	pascal second	Pa· s	$m^{-1}·kg·s^{-1}$	$N·s·m^{-2}$	1 P = 10^{-1} Pa· s =10^{-1} $N·s·m^{-2}$ 1 cP = 1 mPa·s
Kinematic viscosity	*v*	square metre per second	m^2/s	$m^2·\ s^{-1}$	$Pa·s·m^3·kg^{-1}$ $N·m·s·kg^{-1}$	1 St = 1 $cm^2·s^{-1}$ = 10^{-4} $m^2·s^{-1}$
Energy	*W*	joule	J	$m^2·kg·s^{-2}$	N· m	1 erg = 1 $cm^2·g·s^{-2}$ = = 1 dyne·cm = 10^{-7} J 1 cal = 4.1868 J
Power Radiant flow	*P*	watt	W	$m^2·kg·s^{-3}$	$N·m·s^{-1}$ $J·s^{-1}$	1 erg/s = 1 dyne·cm·s^{-1} = = 10^{-7} W = 10^{-7} $N·m·s^{-1}$ = = 10^{-7} $J·s^{-1}$
Absorbed dose of radiant energy	*D*	gray	Gy	$m^2·\ s^{-2}$	J· kg^{-1}	1 rad =10^{-2} Gy
Electric potential, electromotive force	*U*	volt	V	$m^2·kg·s^{-3}·A^{-1}$	W· A^{-1}	
Electric resistance	*R*	ohm	Ohm	$m^2·kg·s^{-3}·A^{-2}$	$V·A^{-1}$	
Quantity of electricity	*Q*	coulomb	C	A· s		
Radioactivity of a substance	*A*	becquerel	Bq	s^{-1}		1 Ci = $37·10^9$ Bq = $37·10^9$ s^{-1}
Molarity	*c, C*	mole per cubic metre	mol/m^3	mol· m^{-3}		1 mol/l = 1 M = = 1 mol/dm^3 = 10^3 mol· m^{-3}
Mass concentration	*ρ*	kilogram per cubic metre	kg/m^3	kg· m^{-3}		1 g/l = 1 g/dm^3 = 1 kg· m^{-3}

Some important and widely used units outside the International System are shown in Table 1.6-3.

The prefixes shown in Table 1.6-4 are used to form the names and symbols of the decimal multiples and submultiples of SI units.

Table 1.6.-3. – *Units used with the International System*

Quantity	Unit		Value in SI units
	Name	Symbol	
Time	minute	min	1 min = 60 seconds
	hour	h	1 h = 60 min = 3600 s
	day	d	1 d = 24 h = 86,400 s
Plane angle	degree	°	1° = (π/180) rad
Volume	litre	L	1 L = 1 dm^3 = 10^{-3} m^3
Weight	ton	t	1 t = 10^3 kg
Rotational frequency	revolution per minute	rpm	1 r/min = (1/60) s^{-1}

Table 1.6.-4. – Decimal multiples and sub-multiples of units

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10^{18}	exa	E	10^{-1}	deci	d
10^{15}	peta	P	10^{-2}	centi	c
10^{12}	tera	T	10^{-3}	milli	m
10^{9}	giga	G	10^{-6}	micro	µ
10^{6}	mega	M	10^{-9}	nano	n
10^{3}	kilo	k	10^{-12}	pico	p
10^{2}	hecto	g	10^{-15}	femto	f
10^{1}	deca	da	10^{-18}	atto	a

NOTES

1. In the Pharmacopoeia of the Union, the Celsius temperature is used (symbol *t*). This is defined by the following equation:

$$t = T - T_0,$$

where T_0 = 273.15 K. The Celsius or centigrade temperature is expressed in degrees Celsius (symbol °C). One degree Celsius is equal to one Kelvin.

2. The practical expressions of concentrations used in the Pharmacopoeia are defined in *1. General Notices.*

3. The radian is the plane angle between two radii of a circle that cut off on the circumference an arc equal in length to the radius.

4. In the Pharmacopoeia of the Union, conditions of centrifugation are defined by reference to the acceleration due to gravity (*g*):

$$g = 9.80665 \text{ m} \cdot \text{s}^{-2}.$$

5. Certain quantities without dimensions are used in the Pharmacopoeia of the Union, for example, relative density (*2.1.2.5*), absorption (*2.1.2.24*), specific absorbance and refractive index (*2.1.2.6*).

6. The microkatal is defined as the enzymic activity that, under defined conditions, produces the transformation (e.g. hydrolysis) of 1 micromole of the substrate per second.

2. GENERAL CHAPTERS

2.1. METHODS OF ANALYSIS

201010001-2019

2.1.1.1. Droppers

The term ‘drops’ means standard drops delivered from a standard dropper (Figure 2.1.1.1.-1). Standard droppers are constructed of colorless glass. The lower extremity has a circular orifice in a flat surface at right angles to the axis.

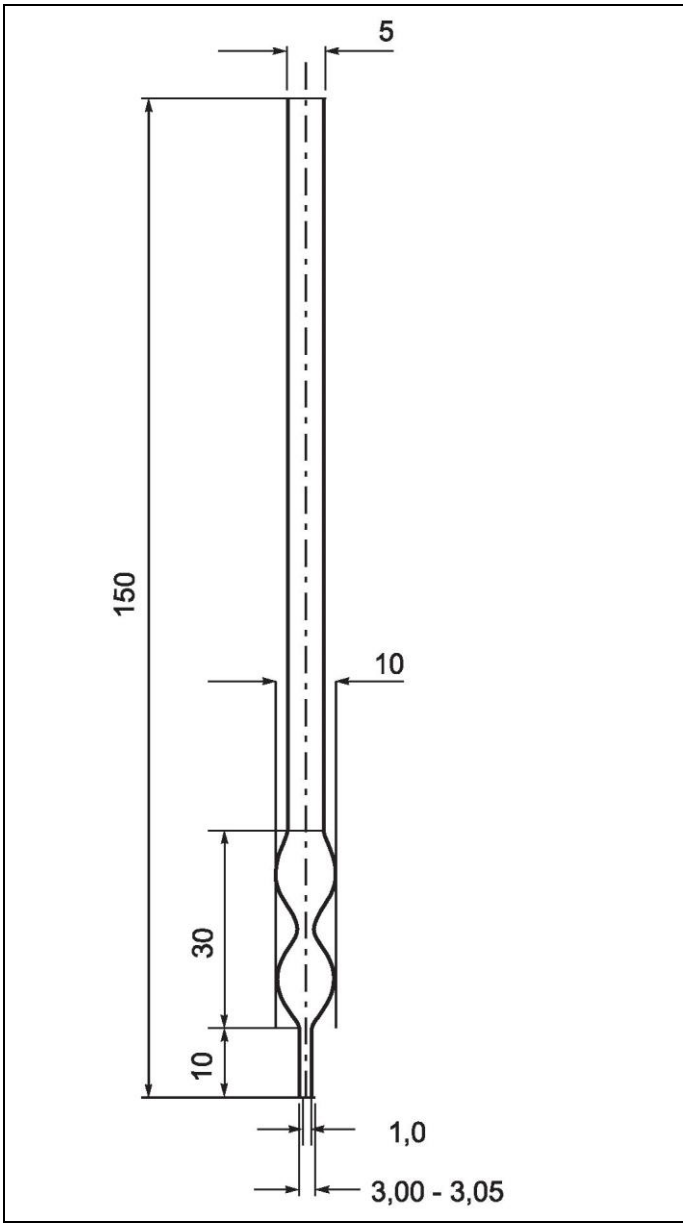

Figure 2.1.1.1.-1 – *Standard dropper. Dimensions in millimetres*

Other droppers may be used provided they comply with the following test.

Twenty drops of water R at a temperature of 20 ± 1 °C flowing freely from the dropper (pipette) held in the vertical position at a constant rate of 1 drop per second weighs 1000 ± 50 mg.

The dropper must be carefully cleaned before use. Carry out three determinations on any given dropper. No result may deviate by greater than 5% from the mean of the three determinations.

201010002-2019

2.1.1.2. Porosity of the Sintered-Glass Filters

Ranges of filters (diameter in micrometres), the limits of which are approximate:

- ≤2.5 – bacterial filtration;

Table 2.1.1.2.-1 – *Porosity of sintered-glass filters*

Porosity number[(1)]	Maximum diameter of pores, µm	Germany	France	United Kingdom
1.6	less than 1.6	5f	—	—
—	1-2.5	5		5
4	1.6-4	—	—	—
—	4-6	—	5	—
10	4-10	4f	—	4
16	10-16	4	4	—
40	16-40	3	3	3
—	40-50	—	—	2
100	40-100	2	2	—
—	100-120	—	—	1
160	100-160	1	1	—
—	150-200	0	0	—
250	160-250	—	—	—
—	200 - 500	—	00	—

[(1)] The European Pharmacopoeia (*Ph. Eur.*) has adopted the system proposed by the International Organisation for Standardisation (*ISO*) .

- 4-10 – ultra-fine filtration, separation of micro-organisms of large diameter;
- 10-40 – analytical filtration, very fine filtration of mercury, very fine dispersion of gases;
- 40-100 – fine filtration, mercury filtration, fine dispersion of gases;
- 100-160 – filtration of coarse materials, dispersion and washing of gases, support for other filter materials;
- 160-500 – filtration of very coarse materials, dispersion and washing of gases.

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2.1.1.3. Ultraviolet Ray Lamps for Analytical Purposes

Mercury vapour in quartz lamps is used as the source of ultraviolet light. A suitable filter may be fitted to eliminate the visible part of the spectrum emitted by the lamp. When the Pharmacopoeia prescribes in a test the use of ultraviolet light of wavelength 254 nm or 365 nm, an instrument consisting of a mercury vapour lamp and a filter which gives an emission band with maximum intensity at about 254 nm or 365 nm is used. The lamp used should be capable of revealing without doubt a standard spot of sodium salicylate with a diameter of about 5 mm on the support of *silica gel G R*, the spot being examined while in a position normal to the radiation.

For this purpose apply 5 µL of a 0.4 g/L solution of *sodium salicylate R* in *96% alcohol R* (alcohol must be free from fluorescence) for lamps of maximum output at 254 nm and 5 µL of a 2 g/L solution of *sodium salicylate R* in *96% alcohol R* for lamps of maximum output at 365 nm. The distance between the lamp and the chromatographic plate under examination used in a pharmacopoeial test should never exceed the distance used to carry out the above test.

201010004-2019

2.1.1.4. Sieves

Sieves are constructed of suitable materials with square meshes. For purposes other than analytical procedures, sieves with circular meshes may be used, the internal diameters of which are 1.25 times the aperture of the square mesh of the corresponding sieve size. There must be no reaction between the material of the sieve and the substance being sifted. Degree of comminution is prescribed in the monograph using the sieve number, which is the size of the mesh in micrometres, given in parenthesis after the name of the substance (Table 2.1.1.4.-1)

Maximum tolerance for an aperture (+*X*): no aperture size shall exceed the nominal size by greater than *X*, where:

$$X = \frac{2(\omega^{0,75})}{3} + 4(\omega^{0,25}),$$

ω is the nominal width of aperture.

Tolerance for mean aperture (±*Y*): the average aperture size shall not depart from the nominal size by greater than ±*Y*, where:

$$Y = \frac{\omega^{0,98}}{27} + 1,6.$$

Intermediary tolerance (+*Z):* not greater than 6% of the total number of apertures shall have sizes between "nominal +*X*" and "nominal +*Z*", where:

$$Z = \frac{X + Y}{2}.$$

The wire diameters *d* given in Table 2.1.1.4.-1 apply to woven metal wire cloth mounted in a frame. The nominal sizes of the wire diameters may depart from these *d* values within the limits d_{max} and d^{min}. The limits define a permissible range of choice ± 15% of the recommended nominal dimensions. The wires in a test sieve shall be of a similar diameter in warp and weft directions.

Table 2.1.1.4.-1. – *Characteristics of sieves*

Sieve number (nominal dimensions of apertures), µm	Tolerances for apertures, µm			Wire diameters, µm		
	Maximum tolerance for an aperture	Tolerance for mean aperture	Intermediary tolerance	Recommended nominal dimensions	Admissible limits	
	$+X$	$\pm Y$	$+Z$	d	d_{max}	d_{min}
11,200	770	350	560	2500	2900	2100
8000	600	250	430	2000	2300	1700
5600	470	180	320	1600	1900	1300
4000	370	130	250	1400	1700	1200
2800	290	90	190	1120	1300	950
2000	230	70	150	900	1040	770
1400	180	50	110	710	820	600
1000	140	30	90	560	640	480
710	112	25	69	450	520	380
500	89	18	54	315	360	270
355	72	13	43	224	260	190
250	58	9.9	34	160	190	130
180	47	7.6	27	125	150	106
125	38	5.8	22	90	104	77
90	32	4.6	18	63	72	54
63	26	3.7	15	45	52	38
45	22	3.1	13	32	37	27
38	—	—	—	30	35	24

2.1.1.5. Tubes for Comparative Tests

Tubes used for comparative tests are matched tubes of colorless glass with a uniform internal diameter and a transparent flat base.

A layer of the liquid is examined down the vertical axis of the tube against a white background, or if necessary, against a black background. The examination is carried out in diffused light.

It is assumed that tubes with an internal diameter of 16 mm will be used. Tubes with a larger internal diameter may be used instead but the volume of liquid examined must then be increased so that the depth of liquid in the tubes is not less than where the prescribed volume of liquid and tubes 16 mm in internal diameter are used.

2.1.1.6. Gas Detector Tubes

Gas detector tubes are cylindrical, sealed tubes consisting of inert transparent material and are constructed to allow the passage of gas. They contain reagents adsorbed onto inert substrates that are suitable for the visualisation of the substance to be detected and, if necessary, they also contain preliminary layers and/or adsorbent filters to eliminate substances that interfere with the substance to be detected. The layer of indicator contains either a single reagent for the detection of a given impurity or several reagents for the detection of several substances (monolayer tube or multilayer tube).

The test is carried out by passing the required volume of the gas to be examined through the indicator tube. The length of the coloured layer or the intensity of a colour change on a graduated scale is a function and a measure of the mass concentration of the analyte.

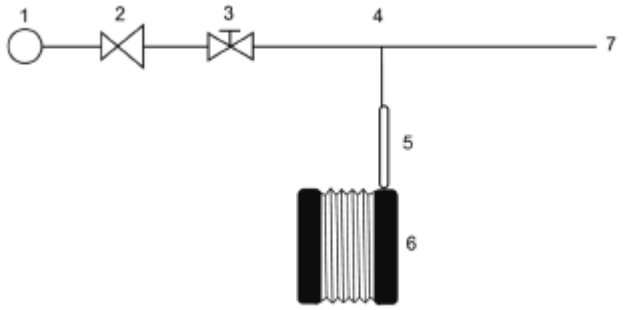

Figure 2.1.1.6.-1 – *Apparatus for gas detector tubes*1. Gas supply; 2. Pressure regulator; 3. Needle valve; 4. "Y"-piece; 5. Indicator tube; 6. Indicator tube pump; 7. End open for gas to escape into the atmosphere.

The calibration of the detector tubes is verified according to the manufacturer's instructions.

Operating conditions. Examine according to the manufacturer's instructions or proceed as follows:

The gas delivery device is connected to a suitable pressure regulator and needle valve. Connect the flexible tubing fitted with a Y-piece to the valve and purge the tubing (Figure 2.1.1.6.-1). Connect the open end of the indicator tube to the short leg of the tubing and operate the pump by the appropriate number of strokes to pass a suitable volume of gas to be examined through the tube. Read the value corresponding to the length of the coloured layer or the intensity of the colour on the graduated scale. If a negative result is achieved, indicator tubes can be verified with a calibration gas containing the appropriate impurity.

In view of the wide variety of available compressor oils, it is necessary to verify the reactivity of the oil detector tubes for the oil used. Information on the reactivity for various oils is given in the leaflet supplied with the tube. If the oil used is not directed in the leaflet, the tube manufacturer must verify the reactivity and if necessary provide a tube specific for this oil.

Carbon dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for hydrazine and crystal violet indicators. The minimum value indicated is 100 ppm with a relative standard deviation of at most ± 15%.

Sulfur dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the iodine and starch indicator. The minimum detectable concentration is 0.5 ppm with a relative standard deviation of ±15%.

Oil detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the sulfuric acid indicator. The minimum value indicated is 0.1 mg/m^3 with a relative standard deviation of at most ± 30%.

Nitrogen monoxide and nitrogen dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for an oxidising layer (Cr(VI) salt) and the diphenylbenzidine indicator. The minimum detectable concentration is 0.5 ppm with a relative standard deviation of ±15%.

Carbon monoxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for di-iodine pentoxide, selenium dioxide, and fuming sulfuric acid indicators. The minimum value indicated is 5 ppm or less, with a relative standard deviation of at most ± 15%.

Hydrogen sulfide detector tube Sealed glass tube containing adsorbent filters and suitable supports for an appropriate lead salt indicator. The minimum value indicated is 1 ppm or less, with a relative standard deviation of at most ± 10%.

Water vapour detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the magnesium perchlorate indicator. The minimum value indicated is 67 ppm or less, with a relative standard deviation of at most ± 20%.

2.1.2. PHYSICAL AND PHYSICOCHEMICAL METHODS

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2.1.2.1. Clarity and Degree of Opalescence of Liquids

VISUAL METHOD

Using identical test-tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with a reference suspension freshly prepared as described below, the depth of the layer being 40 mm. Compare the solutions in diffused daylight 5 min after preparation of the reference suspension (layer height 40 mm), viewing vertically against a black background. The diffusion of light must be such that reference suspension I can readily be distinguished from *water R*, and that reference suspension II can readily be distinguished from reference suspension I.

A liquid is considered clear if its clarity is the same as that of *water R* or of the solvent used when examined under the conditions described above, or if its opalescence is not more pronounced than that of reference suspension I.

Hydrazine sulfate solution. Dissolve 1.0 g of *hydrazine sulfate R* in *water R* and dilute to 100.0 mL with *water R*. Allow to stand for 4-6 h.

Hexamethylenetetramine solution. Dissolve 2.5 g of *hexamethylenetetramine R* in 25.0 mL of *water R* in a 100 mL ground-glass stoppered flask.

Primary opalescent suspension (formazin suspension). To the hexamethylenetetramine solution in the flask add 25.0 mL of the hydrazine sulfate solution; mix, and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension particles can adhere to the glass and must be well mixed before use.

Standard of opalescence. Dilute 15.0 mL of the primary opalescent suspension to 1000.0 mL with *water R*. This standard suspension may be stored for not greater than 24 h.

Reference suspensions. Prepare the reference suspensions according to Table 2.1.2.1.-1. Mix and shake the opalescence standard and *water R* before use.

Turbidity standard. The formazin suspension prepared by mixing equal volumes of the hydrazine sulfate solution and the hexamethylenetetramine solution is defined as a 4000 *NTU* (*nephelometric turbidity units*) primary reference standard. Reference suspensions I, II, III, and IV have values of 3 *NTU*, 6 *NTU*, 18 *NTU*, and 30 *NTU* respectively. Stabilised formazin suspensions that can be used to prepare stable, diluted turbidity standards are available commercially and may be used after comparison with the standards prepared as described.

Formazin has several desirable characteristics that make it an excellent turbidity standard. It can be reproducibly prepared from assayed reagents. The physical characteristics make it a desirable light-scatter calibration standard. The formazin polymer consists of chains of different lengths, which fold into random configurations. This results in a wide assay of particle shapes and sizes, which analytically fits the possibility of different particle sizes and shapes that are found in the real samples. Due to formazin's reproducibility, scattering characteristics, and traceability, instrument calibration algorithms and performance criteria are mostly based on this standard.

Table 2.1.2.1.-1. – *Preparation of reference suspensions*

	I	II	III	IV
Standard of opalescence, mL	5.0	10.0	30.0	50.0
Water R, mL	95.0	90.0	70.0	50.0

INSTRUMENTAL METHODS

INTRODUCTION

The degree of opalescence may also be determined by instrumental measurement of the light absorbed or scattered on account of submicroscopic optical density inhomogeneities of opalescent solutions and suspensions. In practice, 2 techniques are used: nephelometry and turbidimetry. For turbidity measurement of coloured samples, ratio turbidimetry and nephelometry with ratio selection are used.

The light scattering effect of suspended particles can be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry). Ratio turbidimetry combines the principles of both nephelometry and turbidimetry. Turbidimetry and nephelometry are useful for the measurement of slightly opalescent suspensions. Reference suspensions produced under well-defined conditions must be used. For quantitation, the construction of calibration curves is essential, since the relationship between the optical properties of the suspension and the concentration of the dispersed phase is at best semi-empirical.

The determination of opalescence of coloured liquids is done with ratio turbidimeters or nephelometers with ratio selection, since colour provides a negative interference, attenuating both incident and scattered light and lowering the turbidity value. The effect is so great for even moderately coloured samples that conventional nephelometers cannot be used.

The instrumental assessment of clarity and opalescence provides a more discriminatory test that does not depend on the visual acuity of the analyst. Numerical results are more useful for quality monitoring and process control, especially in stability studies. For example, previous numerical data on stability can be projected to determine whether a given batch of dosage formulation or active pharmaceutical ingredient will exceed shelf-life limits prior to the expiry date.

NEPHELOMETRY

When a suspension is viewed at right angles to the direction of the incident light, the system appears opalescent due to the reflection of light from the particles of the suspension (Tyndall effect). A certain portion of the light beam entering a turbid liquid is transmitted, another portion is absorbed and the remaining portion is scattered by the suspended particles. If the measurement is made at 90° to the light beam, the light scattered by the suspended particles can be used for the determination of their concentration, provided the number and size of particles influencing the scattering remain constant. The reference suspension must maintain a constant degree of turbidity and the sample and reference suspensions must be prepared under identical conditions. The Tyndall effect depends upon both the number of particles and their size. Nephelometric measurements are more reliable in low turbidity ranges, where there is a linear relationship between nephelometric turbidity unit (*NTU*) values and relative detector signals. As the degree of turbidity increases, not all the particles are exposed to the incident light and the scattered radiation of other particles is hindered on its way to the detector. The maximum nephelometric values at which reliable measurements can be made lie in the range of 1750-2000 *NTU*. Linearity between turbidity and concentration must be established by constructing a calibration curve using at least four concentrations.

TURBIDIMETRY

The optical property expressed as turbidity is the interaction between light and suspended particles in liquid. This is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted in a straight line through the sample. The quantity of solid material in suspension can be determined by the measurement of the transmitted light.

A linear relationship between turbidity and concentration is obtained when the particle sizes are uniform and homogeneous in the suspension. This is true only in very dilute suspensions containing small particles. Linearity between turbidity and concentration must be established by constructing a calibration curve using at least four concentrations.

RATIO TURBIDIMETRY

In ratio turbidimetry the relationship of the transmission measurement to the 90° scattered light measurement is determined. This procedure compensates for the light that is diminished by the colour of the sample. The influence of the colour of the sample may also be eliminated by using an infrared light-emitting diode (IR LED) at 860 nm as the light source of the instrument. The instrument's photodiode detectors receive and measure scattered light at a 90° angle from the sample as well as measuring the forward scatter (light reflected) in front of the sample along with the measurement of light transmitted directly through the sample. The measuring results are given in *NTU* (ratio) and are obtained by calculating the ratio of the 90° angle scattered light measured to the sum of the components of forward scattered and transmitted light values. In ratio turbidimetry, the influence of stray light becomes negligible. Nephelometers are used for measurements of the degree of opalescence of colorless liquids.

Measurements of reference suspensions I - IV with a ratio turbidimeter show a linear relationship between the concentrations and measured *NTU* values (see Table 2.1.2.1.-2). Reference suspensions I - IV may be used as calibrators for the instrument.

Table 2.1.2.1.-2. – *Opalescent values for various formazin suspensions*

Formazin suspension	Opalescent value (NTU)
Reference suspension I	3
Reference suspension II	6
Reference suspension III	18
Reference suspension IV	30
Standard of opalescence	60
Primary opalescent suspension	4000

INSTRUMENTAL DETERMINATION OF OPALESCENCE

Requirements in monographs are expressed in terms of the visual examination method with the defined reference suspensions. Instrumental methods may also be used for determining compliance with monograph requirements once the suitability of the instrument as described below has been established and calibration with reference suspensions I - IV and with *water R* or the solvent used has been performed.

Apparatus. Ratio turbidimeters or nephelometers with selectable ratio application use as light source a tungsten lamp with spectral sensitivity at about 550 nm operating at a filament colour temperature of 2700 K, or infrared LED having an emission maximum at 860 nm with a 60 nm spectral bandwidth. Other suitable light sources may also be used. Silicon photodiodes and photomultipliers are commonly used as detectors and record changes in light scattered or transmitted by the sample. The light scattered at 90 ± 2.5° is detected by the primary detector. Other detectors are those to detect back and forward scatter as well as transmitted light. The instruments used are calibrated against standards of known turbidity; besides, instruments should be capable of automatic determination of turbidity. The test results expressed in *NTU* units are obtained directly from the instrument and compared to the specifications in the individual monographs.

Instruments complying with the following specifications are suitable.

- *Measuring units*: *NTU*. *NTU* is based on the turbidity of a primary reference standard of formazin.

FTU (*Formazin Turbidity Units*) or *FNU* (*Formazin Nephelometry Units*) are also used and are equivalent to *NTU* in low regions (up to 40 *NTU).* These units are used in all three instrumental methods (nephelometry, turbidimetry and ratio turbidimetry).

- *Measuring range*: from 0.01 *NTU* up to 1100 *NTU*.

- *Resolution*: 0.01 *NTU* within the range of 0-10 *NTU*; 0.1 *NTU* within the range of 10-100 *NTU*; 1 *NTU* for the range > 100 *NTU*. The instrument is calibrated and controlled with reference standards of formazin.

- *Accuracy*: within the range of 0-10 *NTU*: ± (2% of reading + 0.01) *NTU*; within the range of 10-1000 *NTU*: ± 5%.

- *Repeatability*: within the range of 0-10 *NTU*: ± 0.01 *NTU*; within the range of 10-1000 *NTU*: ± 2% of the measured value.

- *Calibration*: with 4 reference suspensions of formazin in the range of interest. Reference suspensions described in this chapter or suitable reference standards calibrated against the primary reference suspensions may be used.

- *Stray light*: stray light is a significant source of error in low-level turbidimetric measurement; stray light reaches the detector of an optical system but does not come from the sample; less than 0.15 *NTU* for the range 0-10 *NTU*, less than 0.5 *NTU* for the range 10-1000 *NTU*.

Instruments complying with the above characteristics and verified using the reference suspensions described under "Visual method" may be used instead of visual examination for determination of compliance with the individual monograph requirements.

Instruments with range or resolution, accuracy, and repeatability capabilities other than those mentioned above may be used provided they are sufficiently validated and are capable for the intended use. The test procedure for the specific active substances/drug preparations to be analysed must also be validated to confirm its applicability. The instrument and methodology should be consistent with the attributes of the product to be tested.

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2.1.2.2. Degree of colouration of Liquids

The examination of the degree of colouration of liquids in the range brown-yellow-red is carried out by one of the two methods below, as prescribed in individual monographs.

The use of instrumental pharmacopoeial methods for determining the colouration and degree of colouration of liquids is allowed if an appropriate validation has been carried out.

A solution is colorless if it has the appearance of *water R* or the solvent or is not more intensely coloured than reference solution B_9.

METHOD I

Using identical tubes of a colourless, transparent, neutral glass of 12 mm external diameter, compare 2.0 mL of the liquid to be examined with 2.0 mL of *water R* or of the solvent or of the reference solution (see Tables of reference solutions) prescribed in the monograph. Compare the colors in diffused daylight, viewing horizontally (perpendicularly to the axis of test tubes) against a white background.

METHOD II

Using identical tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15 mm to 25 mm, compare the liquid to be examined with 40 mm layer of *water R* or the solvent or the reference solution (see Tables of reference solutions) prescribed in the monograph, the depth of the layer being 40 mm. Compare the colors in diffused daylight, viewing vertically against a white background.

REAGENTS

PRIMARY SOLUTIONS

Yellow solution. Dissolve 46 g of *ferric chloride R* in about 900 mL of a mixture of *hydrochloric acid R – water R* (25:975, *V/V*) and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $FeCl_3 \cdot 6H_2O$ per millilitre by adding the same mixture.

Protect the solution from light.

Titration. Place in a 250 mL conical flask fitted with a ground-glass stopper, 10.0 mL of the solution, 15 mL of *water R*, 5 mL of *hydrochloric acid R*, and 4 g of *potassium iodide R*, close the flask, allow to stand in the dark for 15 min. Add 100 mL of *water R*. Titrate the liberated iodine with a *0.1 M solution of sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 27.03 mg of $FeCl_3 \cdot 6H_2O$.

Red solution. Dissolve 60 g of *cobalt chloride R* in about 900 mL of a mixture of *hydrochloric acid R and water R* (25:975, *V/V*) and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $CoCl_2 \cdot 6H_2O$ per millilitre by adding the same mixture.

Titration. Place 5.0 mL of the solution, 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of a 300 g/L solution of *sodium hydroxide R* in a 250 mL conical flask fitted with a ground-glass stopper. Boil gently for 10 min, allow to cool and add 60 mL of *dilute sulfuric acid R* and 2 g of *potassium iodide R*. Close the flask and dissolve the precipitate by shaking gently. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 23.79 mg of $CoCl_2 \cdot 6H_2O$.

Blue solution. Dissolve 63 g of *copper (II) sulfate pentahydrate R* in about 900 mL of a mixture of *hydrochloric acid R and water R* (25:975, *V/V*) and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $CuSO4 \cdot 5H_2O$ per millilitre by adding the same mixture.

Titration. Place 10.0 mL of the solution a 250 mL conical flask fitted with a ground-glass stopper, add 50 mL of *water R*, 12 mL of *dilute acetic acid R* and 3 g of *potassium iodide R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slightly pale brown colour.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 24.97 mg of $CuSO4 \cdot 5H_2O$.

STANDARD SOLUTIONS

Using the three primary solutions, prepare the five standard solutions as specified in Table 2.1.2.2.-1.

Standard solutions

Reference solutions for methods I and II.

Using the 5 standard solutions, prepare the following reference solutions as specified in Tables 2.1.2.2.-2 – 2.1.2.2.-6.

Storage

For Method I, the reference solutions may be stored in sealed tubes of a colourless, transparent, neutral glass of 12 mm external diameter, protected from light.

Table 2.1.2.2.-1.

Standard solution	Volume, mL			
	Yellow solution	Red solution	Blue solution	Hydrochloric acid (10 g/L)
B (brown)	3.0	3.0	2.4	1.6
BY (brownish-yellow)	2.4	1.0	0.4	6.2
Y (yellow)	2.4	0.6	0.0	7.0
GY (greenish-yellow)	9.6	0.2	0.2	0.0
R (red)	1.0	2.0	0.0	7.0

Table 2.1.2.2.-2. – *Reference solutions B*

Reference solution	Volume, mL	
	Standard solution B	Hydrochloric acid (10 g/L HCl)
B_1	75.0	25.0
B_2	50.0	50.0
B_3	37.5	62.5
B_4	25.0	75.0
B_5	12.5	87.5
B_6	5.0	95.0
B_7	2.5	97.5
B_8	1.5	98.5
B_9	1.0	99.0

Table 2.1.2.2.-3. – *Reference solutions BY*

Reference solution	Volume, mL	
	Standard solution BY	Hydrochloric acid (10 g/L HCl)
BY_1	100.0	0.0
BY_2	75.0	25.0
BY_3	50.0	50.0
BY_4	25.0	75.0
BY_5	12.5	87.5
BY_6	5.0	95.0
BY7	2.5	97.5

Table 2.1.2.2.-4. – *Reference solutions Y*

Reference solution	Volume, mL	
	Standard solution Y	Hydrochloric acid (10 g/L HCl)
Y_1	100.0	0.0
Y_2	75.0	25.0
Y_3	50.0	50.0
Y_4	25.0	75.0
Y_5	12.5	87.5
Y_6	5.0	95.0
Y_7	2.5	97.5

Prepare reference solutions for determining the colour intensity of liquids from standard solutions according to method II, immediately before use.

Table 2.1.2.2.-5. – *Reference solutions GY*

Reference solution	Volume, mL	
	Standard solution GY	Hydrochloric acid (10 g/L HCl)
GY_1	25.0	75.0
GY_2	15.0	85.0
GY_3	8.5	91.5
GY_4	5.0	95.0
GY_5	3.0	97.0
GY_6	1.5	98.5
GY_7	0.75	99.25

Table 2.1.2.2.-6. – *Reference solutions R*

Reference solution	Volume, mL	
	Standard solution R	Hydrochloric acid (10 g/L HCl)
R_1	100.0	0.0
R_2	75.0	25.0
R_3	50.0	50.0
R_4	37.5	62.5
R_5	25.0	75.0
R_6	12.5	87.5
R_7	5.0	95.0

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2.1.2.3. Potentiometric Determination of pH

A pH value is a number that represents the negative logarithm of the activity of hydrogen ions contained in an aqueous solution, conventionally expressing the hydrogen ion concentration of a solution. For practical purposes, its definition is an experimental one.

The pH value of a solution to be examined is related to that of a reference solution (pH_s) by the following equation:

$$\mathrm{pH} = \mathrm{pH}_s - \frac{E - E_s}{k},$$

in which E is the potential, expressed in volts, of the cell containing the solution to be examined;

E_s is the potential, expressed in volts, of the cell containing the solution of known pH (pH_s); k is the change in potential per unit change in pH expressed in volts and calculated from the Nernst equation (see Table 2.1.2.3-1).

The potentiometric determination of pH is made by measuring the potential difference of galvanic cell between two appropriate electrodes immersed in the solution to be examined: one of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (for example, a silver-chloride electrode). Often, together with the temperature sensor, they are combined into one compact electrode.

Apparatus. The measuring apparatus is a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Modern pH meters are microprocessor-based and are controlled using the apparatus manufacturer's firmware or software in accordance with the attached instructions.

Handling of electrodes. Store the electrodes properly and in accordance with the manufacturer's recommendations (for example, in an electrolyte solution or a suitable storage solution). Before measuring, check the electrodes visually. For replenishable electrodes, check that there are no air bubbles in the glass bulb, and make sure there is a sufficient level of the internal electrolyte solution. The refill hole must be open when calibrating and measuring. It is also recommended to check the diaphragm of the reference electrode. Before using the electrode for the first time, or if the electrode was stored in the absence of an electrolyte solution, it is usually necessary to condition the electrode in accordance with the manufacturer's recommendations.

Table 2.1.2.3.-1. – *Values of k at different temperatures*

Temperature, °C	*k*, V
15	0.0572
20	0.0582
25	0.0592
30	0.0601
35	0.0611

If the pH value stabilises too slowly (i.e., the response time is too long), or the zero point is shifted, the slope is reduced, or any other problems observed during calibration indicate that the electrode may need to be cleaned or replaced. Cleaning is carried out depending on the type of sample and in accordance with the manufacturer's manual. Regular cleaning is recommended.

Calibration and measurement conditions. Unless otherwise prescribed in the individual monograph, all measurements are made at the same temperature as the pH buffers used in calibration, usually at 20-25 °C (±2.5 °C). Table 2.1.2.3.-2 shows the variation of pH with respect to the temperature of a number of reference buffer solutions used in calibration. For the temperature correction, when necessary, follow the manufacturer's instructions.

Calibration includes determining a slope (e.g. 95-105%) and offset of the measuring system. Most commercially available pH meters allow self-diagnosis or diagnostics when switched on, while, for example, the slope and potential asymmetry are checked compared to the manufacturer's specification. The instrument is calibrated using at least two selected buffer solutions so that the expected pH value of the test solution is between the pH values of the buffer solutions. The range must be at least two pH units. The pH of a buffer solution of intermediate pH read off on the scale must not differ by greater than 0.05 pH unit from the value corresponding to this solution.

It is preferable to use commercial certified reference buffer solutions.

Alternatively, buffer solutions can be prepared in accordance with Table 2.1.2.3.-2. These solutions must be traceable to primary standards.

Calibration should be performed regularly, preferably daily before use or before each series of measurements.

Immerse the electrodes in the solution to be examined and take the reading in the same conditions as for the reference buffer solutions.

If a system calibrated as described above is used to measure the pH in suspensions, emulsions, or non-aqueous or partially aqueous samples, the pH reading can only be considered an approximation of the true value. Suitable electrodes should be used to measure the pH of such mixtures.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

Potassium tetraoxalate 0.05 M. Dissolve 12.61 g of $C_4H_3KO_8 \cdot 2H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium hydrogen tartrate, saturated at 25 °C. Shake an excess of $C_4H_5KO_6$ vigorously with *carbon dioxide-free water R* at 25 °C for 30 min. Filter or decant the supernatant. Prepare immediately before use.

Potassium dihydrogen citrate 0.05 M. Dissolve 11.41 g of $C_6H_7KO_7$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Prepare immediately before use.

Potassium hydrogen phthalate 0.05 M. Dissolve 10.13 g of $C_8H_5KO_4$, previously dried for 1 h at (110 ± 2) °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M. Dissolve 3.39 g of KH_2PO_4 and 3.53 g of Na_2HPO_4, both previously dried for 2 h at (120 ± 2) °C, in *water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M. Dissolve 1.18 g of KH_2PO_4 and 4.30 g of Na_2HPO_4, both previously dried for 2 h at (120 ± 2) °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Disodium tetraborate 0.01 M. Dissolve 3.80 g of $Na_2B_4O_7 \cdot 10H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Sodium carbonate 0.025 M + sodium hydrogen carbonate 0.025 M. Dissolve 2.64 g of Na_2CO_3 and 2.09 g of $NaHCO_3$ in *carbon dioxide-free water* R and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Table 2.1.2.3.-2 – *pH value of reference buffer solutions at various temperatures*

Temperature, °C	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate, saturated at 25 °C	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M + sodium bicarbonate 0.025 M	Calcium hydroxide, saturated at 25 °C
	$C_4H_3KO_8 \cdot 2H_2O$	$C_4H_5KO_6$	$C_6H_7KO_7$	$C_8H_5KO_4$	$KH_2PO4 + N_2HPO_4$	$KH_2PO4 + Na_2HPO_4$	$Na_2B_4O_7 \cdot 10H_2O$	$Na_2CO_3 + NaHCO_3$	$Ca(OH)_2$
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
$\frac{\Delta pH^{(1)}}{\Delta t}$	+0.001	-0.0014	-0.0022	+0.0012	-0.0028	-0.0028	-0.0082	-0.0096	-0.034

(1) — pH variation per degree Celsius.

Calcium hydroxide, saturated at 25 °C. Shake an excess of *calcium hydroxide R* with *carbon dioxide-free water R* and decant at 25 °C. Store protected from atmospheric carbon dioxide.

STORAGE OF BUFFER SOLUTIONS

Store buffer solutions in suitable chemically resistant, tight containers, such as type I glass bottles or plastic containers suitable for aqueous solutions.

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2.1.2.4. Determining the Approximate pH

Use pH indicator strips to find the approximate pH value. In addition, the pH indicators listed in Table 2.1.2.4.-1 can be used.

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Table 2.1.2.4.-1. – *pH Indicators*

Reaction	pH	Indicator
Alkaline	>8	*Red litmus paper R*
Slightly alkaline	8-10	*Phenolphthalein solution R* *Thymol blue solution R*
Strongly alkaline	>10	*Phenolphthalein paper R* *Thymol blue solution R*
Neutral	6-8	*Methyl red solution R* *Phenolic red solution R*
Acid	<6	*Methyl red solution R* *Bromothymol blue solution R*
Slightly acidic	4-6	*Methyl red solution R* *Bromocresol green solution R*
Strongly acidic	<4	*Congo paper R*

2.1.2.5. Relative Density

The relative density $d_{t_2}^{t_1}$ is the ratio of the mass of a certain volume of a substance at temperature t_1 to the mass of an equal volume of water at temperature t_2.

Unless otherwise indicated in the monograph, the relative density d_{20}^{20} is used. Relative density is also commonly expressed as d_4^{20}. Density ρ_{20}, defined as the mass of a unit volume of the substance at 20 °C may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre (1 kg/m^3 = 0.001 g/cm^3). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0{,}998203 \cdot d_{20}^{20} \text{ или } d_{20}^{20} = 1{,}00180 \cdot \rho_{20},$$

$$\rho_{20} = 0{,}999972 \cdot d_4^{20} \text{ или } d_4^{20} = 1{,}00003 \cdot \rho_{20},$$

$$d_4^{20} = 0{,}998230 \cdot d_{20}^{20}.$$

Relative density or density is measured with the precision to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids), or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the 3rd decimal place. When using a density meter, the buoyancy of air has no influence.

Oscillating transducer density meter. The apparatus consists of:

- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;

- a means of measuring the oscillation period (*T*), which may be converted by the apparatus to give a direct reading of density, or used to calculate the density using the constants *A* and *B* described below.

The resonant frequency (*f*) is a function of the spring constant (*c*) and the mass (*m*) of the system:

$$f^2 = \frac{1}{T^2} = \frac{c}{m} \cdot \frac{1}{4\pi^2}.$$

Hence:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \cdot V}{c} \right) \cdot 4\pi^2,$$

where: *M* is the mass of the tube;

V is the internal volume of the tube.

Introduction of two constants *A* - $c / (4\pi^2 \cdot V)$ and *B* - *M* / *V*, leads to the classical equation for the oscillating transducer:

$$\rho = A \cdot T^2 - B.$$

The constants *A* and *B* are determined by operating the instrument with the U-tube filled with 2 different samples of known density, for example, degassed *water R* and air. The following degassing method can be used: heat water with gentle stirring to a temperature of about 41 °C, immediately filter under vacuum, stirring vigorously, through a 0.45 µm filter (or a filter with a smaller pore size), and continue to stir under vacuum for about 5 minutes. A different validated method for removing dissolved gases can also be used.

Control measurements are made daily using degassed *water R.* The results displayed for the control measurement using degassed *water R* shall not deviate from the reference value (ρ_{20} = 0.998203 g/cm^3, d_{20}^{20} = 1.000000) by greater than its specified error. For example, an instrument specified to ±0.0001 g/cm^3 shall display 0.9982 ± 0.0001 g/cm^3 in order to be suitable for further measurement. Otherwise a re-adjustment is necessary. Calibration with certified reference materials is carried out regularly.

Measurements are made using the same procedure as for calibration. The liquid to be examined is equilibrated in a thermostat at 20 °C before introduction into the tube, if necessary, to avoid the formation of bubbles and to reduce the time required for measurement.

Factors affecting accuracy include:

- temperature uniformity throughout the tube;
- non-linearity over a range of density;
- parasitic resonant effects;
- viscosity, whereby solutions with a higher viscosity than the calibrant have a density that is apparently higher than the true value.

The effects of non-linearity and viscosity may be avoided by using calibrants that have density and viscosity close to those of the liquid to be examined (± 5% for density, ± 50% for viscosity). The density meter may have functions for automatic viscosity correction and for correction of errors arising from temperature changes and non-linearity.

Precision is a function of the repeatability and stability of the oscillator frequency, which is dependent on the stability of the volume, mass, and spring constant of the cell.

Density meters are able to achieve measurements with an error of the order of $1 \cdot 10^{-3}$ g/cm^3 to $1 \cdot 10^{-5}$ g/cm^3 and a repeatability of $1 \cdot 10^{-4}$ g/cm^3 to $1 \cdot 10^{-6}$ g/cm^3.

Determination of density using a pycnometer is carried out as specified in Methods 1 and 2, and using a hydrometer as indicated in Method 3, unless otherwise specified in the monograph.

Method 1. Used when determining the density of liquids with an accuracy of 0.001 g/cm^3.

Weigh a clean dry pycnometer to an accuracy of 0.0002 g, fill with *water R* slightly above the mark using a dry funnel, close with a stopper, and thermostat for 20 minutes at a temperature of (20 ±0.1) °C. At this temperature, bring the water level in the pycnometer to volume, quickly selecting excess water using a pipette or a rolled-up strip of filter paper. Close the pycnometer again with a stopper and thermostat for another 10 minutes, checking the position of the meniscus in relation to the mark.

Then remove the pycnometer from the thermostat, wipe the inner surface of the neck of the pycnometer and entire pycnometer outside with filter paper, allow to stand under the glass of the balance for 10 minutes, and weigh with the accuracy specified above.

Remove water from the pycnometer, dry, rinse successively with *96% alcohol R* and *ether R* (it is not allowed to dry the pycnometer by heating), remove the remaining ether by blowing air, fill the pycnometer with the test liquid and then perform the same operations as for *water R*.

The density ρ_{20} (g/cm^3) is taken by the formula:

$$\frac{(m_2 - m) \cdot 0{,}99703}{m_1 - m} + 0{,}0012,$$

where

m is the mass of the empty pycnometer, g;

m_1 is the mass of the pycnometer with *water R*, g;

m_2 is the mass of the pycnometer with the test liquid, g;

0.99703 is the value of water density at 20 °C, g/cm^3 (including air density);

0.0012 is the value of air density at 20 °C, g/cm^3, at a barometric pressure of 101.3 kPa (760 mm Hg).

Method 2. Used for determining the density of solid fats and wax with an accuracy of 0.001 g/cm^3.

Weigh an empty pycnometer, then fill the same pycnometer with *water R* and weigh again. Remove water and dry the pycnometer. All operations are performed under the conditions specified in Method 1.

Using a pipette or a small funnel with a fine-drawn end, fill the pycnometer with melted fat or wax in such quantity that it occupies from 1/3 to 1/2 of the pycnometer volume. Allow the pycnometer to stand for 1 hour w/o a stopper in hot water, then cool to a temperature of 20 °C, weigh, dilute to volume with *water R* at 20 °C, wipe dry, and weigh again. There should be no air bubbles in both phases and on their interface.

Calculate the density ρ_{20} (g/cm^3) using the formula:

$$\frac{(m2 - m) \cdot 0{,}99703}{(m1 + m_2) - (m + m_3)} + 0{,}0012,$$

where: *m* is the mass of the empty pycnometer, g;

*m*1 is the mass of the pycnometer with *water R*, g;

m_2 is the mass of the pycnometer with the test sample, g;

m_3 is the mass of the pycnometer with the test sample and *water R*, g.

Method 3. Used for determining the density of liquids with an accuracy of 0.01 g/cm^3.

Place the test liquid in a cylinder and, at a liquid temperature of 20 °C, carefully lower a clean dry hydrometer into it; use the scale of the device to determine the expected density value. Keep hold of the hydrometer until it floats freely; however, make sure that the hydrometer does not touch the cylinder walls or bottom. 3-4 minutes after immersion of the hydrometer, read the density from its scale at the bottom of the meniscus (when determining dark-coloured liquids, take the measurement from the top of the meniscus). When reading, the eye should be at the level of the meniscus. Do not use the hydrometer for determining density of highly volatile substances.

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2.1.2.6. Refractive Index

The refractive index of a medium with reference to air is equal to the ratio of the sine of the angle of incidence of a beam of light in air to the sine of the angle of refraction of the refracted beam in the given medium.

Unless otherwise prescribed in the individual monograph, the refractive index is measured at a temperature of 20 ± 0.5 °C, with reference to the wavelength of the D-line of sodium (λ = 589.3 nm); the refractive index determined under such conditions has the symbol n_D^{20} 20.

Refractometers normally determine the critical angle. In such apparatus, the essential part is a prism of known refractive index in contact with the liquid to be examined.

Calibrate the apparatus using certified reference materials.

When white light is used, the refractometer is provided with a compensating system. The apparatus gives readings accurate to at least the third decimal place and is provided with a means of operation at the temperature prescribed. The thermometer is graduated at intervals of 0.5 °C or less.

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2.1.2.7. Optical Rotation

Optical rotation is the property displayed by chiral substances of rotating the plane of polarisation of polarised light.

Optical rotation is considered to be positive (+) for dextrorotatory substances (i.e. those that rotate the plane of polarisation in a clockwise direction) and negative (−) for laevorotatory substances.

The specific optical rotation $[\alpha_m]$ is the rotation, expressed in radians (rad), measured at the temperature *t* and at the wavelength λ given by a 1 m thickness of liquid layer or a solution containing 1 kg/m^3 of an optically active substance. For practical reasons, the specific optical rotation is normally expressed in milliradians meter squared per kilogram (mrad·m^2 ·kg^{-1}).

The Pharmacopoeia adopts the following conventional definitions.

The *angle of optical rotation* of liquids is the angle of rotation α, expressed in degrees (°), of the plane of polarisation at the wavelength of the D-line of sodium (λ = 589.3 nm) measured at 20 °C using a layer of 1 dm. For solutions, the method of preparation is prescribed in the monograph.

The *specific optical rotation*[α]*20* of a liquid is the angle of rotation α, expressed in degrees (°), of the plane of polarisation at the wavelength of the D-line of sodium (λ = 589.3 nm) measured at 20 °C in the liquid substance to be examined, calculated with reference to a layer of 1 dm and divided by the density expressed in grams per cubic centimetre.

The *specific optical rotation* $[\alpha]_D^{20}$ of a substance in solution is the angle of rotation α, expressed in degrees (°), of the plane of polarisation at the wavelength of the D-line of sodium (λ = 589.3 nm) measured at 20 °C in the solution of the substance to be examined, and calculated with reference to a layer of 1 dm containing 1 g/mL of the substance in the solution. The specific optical rotation of a substance in solution is always expressed with reference to a given solvent and concentration.

In the Pharmacopoeia the specific optical rotation is expressed by its value without units; the actual units, degree millilitres per decimetre gram [(°)·mL·dm^{-1}·g^{-1}] are understood.

The conversion factor from the International System to the Pharmacopoeia system is the following:

$$[\alpha_m]_\lambda^t = [\alpha]_\lambda^t \cdot 0{,}1745.$$

In certain cases specified in the monograph, the angle of rotation may be measured at temperatures other than 20 °C and at other wavelengths.

The polarimeter must be capable of giving readings to the nearest 0.01°. The scale is usually checked by means of certified quartz plates. The linearity of the scale may be checked by means of sucrose solutions.

Procedure. Determine the zero of the polarimeter and the angle of rotation of polarised light at the wavelength of the D-line of sodium (λ = 589.3 nm) at 20 ± 0.5 °C. Measurements may be carried out at other temperatures only where the monograph indicates the temperature correction to be made to the measured optical rotation. Determine the zero of the apparatus with the tube closed; for liquids the zero is determined with the tube empty and for solids filled with the prescribed solvent.

Calculate the specific optical rotation using the following formulae.

For liquids:

$$[\alpha]_D^{20} = \frac{\alpha}{l \cdot \rho_{20}}.$$

For substances in solution:

$$[\alpha]_D^{20} = \frac{1000 \cdot \alpha}{l \cdot c},$$

where *c* is the concentration of the substance in solution, g/L.

Calculate the content *c* (g/L) or *c′* in (% (*m*/*m*)) of a dissolved substance using the following formulae:

$$c = \frac{1000 \cdot \alpha}{l \cdot [\alpha]_D^{20}}, \quad c' = \frac{100 \cdot \alpha}{l \cdot [\alpha]_D^{20} \cdot \rho_{20}},$$

where a is the angle of rotation in degrees read at 20 ± 0.5 °C;

l is the length in decimetres of the polarimeter tube;

ρ_{20} is the density at 20 °C in grams per cubic centimetre. For the purposes of the Pharmacopoeia, density is replaced by relative density.

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2.1.2.8. Viscosity

The *dynamic viscosity* or *viscosity coefficient* η is the tangential force per unit surface, known as *shearing stress* τ and expressed in pascals (Pa), necessary to move, parallel to the sliding plane, a layer of liquid of 1 m^2 at a rate (*v*) of 1 metre per second (m· s^{-1}) relative to a parallel layer at a distance (*x*) of 1 metre.

The ratio d*v*/d*x* is a speed gradient giving the *rate of shear D* expressed in reciprocal seconds (s^{-1}). Thus, η = τ/*D*.

The unit of dynamic viscosity is the pascal second (Pa·s). The most commonly used submultiple is the millipascal second (mPa·s).

The *kinematic viscosity* V, expressed in square metre per second (m^2· s^{-1}), is obtained by dividing the dynamic viscosity η by the density ρ expressed in kilograms per cubic metre (kg/m^3), of the liquid measured at the same temperature: υ = η / ρ. The kinematic viscosity is usually expressed in square millimetres per second (mm^2· s^{-1}).

A capillary viscometer may be used for determining the viscosity of Newtonian liquids and a rotating viscometer for determining the viscosity of Newtonian and non-Newtonian liquids.

Other viscometers may be used provided that the accuracy and precision are not less than that obtained with the viscometers described below.

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2.1.2.9. Capillary Viscometer Method

The determination of viscosity using a suitable capillary viscometer is carried out at a temperature of 20 ± 0.1 °C, unless otherwise prescribed in the monograph. The time required for the level of the liquid to drop from one mark to the other is measured with a stop-watch to the nearest one-fifth of a second. The data obtained is valid only if two consecutive readings do not differ by greater than 1%.

The average of not fewer than three readings gives the flow time of the liquid to be examined.

Calculate the *dynamic viscosity* η (*2.1.2.8*) in millipascal seconds (mPa· s) using the formula:

$$\eta = k\rho\, t,$$

where *k* is the constant of the viscometer, expressed in square millimetres per second squared (mm^2· s^{-2});

p is the density of the liquid to be examined expressed in milligrams per cubic millimetre (mg· mm^{-3}), obtained by multiplying its relative density (d_{20}^{20}) by 0.9982;

t is the flow time in seconds (s), of the liquid to be examined.

The constant *k* is determined using a suitable viscometer calibration liquid.

To calculate the *kinematic viscosity* ($mm^2 \cdot s^{-1}$), use the following formula:

$$v = kt.$$

The determination may be carried out with an apparatus (proposed by the International Organisation for Standardisation) having the specifications described in Figure 2.1.2.9.-1 and in Table 2.1.2.9.-1.

Table 2.1.2.9.-1. – *Specifications of a suspended level viscometer*

Size number	Nominal constant of viscometer	Kinematic viscosity range	Internal diameter of tube *R*	Volume of bulb *C*	Internal diameter of tube *N*
	$mm^2 \cdot s^{-2}$	$mm^2 \cdot s^{-1}$	mm (±2%)	mL (±5%)	mm
1	0.01	3.5 to 10	0.64	5.6	2.8 to 3.2
1A	0.03	6 to 30	0.84	5.6	2.8 to 3.2
2	0.1	20 to 100	1.15	5.6	2.8 to 3.2
2A	0.3	60 to 300	1.51	5.6	2.8 to 3.2
3	1.0	200 to 1000	2.06	5.6	3.7 to 4.3
3A	3.0	600 to 3000	2.74	5.6	4.6 to 5.4
4	10	2000 to 10,000	3.70	5.6	4.6 to 5.4
4A	30	6000 to 30,000	4.07	5.6	5.6 to 6.4
5	100	20,000 to 100,000	6.76	5.6	6.8 to 7.5

The minimum flow time should be 350 s for size no. 1 and 200 s for all other sizes.

Procedure. Fill the viscometer through a tube (*L*) with a sufficient quantity of the liquid to be examined, previously brought to 20 °C, unless otherwise prescribed in the monograph, to fill bulb (*A*) but ensuring that the level of liquid in bulb (*B*) is below the exit to ventilation tube (*M*). Immerse the viscometer in the bath of water at (20 ± 0.1) °C, unless otherwise prescribed in the monograph, maintain it in the upright position, and allow to stand for not less than 30 min to allow the temperature to reach equilibrium. Close tube (*M*) and raise the level of the liquid in tube (*N*) up to a level of about 8 mm above mark (*E*). Keep the liquid at this level by closing tube (*N*) and opening tube (*M*). Open tube (*N*) and measure, with a stop-watch to the nearest one-fifth of a second, the time required for the level of the liquid to drop from mark (*E*) to (*F*).

When operating the apparatus described above, it is fine to use glass capillary suspended-level viscometers (for example, VPZh-1 type glass capillary viscometers), the parameters of which are similar to those shown in Figure 2.1.2.9.-1. The viscosity is measured in accordance with the viscometer service manual.

To determine the *relative viscosity* of a liquid, measure the average drain time t_{0mean} (between the upper and lower mark of the viscometer) of the liquid relative to which the η_{ratio} is measured.

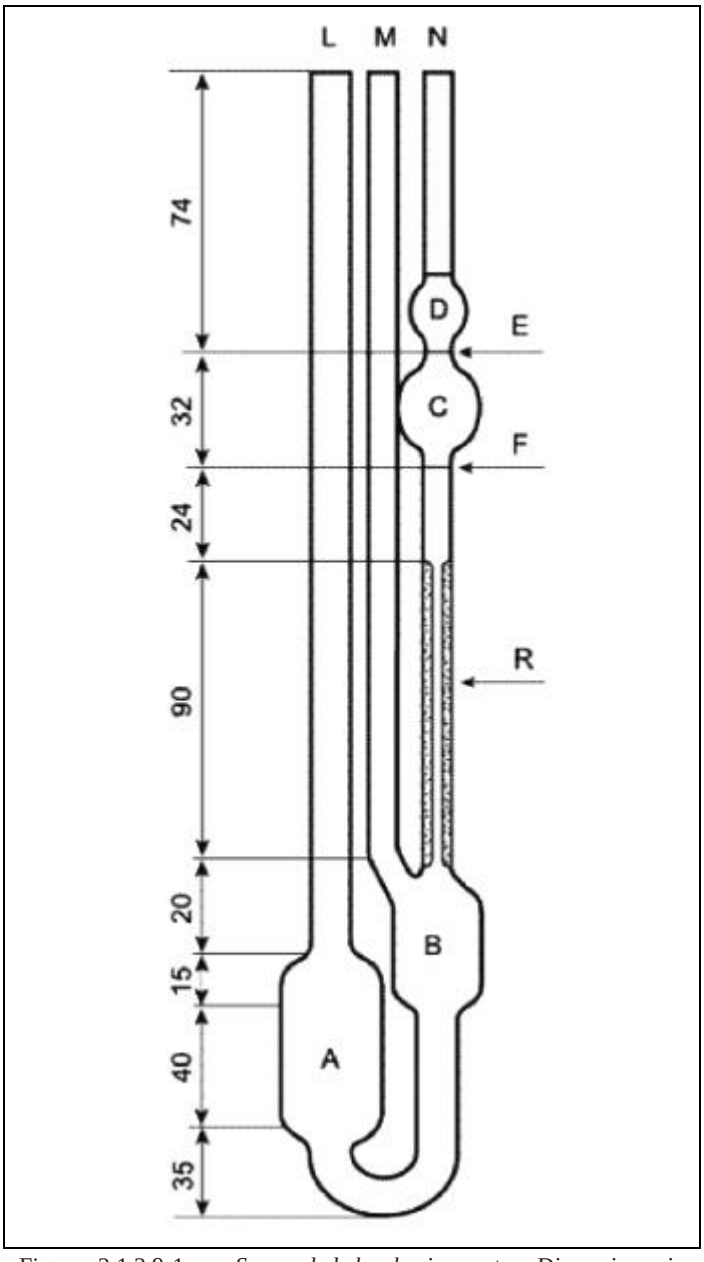

Figure 2.1.2.9-1. – *Suspended level viscometer Dimensions in millimetres*

Then, using the same clean and dry viscometer under the same conditions, determine the drain time t_{mean} of the test liquid.

Simultaneously, using a pycnometer, measure the density of the liquids to be examined (ρ_0 и ρ) at the same temperature at which the viscosity is determined, and calculate the relative viscosity using the following formula:

$$\eta_{ratio} = \frac{t_{mean} \cdot \rho}{t_{0mean} \cdot \rho_0} .$$

To determine the intrinsic viscosity, prepare at least five test solutions of different concentrations. In this case, the condition that the reduced viscosity can be linearly extrapolated to zero concentration must be met, i.e. the minimum solution concentrations must be chosen within the detection limit and level of accuracy of the measurement method. For each concentration of the solution, determine the t_{mean} and calculate the reduced viscosity. Then make a plot of η_{red} vs concentration c and extrapolate the reduced viscosity to zero concentration graphically or by linear least squares, i.e. find the intrinsic viscosity.

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2.1.2.10. Viscosity - Rotating Viscometer Method

The principle of the method is to measure the force acting on a rotor (torque) when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotating viscometers are used for measuring the viscosity of Newtonian (shear-independent viscosity) or non-Newtonian liquids (shear dependent viscosity or apparent viscosity). Rotating viscometers can be divided into two groups, namely absolute and relative viscometers. In absolute viscometers, the flow in the measuring geometry is well defined. The measurements result in absolute viscosity values, which can be compared with any other absolute values.

In relative viscometers the flow in the measuring geometry is not defined. The measurements result in relative viscosity values, which cannot be compared with absolute values or other relative values if not determined by the same relative viscometer method.

Different measuring systems are available for given viscosity ranges as well as several rotational speeds.

APPARATUS

The following types of instruments are most common.

CONCENTRIC CYLINDER VISCOMETERS (ABSOLUTE VISCOMETERS)

In the concentric cylinder viscometer (coaxial double cylinder viscometer or simply coaxial cylinder viscometer), the viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Viscosity measurement can be performed by rotating the internal cylinder (*Searle* type viscometer) or the outer cylinder (*Couette* type viscometer), as shown in Figures 2.1.2.10.-1 and 2.1.2.10.-2, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds (Pa· s) is given by the following formula:

$$\eta = \frac{1}{\omega}\left(\frac{M}{4\pi h}\right)\left(\frac{1}{R_i^2} - \frac{1}{R_0^2}\right) = k\frac{M}{\omega},$$

where M is the torque in newton-metres acting on the cylinder surface;

ω is the angular velocity in radians per second;

h is the height of immersion in metres of the inner cylinder in the liquid medium;

R_i is the radius in metres of the inner cylinder;

R_0 is the radius in metres of the outer cylinder;

k is the constant of the apparatus, expressed in radians per cubic metre.

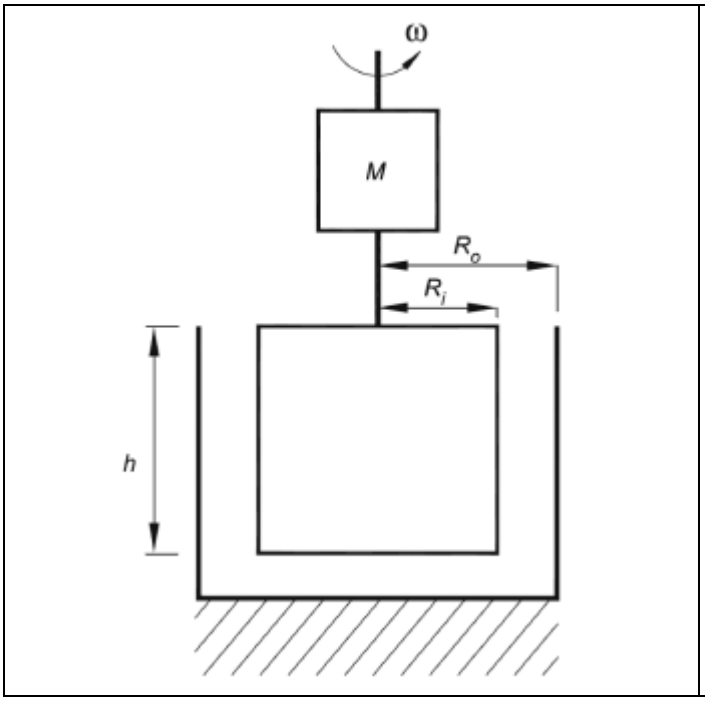


Figure 2.1.2.10.-1.

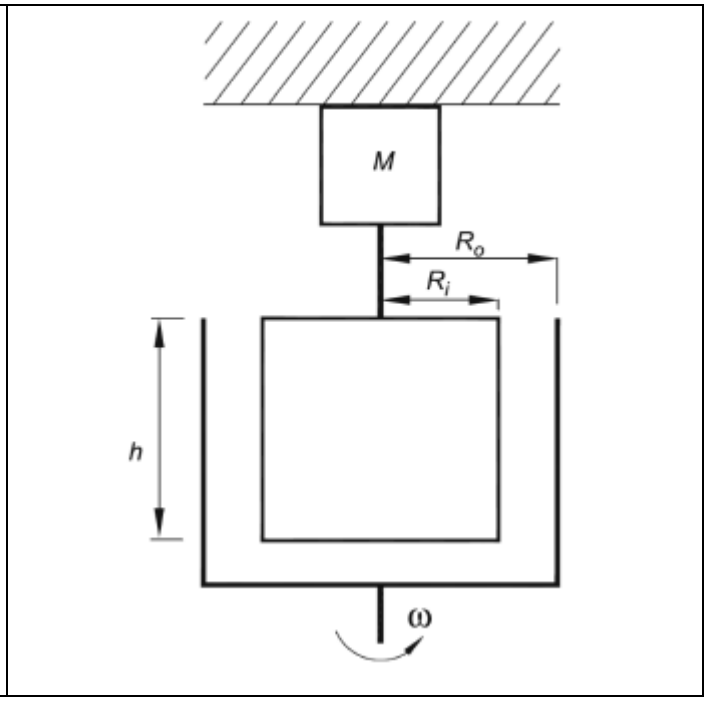


Figure 2.1.2.10.-2.

For non-Newtonian liquids it is indispensable to specify the shear stress (τ) or the shear rate (γ) at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute viscometers), there is a proportional relationship between M and τ and also between ω and γ:

$$\tau = AM;\ \gamma = B\omega,$$

where A and B are constants for the instrument and are calculated from the following expressions:

- *for concentric surface*:

$$A = \frac{1R_i^2 + R_0^2}{4\pi h R_i^2 R_0^2};\ B = \frac{R_i^2 + R_0^2}{R_0^2 - R_i^2},$$

- *for cone-plates*:

$$A = \frac{3}{2\pi R^3};\ B = \frac{1}{\alpha},$$

where M is the torque in Newton-metres acting on the cone or cylinder surface;

ω is the angular velocity in radians per second;

R_i is the radius in metres of the inner cylinder;

R_0 is the radius in metres of the outer cylinder;

R is a radius of the cone, in metres

h is the height of immersion in metres of the inner cylinder in the liquid medium;

α is the angle in radians between the flat disc and the cone;

τ is the shear stress in pascals (Pa);

γ is the shear rate in reciprocal seconds (s^{-1}).

CONE-PLATE VISCOMETERS (ABSOLUTE VISCOMETERS)

In the cone-plate viscometer, the liquid is introduced into the gap between a flat disc and a cone forming a defined angle. Viscosity measurement can be performed by rotating the cone or the flat disc, as shown in Figures 2.2.10.-3 and 2.2.10.-4, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds (Pa· s) is given by the following formula:

$$\eta = \left(\frac{M}{\omega}\right)\left(\frac{3\alpha}{2\pi R^3}\right) = k\frac{M}{\omega},$$

where M is the torque in Newton-metres acting on the flat disc or cone surface;

ω is the angular velocity in radians per second;

α is the angle in radians between the flat disc and the cone;

R is a radius of the cone, in metres

k is the constant of the apparatus, expressed in radians per cubic metre.

Determine the constants A and B of the apparatus similarly as for concentric cylinder viscometers.

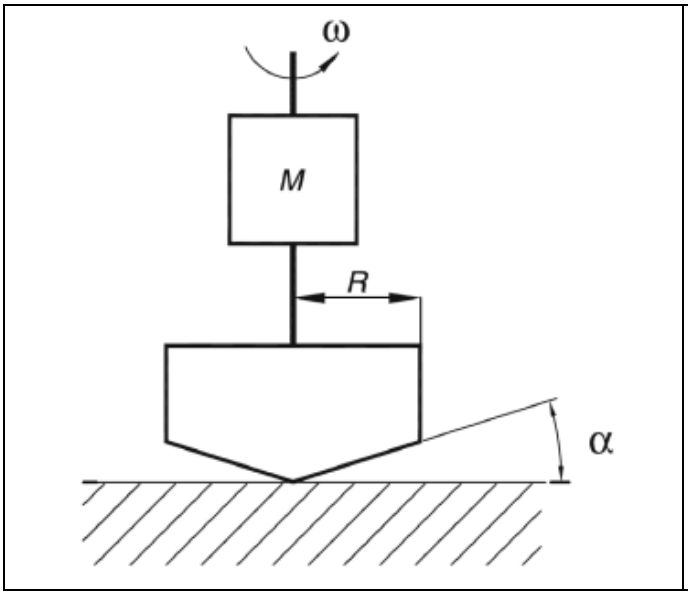


Figure 2.1.2.10.-3.

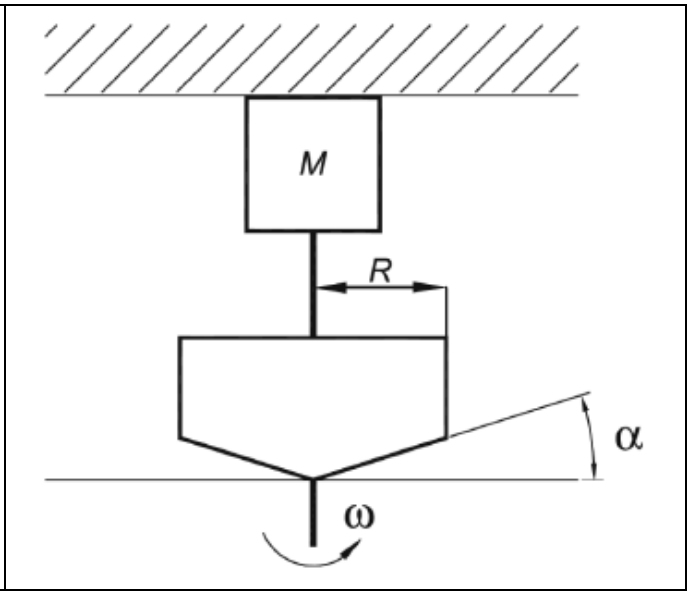


Figure 2.1.2.10.-4.

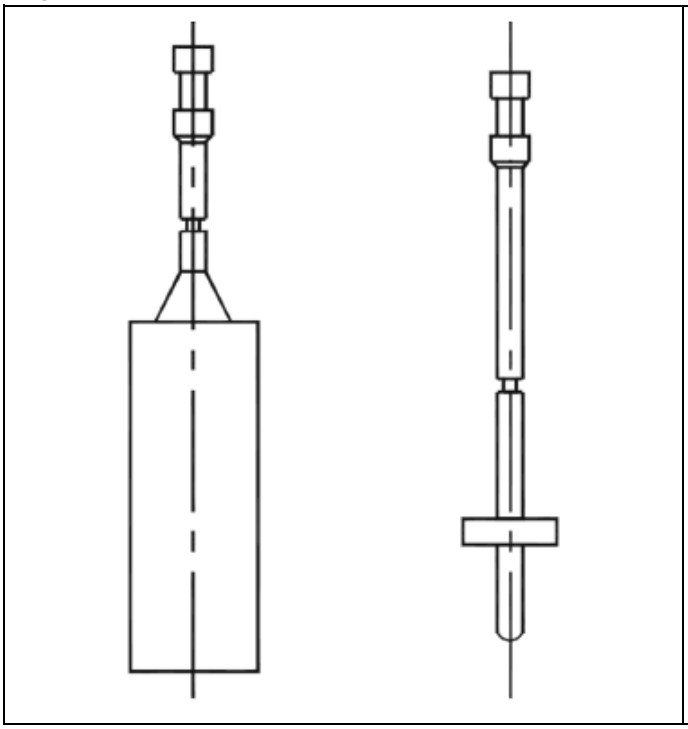


Figure 2.1.2.10.-5.

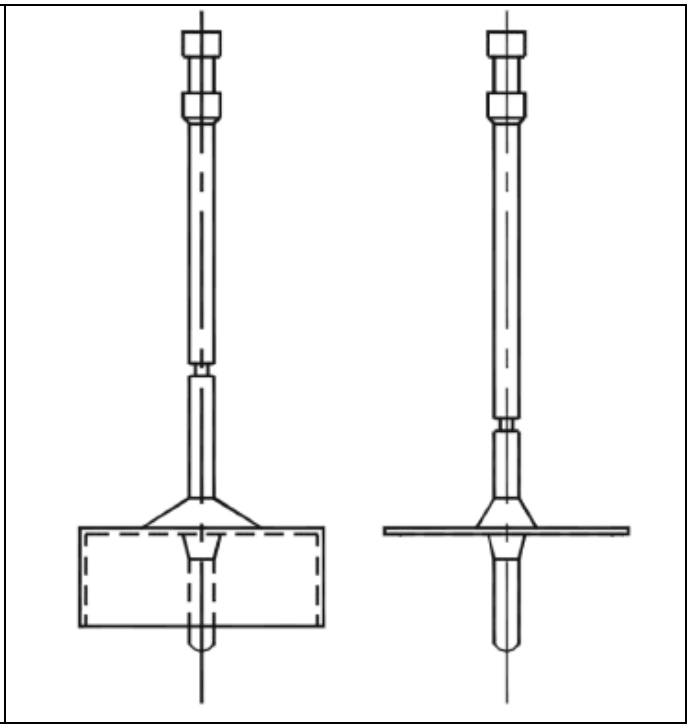


Figure 2.1.2.10.-6.

SPINDLE VISCOMETERS (RELATIVE VISCOMETERS)

In the spindle viscometer, the viscosity is determined by rotating a spindle (for example, cylinder- or disc-shaped, as shown in Figures 2.1.2.10.-5 and 2.1.2.10.-6, respectively) immersed in the liquid. Relative values of viscosity (or apparent viscosity) can be directly calculated using conversion factors from the scale reading at a given rotational speed.

In a general way, the constant *k* of the apparatus may be determined at various speeds of rotation using a certified viscometer calibration liquid. After that the viscosity η then corresponds to the formula :

$$\eta = k\frac{M}{\omega}.$$

Measure the viscosity (or apparent viscosity) according to the instructions for the operation of the rotating viscometer. The temperature for measuring the viscosity is indicated in the monograph. For non-Newtonian systems, the monograph indicates the type of viscometer to be used and if absolute viscometers are used the angular velocity or the shear rate at which the measurement is made. If it is impossible to obtain the indicated shear rate accurately, use a shear rate slightly higher and a shear rate slightly lower and interpolate.

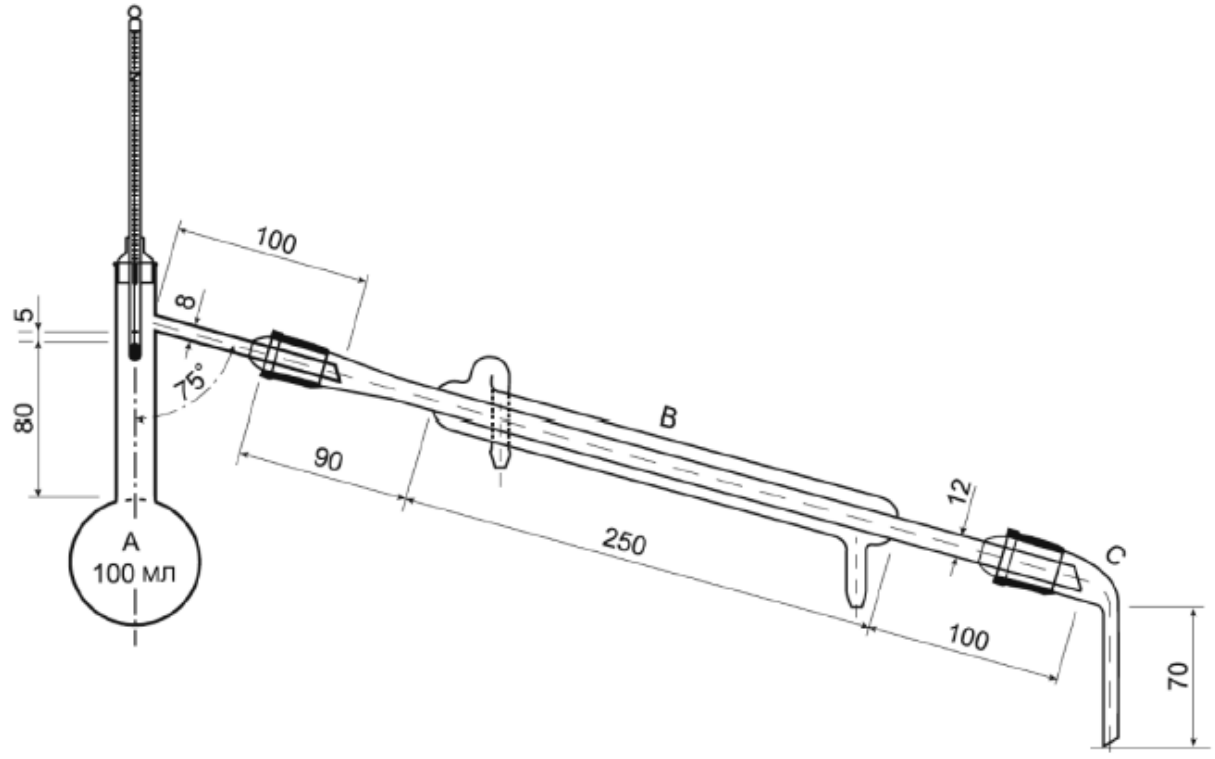
With relative viscometers, the shear rate is not the same throughout the sample and therefore it cannot be defined. Under these conditions, the viscosity of non-Newtonian liquids determined from the previous formula has a relative character, which depends on the type of spindle and the angular velocity as well as the dimensions of the sample container (Ø = minimum 80 mm) and the depth of immersion of the spindle. The values obtained are comparable only if the method is carried out under experimental conditions that are rigorously the same.

2.1.2.11. Distillation Range

The distillation range is the temperature interval, corrected for the pressure of 101.3 kPa (760 mm Hg), within which a liquid, or a specified fraction of a liquid, distills in the following conditions.

Apparatus. The apparatus (see Figure 2.1.2.11.-1) consists of a distillation flask (*A*), a straight tube condenser (*B*) which fits on to the sidearm of the flask, and a plain-bend adaptor (allonge) (*C*) attached to the end of the condenser. The lower end of the condenser may, alternatively, be bent to replace the adaptor. A thermometer is inserted in the neck of the flask so that the upper end of the mercury reservoir is 5 mm lower than the junction of the lower wall of the lateral tube. The thermometer is graduated at 0.2 °C intervals and the scale covers a range of about 50 °C. During the determination, the flask, including its neck, is protected from draughts by a suitable screen.

- Apparatus for the determination of distillation range. Dimensions in millimetres.



	100 mL

Figure 2.1.2.11.-1.

Table 2.1.2.11.-1. – *Temperature correction in relation to the pressure*

Distillation temperature	Correction factor *k*
Up To 100 °C	0.30
Above 100 °C up to 140 °C	0.34
Above 140 °C up to 190 °C	0.38
Above 190 °C up to 240 °C	0.41
Above 240 °C	0.45

Procedure. Place in the flask (*A*) 50.0 mL of the liquid to be examined and a few pieces of porous material. Collect the distillate in a 50 mL cylinder graduated in 1 mL. Cooling by circulating water is essential for liquids distilling below 150 °C. Heat the flask so that boiling is rapidly achieved and note the temperature at which the first drop of distillate falls into the cylinder. Adjust the heating to give a regular rate of the distillation of 2-3 mL/min and note the temperature when the whole or the prescribed fraction of the liquid, measured at 20 °C, has distilled.

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k\,(101{,}3 - b),$$

where t_1 is the corrected temperature;

t_2 is the observed temperature, at barometric pressure b;

k is the correction factor taken from Table 2.1.2.11.-1 unless the factor is given;

b is the barometric pressure, expressed in kilopascals, during the distillation.

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2.1.2.12. Boiling Point

The boiling point is the corrected temperature at which the vapour pressure of a liquid is equal to 101.3 kPa.

Apparatus. The apparatus is that used for Distillation Range (*2.1.2.11*) with the exception that the thermometer is inserted in the neck of the flask so that the lower end of the mercury reservoir is level with the lower end of the neck of the distillation flask and that the flask is placed on a plate of isolating material pierced by a hole 35 mm in diameter.

Procedure. Place in the flask (*A*) 20 mL of the liquid to be examined and a few pieces of porous material. Heat the flask so that boiling is rapidly achieved and record the temperature at which liquid runs from the side-arm into the condenser.

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k\,(101{,}3 - b),$$

where t_1 is the corrected temperature;

t_2 is the observed temperature at barometric pressure b;

k is the correction factor as shown in Table 2.1.2.11.-1;

b is the barometric pressure, expressed in kilopascals, during the distillation.

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2.1.2.13. Determination of Water by Distillation

The *apparatus* (see Figure 2.1.2.13.-1) consists of a glass flask (*A*) connected by a tube (*D*) to a cylindrical tube (*B*) fitted with a graduated receiving tube (*E*) and reflux condenser (*C).* The receiving tube (*E*) is graduated in 0.1 mL. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Procedure. Clean the receiving tube and the condenser of the apparatus, rinse with water, and dry. Introduce 200 mL of *toluene R* and about 2 mL of *water R* into the dry flask, and distill for 2 h. Cool the flask for about 30 min and read the water volume to the nearest 0.05 mL. Place in the flask a quantity of the substance weighed with an accuracy of 1%, expected to give about 2 mL to 3 mL of water. If the substance has a pasty consistency, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil, distil at the rate of about two drops per second until most of the water has distilled over, then increase the rate of distillation to about four drops per second.

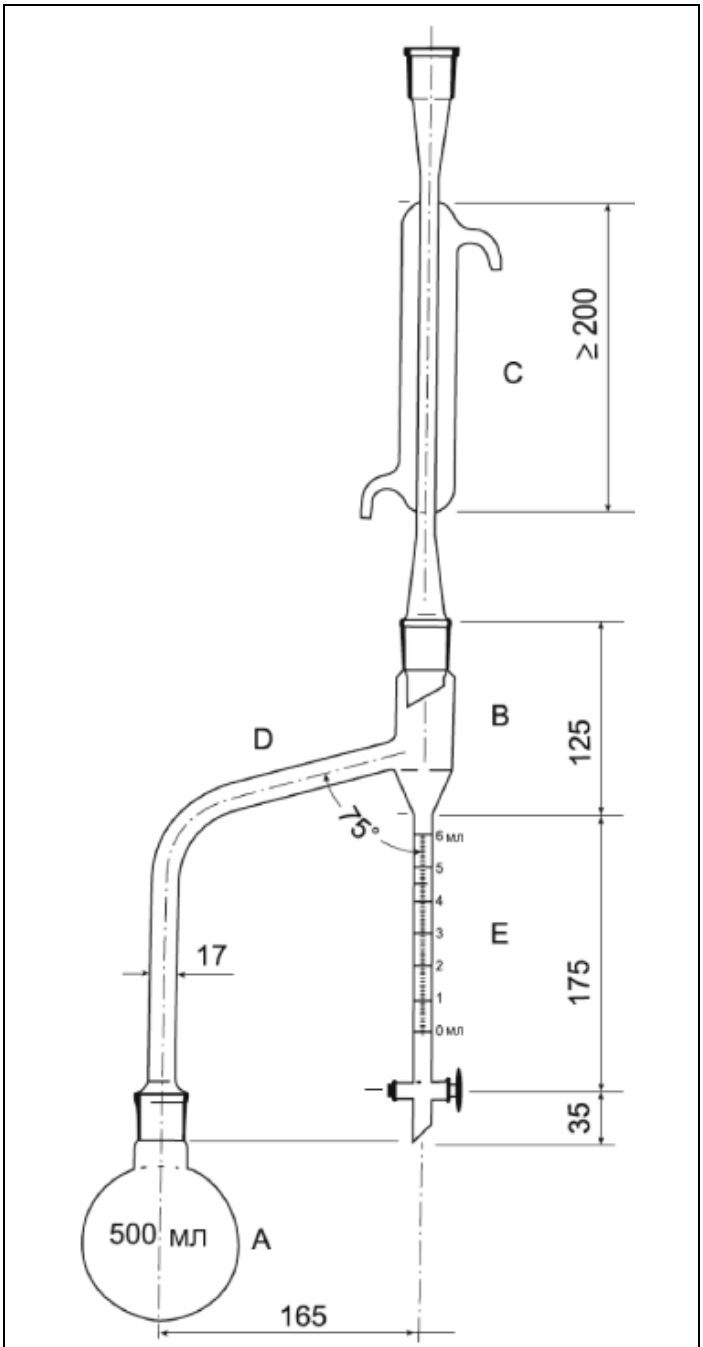

Figure 2.1.2.13.-1 – *Apparatus for the determination of water by distillation. Dimensions in millimetres.*

When the water is completely melted, rinse the inner tube of the condenser with *toluene R*. Continue to heat for another 5 minutes, then remove the heater, allow the receiver to cool to room temperature, and shake off all water drops from the walls of the receiver.

When the water and toluene have completely separated, read the volume of water and calculate the content present in the substance as millilitres per kilogram, using the formula:

$$\frac{1000(n_2 - n_1)}{m},$$

where m is the mass in grams of the substance to be examined;

n_1 is the number of millilitres of water obtained in the first distillation;

n_2 is the total number of millilitres of water obtained in the two distillations.

201020014-2019

2.1.2.14. Melting Point - Capillary Method

The melting point determined by the capillary method is the temperature at which the last solid particle of a compact column of a substance in a tube passes into the liquid phase.

When prescribed in the monograph, the same apparatus and procedure are used for the determination of other factors, such as meniscus formation or melting range, that characterise the melting behavior of a substance.

Apparatus. The apparatus consists of:

- a suitable glass vessel containing a liquid bath (for example, water, liquid paraffin, or silicone oil) and fitted with a suitable means of heating;
- a suitable means of stirring, ensuring uniformity of temperature within the bath;
- a suitable thermometer with graduation at not greater than 0.5 °C intervals and provided with an immersion mark. The range of the thermometer is not greater than 100 °C;
- alkali-free hard-glass capillary tubes of internal diameter 0.9 mm to 1.1 mm with a wall 0.10 mm to 0.15 mm thick and sealed at one end.

Procedure. Unless otherwise prescribed in the monograph, dry the finely powdered substance in vacuo and over *anhydrous silica gel R* for 24 h. Introduce a sufficient substance quantity into a capillary tube to give a compact column 4 mm to 6 mm in height.

Raise the temperature of the bath to about 10 °C below the presumed melting point and then adjust the rate of heating to about 1 °C/min. When the temperature is 5 °C below the presumed melting point, correctly introduce the capillary tube into the instrument. For the apparatus described above, immerse the capillary tube so that the closed end is near the center of the bulb of the thermometer, the immersion mark of which is at the level of the surface of the liquid. Record the temperature at which the last particle passes into the liquid phase.

Calibration of the apparatus. The apparatus may be calibrated using appropriate substances suitable for these purpose.

Other instruments may be used (for example, as specified in monograph *2.1.2.42*) using the capillary method, provided it is shown that the precision and accuracy of measurements will not be worse than in the case of the apparatus described above.

201020015-2019

2.1.2.15. Melting Point - Open Capillary Method

For certain substances, the following method is used to determine the melting point (also referred to as slip point and rising melting point when determined by this method).

Use glass capillary tubes open at both ends, about 80 mm long, having an external diameter of 1.4 mm to 1.5 mm and an internal diameter of 1.0 mm to 1.2 mm.

Introduce a sufficient amount of the substance, previously treated as described, into each of 5 capillary tubes to form a column about 10 mm high in each tube. Allow the tubes to stand for the appropriate time and at the temperature specified in the monograph.

Unless otherwise prescribed, substances with a waxy consistency are carefully and completely melted on a water-bath before introduction into the capillary tubes. Allow the tubes to stand at 2-8 °C for 2 h.

-

Attach one of the tubes to a thermometer graduated in 0.5 °C so that the substance is close to the bulb of the thermometer. Introduce the thermometer with the attached tube into a beaker so that the distance between the bottom of the beaker and the lower part of the bulb of the thermometer is 1 cm. Fill the beaker with water to a depth of 5 cm. Increase the temperature of the water gradually at a rate of 1 °C/min.

The temperature at which the substance begins to rise in the capillary tube is regarded as the melting point.

Repeat the operation with the other four capillary tubes and calculate the result as the mean of the five readings.

201020016-2019

2.1.2.16. Melting Point - Instantaneous Method

The instantaneous melting point is calculated using the expression:

$$\frac{t_1 + t_2}{2},$$

in which t_1 is the first temperature read under the conditions stated below.

t_2 is the second temperature read under the conditions stated below.

Apparatus. The apparatus consists of a metal block resistant to the substance to be examined, of good heat-conducting capacity, such as brass. The upper surface of the block should be plane and carefully polished. The block is uniformly heated throughout its mass by means of a micro-adjustable gas heater or an electric heating device with fine adjustment. The block has a cylindrical cavity, wide enough to accommodate a thermometer, which should be maintained with the mercury column in the same position during the calibration of the apparatus and the determination of the melting point of the substance to be examined. The cylindrical cavity is parallel to the upper polished surface of the block and about 3 mm from it. The apparatus is calibrated using appropriate substances of known melting point.

Procedure. Heat the block at a suitably rapid rate to a temperature about 10 °C below the presumed melting temperature, then adjust the heating rate to about 1 °C/min. At regular intervals drop a few particles of the finely powdered substance, dried in vacuum and over *anhydrous silica gel R* for 24 h, onto the block in the vicinity of the thermometer bulb, cleaning the surface after each test. Record the temperature t_1 at which the substance melts instantaneously for the first time in contact with the metal. Stop the heating. During cooling drop a few particles of the substance at regular intervals on the block, cleaning the surface after each test. Record the temperature t_2 at which the substance ceases to melt instantaneously when it comes in contact with the metal.

Calibration of the apparatus. The apparatus may be calibrated using appropriate substances suitable for these purpose.

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2.1.2.17. Freezing Point

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

Apparatus. The apparatus (see Figure 2.1.2.17.-1) consists of a test-tube about 25 mm in diameter and 150 mm long placed inside a test-tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2 °C fixed in such a way that the bulb is about 15 mm above the bottom of the test tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1 L beaker containing a suitable cooling liquid to within 20 mm of the top. A thermometer is supported in the cooling bath. The cooling bath should also be equipped with a thermometer.

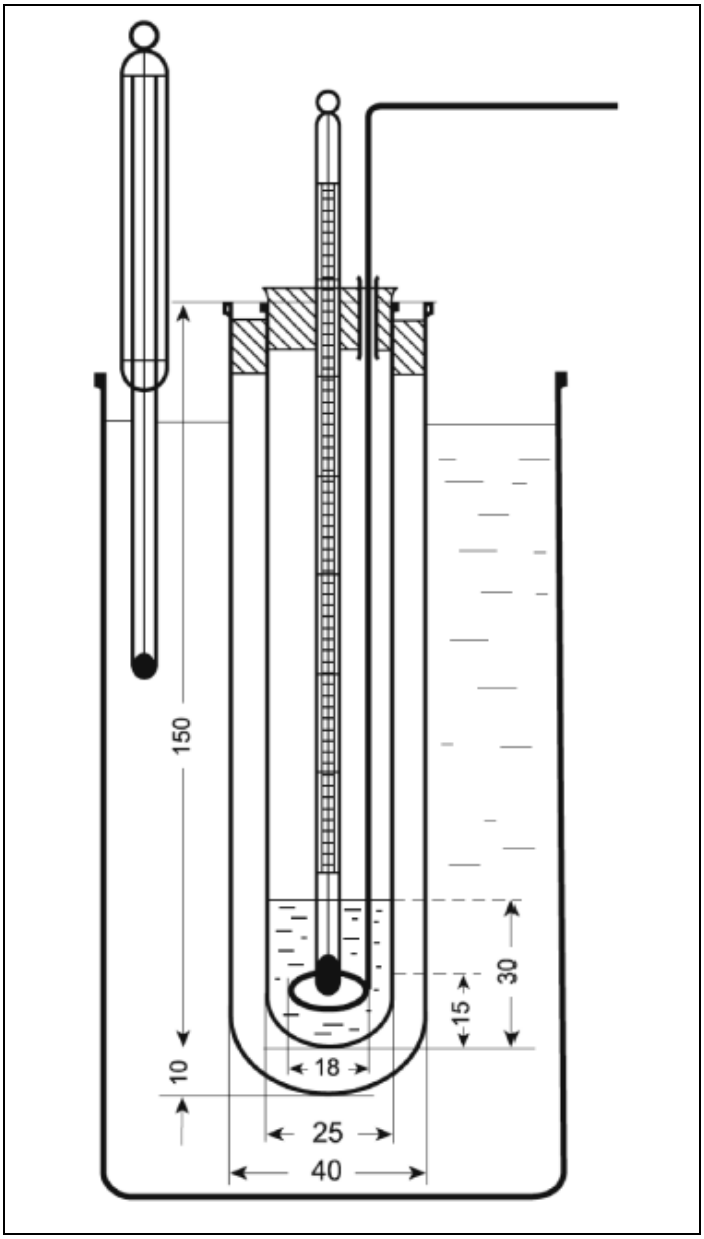


Figure 2.1.2.17.-1. – *Apparatus for the determination of freezing point. Dimensions in millimetres.*

Procedure. Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb (the mercury bulb of the thermometer should be in the middle of the layer of the substance to be examined) and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5 °C above the approximate freezing point until all but the last traces of crystals are melted.

Fill the beaker with water or a saturated solution of sodium chloride, at a temperature about 5 °C lower than the expected freezing point. Insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir the test sample thoroughly until solidification takes place. Note the highest temperature observed during solidification.

201020018-2019

2.1.2.18. Amperometric Titration

Amperometric titration is a quantitation method where the end-point is determined by following the variation of the current measured between two electrodes immersed in the solution to be examined as a function of the quantity of titrant added. One of the electrodes is an indicator, another is a reference electrode maintained at a constant potential. The voltage applied to electrodes should be sufficient for ensuring a diffusion current for the electroactive substances participating in the titrimetric reaction by the potential of the indicator electrode.

A variation of the method is the use of a pair of identical indicator electrodes having a small surface (usually platinum or gold), which are under voltage sufficient for the cathode and anode processes in the presence of a redox couple in the solution. This type of titration is recommended in iodometric and nitritometric definition, and also in the determination of water according to the Karl Fischer method.

Apparatus. The apparatus for amperometric titration comprises an adjustable DC voltage source, microammeter and two electrodes. The detection system generally consists of an inert electrode – a platinum, gold, dropping-mercury, graphite, or glass-carbon electrode, as well as a rotating-disc electrode made of these materials. A calomel electrode or a silver-silver chloride electrode are usually used as reference electrodes.

A three-electrode apparatus is sometimes used for titrations in a highly resistive media. The voltage is applied to the indicator electrode and polarised auxiliary electrode, and the required potential of the indicator electrode is set relative to the reference electrode.

Procedure. For an amperometric titration, set the potential of the indicator electrode as prescribed and plot a graph of the initial current and the values obtained during the titration as functions of the quantity of titrant added. Continue the titration after reaching the presumed equivalence point. At least 3 values on both sides of the equivalence point must fall on a straight line. The point of intersection of the two lines represents the end-point of the titration.

For amperometric titration with two indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

List of parameters specified in monographs. Specific parameters, such as the type of indicator electrode, the potential of the indicator electrode (or the potential difference between two indicator electrodes), the reference electrode, the mass of the analyzed substance, the type and concentration of the titrant, are indicated in monographs.

201020019-2019

2.1.2.19. Potentiometric Titration

In a potentiometric titration (volumetric titration with the potentiometric determination of the end-point), the end-point of the titration is determined by following the variation of the potential difference between 2 electrodes (either 1 indicator electrode and 1 reference electrode or a combination electrode) immersed in the solution to be examined as a function of the quantity of titrant added.

Apparatus. The apparatus represents a millivoltmeter. Commercially available automatic titrators can be used, operated according to the manufacturer's instructions, using the electrodes recommended for the type of titration described.

Table 2.1.2.19.-1. – *Indicators suitable for neutralising a mixture of solvents*

Titrant	Indicator
Perchloric acid	*Crystal violet solution R*
Tetrabutylammonium hydroxide	3 g/L solution of *thymol blue R* in *methanol R*
Ethanolic sodium hydroxide	*Thymolphthalein solution R*

The indicator electrode to be used depends on the substance to be determined and may be a glass or metal electrode (for example, platinum, gold, silver or mercury).

For acid-base titrations, a glass electrode combination is used.

Method. Prepare the sample solution as specified in the monograph. Add suitable titrant aliquots, paying special attention to the rate of addition and the step size near the endpoint. Continue the titration after the expected endpoint to clearly determine it.

The endpoint of titration corresponds to a sharp variation of potential difference as a function of the quantity of the titrant added. Registration of the first or second derivative can help determine the end-point.

In potentiometric titrations of weak acids or bases using non-aqueous solvents, either carry out a blank determination or pre-neutralise the solvent mixture, if necessary, before the dissolution of the substance to be examined. Where it is impracticable to use potentiometric detection for this purpose, the solvent mixture can be pre-neutralised by titration using a suitable indicator. Some examples are given in Table 2.1.2.19.-1.

2.1.2.20. Fluorimetry

Fluorimetry is a procedure which uses the measurement of the intensity of the fluorescent light emitted by the substance to be examined in relation to that emitted by a given standard.

Method. Dissolve the substance to be examined in the solvent or mixture of solvents prescribed in the monograph. Transfer the resulting solution to the cell or the tube of the fluorimeter and illuminate it with an excitant light beam of the wavelength prescribed in the monograph and as near as possible monochromatic.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence. Other types of apparatus may be used provided that the results obtained are identical.

For quantitation, first, introduce into the apparatus the solvent or mixture of solvents used to dissolve the substance to be examined and set the instrument to zero. Introduce the standard solution and adjust the sensitivity of the instrument so that the response is greater than 50. If the second adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Then introduce the test solution of unknown concentration and read the result on the instrument. Calculate the concentration (c_x) of the substance in the solution to be examined, using the formula:

$$c_x = \frac{I_x c_s}{I_s},$$

where c_x is the concentration of the solution to be examined;

c_s is the concentration of the standard solution;

I_x is the intensity of the light emitted by the solution to be examined;

I_s is the intensity of the light emitted by the standard solution.

If the intensity of the fluorescence is not strictly proportional to the concentration, the measurement may be effected using a calibration curve.

In some cases, the measurement can be made with reference to a fixed standard (for example a fluorescent glass or a solution of another fluorescent substance). In such cases, the concentration of the substance to be examined must be determined using a previously drawn calibration curve under the same conditions.

201020021-2019

2.1.2.21. Atomic Emission Spectrometry

GENERAL PRINCIPLE

Atomic emission is a process that occurs when electromagnetic radiation is emitted by excited atoms or ions. In atomic emission spectrometry, the sample is subjected to temperatures high enough to cause not only dissociation into atoms, but also to cause significant amounts of collisional excitation and ionisation of the sample atoms to take place. Once the atoms and ions are in the excited states, they can decay to lower states through thermal or radiative (emission) energy transitions and electromagnetic radiation is emitted. An emission spectrum of an element contains several more lines than the corresponding absorption spectrum.

Atomic emission spectrometry is a technique for determining the concentration of an element in a sample by measuring the intensity of one of the emission lines of the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength corresponding to this emission line.

In this chapter, the only atomisation in a flame is dealt with. The method of inductively coupled plasma-atomic emission spectrometry (ICP-AES) is described in a different general chapter.

APPARATUS

The apparatus consists essentially of the following elements:

- a sample introduction and nebulisation system;
- a flame to generate the atoms to be determined;
- a monochromator;
- a detector;
- a data-acquisition unit.

Oxygen, air, and a combustible gas such as hydrogen, acetylene, propane, or butane may be used in flames. The atomisation source is critical since it must provide sufficient energy to excite and atomise the atoms. The atomic spectra emitted from flames have the advantage of being simpler than those emitted from other sources, the main limitation is that the flames are not powerful enough to cause emission for many elements allowing their determination. Acidified water is the solvent of choice for preparing test and reference solutions, although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

INTERFERENCE

Spectral interference is reduced or eliminated by choosing an appropriate emission line for measurement or by adjusting the slit for spectral band-width. Physical interference is corrected by diluting the sample solution, by matching the matrix or by using the method of standard additions. Chemical interference is reduced by using chemical modifiers or ionisation buffers.

MEMORY EFFECT

The memory effect caused by the deposit of analyte in the apparatus may be limited by thoroughly rinsing between runs, diluting the solutions to be measured if possible, and thus reducing their salt content, and by aspirating the solutions through as swiftly as possible.

METHOD

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

Operate an atomic emission spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Optimise the experimental conditions (flame temperature, burner adjustment, use of an ionic buffer, concentration of solutions) for the specific element to be analyzed and in respect of the sample matrix. Introduce a blank solution into the atomic generator and adjust the instrument reading to zero or to its blank value. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable reading.

It is preferable to use concentrations that fall within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

The use of plastic labware is recommended wherever possible.

METHOD I — DIRECT CALIBRATION

For routine measurements, three reference solutions of the element to be determined and a blank are prepared and examined.

Prepare a solution of the substance to be examined (test solution) as prescribed in the monograph. Prepare not fewer than three reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For quantitation purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are between the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference solutions and to the blank solution at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration; determine the concentration of the element in the test solution from the curve obtained.

METHOD II – STANDARD ADDITIONS

Prepare a solution of the substance to be examined (test solution) as prescribed in the monograph. Add equal volumes of the test solution to at least three similar volumetric flasks. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if at all possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation. Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE PROCEDURE

Satisfactory performance of procedures prescribed in the monograph is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than four reference solutions over the calibration range and a blank solution. Perform not fewer than five replicates.

The calibration curve is calculated by the least-square regression from all measured data. The regression curve, the means, the measured data, and the confidence range of the calibration curve are plotted.

The operating method is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using certified reference materials. Where this is not possible, perform a test for recovery.

Recovery. For quantitation determination procedures, a recovery of 90% to 110% is to be obtained. For other determinations, for example for trace element determination, the test is not valid if recovery is outside of the range 80% to 120% at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

REPEATABILITY

The repeatability is not greater than 3% for a quantitation and not greater than 5% for an impurity test.

QUANTITATION LIMIT

Verify that the quantitation limit (for example, determined using the 10 σ approach) is below the value to be measured.

2.1.2.22. Atomic Absorption Spectrometry

GENERAL PRINCIPLE

Atomic absorption is a process that occurs when a ground state-atom absorbs electromagnetic radiation of a specific wavelength and is elevated to an excited state. The atoms in the ground state absorb energy at their resonant frequency and the electromagnetic radiation is attenuated due to resonance absorption. The energy absorption is virtually a direct function of the number of atoms present.

This chapter provides general information and defines the procedures used in element determinations by atomic absorption spectrometry, either atomisation by flame, by electrothermal vapourisation in a graphite furnace, by hydride generation, or by cold vapour technique for mercury.

Atomic absorption spectrometry is a technique for determining the concentration of an element in a sample by measuring the absorption of electromagnetic radiation by the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength of one of the absorption (resonance) lines of the element concerned. The amount of radiation absorbed is, according to the Lambert-Beer law, proportional to the element concentration.

APPARATUS

This consists essentially of:
- a source of radiation;
- a sample introduction and nebulisation system;
- a sample atomiser;
- a monochromator or polychromator;
- a detector;
- a data-acquisition unit.

The apparatus is usually equipped with a background correction system. Hollow-cathode lamps (*HCL*) and electrodeless discharge lamps (*EDL*) are used as radiation source. The emission of such lamps consists of a spectrum showing very narrow lines with half-width of about 0.002 nm of the element being determined.

There are three types of sample atomisers:

- *Flame technique*

A flame atomiser is composed of a nebulisation system with a pneumatic aerosol production accessory, a gas-flow regulation, and a burner. Fuel-oxidant mixtures are commonly used to produce a range of temperatures from 2000 K to 3000 K; fuel gases include propane, hydrogen, and acetylene; air and nitrous oxide are used as oxidants. The configuration of the burner is adapted to the gases used and the gas delivery rate is adjustable. Samples are nebulised, acidified water being the solvent of choice for preparing test and reference solutions. Organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

- *Electrothermal atomisation technique*

An electrothermal atomiser is generally composed of a graphite tube furnace and an electric power source. Electrothermal atomisation in a graphite tube furnace atomises the entire sample and retains the atomic steam in the light path for an extended period, which improves the detection limit. Samples, liquid as well as solid, are introduced directly into the graphite tube furnace, which is heated in a programmed series of steps to dry the sample and remove major matrix components by pyrolysis and to then atomise all of the analyte. The furnace is cleaned using a final temperature higher than the atomisation temperature. The flow of inert gas during the pyrolysis step in the graphite tube furnace allows a better performance of the subsequent atomisation process.

- *Cold vapour and hydride technique*

The atomic steam may also be generated outside the spectrometer. This is notably the case for the cold-vapour method for mercury or for certain hydride-forming elements such as arsenic, antimony, bismuth, selenium, and tin. For mercury, atoms are generated by chemical reduction with stannous chloride or sodium borohydride, and the atomic steam is swept by a stream of inert gas into a cold quartz cell mounted in the optical path of the instrument. Hydrides thus generated are swept by an inert gas into a heated cell in which they are dissociated into atoms.

INTERFERENCE

Chemical, physical, ionisation, and spectral interferences are encountered in atomic absorption measurements. Chemical interference is compensated by the addition of matrix modifiers, of releasing agents, or by using high temperature produced by a nitrous oxide-acetylene flame. The use of specific ionisation buffers (for example, lanthanum and cesium) compensates for ionisation interference. By dilution of the sample, through the method of standard additions or by matrix matching, physical interference due to high salt content or viscosity is eliminated. Spectral interference results from the overlapping of resonance lines and can be avoided by using a different resonance line. The use of Zeeman background correction also compensates for spectral interference and interferences from molecular absorption, especially when using the electrothermal atomisation technique. The use of multi-element hollow-cathode lamps may also cause spectral interference. Specific or non-specific absorption is measured in a spectral range defined by the band-width selected by the monochromator (0.2-2 nm).

BACKGROUND CORRECTION

Scatter and background in the flame or the electrothermal atomisation technique increase the measured absorbance values. Background absorption covers a large range of wavelengths, whereas atomic absorption takes place in a very narrow wavelength range of about 0.005 - 0.02 nm. Background absorption can in principle be corrected by using a blank solution of exactly the same composition as the sample, but without the specific element to be determined, although this method is frequently impracticable. With the electrothermal atomisation technique, the pyrolysis temperature is to be optimised to eliminate the matrix decomposition products causing background absorption.

Background correction can also be made by using 2 different light sources, the hollow-cathode lamp that measures the total absorption (element + background) and a deuterium lamp with a continuum emission from which the background absorption is measured. Background is corrected by subtracting the deuterium lamp signal from the hollow-cathode lamp signal. This method is limited in the spectral range on account of the spectra emitted by a deuterium lamp (from 190 to 400 nm). Background can also be measured by taking readings of absorption at two wavelengths: at the resonance line and at a non-absorbing wavelength near the resonance line and then subtracting the results from the measurement at the resonance line of the second wavelength.

Another method for the correction of background absorption is the Zeeman effect (based on the Zeeman splitting of the absorption line in a magnetic field). This is particularly useful when the background absorption shows fine structure, which permits an efficient background correction in the range of 185-900 nm.

CHOICE OF THE OPERATING CONDITIONS

After selecting the suitable wavelength and slit width for the specific element, the need for the following has to be ascertained:

- correction for non-specific background absorption;
- chemical modifiers or ionisation buffers to be added to the sample as well as to blank and reference solutions;
- dilution of the sample to minimise, for example, physical interferences;
- details of the temperature program, preheating, drying, pyrolysis, atomisation, post-atomisation with ramp and hold times;
- inert gas flow;
- matrix modifiers for electrothermal atomisation (furnace);
- chemical reducing reagents for measurements of mercury or other hydride-forming elements along with cold vapour cell or heating cell temperature;
- technical requirements of furnace design (chamber, L'vov platform, etc).

METHOD

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

Operate an atomic absorption spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Introduce a blank solution into the atomic generator and adjust the instrument reading so that it indicates maximum transmission. The blank value may be determined by using the solvent to zero the apparatus. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a maximum absorbance reading. Rinse thoroughly the apparatus in order to avoid contamination and memory effects. After completing the analysis, rinse with *water R* or acidified water.

If a solid sampling technique is applied, full conditions of the procedure are provided in the monograph.

It is preferable to use concentrations that fall within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

The use of plastic labware is recommended wherever possible. The preparation of the sample may require a dissolution, digestion (mostly microwave-assisted), an ignition step, or a combination thereof in order to clear up the sample matrix and/or to remove carbon-containing material. If operating in an open system, the ignition temperature should not exceed 600 °C, due to the volatility of some metals, unless otherwise stated in the monograph.

METHOD I — DIRECT CALIBRATION

For routine measurements, three reference solutions of the element to be determined and a blank are prepared and examined.

Prepare the test solution as specified in the monograph. Prepare not fewer than three reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For quantitation purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are between the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference solutions and to the blank solution at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

METHOD II – STANDARD ADDITIONS

Prepare the test solution as specified in the monograph. Add equal volumes of the test solution to at least three similar volumetric flasks. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation. Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE PROCEDURE

Satisfactory performance of procedures prescribed in the monograph is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than four reference solutions over the calibration range and a blank solution. Perform not fewer than five replicates.

The calibration curve is calculated by the least-square regression from all measured data. The regression curve, the means, the measured data, and the confidence range of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using certified reference materials. Where this is not possible, perform a test for recovery.

Recovery. For quantitation determination procedures, a recovery of 90% to 110% is to be obtained. For other determinations, for example, for trace element determination the test is not valid if recovery is outside of the range 80% to 120% at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

REPEATABILITY

The repeatability is not greater than 3% for a quantitation and not greater than 5% for an impurity test.

QUANTITATION LIMIT

Verify that the quantitation limit (for example, determined using the 10 σ approach) is below the value to be measured.

201020023-2019

2.1.2.23. Absorption Spectrophotometry, Infrared

Infrared spectrophotometers are used for recording spectra in the region of 4000 cm^{-1} to 650 cm^{-1} (between 2.5 μm and 15.4 μm) or in some cases down to 200 cm^{-1} (50 μm).

APPARATUS

Spectrophotometers for recording spectra consist of a suitable light source, monochromator or interferometer and detector.

Fourier transform spectrophotometers use polychromatic radiation and calculate the spectrum in the frequency domain from the original data by Fourier transformation. Spectrophotometers fitted with an optical system capable of producing monochromatic radiation in the measurement region may also be used. Normally the spectrum is given as a function of transmittance, the quotient of the intensity of the transmitted radiation and the incident radiation. But it may also be given in absorbance.

The absorbance (*A*) is defined as the logarithm to the base ten of the reciprocal of the transmittance (*T*):

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right),$$

where $T = \frac{I}{I_0}$;

I_0 is the intensity of incident radiation;

I is the intensity of transmitted radiation.

PREPARATION OF THE SAMPLE

FOR RECORDING BY TRANSMISSION OR ABSORPTION

Prepare the sample by one of the following procedures.

Liquids. Examine a liquid either in the form of a film between two plates transparent to infrared radiation or in a cell of suitable path length, also transparent to infrared radiation.

Liquids or solids in solution. Prepare a solution in a suitable solvent. Choose a concentration and a path length of the cell that provide for a satisfactory spectrum. Generally, good results are obtained with concentrations of 10-100 g/L for a path length of 0.5-0.1 mm. Absorption due to the solvent is compensated by placing in the reference beam a similar cell containing the solvent used. If a Fourier transform instrument is used, the absorption is compensated by recording the spectra for the solvent and the sample successively, the solvent absorbance, corrected by a compensation factor, is subtracted.

Solids. Examine solids dispersed in a suitable liquid (mull) or in a solid (halide disc), as appropriate. If prescribed in the monograph, make a film of a molten mass between two plates transparent to infrared radiation.

a) *Suspension.* Triturate a small quantity of the substance to be examined with the minimum quantity of *liquid paraffin R* or other suitable liquid; 5-10 mg of the substance to be examined is usually sufficient to make an adequate mull using one drop of *liquid paraffin R.*

Compress the mull between two plates transparent to infrared radiation.

b) *Discs.* Triturate 1-2 mg of the substance to be examined with 300-400 mg, unless otherwise specified, of finely powdered and dried *potassium bromide R* or *potassium chloride R*. These quantities are usually sufficient to give a disc of 10-15 mm diameter and a spectrum of suitable intensity. If the substance is hydrochloride, it is recommended to use *potassium chloride R.* Carefully grind the mixture, spread it uniformly in a suitable die, and submit it to a pressure of about 800 MPa (8 $t \cdot cm^{-2}$). For substances that are unstable under normal atmospheric conditions or are hygroscopic, the disc is pressed in vacuo. Several factors may cause the formation of faulty discs, such as insufficient or excessive grinding, humidity or other impurities in the dispersion medium or an insufficient reduction of particle size. A disc is rejected if the visual examination shows a lack of uniform transparency or when transmittance at about 2000 cm^{-1} (5 μm) in the absence of a specific absorption band is less than 60% without compensation.

Gases. Examine gases in a cell transparent to infrared radiation and having an optical path length of about 100 mm. Evacuate the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas to be examined.

If necessary, adjust the pressure in the cell to atmospheric pressure using a gas, clear to infrared radiation (for example *nitrogen R* and *argon R*). To avoid absorption interferences due to water, carbon dioxide, or other atmospheric gases, place in the reference beam, if possible, an identical cell that is either evacuated or filled with the gas transparent to infrared radiation.

FOR RECORDING BY DIFFUSE REFLECTANCE

Solids. Triturate a mixture of the substance to be examined with finely powdered and dried *potassium bromide R* or potassium *chloride R.*

Use a mixture containing approximately 5% of the substance, unless otherwise specified in the monograph. Grind the mixture, place it in a sample cup and examine the reflectance spectrum.

The spectrum of the sample in absorbance mode may be obtained after mathematical treatment of the spectra by the Kubelka-Munk function.

FOR RECORDING BY ATTENUATED TOTAL REFLECTION

Attenuated total reflection (including multiple reflection) involves light being reflected internally by a transmitting medium, typically for a number of reflections. However, several accessories exist where only one reflection occurs. Place the substance to be examined in close contact with an internal reflection element (*IRE*) such as diamond, germanium, zinc selenide, thallium bromide-thallium iodide (*KRS-5*) or another suitable material of high refractive index. Ensure close and uniform contact between the substance and the whole crystal surface of the internal reflection element, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the IRE with the obtained solution and evaporating to dryness. Examine the attenuated total reflectance (*ATR*) spectrum.

IDENTIFICATION USING REFERENCE SUBSTANCES

Prepare the substance to be examined and the reference substance by the same procedure and record the spectra between 4000 cm^{-1} and 650 cm^{-1} (2.5-15.4 μm) under the same operational conditions. The transmission minima (absorption maxima) in the spectrum obtained with the substance to be examined meet in position and relative size to those in the spectrum obtained with the reference substance (CRS).

When the spectra recorded in the solid state show differences in the positions of the transmission minima (absorption maxima), treat the substance to be examined and the reference substance in the same manner so that they crystallise or are produced in the same form, or proceed as prescribed in the monograph, then record the spectra.

IDENTIFICATION USING REFERENCE SPECTRA

Control of resolution performance. For instruments having a monochromator, record the spectrum of a polystyrene film approximately 35 µm in thickness. The difference *x* (see Figure 2.1.2.24.-1) between the percentage transmittance at the transmission maximum A at 2870 cm^{-1} (3.48 µm) and that at the transmission minimum B at 2849.5 cm^{-1} (3.51 µm) must be greater than 18. The difference *y* between the percentage transmittance at the transmission maximum C at 1589 cm^{-1} (6.29 µm) and that at the transmission minimum D at 1583 cm^{-1} (6.32 µm) must be greater than 10.

For Fourier-transform instruments, use suitable instrument resolution with the appropriate apodisation prescribed by the manufacturer. The resolution is checked by suitable means, for example by recording the spectrum of a polystyrene film approximately 35 µm in thickness. The difference between the absorbances at the absorption minimum at 2870 cm^{-1} and the absorption maximum at 2849.5 cm^{-1} is greater than 0.33. The difference between the absorbances at the absorption minimum at 1589 cm^{-1} and the absorption maximum at 1583 cm^{-1} is greater than 0.08.

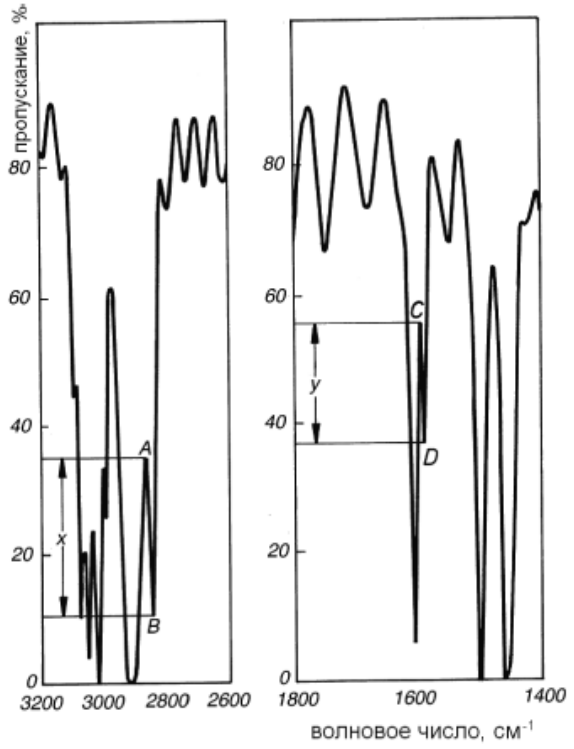
Typical spectrum of polystyrene used to verify the resolution performance.

Verification of the wave-number scale. The wave-number scale may be verified using a polystyrene film, which has transmission minima (absorption maxima) at the wave numbers (in cm^{-1}) shown in Table 2.1.2.23.-1.

Procedure. Prepare the substance to be examined according to the instructions accompanying the reference spectrum/reference substance. Using the operating conditions (usually the same as those for verifying the resolution performance) that were applied to obtain the reference spectrum, record the spectrum of the substance to be examined.

The positions and the relative sizes of the bands in the spectrum of the substance to be examined and the reference spectrum are concordant in the two spectra.

Compensation for water vapour and atmospheric carbon dioxide. For Fourier-transform instruments, spectral interference from water vapour and carbon dioxide is compensated using suitable algorithms according to the manufacturer's instructions.



	transmission, %
	wave number, cm^{-1}

Figure 2.1.2.23.-1.

Alternatively, spectra can be acquired using suitable purged instruments or ensuring that sample and background single beam spectra are acquired under exactly the same conditions.

IMPURITIES IN GASES

For the analysis of impurities, use a cell transparent to infrared radiation and of suitable optical path length (for example, 1 to 20 m).

Table 2.1.2.23.-1. – *Transmission minima and acceptable tolerances of a polystyrene film*

Transmission minima (cm^{-1})	Acceptable tolerance (cm-1)	
	Monochromator instruments	Fourier-transform instruments
3060.0	±1.5	±1.0
2849.5	±2,0	±1.0
1942.9	±1.5	±1.0
1601.2	±1.0	±1.0
1583.0	±1.0	±1.0
1154.5	±1.0	±1.0
1028.3	±1.0	±1.0

Fill the cell as prescribed under Gases section. For detection and quantitation of the impurities, proceed as prescribed in the monographs.

201020024-2019

2.1.2.24. Absorption Spectrophotometry, Ultraviolet and Visible

Determination of absorbance. The absorbance (*A*) of a solution is defined as the logarithm to base 10 of the reciprocal of the transmittance (*T*) for monochromatic radiation and is defined as follows:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$

$$T = \frac{I}{I_0};$$

where

I_0 is the intensity of incident monochromatic radiation;

I is the intensity of transmitted monochromatic radiation.

In the absence of other physico-chemical factors, the absorbance (*A*) is proportional to the path length (*b*) through which the radiation passes and to the concentration (*c*) of the substance in solution in accordance with the equation:

$$A = \varepsilon \cdot c \cdot b,$$

where: ε is the molar absorptivity;

b is the optical path length expressed in centimetres;

c is the concentration of the substance in the solution, in moles per litre.

The expression $A^{1\%}_{1\ cm}$ representing the specific absorbance of a dissolved substance refers to the absorbance of a 10 g/L solution in a 1 cm cell:

$$A^{1\%}_{1\ cm} = \frac{10 \cdot \varepsilon}{mm}\ .$$

Unless otherwise prescribed, measure the absorbance at the prescribed wavelength using a path length of 1 cm.

Unless otherwise prescribed, the measurements are carried out with reference to the same solvent or the same mixture of solvents. The absorbance of the solvent measured against air and at the prescribed wavelength shall not exceed 0.4 and is preferably less than 0.2. Plot the absorption spectrum with absorbance or function of absorbance as ordinate against wavelength or function of wavelength as abscissa.

Where an individual monograph gives a single value for the position of an absorption maximum, it is understood that the value obtained may differ by not greater than ± 2 nm.

Apparatus. Spectrophotometers suitable for measuring in the ultraviolet and visible range of the spectrum consist of an optical system capable of producing monochromatic radiation in the range of 200-800 nm and a device suitable for measuring the absorbance.

Control of wavelengths. Verify the wavelength scale using the absorption maxima of *holmium perchlorate solution R*, the line of a hydrogen or deuterium discharge lamp, or the lines of a mercury vapour arc shown in Table 2.2.2.24.-1. The permitted deviation is ±1 nm for the ultraviolet range and ±3 nm for the visible range. Suitable certified reference materials may also be used.

Control of absorbance.

Check the absorbance using suitable filters or a solution of *potassium dichromate R* at the wavelengths indicated in Table 2.1.2.24.-2. Table 2.1.2.24.-2 gives the exact value and the permitted limits of the specific absorbance for each wavelength. The table is based on a tolerance for the absorbance of ± 0.01.

Table 2.1.2.24.-1. *Absorption maxima for control of wavelength scale*

241.15 nm (Ho)	404.66 nm (Hg)
253.7 nm (Hg)	435.83 nm (Hg)
287.15 nm (Ho)	486.0 nm (Dß)
302.25 nm (Hg)	486.1 nm (HP)
313.16 nm (Hg)	536.3 nm (Ho)
334.15 nm (Hg)	546.07 nm (Hg)
361.5 nm (Ho)	576.96 nm (Hg)
365.48 nm (Hg)	579.07 nm (Hg)

For the control of absorbance, use solutions of *potassium dichromate R* that has been previously dried to constant mass at 130 °C. For the control of absorbance at 235 nm, 257 nm, 313 nm, and 350 nm, dissolve (57.0-63.0) mg of *potassium dichromate R* in *0.005 M sulfuric acid* and dilute to 1000.0 mL with the same solvent. For the control of absorbance at 430 nm, dissolve (57.0-63.0) mg of *potassium dichromate R* in *0.005 M sulfuric acid* and dilute to 100.0 mL with the same solvent. Suitable certified reference materials may also be used.

Limit of stray light. Stray light may be detected at a given wavelength with suitable filters or solutions. For example, the absorbance of a 12 g/L solution of *potassium chloride R* in a 1 cm cell increases steeply between 220 nm and 200 nm and is greater than 2.0 at 198 nm when compared with water as the compensation liquid. Suitable certified reference materials may also be used.

Resolution (for qualitative analysis). When prescribed in a monograph, measure the resolution of the apparatus as follows. Record the spectrum of a 0.02% (*V/V*) solution of *toluene R* in *hexane R*. The minimum ratio of the absorbance at the maximum at 269 nm to that at the minimum at 266 nm is stated in the monograph. Suitable certified reference materials may also be used.

Table 2.1.2.24.-2 – *Specific absorbance values and its maximum tolerances*

Wavelength, nm	Specific absorbance *A* 1%	Maximum tolerance $^{A}1\%$
235	124.5	122.9 to 126.2
257	144.5	142.8 to 146.2
313	48.6	47.0 to 50.3
350	107.3	105.6 to 109.0
430	15.9	15.7 to 16.1

Spectral slit-width (for quantitative analysis). When using an instrument on which the slit-width is variable at the selected wavelength, errors due to spectral slit-width are possible. To avoid them, the slit-width must be small compared with the half-width of the absorption band but it must be as large as possible to obtain a high value of I_0. Therefore, a slit-width is chosen such that further reduction does not result in a change in absorbance reading.

Cells. The tolerance on the path length of the cells used is ± 0.005 cm. When filled with the same solvent, the cells intended to contain the solution to be examined and the compensation liquid must have the same transmittance. If this is not the case, an appropriate correction must be applied.

The cells must be cleaned and handled with care.

DERIVATIVE SPECTROPHOTOMETRY

Derivative spectrophotometry involves the transformation of absorption spectra (zero-order) into first-, second- or higher-order-derivative spectra.

A *first-order-derivative spectrum* is a plot of the gradient of the absorption curve (rate of change of the absorbance with wavelength, $dA/d\lambda$) against wavelength.

A *second-order-derivative spectrum* is a plot of the curvature of the absorption spectrum against wavelength ($d^2A/d\lambda^2$). The second-order-derivative spectrum at any wavelength λ is related to concentration by the following equation:

$$\frac{d^2 A}{d\lambda^2} = \frac{d^2 A^{1\%}_{1cm}}{d\lambda^2} \cdot \frac{c' b}{10} = \frac{d^2 A\varepsilon}{d\lambda^2} \cdot \frac{cb}{10}$$

where c' is the concentration of the absorbing solute, in grams per litre.

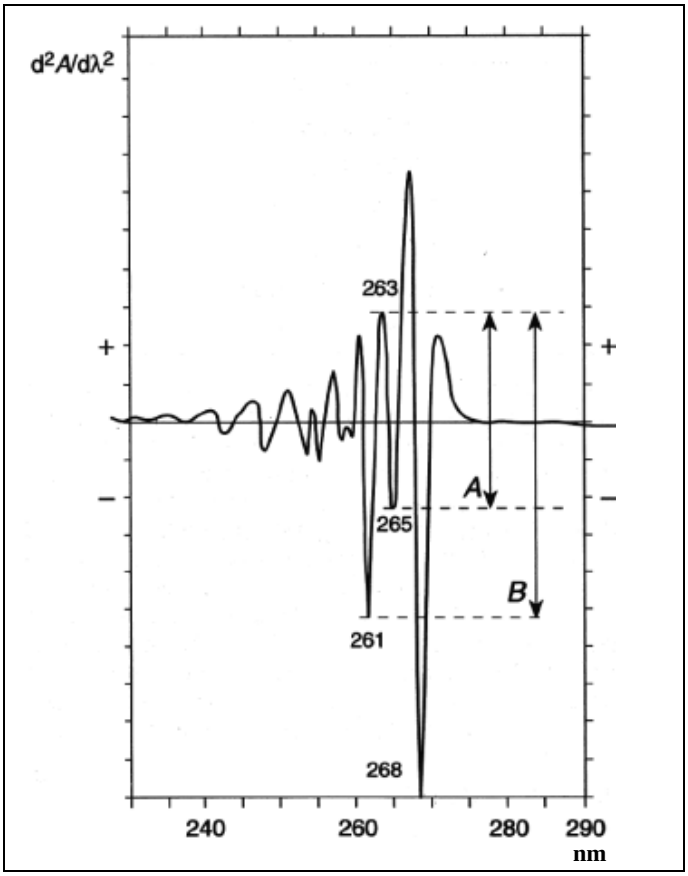


Figure 2.1.2.24.-1. – *Second-order derivative spectrum of a 0.02% (v/v) toluene R solution in methanol R*

Apparatus. Use a spectrophotometer complying with the requirements prescribed above and equipped with an analog resistance-capacitance differentiation module or a digital differentiator or other means of producing derivative spectra. Some methods of producing second-order-derivative spectra produce a wavelength shift relative to the zero-order spectrum and this is to be taken into account where applicable.

Resolution power. When prescribed in a monograph, record the second-order-derivative spectrum of a 0.02% (V/V) solution of *toluene R* in *methanol R*, using *methanol R* as the compensation liquid. The spectrum shows a small negative extremum located between two large negative extrema at 261 nm and 268 nm, respectively, as shown in Figure 2.1.2.24.-1. Unless otherwise prescribed in the monograph, the ratio A/B (see Figure 2.1.2.24.-1) is not less than 0.2.

Procedure. Prepare the solution of the substance to be examined, adjust the various instrument settings according to the manufacturer's instructions, and calculate the amount of the substance to be determined as prescribed in the monograph.

201020025-2019

2.1.2.25. Paper Chromatography

Paper chromatography is a separation method based on the development of the mobile phase through capillaries and the surface of filter paper.

The stationary phase is paper or substances pre-applied to its fibers. The paper chromatography is based on partition or adsorption mechanisms. The development of the mobile phase occurs either through the action of capillary forces only (ascending paper chromatography) or under the action of capillary forces and gravity (descending paper chromatography).

During the chromatographic process, the analytes form adsorption zones on the paper in the form of round or oval spots or stripes, depending on the method of application (as a dot or stripe).

The mobility of a substance during chromatography is characterised by a retardation factor (R_f) (see Chapter *2.2.2.36. Chromatographic separation techniques*).

Paper chromatography can be used for identification, purity testing, and quantitation.

ASCENDING PAPER CHROMATOGRAPHY

Apparatus. The apparatus consists of a glass chamber of a suitable size for the chromatographic paper used, ground at the top to take a closely fitting lid. At the top of the chamber is a device that suspends the chromatographic paper and is capable of being lowered without opening the chamber. At the bottom of the chamber is a dish to contain the mobile phase into which the paper may be lowered. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length, and not less than 2.5 cm wide, the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Procedure. Place in the dish a layer 2.5 cm deep of the mobile phase. If prescribed in the monograph, pour the stationary phase between the walls of the chamber and the dish. For saturation, close the chamber and allow it to stand for 24 h at 20 °C to 25 °C. Maintain the chamber at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper 3 cm from one end, using a micropipette, apply to a spot on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot greater than 10 mm in diameter, apply the solution in portions, allowing each portion to dry before the next application. When greater than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper into the chamber, close the lid, and allow to stand for 1 h 30 min. Lower the paper into the mobile phase and allow elution to proceed for the prescribed distance or time. Remove the paper from the chamber and allow it to dry in air. Protect the chromatographic paper from bright light during the entire elution process.

DESCENDING PAPER CHROMATOGRAPHY

***Apparatus*.** The apparatus consists of a glass chamber of a suitable size for the chromatographic paper used, ground at the top to take a closely fitting lid. The lid has a central hole about 1.5 cm in diameter closed by a heavy glass plate or a stopper. In the upper part of the chamber is suspended a solvent trough. On each side of the trough, parallel to and slightly above its upper edges, are two glass guide rods to support the paper in such a manner that no part of it is in contact with the walls of the chamber. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Procedure. Place a layer 2.5 cm deep of the mobile phase prescribed in the monograph in the bottom of the chamber, close the chamber and allow to stand for 24 h at 20 °C to 25 °C. Maintain the chamber at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper at such a distance from one end that the line is a few centimetres below the guide rod and parallel with it after fixing the end of the paper in the dish. The remainder of the paper is hanging freely over the guide rod. Using a micropipette, apply on the pencil line the volume of the solution specified in the individual monograph. If the total volume to be applied would produce a spot greater than 10 mm in diameter, apply the solution in portions, allowing each portion to dry before the next application. When greater than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart.

Insert the paper in the chamber, close the lid, and allow to stand for 1 h 30 min. Then fill the solvent trough with the mobile phase through the hole in the lid, close the hole, and allow elution to proceed for the distance or time prescribed in the monograph. Remove the paper from the chamber and allow it to dry in air. The chromatographic paper should be protected from bright light during the elution process.

201020026-2019

2.1.2.26. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a separation technique based on adsorption, partition, ion-exchange, or on combinations of these mechanisms and is carried out by the development of solutions of analytes (solutes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase). The stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal, or plastic.

Before chromatography, apply the solutes to the plate.

An enhanced technique that helps in achieving better sensitivity and separation efficiency through the use of special plates with smaller sorbent particle size is called high-performance thin-layer chromatography (HPTLC).

APPARATUS

Plates. The chromatography is carried out using pre-coated plates as described in the chapter *2.2.1.1. Reagents*. When describing tests, the silica gel particle size is indicated after the test name.

Pre-treatment of the plates. It may be necessary to wash the plates prior to separation. This can be done by the migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion, or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120 °C for 20 min.

Chromatographic chamber with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates, used and provided with a tightly fitting lid. For horizontal development, the chromatographic chamber is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipettes, microsyringes, calibrated disposable capillaries, or other application devices suitable for the proper application of the solutions.

Fluorescence detection device. For measuring direct fluorescence or detecting the inhibition of fluorescence.

Visualisation devices and reagents. Suitable devices are used for derivatisation to transfer to the plate reagents (by spraying, immersion, or exposure to vapours) and, where applicable, to facilitate heating for visualisation of separated components.

Documentation. A device may be used to provide documentation of the visualised chromatogram, for example, a photograph or a computer file.

METHOD

Sample application. Apply the prescribed volume of the solutions at a suitable distance from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of circular spots and 5 mm (2 mm on HPTLC plates) between the edges of bands. Apply the solutions in sufficiently small portions to obtain circular spots 2-5 mm in diameter (1-2 mm for HPTLC) or bands 10-20 mm (5-10 mm on HPTLC plates) by 1-2 mm.

In the monograph, where both normal and HPTLC plates may be used, the working conditions for high-performance thin-layer chromatography are given in the brackets [ ] after those for normal plates.

Vertical development.

Line the walls of the chromatographic chamber with filter paper. Pour into the chromatographic chamber a sufficient quantity of the mobile phase for the size of the chamber to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the chromatographic chamber with mobile phase, replace the lid and allow it to stand at 20-25 °C for 1 h. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated chamber. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the chromatographic chamber, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic chamber, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance, measured between the points of application and the solvent front, dry the plate, and visualise the chromatograms as prescribed in the monograph.

For two-dimensional chromatography, dry the plates after the first development and carry out the second development in a direction perpendicular to that of the first development.

Horizontal development. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate in the chamber after verifying that the latter is horizontal and connect the mobile phase delivery device according to the manufacturer's instructions. If prescribed in the monograph, develop the plate starting simultaneously at both ends. Close the chamber and run chromatography at 20-25 °C. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph, dry the plate, and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out the second development in a direction perpendicular to that of the first development.

Stepwise elution chromatography. Apply the prescribed volume of solutions as described above. In the process of chromatography, the composition of the mobile phase or other chromatography conditions are gradually changed. For example, stepwise elution chromatography with different mobile phases is used if chromatography with only one mobile phase is not enough to separate the analysed mixture.

VISUAL EVALUATION

Identification. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by comparing the colour, the size, and the retardation factor (R_F) or R_{st} factor of both spots.

The retardation factor (RF) is defined as the ratio of the distance from the point of application to the center of the spot and the distance traveled by the solvent front from the point of application (see chapter *2.1.2.36.*).

Verification of the separating power for identification. Normally, the performance given by the stationary phase suitability test described in chapter *2.2.1.1.*

Reagents is sufficient. Only in special cases, additional requirements are prescribed in the individual monograph.

Related substances test. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity(ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Verification of the separating power. The requirements for the verification of the separating power are prescribed in the monographs concerned.

Verification of the detecting power. The detecting power is satisfactory if a spot or band is clearly visible in the chromatogram obtained with the most dilute reference solution.

QUANTITATIVE MEASUREMENTS

The requirements for resolution and separation are prescribed in the monographs concerned.

Substances separated by thin-layer chromatography and responding to ultraviolet or visible (UV-Vis) irradiation can be determined quantitatively directly on the plate, using appropriate instrumentation. While moving the plate or the measuring device, examine the plate by measuring the reflectance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in three ways:

- directly by moving the plate alongside a suitable counter or vice versa (see *Radiopharmaceutical preparations*);
- by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter;
- by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail and measuring the radioactivity using a liquid scintillation counter.

Apparatus. The apparatus for direct measurement on the plate consists of:

- a device for exact positioning and reproducible dispensing of the amount of substances onto the plate;
- a mechanical device to move the plate or the measuring device along the *X*-axis or the *Y*-axis;
- a recorder and a suitable integrator or a computer;
- *for substances responding to UV-Vis irradiation*: a photometer with a source of light, an optical device able to generate monochromatic light, and a photocell of adequate sensitivity are used for the measurement of reflectance or transmittance; if fluorescence is measured, a suitable filter is required to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass;
- *for substances containing radionuclides*: a suitable counter for radioactivity; the linearity range of the counting device is to be verified.

Procedure. Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph and, if necessary, prepare the reference solutions of the substance to be determined using the same solvent as in the test solution. Apply the same volume of each solution to the plate and develop.

Substances responding to UV-Vis irradiation. Prepare and apply not fewer than three reference solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (about 80%, 100%, and 120%). Treat with the prescribed reagent, if necessary, and record the reflectance, the transmittance, or fluorescence in the chromatograms obtained with the test and reference solutions. Use the measured results for the calculation of the amount of substance in the test solution.

Substances containing radionuclides. Prepare and apply a test solution containing about 100% of the expected value.

Determine the radioactivity as a function of the path length and report the radioactivity in each resulting peak as a percentage of the total amount of radioactivity.

Criteria for assessing the suitability of the system are described in chapter *2.1.2.36. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

201020027-2019

2.1.2.27. Gas Chromatography

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between two non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. It is applicable to substances or their derivatives which are volatilised under the temperatures employed.

Gas chromatography is based on mechanisms of adsorption, mass distribution, or size exclusion.

APPARATUS

The apparatus consists of an injector, a chromatographic column contained in an oven, a detector and a data acquisition system (or an integrator or a chart recorder). The carrier gas flows through the column at a controlled rate or pressure and then through the detector.

The chromatography is carried out either at a constant temperature or according to a given temperature program.

INJECTION PORT

Direct injections of solutions are the usual mode of injection unless otherwise prescribed in the monograph. An injection may be carried out either directly at the head of the column using a syringe or an injection valve, or into a vapourisation chamber which may be equipped with a stream splitter.

Injections of vapour phase may be carried out *by* static or dynamic head-space injection systems.

Dynamic head-space (purge and trap) injection systems include a sparging device by which volatile substances in solution are swept into an absorbent column maintained at a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the absorbent column.

Static head-space injection systems include a thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow the volatile components of the sample to reach equilibrium between the non-gaseous phase and the vapour phase. After equilibrium has been established, a predetermined amount of the head-space of the vial is flushed into the gas chromatograph.

STATIONARY PHASE

Types of columns filled with stationary phases:

- a capillary column of fused-silica whose wall is coated with the stationary phase;
- a column packed with inert particles impregnated with the stationary phase;
- a column packed with a solid stationary phase.

Capillary columns are 0.1 mm to 0.53 mm in internal diameter (Ø) and 5 m to 60 m in length. The liquid or stationary phase, which may be chemically bonded to the inner surface, is a film 0.1 µm to 5.0 µm thick.

Packed columns, made of glass or metal, are usually 1 m to 3 m in length with an internal diameter of 2 mm to 4 mm. Stationary phases usually consist of porous polymers or solid supports impregnated with a liquid phase. Supports for analysis of polar compounds on columns packed with low-capacity, low-polarity stationary phase must be inert to avoid peak tailing.

The reactivity of support materials can be reduced by silanising prior to coating with a liquid phase. Acid-washed, flux-calcinated diatomaceous earth is often used. Materials are available in various particle sizes, the most commonly used particles are in the ranges of 150 µm to 180 µm and 125 µm to 150 µm.

MOBILE PHASE

Retention time and peak efficiency depend on the carrier gas flow rate; retention time is directly proportional to column length and resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in millilitres per minute at atmospheric pressure and room temperature. Flow rate is measured at the detector outlet, either with a calibrated mechanical device or with a bubble tube, while the column is at operating temperature. The linear velocity of the carrier gas through a packed column is inversely proportional to the square root of the internal diameter of the column for a given flow volume. Flow rates of 60 mL/min in a 4 mm internal diameter column and 15 mL/min in a 2 mm internal diameter column, give identical linear rates and thus similar retention times.

Helium or nitrogen is usually employed as the carrier gas for packed columns, whereas commonly used carrier gases for capillary columns are nitrogen, helium, and hydrogen.

DETECTORS

Flame-ionisation detectors are usually employed but additional detectors that may be used include: electron-capture, nitrogen-phosphorus, mass spectrometric, thermal conductivity, Fourier transform infrared spectrophotometric, and others, depending on the purpose of the analysis.

METHOD

Equilibrate the column, the injector, and the detector at the temperatures and the gas velocity specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the reference solution(s) as prescribed in the monograph. The solutions must be free from solid particles.

Criteria for assessing the suitability of a chromatographic system are described in chapter *2.1.2.36.*

Chromatographic separation techniques. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

Static Head-Space Gas Chromatography

Static head-space gas chromatography is a technique particularly suitable for separating and determining volatile compounds present in solid or liquid samples. The method is based on the analysis of the vapour phase in equilibrium with the solid or liquid phase.

APPARATUS

The apparatus consists of a gas chromatograph provided with a device for introducing the sample that may be connected to a module that automatically controls the pressure and the temperature. If necessary, a device for eliminating solvents can be added.

The sample to be analyzed is introduced into a container fitted with a suitable stopper and a valve-system which permits the passage of the carrier gas. The container is placed in a thermostatically controlled chamber at a temperature set according to the substance to be examined. The container is held at this temperature long enough to allow equilibrium to be established between the solid or liquid phase and the vapour phase.

The carrier gas is introduced into the container and, after the prescribed time, a suitable valve is opened so that the gas expands towards the chromatographic column taking the volatilised compounds with it.

Instead of using a chromatograph specifically equipped for the introduction of samples, it is also possible to use airtight syringes and a conventional chromatograph. Equilibration is then carried out in a separate chamber and the vapour phase is carried onto the column, taking the precautions necessary to avoid any changes in the equilibrium.

METHOD

Using the reference preparations, determine suitable instrument settings to produce an adequate response.

DIRECT CALIBRATION

Separately introduce into identical containers the preparation to be examined and each of the reference preparations, as prescribed in the monograph, avoiding contact between the sampling device and the samples.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph. After equilibration, carry out the chromatography under the prescribed conditions.

STANDARD ADDITIONS

Add equal volumes of the preparation to be examined to a set of identical suitable containers. Add to all but one of the containers, suitable quantities of a reference preparation containing a known concentration of the substance to be determined so as to produce a series of preparations containing steadily increasing concentrations of the substance.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph. After equilibration, carry out the chromatography under the prescribed conditions.

Calculate the linear equation of the graph using a least-squares fit. Derive from it the concentration of the substance to be determined in the preparation to be examined.

Alternatively, determine the concentration using the graphical method. For this purpose, plot on a graph the mean of readings against the added quantity of the substance to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the substance to be determined in the preparation to be examined.

SUCCESSIVE WITHDRAWALS (MULTIPLE HEAD-SPACE EXTRACTION)

If prescribed, the successive withdrawal method is fully described in the monograph.

2.1.2.28. High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid that percolates through a stationary phase contained in a column with high hydraulic resistance. Liquid chromatography, which uses columns with a reduced particle size (for example, less than 2 μm) is called ultra-high-performance liquid chromatography (UHPLC).

In the latter case, equipment is required that is characterised by the following: the ability to use high pressures (usually up to 100 MPa, i.e. about 15,000 psi), reduced extra-column peak broadening, improved gradient mixing parameters, and increased speed of data acquisition by the detection system.

Liquid chromatography, depending on major manifested intermolecular interactions, is based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion, stereochemical interaction, etc. Each of these types can be highly effective if the high-pressure chromatography mode is used.

APPARATUS

The apparatus consists of a mobile phase preparation unit with a degassing system, a pumping system, a mobile phase mixer (if necessary), an injector, a chromatographic column (a column temperature controller may be used), a detector, and a data acquisition system (or an integrator or a chart recorder). In addition, the chromatograph may include a sample preparation system and a pre-column reactor, a column switching system, a post-column reactor, and other equipment. The mobile phase is supplied from one or several reservoirs and flows through the column, usually at a constant rate, and then through the detector.

PUMPING SYSTEMS

Pumping systems are required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimised, e.g. by passing the pressurised solvent through a pulse-dampening device. Tubing and connections are capable of withstanding the pressures developed by the pumping system. Pumping systems used in high-performance liquid chromatography can be equipped with degassers.

Microprocessor controlled systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined program. In the case of gradient elution, pumping systems that deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

INJECTION PORT

The sample solution is introduced into the mobile phase flow at or near the head of the column using an injection system which can operate at high pressure. Fixed-loop and variable volume devices operated manually or by an auto-sampler are used. Manual partial filling of loops may lead to poorer injection volume precision.

STATIONARY PHASE

There are many types of stationary phases employed in high-performance liquid chromatography, including:

- silica gel, alumina, or porous graphite, used in normal-phase chromatography, where the separation is based on differences in adsorption and/or mass distribution (partition chromatography);
- a variety of chemically modified supports prepared from polymers, silica or porous graphite, used in normal-phase (adsorption chromatography) and reversed-phase chromatography, where the separation is based principally on partition of the molecules between the mobile phase and the stationary phase;

- resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;

- porous silica gel or polymers, used in size-exclusion chromatography, where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion;

- special chemically modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins, etc., for the separation of enantiomers (chiral chromatography);

- other stationary phases used in high-performance modifications of various types of liquid chromatography.

Most separations are based upon partition mechanisms utilising chemically modified silica as the stationary phase and polar solvents as the mobile phase. The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Commonly used bonded phases are shown below:

Octyl	$-Si-[CH_2]_7-CH_3$	C_8
Octadecyl	$-Si-[CH_2]_{17}-CH_3$	C_{18}
Phenyl	$-Si-[CH_2]_n-C_6H_5$	C_6H_5
Cyanopropyl	$-Si-[CH_2]_3-CN$	CN
Aminopropyl	$-Si-[CH_2]_3-NH_2$	NH_2
Diol	$-Si-[CH_2]_3-O-$ $-CH(OH)-CH_2-OH$	

Unless otherwise stated by the manufacturer, silica-based reversed-phase columns are considered to be stable in mobile phases having an apparent pH in the range 2.0 to 8.0.

Columns containing porous graphite or particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase chromatography with unmodified silica, porous graphite, or polar chemically modified silica gel (e.g. cyanopropyl or diol), as the stationary phase with a non-polar mobile phase is applicable in certain cases.

For analytical separations, the particle size of the most commonly used stationary phases varies between 2 μm and 10 μm. The particles may be spherical or irregular, of varying porosity and specific surface area. These parameters contribute to the chromatographic behavior of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g. expressed as the carbon loading, and whether the stationary phase is end-capped (i.e. residual silanol groups are silylated) are additional determining factors. Tailing of peaks, particularly of basic substances, can occur when residual silanol groups are present.

In addition to porous particles, surface porous or monolithic materials can be used.

Columns, made of stainless steel unless otherwise prescribed in the monograph, of varying length and internal diameter are used for analytical chromatography. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. The temperature of the mobile phase and the column must be kept constant during an analysis. Most separations are performed at room temperature, but columns may be heated to give higher efficiency.

MOBILE PHASE

For normal-phase chromatography, less polar solvents (hexane, cyclohexane, heptane, etc.) are employed with small additions of polar organic compounds, which regulate the elution power of the mobile phase. The presence of water in the mobile phase is to be strictly controlled to obtain reproducible results in normal-phase chromatography.

In reversed-phase chromatography, aqueous mobile phases, with or without organic modifiers, are employed. Organic additives are usually polar organic solvents (acetonitrile and methanol). To optimise the separation, aqueous solutions with a certain pH value can be used, in particular, buffer solutions, as well as various additives to the mobile phase: phosphoric and acetic acids in the separation of acidic compounds; ammonia and aliphatic amines in the separation of basic compounds, and other modifiers.

Components of the mobile phase are usually filtered to remove particles greater than 0.45 μm (or 0.2 μm, if the stationary phase consists of particles less than 2 μm, and also if special detectors are used, e.g., a light scattering detector). Multicomponent mobile phases are prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by mixing. Alternatively, the solvents may be delivered by individual pumps controlled by proportioning valves by which mixing is performed according to the desired proportion. Solvents are normally degassed before pumping by sparging with helium, sonication or using *on-line* membrane/vacuum modules to avoid the creation of gas bubbles in the detector cell.

Solvents for the preparation of the mobile phase are normally free of stabilisers and are transparent at the wavelength of detection, if an ultraviolet detector is employed. Solvents and other components employed are to be of appropriate quality. Adjustment of the pH, if necessary, is effected using only the aqueous component of the mobile phase and not the mixture. If buffer or saline solutions are used, adequate rinsing of the system is carried out with a mixture of water and the organic modifier of the mobile phase (5% (*V/V*)) to prevent the crystallisation of salts after completion of the chromatography.

Mobile phases may contain other components, e.g. a counter-ion for ion-pair chromatography or a chiral selector for chromatography using an achiral stationary phase.

DETECTORS

Ultraviolet/visible (UV/Vis) spectrophotometers are the most commonly employed detectors. The spectrophotometric detector allows detection at any wavelength in its operating range (usually 190 - 600 nm). Also, multi-wavelength detectors that allow detection at several wavelengths simultaneously and diode array detectors may be used, which allow reading the absorbance values simultaneously in the entire operating wavelength range (usually 190-950 nm). This allows recording the absorption spectra of components passing through the detector cell. In addition, special detectors can be used. Fluorescent detectors are used to detect fluorescent compounds or non-fluorescent compounds in the form of their fluorescent derivatives and have very high sensitivity and selectivity. Refractometric detectors (refractometers) are used to detect compounds that are weakly absorbing in the ultraviolet and visible regions of the spectrum (for example, carbohydrates). The disadvantages of these detectors include their relatively low sensitivity and significant temperature dependence (the temperature of the detector must be controlled to ensure the required signal intensity); moreover, these detectors cannot be used in the gradient elution mode. In addition, electrochemical detectors (amperometric, conductometric) for electroactive compounds, light scattering detectors, charged aerosol detectors, mass spectrometers with very high sensitivity and selectivity, Fourier transform IR spectrophotometers, radioactivity detectors, and other special detectors may be used.

DATA COLLECTION AND PROCESSING SYSTEM

A modern data processing system represents a personal computer connected to the chromatograph with installed software that allows registering and processing the chromatogram, as well as controlling the operation of the chromatograph and monitoring the main parameters of the chromatographic system.

PROCEDURE

Equilibrate the column with the prescribed mobile phase and mobile phase flow rate, at room temperature or at the temperature specified in the monograph, until a stable baseline is achieved. Prepare the solution(s) of the substance to be examined and the reference solution(s) specified in the monograph. The solutions must be free from solid particles. Criteria for assessing the suitability of a chromatographic system are described in chapter *2.1.2.36. Chromatographic separation techniques*. The extents to which adjustments of parameters of the chromatographic system can be made (without general adjustment of the procedures) to satisfy the criteria of system suitability are also given in this chapter.

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2.1.2.29. Size-Exclusion Chromatography

Size-exclusion chromatography is a chromatographic technique which separates molecules in solution according to their size. With organic mobile phases, the technique is known as *gel-permeation chromatography* and with aqueous mobile phases, the term *gel-filtration chromatography* has been used. The sample is introduced into a column, which is filled with a gel or a porous particle packing material, and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the material the column is filled with. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the *total permeation volume* (V_t). Molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the *exclusion volume* (V_0 void volume). Separation according to molecular size occurs between the exclusion volume and the total permeation volume, with useful separation usually occurring in the first two-thirds of this range.

APPARATUS

The apparatus consists essentially of a chromatographic column of varying length and internal diameter, packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes. If necessary, the column is temperature-controlled. Through the column the eluent is passed at a constant rate. One end of the column is usually fitted with an injection port, e.g. a flow adapter, a syringe through a septum, or a flow-blocking injection valve. To the same end of the column, a suitable pump for controlling the flow rate of the eluent may be connected. The sample can also be applied directly to the dry surface of the column material or, if the sample density exceeds the density of the eluent, it can be layered on the surface of the column material under the eluent. The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder which enables the monitoring of the relative concentrations of separated components of the sample. Detectors are usually based on photometric, refractometric, or luminescent properties. An automatic fraction collector may be attached, if necessary.

The stationary phase may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica gel, or a solvent-compatible, cross-linked organic polymer.

Rigid supports usually require pressurised systems giving faster separations. The mobile phase is chosen according to sample type, separation medium, and method of detection. Before carrying out the separation, the packing material is treated, and the column is packed, as described in the monograph, or according to the manufacturer's instructions.

Criteria for assessing the suitability of a chromatographic system are described in chapter *2.1.2.36. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURES

Carry out the separation as stated in the monograph. If possible, monitor the elution of the components continuously and measure the corresponding peak areas. If the sample is monitored by a physicochemical property to which all the components of interest exhibit equivalent responses (for example if they have the same specific absorption), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components of interest. If the responses to the property used for detection of the components of interest are not equivalent, calculate the content by means of calibration curves obtained with the calibration standards prescribed in the monograph.

DETERMINATION OF MOLECULAR MASSES

Size-exclusion chromatography may be used to determine molecular masses by comparison with appropriate calibration standards specified in the monograph. The retention volumes of the calibration standards may be plotted against the logarithm of their molecular masses. The graph plotted within the exclusion volume limits and the total permeation limits usually approximates a straight line for the column under the given experimental conditions. From the calibration curve, molecular masses may be estimated. The molecular-mass calibration is valid only for the particular macromolecular substance/solvent system used under the specified experimental conditions.

DETERMINATION OF MOLECULAR SIZE DISTRIBUTION OF POLYMERS

Size-exclusion chromatography may be used to determine the distribution of the molecular size of polymers. However, sample comparison may be valid only for results obtained under the same experimental conditions. The reference substances used for the calibration and the procedure for determination of the distribution of molecular sizes of polymers are specified in the monographs.

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2.1.2.30. Electrophoresis

1. GENERAL PRINCIPLE

Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. For gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration velocities according to sizes, shapes, and charges of particles. Because of their different physicochemical properties, different macromolecules of a mixture will migrate at different velocities during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g. free solution separation in capillary electrophoresis) and in stabilising media such as thin-layer plates, films, or gels.

2. FREE OR MOVING BOUNDARY ELECTROPHORESIS

This method is mainly used for the determination of mobility, the experimental characteristics being directly measurable and reproducible. It is chiefly employed with substances of high relative molecular mass and low diffusibility. The boundaries are initially located by a physical process such as refractometry or conductimetry. After applying a given electric field for an accurately measured time, the new boundaries and their respective positions are observed. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

3. ZONE ELECTROPHORESIS USING A SUPPORTING MEDIUM

This method requires the use of small samples only.

The nature of the support, such as paper, agar gel, cellulose acetate, starch, agarose, methacrylamide, mixed gel, introduces a number of additional factors modifying the mobility:

a) owing to channeling in the supporting medium, the apparent distance covered is less than the real distance;

b) some supporting media are not electrically neutral. As the medium is a stationary phase it may sometimes give rise to a considerable electro-endosmotic flow;

c) any heating due to the joule effect may cause some evaporation of the liquid from the supporting medium which, by capillarity, causes the solution to move from the ends towards the centre. The ionic strength, therefore, tends to increase.

The migration velocity then depends on four main factors: the mobility of the charged particle, the electro-endosmotic flow rate, the evaporation flow, and the field strength. Hence it is necessary to operate under clearly defined experimental conditions and to use, wherever possible, reference substances.

An apparatus for electrophoresis mainly consists of:

- A *generator supplying direct current* whose voltage can be controlled and, preferably, stabilised.

- An *electrophoresis chamber* . This chamber is usually rectangular and made of glass or rigid plastic, with two separate compartments, the anodic and the cathodic, containing the electrolyte solution. In each compartment is immersed an electrode, for example of platinum or graphite. These are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the two compartments is kept equal to prevent siphoning.

The electrophoresis chamber is fitted with an airtight lid that maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is preferable to cool the support.

- A *support-carrying device* :

Strip electrophoresis. The supporting strip, previously wetted with the same conducting solution and dipped at each end into an electrode compartment is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte. Such carriers can be a horizontal frame, inverted-V stand, or a uniform surface with contact points at suitable intervals.

Gel electrophoresis. The device consists essentially of a glass plate (for example, a microscope slide) over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The compound between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions must be taken to avoid condensation of moisture or drying of the solid layer.

- *measuring device or means of detection*.

Procedure. Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. After the current has been switched off, remove the support from the chamber, dry, and visualise.

4. POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N*,*N*′-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution is applied to each rod.

Apparatus. This consists of two buffer solution reservoirs made of a suitable material such as poly(methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply allowing operation either at a constant current or at a constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

Procedure. The solutions should usually be degassed before polymerisation and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to equal height in each tube, and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water R* to exclude air and allow it to set. Gel formation usually takes about 30 min and is complete when a sharp interface appears between the gel and the water layer. Remove the aqueous layer. Fill the lower reservoir with the prescribed buffer solution. Open the glass tubes (remove the stoppers from the bottom end) and fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution.

Prepare test and reference samples containing the prescribed marker dye, and make them dense by dissolving in tem *sucrose R*, for example.

Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current.

Turn off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electropherogram as prescribed.

5. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

5-1. SDS-PAGE—GELS WITH A HOMOGENEOUS CONCENTRATION

Scope. Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and quantitation.

Purpose. Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins.

Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

5-1-1. CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains.

Polymerisation is catalysed by a free radical-generating system composed of ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration velocity of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition in acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. The mobility is influenced by the type, concentration, and pH of the buffer, by the temperature and the field strength as well as by the nature of the support material.

5-1-2. DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14,000 to 100,000 daltons. It is possible to extend the boundaries of molecular masses using various procedures (for example, by using gradient gels, certain buffer systems). For example, tricin-PAAG gels containing tricin as a lagging ion in a separating buffer solution for electrophoresis (instead of glycine, as in the method described above) allow the separation of very small proteins and peptides from less than 10,000 to 15,000 daltons.

Denaturing polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method.

Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent sodium dodecyl sulfate (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with the velocity dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent- polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained.

The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

Reducing conditions. Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS.

In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression (to obtain a more accurate result – by the method of non-linear regression) in the presence of suitable molecular-mass standards.

Non-reducing conditions. For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-mercaptoethanol or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein, and no dissociation into subunit polypeptides occurs. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. Disulfide bonds within the chain strengthen the structure of the polypeptide, usually by decreasing the hydrodynamic radius of the molecule, and thus decreasing the apparent average molecular weight. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

5-1-3. CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS (DISC ELECTROPHORESIS)

The most popular electrophoretic method for the characterisation of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution.

When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases.

Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface between the concentrating and resolving gels, there is a sharp increase in the effect of protein retention due to the limited pore size of the resolving gel and the discontinuity of the buffer solution, which also contributes to the focusing of proteins. Once in the resolving gel, proteins continue to be slowed by the sieving (separating ability) of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

5-1-4. PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

This section describes the preparation of gels using special instruments. This does not apply to precast gels. When using pre-prepared or any other commercial gels or other equipment, follow the manufacturer's instructions.

It is recommended to use commercial reagents purified in solution. Otherwise, as well as when using insufficiently purified reagents, pre-treatment is carried out. For example, a solution that has such a degree of contamination that it needs to be filtered must also be deionised using a mixed-type ion exchange resin (cation exchange/anion exchange) to remove acrylic acid and other charged degradation products. Solid-state solutions of acrylamide/bisacrylamide and persulfate remain stable for a long period if stored according to the guidance.

Assembling of the gel moulding cassette. Clean the two glass plates (size: e.g. 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tubing (diameter e.g. 0.6 mm × 35 cm) with mild detergent and rinse extensively with water, and dry at a room temperature. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with one finger along the long side twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

Preparation of the gel. In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

Preparation of the resolving gel. In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 2.1.2.30.-1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 μm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.1.2.30.-1, swirl and pour immediately into the gap between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position and allow to polymerise at room temperature.

Preparation of the stacking gel. After polymerisation is complete (about 30 min), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2.1.2.30.-2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 μm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution.

Table 2.1.2.30.-1. – *Preparation of resolving gel*

Solution components	Component volumes (mL) per gel mould volume of							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6% acrylamide								
Water R	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution(1)	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% acrylamide								
Water R	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution(1)	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% acrylamide								
Water R	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution(1)	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% acrylamide								
Water R	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution(1)	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% acrylamide								
Water R	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution(1)	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8)(2)	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% acrylamide								
Water R	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution(1)	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
A 100 g/L SDS solution(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
A 100 g/L APS solution(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution*: 30% solution of acrylamide-bisacrylamide (29:1) R.*

(2) 1.5 M Tris (pH 8.8): *1.5 M Tris hydrochloride buffer solution* pH *8.8 R.*

(3) 100 g/L SDS solution: 100 g/L solution of *sodium dodecyl sulfate R.*

(4) 100 g/L APS solution: 100 g/L solution of *ammonium persulfate R*. Due to ammonium persulfate, free radicals appear that accelerate the polymerisation of acrylamide and bisacrylamide. Since the ammonium persulfate solution decomposes quickly, fresh solutions should be prepared daily.

(5) TEMED*: tetramethylethylenediamine R.*

Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.1.2.30.-2, swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerised resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerise at room temperature.

Sample preparation. Unless otherwise specified in the monograph, samples may be prepared by the following methods.

Sample preparation (non-reducing conditions). Mix equal volumes of a mixture consisting of *water R* and the test drug or comparison drug, and a *concentrated buffer solution for preparing samples for electrophoresis in polyacrylamide gel with sodium dodecyl sulfate R.*

Sample preparation (reducing conditions). Mix equal volumes of a mixture consisting of *water R* and the test drug or reference drug, and a *concentrated buffer solution for preparing samples under reducing conditions during polyacrylamide gel electrophoresis with sodium dodecyl sulfate R* containing 2-mercaptoethanol (or dithiothreitol) as a reducing agent.

The concentration indicated in the monograph may vary depending on the protein and the method of staining.

Sample treatment: keep the sample in a boiling water bath for 5 min or in a heating unit at 100 °C, then allow to cool. The temperature and time specified in the monograph may vary due to the possible destruction of the protein that may occur during heat treatment.

Install the gel in the apparatus for electrophoresis and electrophoretic separation. After polymerisation is complete (about 30 min), carefully remove the polytetrafluoroethylene comb. Wash the wells immediately with *water or buffer working solution for polyacrylamide gel electrophoresis with sodium dodecyl sulfate R* to remove unpolymerised acrylamide. If necessary, align the partitions of the concentrating gel with a blunt hypodermic needle attached to the syringe.

Table 2.1.2.30.-2. – *Concentrating gel preparation*

Solution components	Component volumes (mL) per gel mould volume of							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water R	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution(1)	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)(2)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
A 100 g/L SDS solution(3)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
A 100 g/L APS solution(4)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED(5)	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: *30% solution of acrylamide/bisacrylamide (29:1) R.*
(2) 1 M Tris (pH 6,8): *1 M Tris hydrochloride buffer solution* pH *6,8 R.*
(3) 100 g/L SDS solution: 100 g/L solution of *sodium dodecyl sulfate R*.
(4) 100 g/L APS solution: 100 g/L solution of *ammonium persulfate R*. Due to ammonium persulfate, free radicals appear that accelerate the polymerisation of acrylamide and bisacrylamide. Since the ammonium persulfate solution decomposes quickly, fresh solutions should be prepared daily.
(5) TEMED*: tetramethylethylenediamine R.*

Remove the clips from one short side, carefully pull out the tube and return the clips to their place. Repeat these operations on the other short side. Remove the tube from the bottom of the gel. Place the gel in the electrophoresis device. The upper and lower reservoirs are filled with buffer solution for electrophoresis. Remove all bubbles formed at the bottom of the gel between two glass plates. For these purposes, it is best to use a curved needle attached to a syringe. Do not perform the initial electrophoresis before applying the samples, as this will cause discontinuity of the buffer system. Before applying the samples, carefully rinse each well with a *working solution for electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate*. Prepare the test and reference samples in the recommended buffer solution for samples and process them as indicated in the monograph. Apply the required amount of each solution to the wells of the concentrating gel.

Perform electrophoresis under the conditions recommended by the equipment manufacturer. Manufacturers of equipment for SDS-PAGE can provide gels of various size and thickness. The electrophoresis time and current/voltage values may vary when optimal separation is achieved. Make sure that the dye front moves into the separating gel. When the dye reaches the lower edge of the gel, stop electrophoresis. Remove the gel cartridge from the device and carefully separate the glass plates. Remove the pads, cut off and discard the concentrating gel, and immediately begin discolouring the remaining gel.

Table 2.1.2.30.-3. – *Gel separating capacity with a concentration gradient.*

Acrylamide (%)	Range of protein molecular masses (kDa)
5-15	20-250
5-20	10-200
10-20	10-150
8-20	8-150

5-2. SDS-PAGE GRADIENT CONCENTRATING GELS

Prepare gradient gels (separating gels) with an increase in the concentration of acrylamide in the top-down direction. For the preparation of gradient gels, a gradient forming apparatus is required. Ready-made gradient gels are available In the market according to the recommended protocols.

Gradient gels have some advantages as they allow the separation of proteins that migrate together in gels with a fixed concentration of acrylamide. In electrophoresis, proteins migrate until the size of the pores prevents them from moving further, and therefore there is a stacking effect that leads to sharper bands. As shown in Table 2.1.2.30.-3, gradient gels also allow the separation of proteins with a wider range of molecular masses compared to gels with a fixed concentration.

The table shows the recommended linear gradient compositions, with a comparison of the range of acrylamide concentrations to the corresponding ranges of protein molecular mass. Keep in mind that other gradient shapes can be prepared (for example, concave) for special applications.

Gradient gels are also used to determine the molecular weight and purity grade of the protein.

5-3. DETECTION OF PROTEINS IN GELS

The most widely used methods for staining proteins with Coomassie dye and silver are described in detail below. Other commercial dyes, detection methods, and kits are also available. For example, fluorescent dyes are co-used with a fluorescent imaging device and often provide a linear dependence of the analytical signal over a wide range of protein concentrations, often within several orders of magnitude depending on the nature of the protein.

Coomassie dye staining allows determining 1 μg to 10 μg of proteins in a single band. Silver staining is a more sensitive method of protein staining, and bands containing from 10 ng to 100 ng of protein can be detected. These figures are considered really achievable for this gel staining method.

Sometimes in the litreature, the sensitivity of silver staining by 1 or 2 orders of magnitude higher is indicated.

Coomassie dye staining provides a more linear signal than silver staining; however, the signal and range depend on the nature of the protein and the time of electrophoretic separation. Reproducibility when stained with Coomassie dye and silver may decrease if the staining is stopped for subjective reasons, i.e. at a time when it seems sufficient. It is very important to use dynamic ranges of standard proteins, as they help to assess sensitivity and linearity during the test. All stages of gel staining are performed with disposable gloves, at room temperature, with gentle mixing (for example, using an orbital shaker), and in any suitable container.

Staining with Coomassie dye. Immerse the gel in a large volume of *Coomassie staining solution R* and allow it to stand for at least 1 h. Then drain the staining solution.

The gel is discoloured with a large volume of washing solution *R*. Change the washing solution several times until the protein bands are distinguishable on a clear background. The more thoroughly the gel is washed, the less protein can be found by this method. Discolouration can be accelerated by adding a few grams of anion-exchange resin or a small piece of porous material to the *washing solution R.*

NOTE. Acid-alcohol solutions used in this procedure do not allow the complete fixation of proteins in the gel. This can lead to the loss of some low-molecular-weight proteins during the staining and discolouration of thin gels. Complete fixation is possible when the gel is kept in a mixture of trichloroacetic acid R/methanol R/water R (1:4:5, V/V/V) for 1 h before immersing the gel in the Coomassie staining solution R.

Silver staining. Immerse the gel in a large volume of *fixing solution R* and allow it to stand for 1 h. Drain the fixing solution, add a new portion of the fixing solution, and allow it to stand for at least 1 h or, if possible, overnight.

Then drain the fixing solution and wash the gel with a large volume of *water R* for 1 h. Next, keep the gel in a *1% solution of glutaraldehyde R* (*V/V*) for 15 min, wash twice with large volume of *water R*, each time for 15 min, place in a freshly prepared *silver nitrate reagent R*, and allow to stand in a dark place for 15 min. Then wash thrice with large volume of *water R*, each time for 5 min. Keep the gel in *developing solution R* for about 1 min until sufficient staining. The development is stopped by placing the gel in a *blocking solution R* for 15 min. Rinse the gel with *water R*.

5-4. PRESENTATION OF RESULTS

Gels are photographed and scanned while they are still wet or after an appropriate drying method. Currently, gel scanning systems are available that are equipped with data analysis software and allow to photograph and analyse wet gels immediately.

Treat the gels depending on the staining method used. Allow Coomassie-stained gels after the discolouration stage to stand in a 100 g/L solution of *glycerol R* for at least 2 h (incubation during the night is possible). In the case of silver staining at the final stage of washing, the gels are kept in a 20g/L solution of *glycerol R* for 5 min.

Drying of stained DSN-polyacrylamide gels is one of the methods used for long-term documentation. This method often causes the gel cracking in the process of drying between the cellulose films.

Immerse two sheets of porous cellulose film in *water R* and allow to stand for 5-10 min. Stretch one of the film sheets on the frame for drying, carefully place the gel soaked in the solution on the stretched cellulose film, remove all accidentally trapped air bubbles, pour several millilitres of *water R* around faces of the gel, place the second film sheet on top and again remove all air bubbles, finish assembling the frame for drying the gel, and place it in the drying cabinet or leave it at room temperature until the gel dries completely.

5-5. DETERMINATION OF MOLECULAR WEIGHT

Determine molecular weights of proteins by comparing their mobility with the mobility of several marker proteins with a known molecular weight. For gel calibration, mixtures of previously stained and unstained proteins with accurately known molecular weights are available, mixed for uniform staining. Mixtures for different ranges of molecular weights were obtained. Dilute concentrated solutions of proteins with a known molecular weight with the appropriate sample buffer and apply to the same gel as for the test sample.

Immediately after electrophoresis, note the position of the bromophenol blue dye band, which corresponds to the leading edge of the electrophoretic ion front. This can be done by applying an incision to the edge of the gel or by puncturing the gel in the stained area with a needle previously dipped in black ink or other suitable contrast dye. After gel staining, measure the development for each protein band (markers or test sample) from the top of the resolving gel. Calculate the ratio of the distance traveled by each protein to the distance traveled by the dye. The normalised development distance calculated in this way is called relative mobility of proteins (relative to the dye front), denoted as R_F. Plot the dependence of the logarithm of the relative molecular masses (*Mr*) of protein reference samples against the *RF values obtained*. Unknown molecular weights are determined using the linear regression method (for a more accurate result, using the nonlinear regression method) or by interpolating the log M_r - *RF* curves, if the values obtained for the test samples are located on the linear part of the graph.

5-6. VALIDATION OF THE TEST

The test results are considered reliable if the required range of gel resolution is confirmed by the distribution of marker proteins with known molecular weights, for example, over 80% of the gel length.

The separation obtained for marker proteins should show a linear dependence of the logarithm of molecular weight on *RF*. If the graph has a sigmoid shape, then only data from a section of the linear area of the curve can be used in calculations. A monograph may specify additional validation requirements for the test sample.

Sensitivity should also be validated. To test the suitability of the system, a protein reference standard with a concentration corresponding to the required limit can be used, analysed simultaneously with the test samples.

5-7. QUANTITATION OF IMPURITIES

SDS-PAGE is often used when testing for impurity limits. If the impurity content is determined by normalisation and relative to the baseband using densitometric integration or image analysis, the linearity of the signal must be validated. Note that the linearity range may vary depending on the detection method, but it must be determined at each test using one or more reference samples containing proteins in a suitable concentration range.

In cases where the impurity limit is specified in the monograph, a reference solution corresponding to this level of impurity content, prepared by diluting the test solution, should be used in the test. For example, if the maximum impurity content is 5%, it is necessary to prepare a reference solution by diluting the test solution in a ratio of 1:20. On the electrophoregram with the test solution, no band other than the base one should be more intense than the band obtained with the reference solution.

For a validated procedure, the impurity content can be quantified by internal normalisation with respect to the main band using an integrating densitometer or by image analysis. When validating the procedure, linearity must be confirmed.

2.1.2.31. Loss on drying

Determination of the loss on drying is carried out using one of the above methods and expressed as a percentage (*m/m*).

Procedure. Place the quantity of the test sample specified in the monograph in a tared weighing bottle, pre-dried under the conditions described for the test sample. Dry the sample in a weighing bottle with the lid open to constant weight by one of the following methods, if necessary, cool down in a desiccator and weigh with the lid closed. If the temperature range is not specified, then dry at the specified temperature ±2 °C.

Method 1. Dry the sample in an oven, the suitability of which is tested in accordance with established quality system methods, for example, using suitable certified reference standards. Unless otherwise specified in the monograph, dry for 2 hours at 105 °C, then place the open weighing bottle together with the lid in a desiccator to cool down for 50 minutes, after which close it with a lid and weigh. Carry out subsequent weighing after each hour of further drying to a constant mass.

Method 2. Dry over *phosphorus (V) oxide R* by one of the following methods:

- in a desiccator at atmospheric pressure and room temperature

- in vacuo at a pressure between 1.5 kPa and 2.5 kPa and at room temperature or the temperature specified in the monograph or in a regulatory document on quality;

- under "high vacuum": at a pressure of no greater than 0.1 kPa and temperature specified in the monograph or regulatory documentation.

If other conditions are used, the procedure employed is fully described in the monograph.

2.1.2.32. Osmolality

Osmolality is an indicator that helps estimate the total contribution of various solutes to the osmotic pressure of a solution.

An approximate calculation of the osmolality ξ_m of an aqueous solution is carried out using the following formula:

$$\xi_m = \upsilon \, m \, \Phi,$$

where υ is the total number of ions formed from a single solute molecule as a result of dissociation. If the solute does not dissociate into ions, $\upsilon = 1$;

m is the molality of the solution, i.e. the number of moles of solute per kilogram of solvent;

ϕ is the molal osmotic coefficient, which takes into account the interaction between ions of the opposite sign in the solution and depends on the value of *m*. As the composition of the solution becomes more complex, the determination of the value ϕ becomes more complicated.

The unit of osmolality is osmole per kilogram (Osmol/kg), however, as a rule, the unit of milliosmole per kilogram (mOsmol/kg) is used.

Unless otherwise specified in the monograph, determine osmolality by the freezing-point depression of the solution. The relationship between osmolality and a decrease in the freezing point ΔT is expressed as follows:

$$\xi_m = \frac{\Delta T}{1.86} \cdot 1000 \text{ mOsmol/kg}.$$

Apparatus. The apparatus (osmometer) cosists of:

- a device for cooling the vessel with a measuring cell;

- a temperature measuring system consisting of a temperature-sensitive resistor (thermistor) with an appropriate device for measuring current or potential difference. The measuring instrument can be calibrated in degrees of temperature drop or directly in osmolality units;

Table 2.1.2.32.-1. – *Reference solutions for osmometer calibration*

Mass of *sodium chloride R*, in grams per kilogram of *water R*	Actual osmolality (mOsmol/kg)	Expected osmolality of an ideal solution (mOsmol/kg)	Molal osmotic coefficient	Cryoscopic lowering of the temperature (°C)
3.087	100	105.67	0.9463	0.186
6.260	200	214.20	0.9337	0.372
9.463	300	323.83	0.9264	0.558
12.684	400	434.07	0.9215	0.744
15.916	500	544.66	0.9180	0.930
19.147	600	655.24	0.9157	1.116
22.380	700	765.86	0.9140	1.302

- usually a device for mixing the sample.

Procedure. Prepare standard solutions in accordance with Table 2.1.2.32.-1. Set the zero value on the scale of the apparatus using *water R*. Calibrate the apparatus using standard solutions: place the corresponding volumes of standard solution in the measuring cell and start cooling the system. To prevent overcooling, the measuring instrument is usually programmed to operate at temperatures lower than the expected cryoscopic drop. A suitable instrument indicates that system is equilibrated. Before each measurement, rinse the measuring cell with the appropriate reference solution.

Perform the same operations with the test solution. Before each measurement, rinse the measuring cell with the test solution. The results are either determined directly from the instrument scale, or calculated from the measured freezing-point depression. The results are considered reliable if the obtained osmolality value of the test solution does not exceed the osmolality values of the two reference solutions used for calibration.

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2.1.2.33. Conductivity

The current I (in amperes) flowing through the conductor is directly proportional to the applied electromotive force E (in volts) and inversely proportional to the resistance of the conductor R (in ohms):

$$I = \frac{E}{R}.$$

The conductivity (formerly called specific conductance) of a solution (κ) is, by definition, the reciprocal of resistivity (ρ). Resistivity is defined as the quotient of the electric field and the density of the current. The resistance R (in Ω) of a conductor of cross-section area S (in cm^2) and length L (in cm) is given by the expression:

$$R = \rho \frac{L}{S}.$$

Thus:

$$R = \frac{1}{\kappa} \cdot \frac{L}{S}$$

or

$$\kappa = \frac{1}{R} \cdot \frac{L}{S},$$

where L/S corresponds to the ideal cell constant.

The unit of conductivity in the International System is α the siemens per metre ($S \cdot m^{-1}$). In practice, the electrical conductivity of a solution is expressed in siemens per centimetre ($S \cdot cm^{-1}$) or in microsiemens per centimetre ($\mu S \cdot cm^{-1}$). The unit of resistivity in the International System is the ohm-metre (Ω·m). The resistivity of a solution is generally expressed in ohm-centimetres (Ω·cm). Unless otherwise prescribed in the monograph, the reference temperature for the expression of conductivity or resistivity is 25 °C.

The apparatus and operating procedure described below are applicable to laboratory measurement of conductivity greater than 10 $\mu S \cdot cm^{-1}$.

The measurement of conductivity of water is dealt with in the relevant monographs.

APPARATUS

The apparatus used (conductivity meter or resistivity meter) measures the resistance of the column of liquid between the electrodes of the immersed measuring device (conductivity cell). The apparatus is supplied with alternating current to avoid the effects of electrode polarisation. It is equipped with a temperature probe and a temperature compensation device.

The conductivity cell contains 2 parallel platinum electrodes coated with platinum black, each with a surface area *S*, and separated from the other by a distance *L*. Both are generally protected by a glass tube. Other types of cells may also be used.

OPERATING PROCEDURE

Determination of the cell constant

Choose a conductivity cell that is appropriate for the properties and conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen (low ρ). Commonly used conductivity cells have cell constants of the order of 0.1 cm^{-1}, 1 cm^{-1} and 10 cm^{-1}. Use a certified reference substance, for example, a solution of potassium chloride, that is appropriate for the measurement. The conductivity value of the certified reference substance should be near the expected conductivity value of the test solution. Other certified reference materials may be used especially for cells having a constant of 0.1 cm^{-1}. Wash the cell several times with *distilled water R* and at least twice with a certified reference standard used to determine the constant of the conductivity cell. Measure the resistance of the conductometric cell using a certified reference standard at a temperature of 25 ± 1 °C.

The cell constant K_{cell} (cm^{-1}) depends on the geometric shape of the conductometric cell and is calculated using the formula:

$$K_{cell} = R_{CRS} - K_{CRS}$$

where R_{CRS} is the measured resistance in megaohms;

K_{CRS} is the electrical conductivity of the certified reference standard in microsiemens per centimetre.

The measured cell constant value K_{cell} of the conductometric cell must not differ from the specified value by greater than 5%.

If the constant of the conductometric cell is determined at a temperature different from that specified for a certified reference standard, calculate the electrical conductivity value using the formula:

$$K_T - K_{TCRS} \cdot [1 + a\ (T - TCRS\ )1$$

where K_T is the value of electrical conductivity at a different temperature;

K_{TCRS} is the value of electrical conductivity of the certified reference standard;

T is the temperature for calibration;

T_{CRS} is the temperature specified for the certified reference standard;

α is the temperature coefficient for the electrical conductivity of the certified reference standard; for potassium chloride, α = 0.021.

Determination of the specific electrical conductivity of the test solution

After calibration of the apparatus using a solution of the certified reference standard, wash the conductometric cell several times with *distilled water R* and at least twice with the aqueous solution to be examined. Carry out subsequent measurements as specified in the monograph.

2.1.2.34. Near-Infrared Spectroscopy

Near-infrared (NIR) spectroscopy is a technique with wide and varied applications in pharmaceutical analysis. The NIR spectral range extends from 780 nm to 2500 nm (wavelength range from 12,800 cm^{-1} to 4000 cm^{-1}). NIR spectra are dominated by C-H, N-H, O-H, and S-H overtones and combinations of fundamental mid-infrared (MIR) vibrations. They contain composite chemical and physical information and, in most cases, this information can be extracted by suitable mathematical data treatment. NIR bands are much weaker than the fundamental MIR vibrations from which they originate. Because absorptivities in the NIR range are low, radiation can penetrate up to several millimetres into materials (including solids). Furthermore, many materials such as glass are relatively transparent in this region.

Measurements can be made directly *in situ*, in addition to standard sampling and testing procedures. NIR measurements can be performed *off-line*, and also *at-line* or *in-line*, and *on-line* for process analytical technology (PAT). Suitable chemometric methods may be required for identification. However, when the specificity criteria for a qualitative method are met, chemical identification or solid-state characterisation is possible by direct comparison of the untreated or pre-treated spectra obtained with the chemical substance being examined with a spectrum of a reference substance.

NIR spectroscopy has a wide variety of applications for chemical, physical and process analysis, for example:

Chemical analysis:

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical materials and packaging materials;
- qualification of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;
- quantitation of active substances in a sample matrix, determination of chemical values such as hydroxyl value, determination of absolute water content, determination of the degree of hydroxylation, and control of solvent content.

Physical analysis:

- crystalline form and crystallinity, polymorphism, solvates, particle size;
- disintegration, hardness;
- film properties.

Process analysis:

- monitoring of unit operations such as synthesis, crystallisation, blending, drying, granulation and coating, for the purpose of process control;
- control and endpoint detection.

Measurements in the NIR region are influenced by many chemical and physical factors as described below; the reproducibility and relevance of results depend on control of these factors and measurements are usually valid only for a defined calibration model.

APPARATUS

All NIR measurements are based on passing light through or into a sample and measuring the attenuation of the emerging (transmitted or reflected) beam. Spectrometers for measurement in the NIR region consist of a suitable light source (such as a highly-stable quartz-tungsten lamp), a monochromator or interferometer, and a detector. Common monochromators are acousto-optic tunable filters (*AOTF*), gratings, or prisms. Traditionally, many NIR instruments have a single-beam design, though some process instruments use internal comparison and can therefore be dual-beam (for example in diode array instruments). Silicon, lead sulfide, and indium gallium arsenide are examples of detector materials.

Conventional cuvette sample holders, fiber-optic probes, transmission dip cells, neutral borosilicate vials, and spinning or traversing sample holders are a few examples of sampling devices. The selection is based on the intended application, paying particular attention to the suitability of the sampling system for the type of sample to be analysed. Suitable data processing and evaluation units (e.g. software and computer) are usually part of the system.

It is common to express the wavelength (λ) in nanometres and the wavenumber (ν) in reciprocal centimetres (cm^{-1}), depending on the measurement technique and apparatus. Conversion between nanometres and inverse centimetres is performed according to the following expression:

$$\nu_{cm}^{-1} = 10^7 \cdot \frac{1}{\lambda_{nm}}.$$

MEASUREMENT METHODS

Transmission mode. Transmittance (*T*) is a measure of the decrease in radiation intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and the detector. The arrangement is analogous to that in many conventional spectrometers. The resulting spectrum can be presented directly in terms of transmittance (*T*) and/or absorbance (*A*) (*y*-axis) versus the wavelength or wavenumber (*x*-axis).

$$T = \frac{I}{I_0},$$

where *I* is the intensity of incident radiation;
I_0 is the intensity of the transmitted radiation.

$$A = -\log_{10} T = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right).$$

Diffuse reflection mode. The diffuse reflection mode gives a measure of reflectance (*R*), the ratio of the intensity of light reflected from the sample (*I*) to that reflected from a background or reference reflective surface (I_r).

Depending on the chemical composition and physical characteristics of the sample, NIR radiation can penetrate a more or less substantial distance into the sample, where it can be absorbed by the overtones and combinations of the fundamental vibrations of the analyte species present in the sample. Non-absorbed radiation is reflected back from the sample to the detector. NIR reflectance spectra are typically obtained by calculating and plotting $\log_{10}$ (1/R) (*y*-axis) versus the wavelength or wavenumber (*x*-axis).

$$R = \frac{I}{I_r},$$

where *I* is the intensity of light diffusively reflected from the sample;

I_0 is the intensity of light reflected from the background or reference reflective surface.

$$A_R = \log_{10}\left(\frac{1}{R}\right) = \log_{10}\left(\frac{I_r}{I}\right).$$

Transflection mode. This mode is a combination of transmittance and reflectance. In the measurement of transflectance (*T**), a mirror or a diffuse reflectance surface is used to reflect the transmitted radiation back through the sample, thus doubling the pathlength. Non-absorbed radiation is reflected back from the sample to the detector.

$$T^* = \frac{I}{I_T},$$

where I_T is the intensity of transflected radiation measured from the sample;

I is the intensity of transflected radiation of the reference material as background.

$$A^* = \log_{10}\left(\frac{1}{T^*}\right) = \log\left(\frac{I_T}{I}\right).$$

SAMPLE PREPARATION/PRESENTATION

Sample preparation and presentation may vary according to the measurement mode. The following requirements are necessary for all sampling procedures:

- optimise the measuring time and number of scans to optimise the signal-to-noise ratio;

- find the best suitable measurement mode for the intended application (transmission, diffuse reflection, or transflection);

- find the best orientation of the sample (e.g. to minimise the impact of debossing on tablets);

- find the best suitable accessory (e.g. transmission cell or immersion probe);

- optimise pathlength in transmission and transflection modes;

- find a suitable spectroscopic background reference standard;

- show that the background reference standard is reliable over time and that the measurement of the background is reproducible and stable over time;

- when measuring moving materials or samples (for process-related measurements) it is important to obtain a representative spectrum (e.g. by adjusting the measuring time, the number of scans, co-adding individual spectra, or increasing the beam size);

- ensure there is no fouling of the sensor, for example with a build-up of material or contamination;

- the measuring conditions (measuring time, beam size) in relation to the minimal sample size should be justified.

In some process analysis situations, it may be impossible to remove a probe for reference background data collection; various options are therefore to be considered, including internal comparison, measurement of a background reference using a second detector, etc. Only spectra measured against a background possessing the same optical properties can be directly compared with one another.

Transmission mode. The measurement of transmittance (*T*) is dependent on a background transmittance spectrum for its calculation. Examples of background references include air, a polymeric disc, an empty cell, a solvent blank or in special cases a reference standard. The method generally applies to liquids (diluted or undiluted), dispersions, solutions and solids (including tablets and capsules).

For transmittance measurements of solids, a suitable sample accessory is used. Liquid samples are examined in a cell of suitable pathlength (typically 0.5-4 mm), transparent to NIR radiation, or alternatively by immersion of a fibre-optic probe of a suitable configuration.

Diffuse reflection mode. This mode generally applies to solids. The sample is examined directly, or in a suitable device (for example a sample holder), or in direct contact with a fiber-optic probe. For process monitoring, the material can be analyzed through a polished window interface (e.g. sapphire) or using an in-line fiber-optic probe. Care must be taken to ensure that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references include ceramic, thermoplastic resins and gold. Other suitable materials may be used.

Transflection mode. This mode generally applies to liquids, suspensions, and clear plastic materials. A reflector is placed behind the sample so as to double the pathlength. This configuration can be adopted to share the same instrument geometry with reflectance and fiber-optic probe systems where the source and the detector are on the same side of the sample. The sample is examined through a cell with a mirror or a suitable diffusive reflector, made either of metal or of an inert substance (for example, dried titanium dioxide) not absorbing in the NIR region. Liquids can also be measured using *in-line* transflectance probes.

FACTORS AFFECTING SPECTRAL RESPONSE

Environment. The environment temperature and humidity must be taken into consideration before carrying out measurements.

Sample presentation area. The sample presentation area or probe end must be clean of residue prior to measurement. Similarly, the *in-line* or *on-line* interface to the sample should not have a significant product or contamination build-up, which would interfere with the desired measurement.

Sample temperature. This parameter is important for aqueous solutions and many liquids, where a difference of a few degrees can result in measurable spectral changes which may have a significant effect on the analysis. Temperature is also an important parameter for solids and powders containing water.

Moisture and solvent residues. Moisture and solvent residues present in the samples will contribute significant absorption bands in the NIR region.

Sample thickness. Sample thickness is a known source of spectral variability and must be assessed and/or controlled, particularly for tablet and capsule analysis in transmittance mode. For the measurement of compressed powders, an infinite thickness is typically reached after 5 mm of sample depth (e.g. in a vial).

Sample optical properties. In solids, both surface and bulk scattering properties of samples must be taken into account. Spectra of physically, chemically, or optically heterogeneous samples may require increasing the beam size, or examining multiple samples, or spinning the sample to obtain a representative spectrum of the sample. Certain factors such as differing degrees of compaction or particle size in powdered materials and surface finish can cause significant spectral differences.

Solid-state forms. Variations in solid-state forms (polymorphs, hydrates, solvates and amorphous forms) influence vibrational spectra. Hence, different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration samples have a distribution of forms relevant to the intended application.

Age of samples. Samples may exhibit changes in their chemical, physical or optical properties over time. Depending on the storage conditions, solid samples may either absorb or desorb water, and portions of amorphous material may crystallise. Materials used for NIR calibration should be representative of future samples and their matrix variables.

PRE-TREATMENT OF NIR SPECTRAL DATA

In many cases, and particularly for reflection mode spectra, some form of mathematical pre-treatment of the spectrum may be useful prior to the development of a classification or calibration model. The aim can be, for example, to reduce baseline variations, to reduce the impact of known variations that are interfering in the subsequent mathematical models, or to simplify data before use. In some cases, spectra may also be normalised or corrected for scattering, for example using standard normal variate (SNV) transformation. Spectral pre-treatment techniques may include, for example, windowing and noise reduction and the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended because of increased spectral noise.

CONTROL OF INSTRUMENT PERFORMANCE

Use the apparatus according to the manufacturer’s instructions and carry out the prescribed verification at regular intervals, according to the use of the apparatus and the application. For *in-line* and *on-line* applications, the use of alternative means of control of instrument performance must be scientifically justified. For example, utilise the standards built into the instrument or separate channels/probes to demonstrate instrument performance (pending practicality).

Table 2.1.2.34.-1. – *Control of instrument performance*

Measurement mode	Reflection	Transflection	Transmission
Verification of wavelength scale (except for filter apparatus)	Typical tolerances for agreement with standard values are: ±1.0 nm at 780 nm (±16 cm^{-1} at 12,800 cm^{-1}); ±1.0 nm at 1200 nm (±8 cm^{-1} at 8300 cm^{-1}); ±1.0 nm at 1600 nm (±6 cm^{-1} at 6250 cm^{-1}); ±1.5 nm at 2000 nm (±4 cm^{-1} at 5000 cm^{-1}); ±1.5 nm at 2500 nm (±2 cm^{-1} at 4000 cm^{-1}). For the reference material used, apply the tolerance for the nearest wavelength or wavenumber for each peak used. For diode array instruments, most often the pixel resolution (wavelength between pixels) can be as large as 10 nm. The pixel resolution must be adapted to match the spectral resolution. The peak-finding algorithms are critical to wavelength accuracy. Practically, ± 2 nm is appropriate for peak wavelength accuracy using such instrumentation. Alternatively, refer to the manufacturer's specifications for acceptance.		
Bench/mobile instrument	Measure *talc R* via a suitable medium or by a fiber-optic probe. *Talc R* has characteristic peaks at 948 nm, 1391 nm, and 2312 nm suitable for calibration. Alternatively, other suitable reference standards may also be used that ensure wavelength accuracy in the procedure working range. For example, measure an internal polystyrene standard if built-in, or measure a *NIST* reference standard or other traceable material, and assess 3 peaks across the wavelength range for calibration.	A suspension of 1.2 g of dry *titanium dioxide R* in about 4 mL of *methylene chloride R* is used directly with a cell or a probe. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 cm^{-1} at 4000 cm^{-1}). Methylene chloride has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose three peaks across the wavelength range for calibration. Other suitable reference standard may also be used, such as a liquid transflection standard mixed with titanium dioxide or some other reflective medium.	*Methylene chloride R* may be used and has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose three peaks across the wavelength range for calibration. Other suitable reference standard may also be used.
Process instrument	If it is not practically possible to measure a traceable reference standard at the point of sample measurement, use internal material such as polystyrene, fiberglass, or solvent and/or water steam. Alternatively, adopt a second external channel/probe. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapour line, for example, the line at 7306.74 cm^{-1}, or 7299.45 cm^{-1}, or 7299.81 cm^{-1}, or a narrow line from a certified reference material.		
Verification of wavelength repeatability (except for filter apparatus)	The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified.		

Table 2.1.2.34.-1. – *(continued)*

Measurement mode	Reflection	Transflection	Transmission
Bench/mobile instrument	Check the repeatability of the wavelengths using a suitable external or internal reference standard.		
Process instrument	Check the repeatability of the wavelengths using a suitable external or internal reference standard.		
Verification of photometric linearity and response stability(1)	Measure 4 photometric standards across the procedure absorbance working range.		
Bench/mobile instrument	Analyze 4 reflectance standards, for example in the range of (10-99)%, including 10%, 20%, 40%, and 80%. In some circumstances, 2% may be appropriate. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.	Transflection measurements can use appropriate reflectance or transmittance standards and criteria.	Analyze 4 transmittance standards to cover the absorbance values over the working absorbance range of the modeled data. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.
Process instrument	If photometric reflectance and transmittance standards cannot be measured at the point of sample measurement, use the photometric standards built into the instrument. Process instruments can use internal photometric standards for photometric linearity. Follow the manufacturer's verified tolerances in such cases.		
Verification of photometric noise(1)	Determine the photometric noise at a relevant photometric region of the spectrum using a suitable reflectance standard, for example, white reflective ceramic tiles or carbon-doped polymer standards. Follow the manufacturer's methodology and specifications.		
Bench/mobile instrument	Scan the reflectance low flux reference standard (e.g. 5% or 10%, carbon-doped polymer standard) over a suitable wavelength range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.		Scan the transmittance high flux standard (e.g. 90% or 99%, carbon-doped polymer standard) over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.

Table 2.1.2.34.-1. – *(cont.)*

Measurement mode	Reflection	Transflection	Transmission
Process instrument	As above, or if not practically possible, use the reference standard built into the instrument for noise testing and manufacturer specifications.		As above, or if not practically possible, use the reference standard built into the instrument for noise testing and manufacturer specifications.

(1) *Verification of photometric linearity* and *Verification of photometric noise* are not required for instruments using methods to perform simple identification test which do not use the photometric absorption as part of the model strategy (for example, simple correlation with absorption wavelengths).

System suitability tests may be required prior to sample scanning, and the instrument attributes with potential impact on the suitability of the final measurement (typically photometric noise and wavelength accuracy) must be tested. The frequency at which each performance test is carried out must be risk-assessed depending on the instrument type and its environment. For example, instruments placed in harsh environments with variations in temperature and humidity may need frequent performance testing. Cases where the measurement system cannot be removed such as an *in-line* probe or flow cell are also to be considered.

Some accessories are custom designed and therefore require adequate performance testing.

Verification and calibration of the wavelength or wavenumber scale (except for filter apparatus). Verify the wavelength scale employed, generally in the region between 780 nm and 2500 nm (12,800 cm^{-1} to 4000 cm^{-1}) or in the intended spectral range using one or more suitable wavelength standards which have characteristic maxima or minima within the wavelength range to be used. For example, *methylene chloride R, talc R*, wavelength reference lamps, or a mixture of rare-earth oxides are suitable reference materials. Other suitable standards may also be used. Obtain a spectrum and measure the position of at least 3 peaks distributed over the range used. For rare-earth oxides, suitable reference materials are provided by the National Institute of Standards and Technology (*NIST).* Fourier transform (FT) instruments have a linear frequency range, therefore wavelength certification at a single frequency is sufficient.

Verification and calibration of photometric linearity. The photometric linearity is demonstrated with a set of transmittance or reflectance standards with known percentage values of transmittance or reflectance. For reflectance measurements, carbon-doped polymer standards are available. Ensure that the absorption of the materials used is relevant to the intended linear working range of the procedure. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. Non-linear calibration models and hence non-linear responses are acceptable with understanding demonstrated by the user.

Spectra obtained from reflectance and transmittance standards are subject to variability due to the differences between the experimental conditions under which they were factory-calibrated and those under which they are subsequently put to use. Hence, the percentage reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an “absolute” calibration for a given instrument.

As long as the standards do not change chemically or physically and the same reference background is used as was used to obtain the certified values, subsequent measurements of the same standards under identical conditions, including precise sample positioning, give information on long-term stability of the photometric response. Deviation of ± 2% of the absorbance value is acceptable for long-term stability; this verification is only necessary if the spectra are used without pre-treatment.

Recommendations for the conditions used to control instrument performance for the various measurement modes are summarised in Table 2.1.2.34.-1.

QUALITATIVE ANALYSIS (IDENTIFICATION AND CHARACTERISATION)

Establishment of a spectral reference library. Record the spectra of a suitable number of representative samples of the substance which have known, traceable identities and exhibit typical variability (for example, solid-state form, particle size, etc.). Libraries are built using representative samples under appropriate environmental conditions. The set of spectra obtained represents the information that can be used for the identification of the sample to be analysed.

The collection of spectra in the library may be represented in different ways defined by the mathematical technique used for identification. These may be:

- all individual spectra representing the substance;
- a mean spectrum of the measured batches for each chemical substance;
- if necessary, a description of the variability within the substance spectra.

The number of substances in the library depends on the specific application. All spectra in the used library have the same:

- spectral range and number of data points;
- technique of measurement;
- data pre-treatment.

If sub-groups (sub-libraries) are created, the above criteria are applied independently for each group. Sub-libraries are individually validated. Original spectral data for the preparation of the spectral library must be archived. Caution must be exercised when performing any mathematical transformation, as artifacts can be introduced or essential information (important with qualification methods) can be lost. The suitability of the algorithm used should be demonstrated by successful procedure validation and in all cases, the rationale for the use of transform must be documented.

Direct comparison of substance and reference spectra. Direct comparison of representative spectra of the substance to be examined and of a reference substance for qualitative chemical or physical identification purposes may not require use of a reference spectral library where specificity permits.

Data evaluation. A direct comparison of the representative spectrum of the substance to be examined is made with the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification; alternatively, this can be achieved visually by overlaying spectral data if specificity is inherent. There are different techniques available, such as principal component analysis, cluster analysis, and soft independent modelling by class analogy. The reliability of the procedure chosen for a particular application has to be validated.

Validation of the model. Identification procedures using direct spectral comparison must be validated in accordance with identification procedure validation process. The validation parameters for qualitative procedures are robustness and specificity.

LIMIT ANALYSIS

Relative comparison of spectra. Calibration is not required when comparing a set of spectra for limit analysis purposes, such as the maximum or minimum absorbance at which an analyte absorbs.

Also, dryer endpoint control may use a qualitative approach around a specific absorbing wavelength. Suitability of spectral ranges and pre-treatments (if used) must be shown to be fit for purpose.

Specificity. The relative discriminatory power for a limit test must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the measurement.

TREND ANALYSIS

Relative comparison of spectra. Calibration is not necessarily required when comparing a set of spectra for trend analysis purposes, such as the moving block approach to estimate statistical parameters such as mean, median, and standard deviation. For example, blend uniformity monitoring using NIR spectroscopy has adopted such data analysis approaches. Appropriate spectral ranges and algorithms must be used for trend analyses.

Specificity. The relative discriminatory power for trend analysis must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the trend analysis.

QUANTITATIVE ANALYSIS

Establishment of a spectral reference library for a calibration model. Calibration is the process of constructing a mathematical model to relate the response from a sample scanned using an analytical instrument to the properties of the samples. Any calibration model that can be clearly defined in a mathematical expression and gives suitable results can be used.

Record the spectra of a suitable number of representative samples with known or future-established values of the attribute of interest throughout the range to be measured (for example, the content of water). The number of samples for calibration will depend on the complexity of the sample matrix and interferences (e.g. temperature, particle size, etc.). All samples must give quantitative results within a calibration interval as defined by the intended purpose of the procedure. Multiple linear regression (MLR), principal component regression (PCR), and partial least squares regression (PLS) are commonly used algorithms. For PLS or PCR calibrations, the regression coefficients and/or the loadings should be plotted and the regions of large coefficients or loadings compared with the spectrum of the analyte. Predicted residual error sum of squares (PRESS) plots (or similar) are useful to facilitate the optimising of the number of PCR or PLS factors.

Pre-treatment of data. Wavelength selection or excluding certain wavelength ranges may enhance the accuracy and robustness of calibration models. Wavelength compression (wavelength averaging) techniques may be applied to the data.

Model validation parameters. Analytical performance characteristics to be considered for demonstrating the validation of NIR procedures are similar to those required for any other analytical procedure. Specific acceptance criteria for each validation parameter must meet the intended use of the procedure. Validation parameters for quantitative procedures are accuracy, linearity, precision (repeatability and intermediate precision), robustness, and specificity.

ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters.

TRANSFER OF DATABASES

When databases are transferred to another instrument, spectral range, number of data points, spectral resolution and other parameters have to be taken into consideration. Further procedures and criteria must be applied to demonstrate that the model remains valid with the new database or new instrument.

201020035-2019

2.1.2.35. Total Organic Carbon in Water for Pharmaceutical Use

Total organic carbon (TOC) determination is an indirect measure of organic substances present in water for pharmaceutical use. TOC determination can also be used to monitor the performance of various operations in the preparation of medicinal products.

A variety of acceptable procedures is available for determining TOC, rather than prescribing a given method to be used, this general chapter describes the procedures used to qualify the chosen method and the interpretation of results in limit tests. A standard solution is analysed at suitable intervals, depending on the frequency of measurements; the solution is prepared with a substance that is expected to be easily oxidisable (for example, sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured. The suitability of the system is determined by analysis of a solution prepared with a substance expected to be oxidisable with difficulty (for example, 1,4-benzoquinone).

The various types of apparatus used to measure TOC in water for pharmaceutical use have in common the objective of completely oxidising the organic molecules in the sample water to produce carbon dioxide followed by measurement of the amount of carbon dioxide produced, the result is used to calculate the carbon concentration in water.

The apparatus used must discriminate between organic and inorganic carbon, the latter being present as carbonate.

The discrimination may be effected either by measuring the inorganic carbon and subtracting it from the total carbon or by purging inorganic carbon from the sample before oxidisation. Organic molecules may also be removed from the test sample during the purging process, but some of the associated carbon in water for pharmaceutical use is insignificant.

Apparatus. Use a calibrated instrument installed either *on-line* or *off-line*. Verify the system suitability at suitable intervals as described below. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

TOC water. Use highly purified water complying with the following specifications:

- conductivity: not greater than 1.0 $\mu S \cdot cm^{-1}$ at 25 °C;
- total organic carbon: not greater than 0.1 mg/L.

Depending on the type of apparatus used, the content of heavy metals and copper may be critical, the manufacturer's instructions should be followed.

Glassware preparation. Use glassware that has been scrupulously cleaned by a method that will remove organic matter. Use *TOC water* for the final rinse of glassware.

Reference solution. Dissolve sucrose R dried at 105 °C for 3 h in *TOC water* to obtain a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

Test solution. Using all due care to avoid contamination, collect water to be tested in an airtight container leaving minimal head-space. Examine the water with minimum delay to reduce contamination from the container and its closure.

*System suitability solution.*Dissolve *1,4-benzoquinone R in TOC water* to obtain a solution having a concentration of 0.75 mg of *1,4-benzoquinone* per litre (0.50 mg of carbon per litre).

TOC water control.

Use *TOC water* obtained at the same time as that used to prepare the *standard solution and the system suitability solution*.

Control solutions. In addition to the TOC water control, prepare suitable blank solutions or other solutions needed for establishing the baseline or for calibration adjustments following the manufacturer's instructions; run the appropriate blanks to zero the instrument.

System suitability. Run the following solutions and record the responses: *TOC water, standard solution, system suitability solution*. Calculate the percentage response efficiency using the following formula:

$$\frac{r_{ss} - r_w}{r_s - r_w} \cdot 100,$$

where r_w is the response of the instrument for *TOC water*;

r_s is the response of the instrument for the *standard solution*;

r_{ss} *is the* response of the instrument *for the system suitability solution.*

The system is suitable if the response efficiency is not less than 85% and not greater than 115% of the theoretical response.

Procedure. Run the test solution and record the response (r_u). The test solution complies with the test if r_u is not greater than r_s - r_w.

The procedure can also be applied using *on-line* instrumentation that has been adequately calibrated and shown to have acceptable system suitability. The location of instrumentation must be chosen to ensure that the responses are representative of the water used.

201020036-2019

2.1.2.36. Chromatographic Separation Techniques

Chromatographic separation techniques are multi-stage separation methods in which the components of a sample are distributed between two phases, one of which is stationary, while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc.

The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion-exchange, etc., or based on differences in the physicochemical properties of the molecules such as size, mass, volume, etc.

This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. Principles of separation, apparatus and procedures are given in the following general methods:

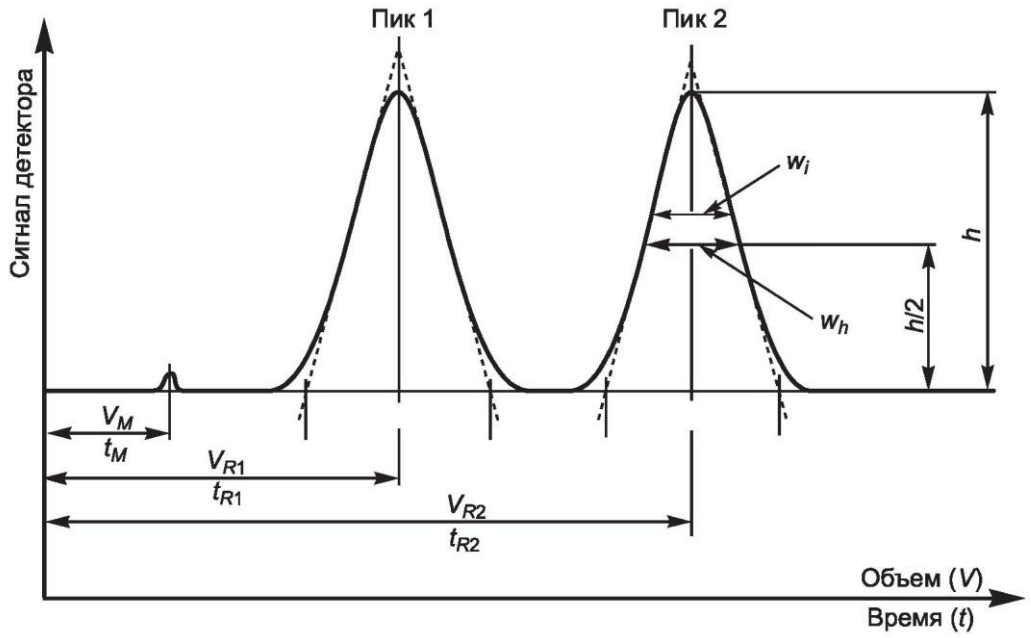
- Paper chromatography (*2.1.2.25*);
- Thin-layer chromatography (*2.1.2.26);*
- Gas chromatography (*2.1.2.27);*
- High-performance liquid chromatography (*2.1.2.28*);
- Size exclusion chromatography (*2.1.2.29*);
- Supercritical fluid chromatography

DEFINITIONS

The system suitability and acceptance criteria in monographs have been set using parameters as defined below. With some equipment, certain parameters, such as the signal-to-noise ratio and resolution, can be calculated using software provided by the manufacturer. It is the user's responsibility user to ensure that the calculation methods used in the software are equivalent to the requirements of the Pharmacopoeia and to make any necessary corrections if this is not the case.

Chromatogram is a graphical or other representation of detector response, effluent concentration or other quantity used as a measure of effluent concentration, versus time or volume. Idealised chromatograms are represented as a sequence of Gaussian peaks on a baseline (Figure 2.1.2.36.-1).

A *peak* is a portion of a chromatogram recording the detector response when a single component (or two or more unseparated components) is eluted from the column.



	Detector response
	Peak 1
	Peak 2
	Volume (V)
	Time (t)

Figure 2.1.2.36-1. – *Schematic representation of the chromatogram.*

The peak may be defined by the peak area, or the peak height (h) and the peak width at half-height (w_h), or the peak height (h) and the peak width between the points of inflection (w_i). In Gaussian peaks (Figure2.1.2.36.-1), there is the following relationship:

$$w_h = 1{,}18\ w_i.$$

An *adsorption zone* forms part of a chromatographic plate containing the adsorbed substance to be determined and visualised as a spot (round or elliptical) or band.

Retention time (t_R) is the time required for elution of a component (Figure 2.1.2.36.-1, baseline scale measured in minutes).

Retention volume (V_R) is the volume of the mobile phase required for the elution of a component. Retention volume may be calculated from the retention time and the mobile phase flow rate (F) in millilitres per minute using the following equation:

$$VR = T_R \times F.$$

Hold-up time (t_M) is the time required for elution of an unretained component (Figure 2.1.2.36.-1, baseline scale measured in minutes). In size-exclusion chromatography, the symbol *t*o (see below) is used.

Hold-up volume (V_M) is the volume of the mobile phase required for the elution of an unretained component. Hold-up volume may be calculated from the hold-up time and the mobile phase flow rate (F) (in millilitres per minute) using the following equation:

$$V_M = T_M \times F$$

In size-exclusion chromatography, the symbol V_0 (see below) is used.

Retention factor (k) is the characteristics defined as:

$$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_C \frac{V_s}{V_M},$$

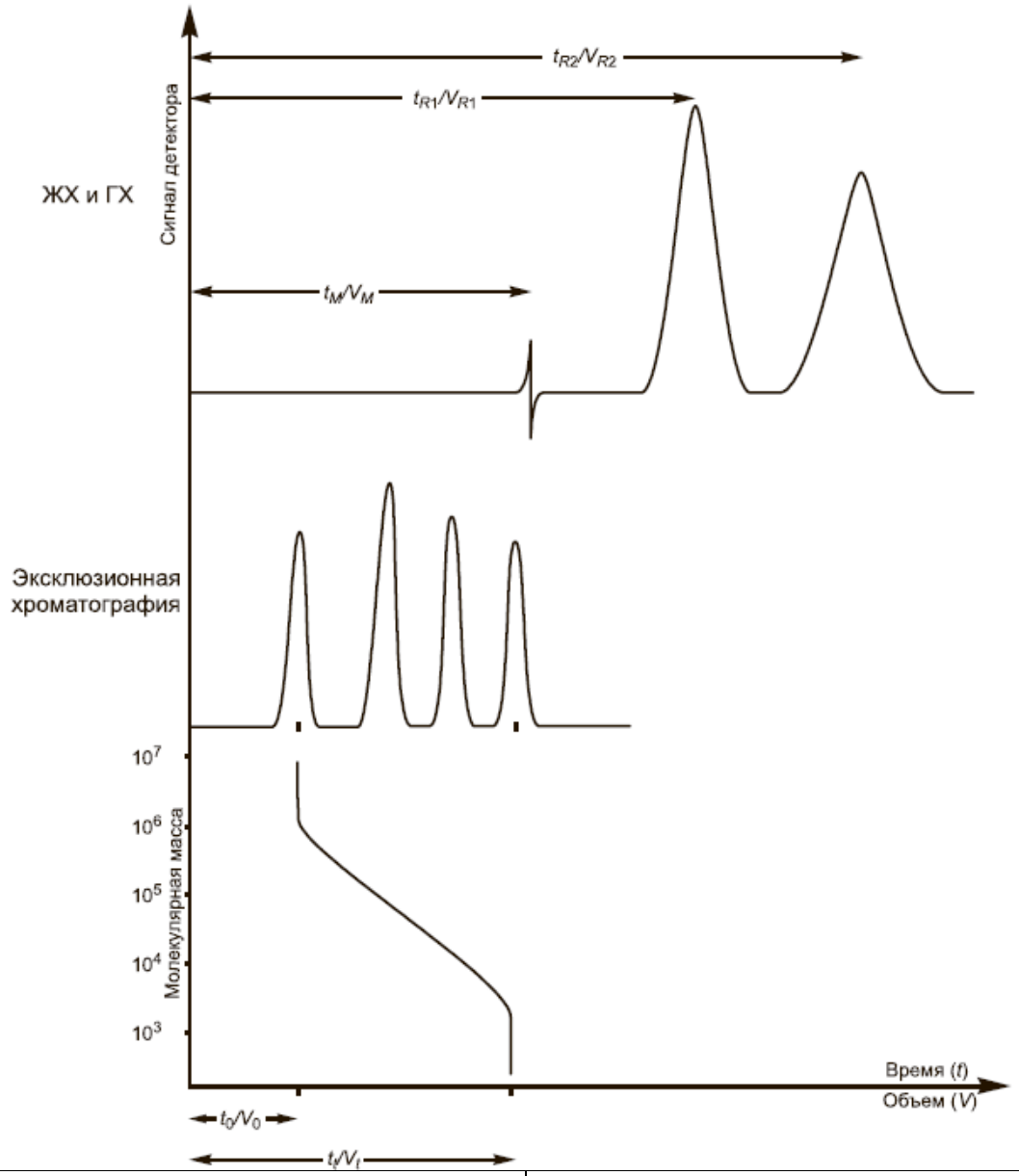
where K_C is the distribution constant (also known as equilibrium distribution coefficient);

V_s is the volume of the stationary phase;

V_M is the volume of the mobile phase.

The retention factor of a component may be determined from the chromatogram using the following equation:

$$k = \frac{t_R - t_M}{t_M}.$$



	LC and GC	
	Detector response	
	Size-Exclusion Chromatography	
	Molecular mass	
	Time (t)	
	Volume (V)	

Figure 2.1.2.36. – *Time/volume indicators of chromatograms.*

Total mobile phase time (t_t) in size-exclusion chromatography is the retention time of a component whose molecules are smaller than the smallest gel pores (Figure 2.1.2.36.-2).

Total mobile phase volume (V_t) in size-exclusion chromatography is the retention volume of a component whose molecules are smaller than the smallest gel pores. Total mobile phase volume may be calculated from the total mobile phase time and the mobile phase flow rate (F) (in millilitres per minute) using the equation:

$$Vt = t_t \times F.$$

Retention time of an unretained component ($t0$) in size-exclusion chromatography is the retention time of a component whose molecules are larger than the largest gel pores (Figure 2.1.2.36.-2).

Retention volume of an unretained component (V_0) in size-exclusion chromatography is the retention volume of a component whose molecules are larger than the largest gel pores.

The retention volume of an unretained component may be calculated from the retention time of an unretained component and the mobile phase flow rate (*F*) (in millilitres per minute) using the equation:

$$V_0 = t_0 \times F.$$

Distribution constant (*K*0) in size-exclusion chromatography is the elution characteristics of a component in a particular column, which is calculated using the equation:

$$K_0 = \frac{t_R - t_0}{t_t - t_0}.$$

Retardation factor (*RF*) is the characteristics of the relative rate of movement of a component in a thin layer (also known as retention factor (*Rf*) in planar chromatography). Retardation factor is equal to the ratio of the distance from the point of application to the center of the spot and the distance traveled by the solvent front from the point of application (Figure 2.1.2.36.-3):

$$R_F = \frac{b}{a},$$

where *b* is the migration distance of the component;

a is the migration distance of the solvent front.

Number of theoretical plates (*N*) is the characteristics of the column performance (apparent efficiency). The number of theoretical plates may be calculated from data obtained under either isothermal, isocratic, or isodense conditions, depending on the procedure, using the equation, where the values of t_R and w_h have to be expressed in the same units:

$$N = 5{,}54\left(\frac{t_R}{w_h}\right)^2,$$

where $t_R$is the retention time of the peak corresponding to the component; w_h is the width of the peak at half-height.

The number of theoretical plates varies with the component as well as with the column, the column temperature, the mobile phase, and the retention time.

Dwell volume (*D*) is the volume between the point at which the two eluents meet and the top of the column (also known as gradient delay volume).

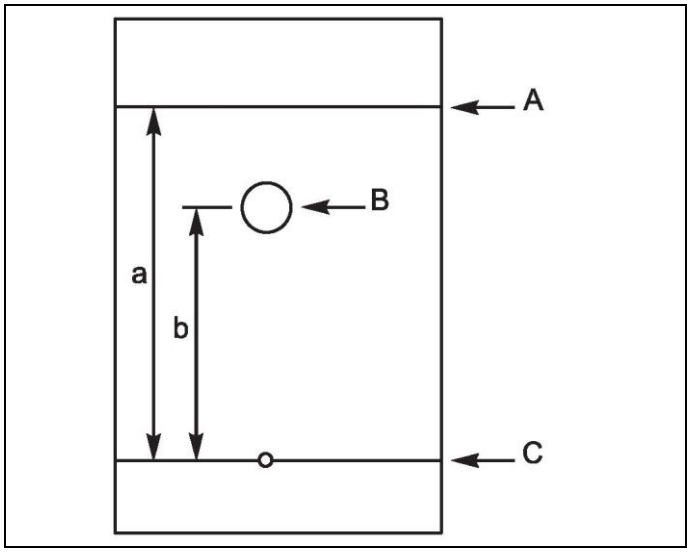


Figure 2.1.2.36. – *Schematic representation of a thin-layer chromatogram. A is the mobile phase front; B is the spot; C is the line of sample application (start line).*

Dwell volume can be determined in the chromatographic conditions below.

Column: replace the chromatographic column with an appropriate capillary tubing (e.g. 1 m long and of 0.12 mm inner diameter).

Mobile phase:

- *mobile phase A*: *water R*;
- *mobile phase B:* 0.1% *(V/V)* solution of *acetone R*;

Time (min)	Mobile phase A (%, *V/V*)	Mobile phase B (%, *V/V*)
0-20	100→0	0→100
20-30	0	100

Mobile phase flow rate: set to sufficient back pressure (for example, 2 mL/min);

Detection: spectrophotometer, at a wavelength of 265 nm.

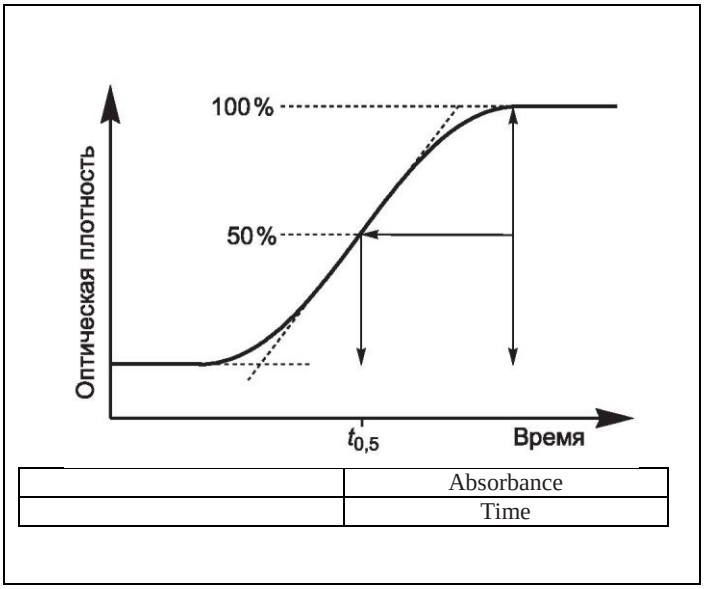
Determine the time ($t_{0.5}$) (in minutes) at which the absorbance increases by 50% (Figure 2.1.2.36.-4).

$$D = t_D \times F,$$

where $t_D = t_{0,5} - 0{,}5t_G$ (in minutes);

t_G is the previously found gradient time (equal to 20 min);

F is the mobile phase flow rate (in millilitres per minute).



	Absorbance
	Time

Figure 2.1.2.36.-4. – *Determination of the gradient delay volume.*

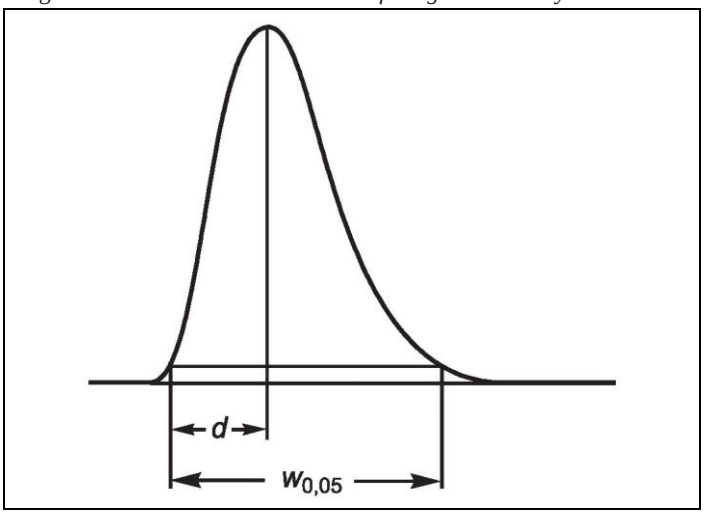


Figure 2.1.2.36.-5. – *Schematic representation of an excessive peak tailing.*

Symmetry factor (A_s) is the characteristics of the symmetry of a peak (Figure 2.1.2.36.-5) calculated using the following equation:

$$A_s = \frac{w_{0,05}}{2d},$$

where $w_{0.05}$is the width of the peak at one-twentieth of the peak height;

d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

An A_s value of 1.0 signifies symmetry. When A_s > 1.0, the peak is tailing; when A_s < 1.0, the peak is fronting.

Resolution (R_s) is the characteristics of the resolution between peaks of 2 components (Figure 2.1.2.36.-1), which may be calculated using the following equation :

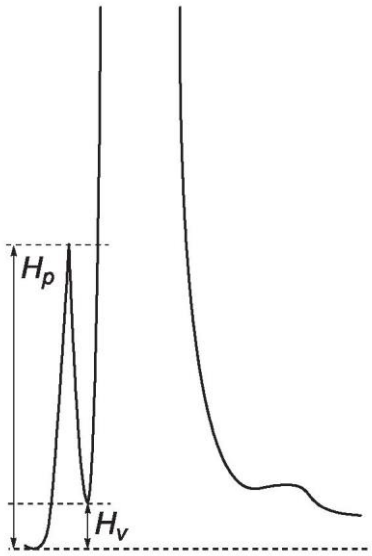


Figure 2.1.2.36.-6. – *Schematic representation of undivided peaks.*

$$R_s = \frac{1{,}18\,(t_{R2} - t_{R1})}{w_{h1} + w_{h2}},$$

where $t_{R2} > t_{R1}$

t_{R1} and t_{R2} are the retention times of the peaks;

w_h1 and w_{h2} are the peak widths at half height.

In quantitative planar chromatography with the aid of densitometry, the development length is used instead of retention time, and the resolution between the peaks of the two components is calculated by the formula:

$$R_s = \frac{1{,}18a\,(R_{F2} - R_{F1})}{w_{h1} + w_{h2}},$$

where R_{F1} и R_{F2} are the retardation factors of the peaks;

w_{h1} and w_{h2} are the peak widths at half height;

a is the migration distance of the solvent front.

Peak-to-valley ratio (P/V) is a characteristic used as a criterion for the suitability of a chromatographic system in the test on related substances when the separation of two peaks to the baseline is not achieved

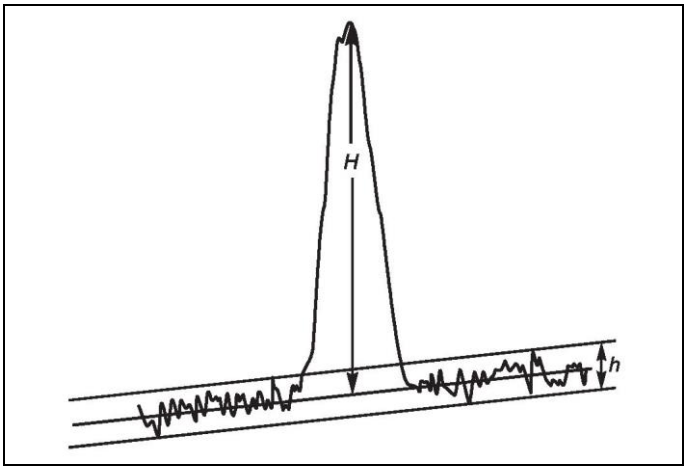


Figure 2.1.2.36.-7. – *Schematic representation of parameters for calculating the signal-to-noise ratio.*

(Figure 2.1.2.36. -6). The peak-to-valley ratio is calculated using the formula:

$$p/v = \frac{H_p}{H_v},$$

where H_p is the height of the smaller peak relative to the extrapolated baseline;

H_v is the height above the extrapolated baseline of the lowest point on the curve that separates the smaller and larger peaks.

Relative retention (r) is the characteristic calculated by the formula:

$$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M},$$

where t_{Ri} is the retention time of the analyte peak;

t_{Rst} is the retention time of the comparison peak (usually the peak of the substance to be examined);

t_M is the hold-up time.

Unadjusted relative retention (r_G) is calculated using the formula:

$$r_G = \frac{t_{Ri}}{t_{Rst}}.$$

Unless otherwise prescribed, the relative retention values specified in monographs correspond to the unadjusted relative retention.

In planar chromatography, the retardation factors R_{Fst} and R_{Fi}. are used instead of t_{Rst} and t_{Ri}. The resulting value is the factor R_{St}.

Signal-to-noise ratio (*S/N*) is the characteristics of the influence of the short-term noise on the precision of quantitation. The signal-to-noise ratio is calculated using the following equation:

$$S/N = \frac{2H}{h},$$

where H is the height of the peak (Figure 2.1.2.36.-7) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to at least five times the width at half-height;

h is the range of the noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

System repeatability is the characteristics of response expressed as an estimated percentage relative standard deviation (s_r(%)) of a consecutive series of measurements for not fewer than 3 injections or applications of a reference solution, and is calculated using the following equation:

$$s_r(\%) = \frac{100}{\bar{y}} \sqrt{\frac{\sum (y_i - \bar{y})^2}{n-1}},$$

where *yi* is the individual values expressed as peak area, peak height, or ratio of areas by the internal standardisation method;

y is the mean of individual values;

n is the number of individual values.

Disregard limit is the limit at which and below which peaks are disregarded.

SYSTEM SUITABILITY

The various components of the equipment employed must be qualified and be capable of achieving the performance required to conduct the test or quantitation.

The system suitability tests represent an integral part of the procedure and are used to ensure adequate performance of the chromatographic system. Apparent efficiency, retention factor (mass distribution ratio), resolution, relative retention and symmetry factor are the parameters that are usually employed in assessing the performance of the column.

Factors that may affect the chromatographic behaviour include:

- the composition, ionic strength, temperature and apparent pH of the mobile phase;
- mobile phase flow rate, column dimensions, column temperature and pressure;
- stationary phase characteristics including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, specific surface area;
- reversed-phase and other surface-modification of the stationary phases (reversed-phase and other modifications), the extent of chemical modification (blocking of the end groups, i.e. end-capping, carbon loading etc.).

The following requirements and any supplementary requirements given in the individual monograph are to be fulfilled unless otherwise prescribed:

- in a related substances test or quantitation, for a peak in the chromatogram obtained with a reference solution used for quantification, the symmetry factor is 0.8 to 1.5, unless otherwise prescribed;
- in a quantitation of an active substance where the value is 100 per cent for a pure substance, the maximum permitted relative standard deviation (s_r(%)max) for the defined limits is calculated for a series of injections of the reference solution using the following equation:

$$s_r(\%)_{\max} = \frac{KB\sqrt{n}}{t_{90\%,\,n-1}},$$

where K is constant (0.349), obtained from the expression $K = \frac{0{,}6}{\sqrt{2}} \cdot \frac{t_{90\%,\,5}}{\sqrt{6}}$ in which $\frac{0{,}6}{\sqrt{2}}$ represents the required percentage relative standard deviation after six sample injections for B = 1.0;

B is upper quantitation limit specified in the the definition of the individual monograph minus 100%;

n is number of replicate injections of the the reference solution ($3 \leq n \leq 6$);

$t_{90\%,\,n\text{-}1}$ — Student's t at the 90% double sided probability level with n-*1* degrees of freedom.

Unless otherwise prescribed, the maximum permitted relative standard deviation does not exceed the appropriate value given in Table 2.1.2.36.-1. This requirement does not apply to tests for related substances.

- in a related substances test, the quantitation limit (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. Depending on various factors, such as the frequency of use of the procedure and experience with the chromatographic system, the analyst chooses an appropriate verification scheme to monitor this compliance.

Table 2.1.2.36.-1. – *Repeatability requirements*

B (%)	Number of individual injections			
	3	4	5	6
	Maximum permitted relative standard deviation			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the procedures are listed below.

Adjustment of conditions with gradient elutions is more critical than with isocratic elutions, since it may lead to shifts in peaks to a different step of the gradient, thus leading to the incorrect assignment of peaks, and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time.

Changes other than those indicated require revalidation of the procedure. The chromatographic conditions described have been validated during the elaboration of the monograph.

The system suitability tests are included to verify that the separation required for satisfactory performance of the test or quantitation is achieved. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behavior, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reversed-phase liquid chromatographic procedures in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel), which exhibits the desired chromatographic behavior.

For critical parameters, the adjustments are defined clearly in the monograph to ensure the system suitability.

Thin-layer chromatography and paper chromatography

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30% (relative value) or ± 2% (absolute value), whichever is the larger. For example, for a minor component at 10% of the mobile phase, a 30% relative adjustment allows a range of 7-13% whereas a 2% absolute adjustment allows a range of 8-12%, i.e. the relative value therefore being the larger. For a minor component at 5% of the mobile phase, a 30% relative adjustment allows a range of 3.5-6.5% whereas a 2% absolute adjustment allows a range of 3-7%, i.e. the absolute value being the larger in this case. No other component is altered by greater than 10% absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10%.

Application volume: 10-20% of the prescribed volume if using fine particle size plates (2-10 µm).

Liquid chromatography: isocratic elution

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30% relative or ± 2% absolute, whichever is the larger (see example above). No other component is altered by greater than 10% absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10%.

Mobile phase flow rate: ± 50%; a larger adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters:

Stationary phase:

- no change of the identity of the substituent of the stationary phase permitted (i.e. no replacement of C18 by C8);

- *particle size*: maximum reduction of 50%, no increase permitted.

Column dimensions::

- *length*: ± 70%;
- *internal diameter:* ± 25%.

When column dimensions are changed, the mobile phase flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 \cdot d_2^2}{l_1 \cdot d_1^2},$$

where $F1$ is the mobile phase flow rate specified in the monograph, in millilitres per minute;

F_2 is the adjusted mobile phase flow rate, in millilitres per minute;

$l1$ is the length of the column specified in the monograph, in millimetres;

l_2 is the length of the column used, in millimetres;

d_1 — internal diameter of the column specified in the monograph, in millimetres;

d_2 is the internal diameter of the column used, in millimetres.

Temperature: ± 10 °C, where the operating temperature is controlled, unless otherwise prescribed in the monograph.

Detector wavelength: no adjustment permitted.

Injection volume: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

Liquid chromatography: gradient elution

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

Composition of the mobile phase/gradient elution: minor adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- the system suitability requirements are fulfilled;
- the principal peak(s) elute(s) within ± 15% of the indicated retention time(s);
- the final composition of the mobile phase is not weaker in elution power than the composition prescribed in the monograph.

Where compliance with the system suitability requirements cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

Dwell volume. The configuration of the equipment employed may significantly alter the resolution, retention time and relative retentions described in the procedure, should this occur, it may be due to excessive dwell volume. Monographs preferably include an isocratic step before the start of the gradient program so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points (t min) stated in the gradient table may be replaced by adapted time points (t_c min), calculated using the following equation:

$$t_c = t - \frac{(D - D_0)}{F},$$

where D is the dwell volume, in millilitres;

D_0 is the dwell volume used for development of the procedure, in millilitres;

F is the mobile phase flow rate, in millilitres per minute.

The isocratic step introduced for this purpose may be omitted if validation data for application of the procedure without this step is available.

pH of the aqueous component of the mobile phase: no adjustment permitted.

Concentration of salts in the buffer component of a mobile phase: no adjustment permitted.

Mobile phase flow rate: the adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters:

Stationary phase:

- no change of the identity of the substituent of the stationary phase permitted (i.e. no replacement of C18 by C_8);

- *particle size*: no adjustment permitted.

Column dimensions::

- *length*: ±70%

- *internal diameter:* ±25%.

When column dimensions are changed, the mobile phase flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 \cdot d_2^2}{l_1 d_1^2},$$

where *F*1 is the mobile phase flow rate specified in the monograph in millilitres per minute;

F_2 is the adjusted mobile phase flow rate, in millilitres per minute;

l_1 is the length of the column specified in the monograph in millimetres;

l_2 is the length of the column used in millimetres;

d_1 — internal diameter of the column specified in the monograph in millimetres;

d_2 is the internal diameter of the column used, in millimetres.

Temperature: ±5 °C, where the operating temperature is controlled, unless otherwise prescribed in the monograph.

Detector wavelength: no adjustment permitted.

Injection volume: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

Gas Chromatography

Column parameters:

Stationary phase:

- *particle size*: maximum size reduction of 50% is allowed, no increase is allowed (packed columns)

- *film thickness:* -50% to +100% (capillary columns).

Column dimensions::

- *length*: ± 70%;

- *internal diameter:* ±50%.

Flow rate: ±50%.

Temperature: ±10%.

Injection volume: may be adjusted, provided detection and repeatability are satisfactory.

Supercritical fluid chromatography

Composition of the mobile phase: for packed columns, the amount of the minor solvent component may be adjusted by ± 30% relative or ± 2% absolute, whichever is the larger. No adjustment is permitted for a capillary column system.

Detector wavelength: no adjustment permitted.

Column parameters:

Stationary phase:

- *particle size*: maximum size reduction of 50% is allowed, no increase is allowed (packed columns)

Column dimensions::

- *length*: ±70%

- *internal diameter*:

±25% (packed columns);

±50% (capillary columns).

Flow rate: ±50%.

Temperature: ±5 °C for controlled operating temperature.

Injection volume: may be decreased, provided detection and repeatability are satisfactory; no increase permitted.

QUANTITATION

Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are disregarded during the quantitation.

- *Detector sensitivity.* The detector sensitivity is the output signal per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative sensitivity coefficient of the detector, usually called the sensitivity coefficient, expresses the sensitivity of the detector to a given substance relative to a standard substance. The *correction factor* is the reciprocal of the response factor.

- *External standard method.* The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.

- *Internal standard method.* Equal amounts of a component that will be resolved from the substance to be examined (the internal standard) are introduced into the test solution and a reference solution. The internal standard is chosen such that it does not react with the substance to be examined, is stable and does not contain impurities with the same retention time as that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the reference solution.

- *Normalisation procedure.* The percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix, and those at or below the disregard limit.

- *Calibration procedure.* The relationship between the measured or evaluated signal (*y*) and the quantity (concentration, mass, etc.) of substance (*x)* is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

In tests for related substances for both the external standard method, when a dilution of the test solution is used for comparison, and the normalisation procedure, any correction factors indicated in the monograph are applied (i.e. when the response factor is outside the range 0.8-1.2).

When the related substances test prescribes the total of impurities or there is a quantitation of an impurity, it is important to choose an appropriate threshold setting and appropriate conditions for the integration of the peak areas. In such tests the *disregard limit* is generally 0.05%. Integration of the peak area of any impurity that is not completely separated from the principal peak is preferably performed by valley-to-valley extrapolation (tangential skim).

201020037-2019

2.1.2.37. Capillary Electrophoresis

GENERAL PRINCIPLES

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity *E*, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} \cdot E = \left(\frac{q}{6\pi \cdot r \cdot \eta} \right) \cdot \left(\frac{V}{L} \right),$$

where *q* is the effective charge of the solute;
r is the Stoke's radius of the solute;
η is the viscosity of the electrolyte solution;
V is the applied voltage;
L is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow.

The electro-osmotic flow rate depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} \cdot E = \left(\frac{\varepsilon \cdot \zeta}{\eta}\right) \cdot \left(\frac{V}{L}\right),$$

where ε is the dielectric constant of the buffer solution;

ζ is the zeta potential of the capillary surface.

The velocity of the substance (v) is given by:

$$v = v_{ep} + v_{eo}.$$

The electrophoretic mobility of the substance and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the substance to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{I}{v_{ep} + v_{eo}} = \frac{I \cdot L}{(\mu_{ep} + \mu_{eo}) \cdot V}.$$

In general, uncoated fused-silica capillaries above pH 3 have a negative charge due to ionised silanol groups in the inner capillary wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \cdot V \cdot I}{2 \cdot D \cdot L},$$

where D is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between two bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\overline{\mu}_{ep} + \mu_{eo})},$$

where μ_{epa} и μ_{epb} are the electrophoretic mobilities of the two analytes being separated;

μ_{ep} is the mean electrophoretic mobility of the two analytes $\overline{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa})$.

APPARATUS

An apparatus for capillary electrophoresis is composed of:

- a high-voltage, controllable direct-current power supply;

- two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;

- two electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply;

- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector; the ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;

- a suitable injection system ;

- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds;

- a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility;

- a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance.

The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical procedure to achieve reproducible migration times of the solutes.

CAPILLARY ZONE ELECTROPHORESIS

PRINCIPLE

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer solution without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small (M_r < 2000) and large molecules (2000 < Mr < 100 000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

OPTIMISATION

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separation procedures are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage. A Joule heating plot is useful in optimising the applied voltage and capillary temperature.

Separation time is inversely proportional to the applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity. Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

Temperature. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation procedure. In the specific case of proteins, several strategies have been devised to avoid adsorption. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolytic solution parameters

Buffer type and concentration. Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

The selection of a buffer ion with a mobility corresponding to that of the solute in all possible cases is important to minimise distorted banding. Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion, the type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute migration velocity.

Buffer pH. The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the substance from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents. Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the substances or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow. Additives for chiral separations.

Additives for chiral separations. For the separation of optical isomers, a chiral selector is added to the separation buffer.

The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation procedure it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionisable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

CAPILLARY GEL ELECTROPHORESIS

PRINCIPLE

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Thus, different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

CHARACTERISTICS OF GELS

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer solution usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the substances. Due to the rigidity of these gels, only electrokinetic injection can be used. Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the substance for the same buffer.

Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

CAPILLARY ISOELECTRIC FOCUSING

PRINCIPLE

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer. The three basic steps of isoelectric focusing are loading, focusing and mobilisation.

Loading step. Two methods may be employed:

- loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;

- sequential loading: a leading buffer solution, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer solution is introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

Focusing step. When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

Mobilisation step. If mobilisation is required for detection, use one of the following methods:

- in the first method, mobilisation is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;

- in the second method, mobilisation is accomplished by applying positive pressure after the focusing step;

- in the third method, mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilised in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as Δpl, depends on the pH gradient (dpH/dx), the number of ampholytes having different pl values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/d$pH):

$$\Delta pI = 3 \cdot \sqrt{\frac{D \cdot (d\mathrm{pH}/dx)}{E \cdot (-d\mu/d\mathrm{pH})}}.$$

OPTIMISATION

The main parameters to be considered in the development of separation procedures are:

Voltage. Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary. The electro-osmotic flow must be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions. The anode buffer reservoir is filled with a solution with a pH lower than the pl of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pl of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity.

Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers. During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY

PRINCIPLE

In micellar electrokinetic chromatography (*MEKC*), separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, velocity and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate. Although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionised solutes is essentially chromatographic, migration of the solute and resolution can be rationalised in terms of the retention factor of the solute (k'), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase.

For neutral compounds, k' is defined as:

$$k' = \frac{t_R - t_0}{t_0 \cdot \left(1 - \frac{t_R}{t_{mc}}\right)} = K \cdot \frac{V_S}{V_M},$$

where t_R is the migration time of the solute;

t_0 is the analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol);

t_{mc} is the micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle);

K is the partition coefficient of the solute;

V_S is the volume of the micellar phase;

V_M is the volume of the mobile phase.

Likewise, the resolution between 2 closely-migrating solutes (R_s) is given by :

$$R_S = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k'_b}{k'_b + 1} \cdot \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k'_a \cdot \left(\frac{t_0}{t_{mc}}\right)},$$

where N is the number of theoretical plates for one of the solutes;

a is the selectivity;

k'_a и k'_b are the retention factors for both solutes, respectively ($k'_b > k'_a$).

Similar, but not identical, equations give k' and R_s values for electrically charged solutes.

OPTIMISATION

The main parameters to be considered in the development of separation procedures by MEKC are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage. Separation time is inversely proportional to the applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature. Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary. As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both the effective length and total length can decrease the electric fields (working at constant voltage), increase migration time, and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the substance band broadening.

Electrolytic solution parameters

Surfactant type and concentration. The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log k' of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k' approaches the value of $\sqrt{t_{mc}/t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

Buffer pH. Although pH does not modify the partition coefficient of non-ionised substances, it can modify the electro-osmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electro-osmotic flow and therefore increases the resolution of the neutral substances in MEKC, resulting in a longer analysis time.

Organic solvents. To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation.

Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellisation is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of high content of organic solvent does not always mean that the separation will no longer be possible; in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations. For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellised achiral surfactants.

Other additives. Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic substances with the micelle, thus increasing the selectivity for this type of compound.
The addition of substances able to modify solute-micelle interactions by adsorption on the latter is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

QUANTITATION

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

- compensate for the shift in migration time from run to run, thus reducing the variation of the response;
- compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalisation procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor (k') (only for micellar electrokinetic chromatography), the apparent number of theoretical plates (N), symmetry factor (A_s), and resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

APPARENT NUMBER OF THEORETICAL PLATES

The apparent number of theoretical plates (*N*) may be calculated using the expression:

$$N = 5{,}54 \cdot \left(\frac{t_R}{w_h} \right)^2 ,$$

where t_R is the migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component;

w_h is the width of the peak at half-height.

RESOLUTION

The resolution (R_s) between peaks of the similar height of two components may be calculated using the expression:

$$R_s = \frac{1{,}18 \cdot (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}, \; t_{R2} > t_{R1} ,$$

where t_{R1} и t_{R2} are the migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks;

w_{h1} and w_{h2} are the peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (*Hv*) between two partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

$$p / v = \frac{H_p}{H_v}.$$

SYMMETRY FACTOR

The symmetry factor (A_s) of a peak may be calculated using the expression:

$$A_s = \frac{w_{0,05}}{2d},$$

where $w_{0\cdot05}$ is the width of the peak at one-twentieth of the peak height,

d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the quantitation limit) may also be useful for the determination of related substances.

SIGNAL-TO-NOISE RATIO

The detection limit and quantitation limit correspond to signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio (*S*/*N*) is calculated using the expression:

$$S/N = \frac{2H}{h},$$

where *H* is the height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height;

h is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

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2.1.2.38. Isoelectric Focusing

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes).

When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating this substance. This concentrating effect is called "focusing". Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation is estimated by determining the minimum pl difference (Δpl), which is necessary to separate two neighboring bands:

$$\Delta pl = 3 \cdot \sqrt{\frac{D(d\mathrm{pH}/dx)}{E(-d\mu/d\mathrm{pH})}},$$

where D is the diffusion coefficient of the protein;

$\frac{d\mathrm{pH}}{dx}$ is the pH gradient;

E is the intensity of the electric field, in volts per centimetre;

$-\frac{d\mu}{d\mathrm{pH}}$ is the variation of the solute mobility with the pH in the region close to the pl.

Since D and $-\frac{d\mu}{d\mathrm{pH}}$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilised pH gradients where the buffering substances, which are analogous to carrier ampholytes, are copolymerised within the gel matrix. Proteins exhibiting pls differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilised pH gradients can resolve proteins differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2% ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some cases, the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identification test. In this case, the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins. The IEF gel can be used as a limit test. Wherein the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation. The IEF can also be used as a quantitative test.

APPARATUS

An apparatus for IEF consists of:

- a controllable generator for constant potential, current, and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended;
- a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;
- a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following procedure is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

PREPARATION OF THE GELS

Mould. The mould (see Figure 2.1.2.38.-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together.

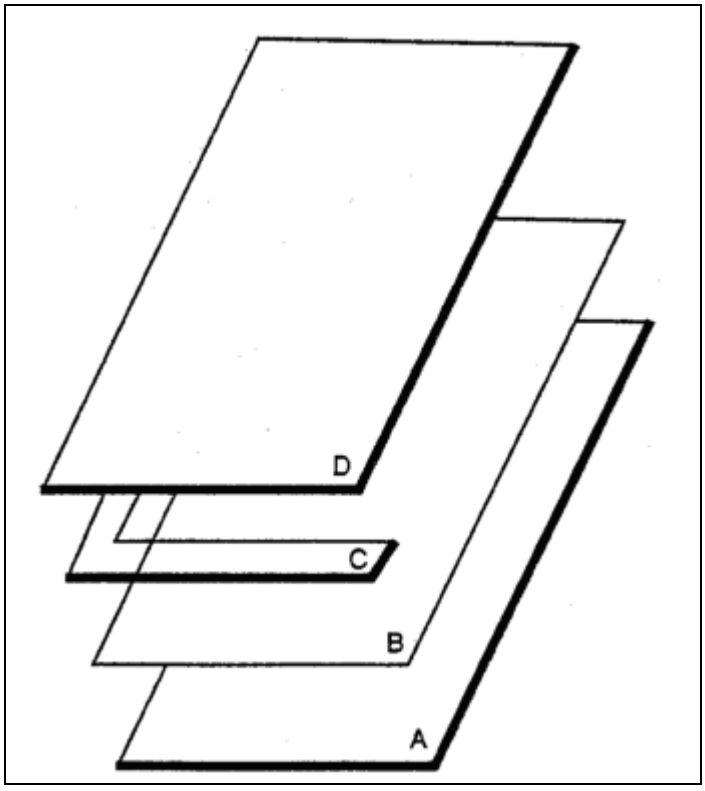

Figure 2.1.2.38.-1. – Mould

7.5% polyacrylamide gel. Dissolve 29.1 g of *acrylamide R* and 0.9 g of *methylenebisacrylamide R* in 100 mL of *water R*. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water R*. Mix carefully and degas the solution.

Preparation of the mould. Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g/L solution of *ammonium persulfate R* and 0.25 volumes of *tetramethylethylenediamine R*. Immediately fill the space between the glass plates of the mould with the solution.

PROCEDURE

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for the sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode (the compositions of the anode and cathode solutions are given in the monograph).

Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in *fixing solution for isoelectric focusing in polyacrylamide gel R.* Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and add 200 mL of *destaining solution R.* Incubate with shaking for 1 h. Dry the gel, add *Coomassie staining solution R*. Incubate for 30 min. Destain the gel by passive diffusion with *destaining solution R* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where a reference to the general procedure on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available precast gels and of commercial staining and destaining kits;
- the use of immobilised pH gradients;
- the use of rod gels;
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels;
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper;
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than a subjective interpretation of band stability;
- the inclusion of a pre-focusing step;
- the use of automated instrumentation;
- the use of agarose gels.

VALIDATION OF ISO-ELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using coloured pH markers of known isoelectric points;
- comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined;
- any other validation criteria as prescribed in the monograph.

SPECIFIED VARIATIONS TO THE GENERAL PROCEDURE

Variations to the general procedure required for the analysis of specific substances may be specified in detail in monographs. These include:

- the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;

- the use of alternative staining methods;

- the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., *CHAPS* or *CHAPSO*), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During the procedure development, the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends). The pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

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2.1.2.39. Peptide Mapping

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner.

It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterise the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analysed. Four principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds ; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance.

Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

ISOLATION AND PURIFICATION

Isolation and purification are necessary for the analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

SELECTIVE CLEAVAGE OF PEPTIDE BONDS

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 2.1.2.39.-1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Pretreatment of sample. Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the cleavage agent. Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin.

Table 2.1.2.39.-1. – *Examples of cleavage agents*

Type	Agent	Specificity
Enzymatic	Trypsin (*EC* 3.4.21.4)	*C*-terminal side of arginine and lysine
	Chymotrypsin (*EC* 3.4.21.1)	*C*-terminal sides of hydrophilic residues (e.g., leucine, methionine, alanine, aromatic amino acids)
	Pepsin (*EC* 3.4.23.1 and 2)	Non-specific digest
	Lysyl endopeptidase (Lys-C endopeptidase) (*EC* 3.4.21.50)	*C*-terminal side of lysine
	Glutamyl endopeptidase (fromS. *aureus* strainV8) (*EC* 3.4.21.19)	*C*-terminal side of glutamine and asparagine
	Peptidyl-Asp metallo-endopeptidase (endoprotease Asp-N)	*N*-terminal side of asparagine
	Clostripain (*EC* 3.4.22.8)	*C*-terminal side of arginine
Chemical	Cyanogen bromide	*C*-terminal side of methionine
	2-Nitro-5-thio-cyanobenzoic acid	*N*-terminal side of cysteine
	O-Iodosobenzoic acid	*C*-terminal side of tryptophan and tyrosine
	Dilute acid	Asparagine and proline
	BNPS-skatole	Tryptophan

Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the protein. Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from excipients and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography and lyophilisation. In order to perform protein denaturation prior to the peptide mapping method, other preparation methods may be used, such as the addition of chaotropic agents (for example, urea). To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion. Digestion with trypsin can introduce ambiguities in the peptide map due to side reactions occurring during the digestion reaction, such as non-specific cleavage, deamidation, disulfide isomerisation, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g. at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of optimal digestion conditions. Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH of the reaction medium. The pH of the digestion mixture is empirically determined to ensure the optimisation of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion and must not change during the course of the fragmentation reaction.

Temperature. For most cleavage reactions, the most appropriate temperature is between 25 °C and 37 °C. The created temperature should help minimise side chemical reactions. The type of protein under test will dictate the temperature of the reaction medium, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4 °C, because at higher temperatures it will precipitate during digestion.

Time. If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 h to 30 h. The reaction is stopped by the addition of an acid which does not interfere in the map or by freezing.

Amount of cleavage agent used. Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e. 6-20 hours), the amount of cleavage agent is minimised to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent is added in 2 or more stages to optimise cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping, the separation step.

To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

CHROMATOGRAPHIC SEPARATION

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2.1.2.39.-2. In this section, a most widely used reversed-phase HPLC method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available, are recommended for reversed-phase HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column.

Table 2.1.2.39.-2. – *Techniques used for the separation of peptides*

Reverse-phase high-performance liquid chromatography
Ion-exchange chromatography
Hydrophobic interaction chromatography
Polyacrylamide gel electrophoresis, non-denaturing
Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Capillary electrophoresis
Paper chromatography // High voltage-paper electrophoresis

Both pre- and post-pump filtration is also recommended.

Chromatographic column. The selection of a column is empirically determined for each protein. Columns with 10 nm or 30 nm pore size with silica support can give optimal separation. For smaller peptides, *octylsilyl silica gel for chromatography R* (3-10 μm) and *octadecylsilyl silica gel for chromatography R* (3-10 μm) column packings are more efficient than *butylsilyl silica gel for chromatography R* (5-10 μm).

Solvent. The most commonly used solvent is water with acetonitrile as the organic modifier to which not greater than 0.1% trifluoroacetic acid is added. If necessary, add propyl alcohol or isopropyl alcohol to solubilise the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile phase. Buffered mobile phase containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Gradient. Gradients can be linear, nonlinear, or include step change in composition. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimised to provide clear resolution of 1 or 2 peaks that will become "marker" peaks for the test.

Isocratic elution. Isocratic HPLC systems employing a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain a clear resolution of each peak is sometimes difficult to establish.

Mobile phases for which slight changes in component ratios or in pH significantly affect retention time of peaks in peptide maps must not be used in isocratic HPLC systems.

Other parameters. Temperature control of the column is usually necessary to achieve good reproducibility. The mobile phase flow rate ranges from 0.1-2.0 mL/min, and the detection of peptides is performed with a UV detector at 200-230 nm. Other methods of detection have been used (e.g. post-column derivatisation), but they are not as robust or versatile as UV detection.

Validation. This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint has been achieved is shown by comparison with a reference standard, which is treated in the same manner as the test protein. The use of a reference substance in parallel with the test protein is critical in the development and establishment of system suitability limits. In addition, a chromatogram is included with the reference substance for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when the identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference substance for the specified protein.

The use of a digested reference substance for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterised mixture of a variant and a reference substance can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters, such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency, may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference substance for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation. Differences in the recovery and precision of the identified peptides are to be expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the monograph.

Visual comparison of the relative retentions, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (*V/V*) mixture of sample and reference substance digest.

The identity of the analysed sample is confirmed if all peaks of the analysed protein and the reference substance digests have similar retention times and the ratio of the estimated peak responses.

If all peaks that initially eluted with significantly different relative retentions are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separated peaks are observed in a 1:1 mixture, this is evidence of the presence of different peptides in each of the peaks. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and reference substance digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. One of such approaches is, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference substance and sample digest fragments.

The numerical comparison of the peak retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference substance. The possibility of auto-hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

ANALYSIS AND IDENTIFICATION OF PEPTIDES

This section gives guidance on the use of peptide mapping during development of monographs and regulatory quality documents in support of marketing authorisation applications.

The use of a peptide map as a qualitative tool does not require the complete characterisation of the individual peptide peaks. However, validation of peptide mapping procedure during development of monographs and regulatory quality documents in support of marketing authorisation applications requires accurate characterisation of each of the individual peaks in the peptide map. Methods to characterise peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy.

For characterisation purposes, when *N*-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary.

Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be released before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase and matrix-assisted laser desorption ionisation coupled to time-of-flight analyser can also be used for characterisation purposes.

The use of mass spectroscopy for characterisation of peptide fragments is by direct infusion of isolated peptides or by the use of *on-line* HPLC with mass spectrometer for structure analysis. In general, it includes electrospray and matrix-assisted laser desorption ionisation coupled to time-of-flight analyser, as well as fast-atom bombardment. Tandem mass spectroscopy has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated procedure of characterisation of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

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2.1.2.40. Amino Acid Analysis

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other drug preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organised as a linear polymer.

The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify proteins and peptides, to quantify the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyse a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other drug preparations. The amino acid constituents of the test sample typically undergo chemical modification (are derivatised) for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current procedures take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have post-column derivatisation capability, unless the sample is analysed using pre-column derivatisation. The detector is usually an ultraviolet/visible or fluorescence detector depending on the derivatisation method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitative calculations.

It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with naked hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARDS

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analysed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF THE APPARATUS

Calibration of amino acid analysis instrumentation typically involves analysing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the amino acid reference standard is diluted to several different analyte levels within the expected linear range of the amino acid analysis procedure. Then, replicates at each of the different analyte levels can be analysed. Peak areas obtained for each amino acid are plotted on the y-axis versus the known concentration for each of the amino acids in the standard dilution – on the x-axis. These results will allow determining the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important to prepare the samples for amino acid analysis so that they are within the analytical limits (i.e. linear working range) of the procedure employed in order to obtain accurate and repeatable results.

4 to 6 amino acid standard levels are analysed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per 1 nmol (10^{-9} mol) of amino acid present in the standard.

A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the quantity in nanomoles (nmol) of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the quantitation. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that correspond to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitative calculations. A typical repeatability evaluation involves preparing a standard amino acid solution and analysing many replicates (e.g., 6 analyses or more) of the same standard solution. The relative standard deviation is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple quantitations conducted over several days by different analysts. Multiple quantitations include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. The amino acid composition of a standard protein (e.g., bovine serum albumin) is often analysed as part of the repeatability evaluation. By evaluating the relative standard deviations, the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results.

Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst competence and condition. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Procedures that utilise post-column derivatisation of the amino acids are generally not affected by buffer components to the extent seen with pre-column derivatisation procedures. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis.

The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis, whose recovery rates are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results that is necessary to take into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and mobile phase flow rates. Ideally, an internal standard is an commercial amino acid that differ from natural amino acids. Besides, an internal standard should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

For amino acid analysis of protein and peptide samples, the conduction of hydrolysis is necessary. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders as well as fingerprints on the hydrolysis glassware may cause contamination. To clean glass hydrolysis tubes, boil them for 1 h in 1 M hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of equal volumes of concentrated hydrochloric acid and nitric acid. Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Ignition of clean glassware at 500 °C for 4 h may also be used to eliminate contamination from hydrolysis tubes.

Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolysing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the deviation of the analysis due to the complete or partial destruction of several amino acids: tryptophan is destroyed, serine and threonine are partially destroyed, methionine might undergo oxidation, and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 μm of mercury or 26.7 Pa) or the introduction of inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative decomposition. In peptide bonds involving isoleucine and valine the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during acid hydrolysis limits the quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (e.g., Methods 4-11) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis procedure are weighed against the concerns with the procedure and are tested adequately before employing a procedure other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24 h, 48 h, and 72 h) is often employed to analyse the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (e.g., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. The level of residues of amino acids that are slow to cleave (e.g., isoleucine and valine) is taken equal to the height of the plateau on the resulting plot of the residue concentration versus the hydrolysis time.

If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard with known concentrations to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the decomposition rate of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this procedure will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins.

During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

METHOD 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution. 6 M hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure

Liquid phase hydrolysis. Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 μL of hydrolysis solution per 500 μg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuo. Samples are typically hydrolyzed at 110 °C for 24 h in vacuo or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 h and 72 h) are investigated if there is a concern that the protein is not completely hydrolysed.

Vapour phase hydrolysis. This is one of the most common acid hydrolysis methods. It is preferred for microanalysis when only small amounts of the sample are available. The contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution must not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 μm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110 °C for a 24 h hydrolysis time. Acid vapour hydrolyses the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample in vacuo to remove any residual acid.

METHOD 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid as the reducing acid.

Hydrolysis solution. 2.5 M mercaptoethanesulfonic acid solution.

Vapour phase hydrolysis. Dry about 1 μg to 100 μg of the protein/peptide under test in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 μL of the hydrolysis solution. Seal the larger tube in vacuo (about 50 μm of mercury or 6.7 Pa) to vapourise the hydrolysis solution.

Heat the hydrolysis tube to 170-185 °C for about 12.5 min. After hydrolysis, dry the sample tube in vacuo for 15 min to remove the residual acid.

METHOD 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis solution. 7 M hydrochloric acid containing 1% of phenol, 10% of trifluoroacetic acid, and 20% of thioglycolic acid.

Vapour phase hydrolysis. Dry about 10 µg to 50 µg of the protein/peptide under test in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 µL of the hydrolysis solution. Seal the larger tube in vacuo (about 50 µm of mercury or 6.7 Pa). Heat the sample tube to 166 °C for about 15-30 min. After hydrolysis, dry the sample tube in vacuo for 5 min to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

METHOD 4

Cysteine/cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation solution. Mix 1 volume of 30% hydrogen peroxide solution and 9 volumes of anhydrous formic acid and incubate at room temperature for 1 h. Use the resulting performic acid freshly prepared.

Procedure. Dissolve the protein/peptide sample in 20 µL of anhydrous formic acid and heat at 50 °C for 5 min, then add 100 µL of the oxidation solution. Allow the oxidation to proceed for 10-30 min. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method 1 or Method 2. This procedure may cause modifications to tyrosine residues in the presence of halides.

METHOD 5

Cysteine/cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis solution. To 6 M hydrochloric acid containing 0.2% of phenol, add sodium azide to obtain a final concentration of 2 g/L. The added phenol prevents halogenation of tyrosine.

Liquid phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the sodium azide present in the hydrolysis solution. This procedure allows better tyrosine recovery than Method 4, but it is not suitable for methionine quantitation. Methionine is converted to a mixture of the parent methionine and its 2 oxidative products, methionine-sulfoxide and methionine-sulfone.

METHOD 6

Cysteine/cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis solution. To 6 M hydrochloric acid containing 0.1% to 1.0% of phenol, add dimethyl sulfoxide to obtain a final concentration of 2% (*V/V*).

Vapour phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the DMSO present in the hydrolysis solution. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolysis of standard proteins containing 1–8 cysteine residues. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for non-hydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this procedure.

METHOD 7

Cysteine/cystine reduction and alkylation are accomplished by a vapour phase pyridylethylation reaction.

Reducing solution. Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container and mix.

Procedure. Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place it in a larger tube. Transfer the reducing solution to the large tube, seal in vacuo (about 50 µm of mercury or 6.7 Pa), and heat at about 100 °C for 5 min. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 min to remove residual reagents. The pyridylethylated sample can then be acid hydrolysed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1-8 cysteine residues to evaluate the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

METHOD 8

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock solutions. Prepare and filter 3 solutions: 1 M Tris-hydrochloride pH 8.5 containing 0.004 M disodium edetate (stock solution A), 8 M guanidine hydrochloride (stock solution B), and 10% of 2-mercaptoethanol (stock solution C).

Reducing solution. Prepare a mixture of 1 volume of stock solution A and 3 volumes of stock solution B to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M tris-hydrochloride.

Procedure. Dissolve about 10 µg of the test sample in 50 µL of the reducing solution, and add about 2.5 µL of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 h at room temperature in the dark. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

METHOD 9

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock solutions. Prepare as directed for Method 8.

Carboxymethylation solution.. Prepare a 100 g/l iodoacetamide solution in 96% alcohol.

Buffer solution. Use a reducing solution prepared as specified in Method 8.

Procedure. Dissolve the test sample in 50 µl of buffer solution and add about 2.5 µl of the stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. Add the carboxymethylation solution in a ratio of 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 min at room temperature in the dark. If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present. The reaction is stopped by adding excess of 2-mercaptoethanol. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethyl-cysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

METHOD 10

Cysteine/cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis procedure.

Reducing solution. A 10 g/L solution of dithiodiglycolic acid (or dithiodipropionic acid) in 0.2 M sodium hydroxide.

Procedure. Transfer about 20 μg of the test sample to a hydrolysis tube, and add 5 μL of the reducing solution. Add 10 μL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolyzed using Method 1. This method has the advantage that other amino acid residues are not derivatised by side reactions and that the sample does not need to be desalted prior to hydrolysis.

METHOD 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (*BTI*) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow determining the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing solutions. Prepare and filter 3 solutions: a solution of 0.01 M trifluoroacetic acid (Solution A), a solution of 5 M guanidine hydrochloride with 0.01 M trifluoroacetic acid (Solution B); a freshly prepared solution of dimethylformamide containing 36 mg/mL of *BTI* (Solution C).

Procedure. In a clean hydrolysis tube, transfer about 200 μg of the test sample, and add 2 mL of Solution A or Solution B and 2 mL of Solution C. Seal the hydrolysis tube in vacuo. Heat the sample at 60 °C for 4 h in the dark. The sample is then dialysed with water to remove the excess reagents. Extract the dialysed sample 3 times with equal volumes of butyl acetate, and then lyophilise. The protein can then be acid hydrolysed using previously described procedures.

The α,β-diaminopropionic and α,γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on the amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content quantified with underivatised and *BTI*-derivatised acid hydrolysis. The threonine, methionine, cysteine, tyrosine, and histidine quantified content can be altered by *BTI* derivatisation; hydrolysis without *BTI* will have to be performed if it is necessary to determine these other amino acid residues of the protein/peptide.

METHODOLOGIES OF AMINO ACID ANALYSIS: GENERAL PRINCIPLES

Many amino acid analysis techniques exist. The choice of any one technique often depends on the sensitivity required from the quantitation. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by post-column derivatisation (e.g., with ninhydrin or *o*-phthalaldehyde). Post-column derivatisation techniques can be used with samples that contain small amounts of buffer components, (such as salts and urea) and generally require between 5 μg and 10 μg of protein sample per analysis. The remaining amino acid techniques typically involve pre-column derivatisation of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenylmethyl chloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Pre-column derivatisation techniques are very sensitive and usually require between 0.5 μg and 1.0 μg of protein sample per analysis but may be influenced by buffer salts in the samples. Pre-column derivatisation techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation.

The following methods may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will use more than one amino acid analysis procedure to exploit the advantages offered by each. In each of these procedures, the analog signal is visualised by means of a data acquisition system, and the peak areas are integrated for quantitation purposes.

METHOD 1. POST-COLUMN NINHYDRIN DERIVATISATION

Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically 17 amino acids). The separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and ionic strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has a characteristic violet or yellow colour. Amino acids, except imino acid, give a violet colour and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour and show an absorption maximum at 440 nm.

The post-column reaction between ninhydrin and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 nmol for most of the amino acid derivatives, but 50 nmol for the proline derivative. Response linearity is obtained in the range of 20-500 nmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 μg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

METHOD 2. POST-COLUMN O-PHTHALALDEHYDE DERIVATISATION

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is used for the post-column derivatisation in the analysis of amino acids by ion-exchange chromatography. The separation conditions are the same as Method 1.

To form fluorescent substances with OPA, the oxidation of secondary amines (imino acids such as proline) with sodium hypochlorite or chloramine T is conducted first. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by post-column oxidation with sodium hypochlorite or chloramine T and post-column derivatisation using OPA and a thiol compound such as *N*-acetyl-L-cysteine or 2-mercaptoethanol. The derivatisation of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite or chloramine T.

The separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and ionic strength. After the post-column derivatisation of eluted amino acids with OPA, the reactant passes through the fluorometric detector. The fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of picomole levels for most of the OPA-derivatised amino acids. Response linearity is obtained in the range of a few picomole levels to a few tens of nanomole levels.

To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 3. PRE-COLUMN PHENYLISOTHIOCYANATE DERIVATISATION

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 254 nm. Therefore, pre-column derivatisation of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyse the amino acid composition.

After the reagent is removed under vacuum, the derivatised amino acids can be stored dry and frozen for several weeks with no significant decomposition. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after 3 days.

Separation of the PTC-amino acids on a reversed-phase HPLC with an octadecylsilyl (ODS) column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 nmol for most of the PTC-amino acids. Response linearity is obtained in the range of 20-500 nmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 4. PRE-COLUMN 6-AMINOQUINOLYL-N-HYDROXYSUCCINIMIDYL CARBAMATE DERIVITISATION

Pre-column derivatisation of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used. AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are freely amenable to analysis by reversed-phase HPLC with fluorometric detection.

Separation of the AQC-amino acids on a reversed-phase HPLC with an octadecylsilyl silica gel column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. The excess reagent is rapidly hydrolyzed ($t_{1/2}$<15 s) to yield 6-aminoquinoline, *N*-hydroxysuccinimide, and carbon dioxide, and after 1 min no further derivatisation can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature. Therefore AQC-amino acids have greater than sufficient stability to allow for overnight automated chromatographic analysis. The detection limit is considered to range from about 40 fmol to 320 fmol for each amino acid, except for cystein.

The detection limit for cystein is approximately 800 fmol. Response linearity is obtained in the range of 2.5-200 μmol with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatised protein hydrolysates derived from at least 30 ng of protein/peptide.

METHOD 5. PRE-COLUMN O-PHTHALALDEHYDE DERIVATISATION

Pre-column derivatisation of amino acids with *o*-phthalaldehyde (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as the thiol compound. OPA itself does not fluoresce and consequently produces no interfering peaks.

In addition, its solubility and stability in aqueous solution, along with the rapid reaction rate, make it amenable to automated derivatisation and analysis using an autosampler to mix the sample with the reagent. The lack of reactivity with secondary amino acids (e.g., proline) is the main drawback of OPA. To compensate for this drawback, this procedure may be combined with another technique described in Method 7 or 8.

Pre-column derivatisation of amino acids with OPA is followed by a reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatisation. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatised amino acids. The fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 nmol.

METHOD 6. PRE-COLUMN (DIMETHYLAMINO)AZOBENZENESULFONYL CHLORIDE DERIVATISATION

Pre-column derivatisation of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show an absorption maximum at 436 nm.

DABS-amino acids, all naturally occurring amino acid derivatives, can be separated on an octadecylsilyl silica gel column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

The method allows analysing of both amino acids and imino acids (such as proline) together with the amino acids at the same degree of sensitivity. DABS-Cl derivatisation method permits the simultaneous quantitation of tryptophan residues by the previous hydrolysis of the protein/peptide with sulfonic acids such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid described in Method 2 under Protein hydrolysis. The other acid-labile residues, asparagine and glutamine, can also be analysed by the previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI described in Method 11 under Protein hydrolysis section.

The non-proteinogenic amino acid norleucine cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. The DABS-amino acid detection limit is 2-5 nmol. - To obtain reliable data, it is recommended to use 10 - 30 ng of DUBS-derivatives of protein hydrolysate for one analysis.

METHOD 7. PRE-COLUMN 9-FLUOROENYLMETHYL CHLOROFORMATE DERIVATISATION

Pre-column derivatisation of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction proceeds under mild conditions in aqueous solution and is completed in 30 s. The derivatives are stable, only the histidine derivative showing any decomposition. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an octadecylsilyl silica gel column. The separation is carried out by gradient elution with the mobile phase composition varied linearly from a mixture of acetonitrile - methanol - acetic acid buffer solution (10:40:50, *V/V*) to a mixture of acetonitrile - acetic acid buffer solution (50:50, *V/V*). As a result of this elution, 20 amino acid derivatives are separated in 20 min. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low femtomole range. A linearity range of 0.1-50 μmol is obtained for most of the amino acids.

METHOD 8. PRE-COLUMN 7-FLUORO-4-NITROBENZO-2-OXA-1,3-DIAZOLE DERIVATISATION

Pre-column derivatisation of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatised with NBD-F by heating to 60 °C for 5 min.

NBD-amino acid derivatives are separated on an octadecylsilyl silica gel column of a reversed-phase HPLC by employing a gradient elution system and mobile phase consisting of acetonitrile and aqueous buffer mixture. As a result of this elution, 17 amino acid derivatives are separated in 35 min. ε-Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for the pre-column OPA derivatisation method (Method 5), excluding proline to which OPA is not reactive (advantageous for NBD-F against OPA). The detection limit for each amino acid is about 10 fmol. Profile analysis can be achieved with about 1.5 mg of protein hydrolysates in the pre-column reaction mixture.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine, serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants for samples. Application of adequate vacuum (less than 200 μm of mercury or 26.7 Pa) or the introduction of inert gas (argon) in the headspace of the reaction vessel during vapour phase hydrolysis can reduce the level of oxidative decomposition. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Amino acid mole percent is the number of specific amino acid residues per 100 residues in a protein. This value may be useful for evaluating amino acid analysis data when the molecular mass of the protein under investigation is unknown. This information can be used to confirm the identity of a protein/peptide and has other applications. Calculate the mole percent for each amino acid present in the test sample using the formula:

$$\frac{100 \cdot n_U}{r},$$

in which *rU* is the peak response, in nanomoles, of the amino acid under test;

r is the sum of peak responses, in nanomoles, for all amino acids present in the test sample.

A comparison of the mole percent of the amino acids under test with data from known proteins can help establish or corroborate the identification of the protein sample.

Unknown protein samples. This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in micrograms, of each recovered amino acid using the formula:

$$\frac{m \cdot M_r}{1000},$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test;

M_r is the average molecular mass for that amino acid, corrected for the mass of the water molecule that was eliminated during peptide bond formation.

The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analysed after appropriate correction for partially and completely destroyed amino acids. If the molecular mass of the unknown protein is available (e.g., by sodium dodecyl sulfate polyacrylamide gel electrophoresis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid using the formula:

$$\frac{m}{\left(\frac{1000 \cdot M}{M_{rt}}\right)},$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test ;

M is the total mass, in micrograms, of the protein;

M_{rt} is the molecular mass of the unknown protein.

Known protein samples. This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular mass and amino acid composition using the amino acid analysis data.

When analysing the composition of the known protein, remember that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (e.g., isoleucine and valine), and free amino acid contamination (e.g., glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nanomoles, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein quantity based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation (typically greater than 5%) from the mean. Recalculate the mean protein content from the remaining values to obtain the protein content of the test sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis. Calculate the relative compositional error, in percentage, using the formula:

$$\frac{100 \cdot m}{m_s},$$

where m is the experimentally determined quantity of the amino acids, in nanomoles per amino acid residue;

m_s is the known residue value for that amino acid.

The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation.

The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

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2.1.2.41. Inductively Coupled Plasma-Atomic Emission Spectrometry

GENERAL PRINCIPLE

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) is an atomic emission spectrometry technique that uses the inductively coupled plasma (ICP) as excitation source.

A highly ionised inert gas (typically argon) with equal numbers of electrons and ions sustained by a radio-frequency (RF) field is used to create the inductively coupled plasma. The high temperature reached in the plasma successively desolvates, turns into steam, excites (atomic emission spectrometry (AES) detection), and ionises (mass spectrometry (MS) detection) atoms from the sample. Detection limits are, generally, in the lower nanogram (ICP-MS) to microgram (ICP-AES) per litre range.

The plasma is formed by a tangential stream of support gas through a "torch", i.e. a system consisting of three concentric quartz tubes. A metal coil (induction coil) surrounds the top end of the torch and is connected to a radio-frequency (RF) generator. Power (usually 700-1500 W) is applied through the coil and an oscillating magnetic field corresponding to the frequency of the generator (in most cases 27 MHz, 40 MHz) is formed. The plasma forms when the support gas is made conductive by, and seed electrons and ions appear. In the induced magnetic field, the charged particles (electrons and ions) are forced to flow in a closed annular path. As they meet resistance to their flow, heating takes place producing additional ionisation. The process occurs almost instantaneously, and the plasma expands to its full strength and dimensions. The radio-frequency oscillation of the power applied through the coil causes radio-frequency electric and magnetic fields to be set up in the area at the top of the torch. When a spark (produced by a Tesla tube or some other device) is applied to the support gas flowing through the torch, some electrons are stripped from the support gas atoms. These electrons are then caught up in the magnetic field and accelerated. Adding energy to the electrons by the use of a coil is known as inductive coupling. These high-energy electrons collide with other carrier gas atoms, knocking out more and more electrons. The collisional ionisation of the support gas continues in a chain reaction, breaking down the gas into a physical plasma consisting of support-gas atoms, electrons, and support-gas ions. The plasma is then sustained within the torch and load coil as radio-frequency energy is continually transferred to it through the inductive coupling process.

The ICP appears as an intense, very bright, plume-shaped plasma. At the base the plasma is toroidal, and this is referred to as the induction region (IR), i.e. the region in which the inductive energy transfer from the load coil to the plasma takes place. The sample is introduced through the IR into the center of the plasma.

APPARATUS

The apparatus consists essentially of the following elements:

- sample-introduction system consisting of a peristaltic pump delivering the solution at a constant rate into a nebuliser;
- radio-frequency (RF) generator;
- plasma torch;
- transfer optics focussing the image of the plasma at the entrance slit of the spectrometer; radial viewing is better for difficult matrices (alkalis, organic substances), whereas axial viewing gives more intensity and better detection limits in simple matrices;

- dispersive devices consisting of diffraction gratings, prisms, filters, or interferometers;
- a detector converting radiant energy into electrical energy;
- a data-acquisition unit.

INTERFERENCE

Interference is anything that causes the signal from an analyte in a sample to be different from the signal for the same concentration of that analyte in a calibration solution. The well-known chemical interference that is encountered in flame atomic absorption spectrometry is usually weak in ICP-AES. In rare cases where interference occurs, it may be necessary to increase the radio-frequency power or to reduce the inner support-gas flow to eliminate it. The interference in ICP-AES can be of spectral origin or even the result of high concentrations of certain elements or matrix compounds. Physical interference (due to differences in viscosity and surface tension of the sample and calibration standards) can be minimised by dilution of the sample, matrix matching, use of internal standards, or through the application of the method of standard additions.

Another type of interference occasionally encountered in ICP-AES is the so-called "easily ionised elements effect". The easily ionised elements are those elements that are ionised much more easily, for example, alkaline metals and alkaline earth metals. In samples that contain high concentrations of such elements (greater than 0.1%), suppression or enhancement of emission signals is likely to occur.

Spectral interference. This may be due to other lines or shifts in background intensity. These lines may meet to argon (observed above 300 nm), OH bands due to the decomposition of water (at about 300 nm), NO bands due to the interaction of the plasma with the ambient air (between 200 nm and 300 nm), and other elements in the sample, especially those present at high concentrations. The interference falls into four different categories: simple background shift; sloping background shift; direct spectral overlap; and complex background shift.

Absorption interference. This arises when part of the emission from an analyte is absorbed before it reaches the detector. This effect is observed particularly when the concentration of a strongly emitting element is so high that the atoms or ions of that element that are in the lower energy state of transition absorb significant amounts of the radiation emitted by the relevant excited species. This effect, known as self-absorption, determines the upper end of the linear working range for a given emission line.

Multicomponent spectral fitting. Multiple emission-line determinations are commonly used to overcome problems with spectral interferences. A better, more accurate method for performing spectral interference corrections is to use the information obtained with advanced detector systems through multicomponent spectral fitting. This quantifies not only the interference but also the background contribution from the matrix, thereby creating a correction formula. Multicomponent spectral fitting utilises a multiple linear-squares model based on the analysis of pure analyte, the matrix, and the blank, creating an interference-corrected mathematical model. This permits the determination of the analyte emission in a complex matrix with improved detection limits and accuracy.

PROCEDURE

SAMPLE PREPARATION AND SAMPLE INTRODUCTION

The basic goal for the sample preparation is to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulfuric and phosphoric acids can contribute to background emission observed in the ICP spectra. Nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid.

In selecting a sample-introduction method, the requirements for sensitivity, stability, rate, sample size, corrosion resistance, and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps used for ICP-AES usually deliver the standard and sample solutions at a rate of 1 mL/min or less.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

CHOICE OF OPERATING CONDITIONS

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen:

- wavelength selection;
- support-gas flow rates (outer, intermediate, and inner tubes of the torch);
- radio-frequency power;
- viewing position (radial or axial);
- pump speed;
- conditions for the detector (gain/voltage for photomultiplier tube detectors, others for array detectors);
- integration time (time set to measure the emission intensity at each wavelength).

CONTROL OF INSTRUMENT PERFORMANCE

System suitability

The following tests may be carried out with a multi-element control solution to ensure the adequate performance of the ICP-AES system:

- energy transfer (generator, torch, plasma); measurement of the ratio Mg II (280.270 nm)/Mg I (285.213 nm) may be used;
- sample transfer, by checking nebuliser efficiency and stability;
- resolution (optical system), by measuring peak widths at half height, for example As (189.042 nm), Mn (257.610 nm), Cu (324.754 nm) or Ba (455.403 nm);
- analytical performance, by calculating detection limits of selected elements over the wavelength range.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than four reference solutions over the calibration range and a blank solution. Perform not fewer than five replicates.

The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence range of the calibration curve are plotted. The operating procedure is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using certified reference materials. Where this is not possible, perform a test for recovery.

Recovery. For quantitation procedures, a recovery of 90% to 110% is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside of the range 80% to 120% of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

REPEATABILITY

The repeatability is not greater than 3% for a quantitation and not greater than 5% for an impurity test.

QUANTITATION LIMIT

Verify that the quantitation limit (for example, determined using the 10 σ approach) is below the value to be measured.

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2.1.2.42. Melting Point – Instrumental Method

This chapter describes the measurement of a melting point by the capillary method using an instrumental method of determination.

APPARATUS

There are two modes of automatic observation arrangements:

- mode A: by light transmission through the capillary tube loaded with the sample;
- mode B: by light being reflected from the sample in the capillary tube.

In both modes, the capillary tube sits in a hollow of a metal block, which is heated electrically and controlled by a temperature sensor placed in another hollow of the metal block.

The heating block is capable of being maintained at a pre-defined temperature with accuracy to ± 0.1 °C by the heating element, and of being heated at a slow and steady rate of 1 °C/min, after an initial isothermal period.

In mode A, a beam of light shines through a horizontal hollow and crosses the capillary tube. A sensor detects the beam at the end of the cylindrical hole after the capillary tube.

In mode B, a beam of light illuminates the capillary tube from the front and the sensor records the image of the reflected signal.

Some apparatuses allow for the visual determination of the melting point.

The temperature at which the sensor signal first leaves its initial value is defined as the beginning of melting, and the temperature at which the sensor signal reaches its final value is defined as the end of melting, or the *melting point*.

Use glass capillary tubes that are open at one end, about 100 mm long, with an external diameter of 1.3 - 1.5 mm and an internal diameter of 0.8 - 1.3 mm. The wall thickness of the tube is 0.1-0.3 mm.

Some apparatuses allow for the determination of the melting point on greater than 1 capillary tube.

PROCEDURE

Introduce into the capillary tube a sufficient amount of the test sample, previously treated as described in the monograph, to form a compact column about 4 mm high in each tube, and allow the tubes to stand for the appropriate time at the prescribed temperature.

Then proceed according to the manufacturer’s instructions or as follows. Heat the heating block until the temperature is about 5 °C below the expected melting point. Place the capillary tube in the heating block with the closed end downwards. Start the temperature program.

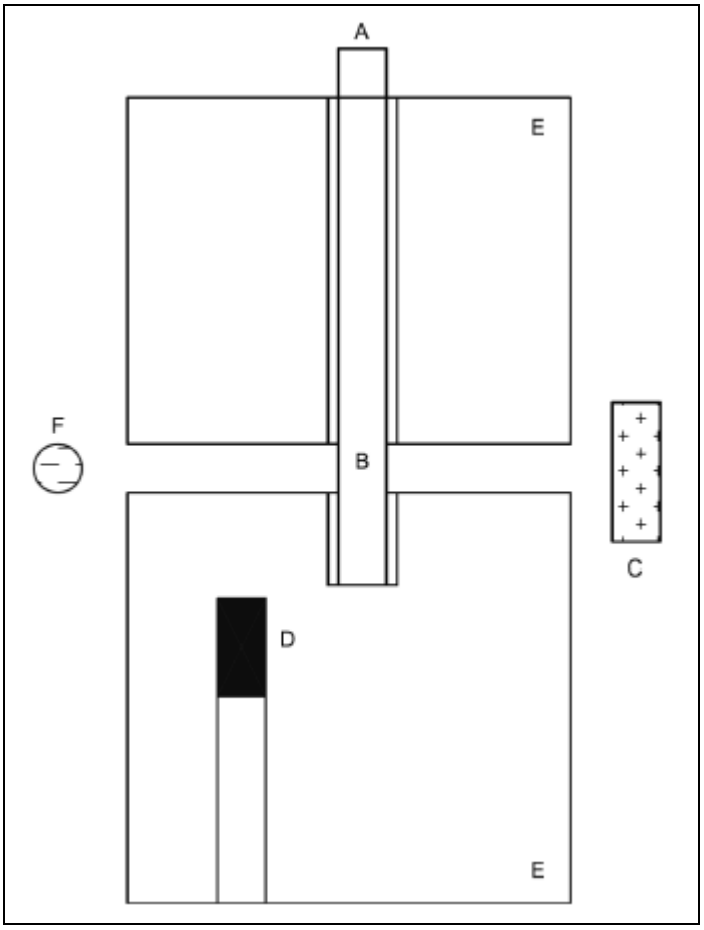


Figure 2.1.2.42.-1. – *Mode A – transmission.* A. Glass capillary tube; B. Sample; C. Photosensor; D. Temperature sensor; E. Heating block; F. Light source.

When the test sample starts melting, it changes its appearance in the capillary tube, as a result, the temperature of the heating block is recorded automatically following the signal changes from the photosensor due to light transmission (Figure 2.1.2.42.-1), or following image processing (Figure 2.1.2.42.-2).

Carry out the test on two other samples and calculate the mean value of the three results.

CALIBRATION

The temperature scale of the apparatus is checked periodically by measuring the melting point of certified reference materials. Use capillary tubes having the same dimensions as those used for the determination of the melting point of test samples (see "Apparatus").

Prepare three capillary tubes for each of at least two certified reference materials.

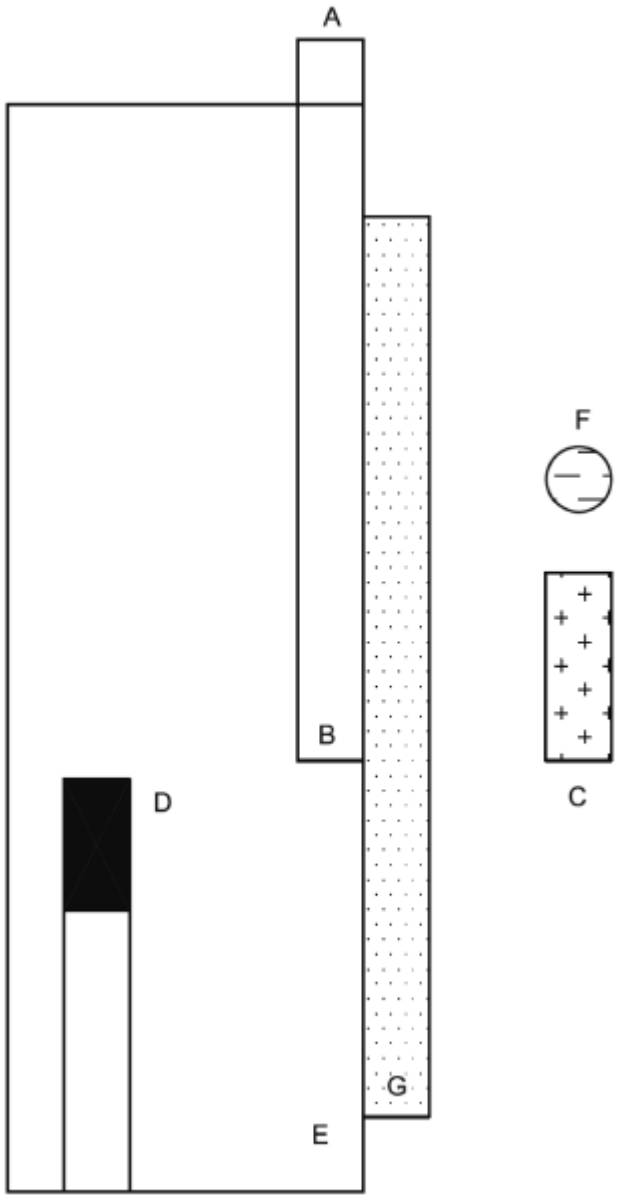


Figure 2.1.2.42.-2. – *Method B – reflection.* A. Glass capillary tube; B. Sample; C. Imagesensor; D. Temperature sensor; E. Heating block; F. Light source; G. Transparent plate.

Carry out the test and calculate the mean value of the three results for each reference material.

SYSTEM SUITABILITY

In addition to the calibration, carry out verification, before the measurements, using a suitable certified reference material whose melting point is close to that expected for the substance.

Prepare three capillary tubes, carry out the test, and calculate the mean value of the three results.

The mean value is within the tolerance given on the certificate supplied with the certified reference material.

201020043-2019

2.1.2.43. Detection and Measurement of Radioactivity

INTRODUCTION

Within the context of the Pharmacopoeia, the term "radioactivity" is used both to describe the phenomenon of radioactive decay and to express the physical quantity of this phenomenon. In the monographs on radiopharmaceutical drug preparations (RPDP), the detection and measurement of radioactivity are performed for different purposes: verification of the characters, identification, determination of radionuclidic and radiochemical purity, as well as determination of the radioactivity in a substance (quantitation).

Under these assumptions, the measurement can be qualitative, quantitative, or both, depending on whether it is directed to the identification of the radionuclide or the determination of its activity (rate of decay) or both of them.

Radioactive sources can produce various types of emissions, such as alpha particles, electrons, positrons, gamma- and X-rays, according to the radionuclidic composition.

Each radionuclide yields characteristic emissions, with specific energies and relative intensities. Such radiations can be detected as a result of their ionising properties in an ionisation chamber but without further characterisation; when they are detected and analysed using a spectrometer, an energy spectrum is obtained. A detailed spectrum analysis is typically used to identify radionuclides present in a sample. Spectrometry can also be used for the quantitation of the radioactivity in sources made of a single radionuclide or radionuclide mixtures or of the individual radionuclides present.

A measurement of radioactivity is generally performed by counting the number of detected decay events (emissions). Therefore, the geometry of the sample during the measurement of radioactivity and the acquisition time strongly influence the result. In general, the measurement geometry must meet to a calibrated geometry and the acquisition time must be long enough to reach sufficient counting statistics.

Measurement of radioactivity can be done in a stand-alone mode (e.g. using an ionisation chamber or a spectrometer) or in combination with a separation technique (e.g. radiochromatography) to account for relative contributions from different radioactive chemical species that may be present in a mixture.

MEASUREMENT OF RADIOACTIVITY

A direct determination of the radioactivity of a given sample, in becquerel (Bq), may be carried out if the decay scheme of the radionuclide is known, but in practice, many corrections are required to obtain accurate results. For this reason, it is possible to carry out the measurement with the aid of a primary standard source or by using measuring instruments such as an ionisation chamber or a spectrometer calibrated using suitable standards for the particular radionuclides.

A spectrometer is used when measuring the radioactivity of radionuclides in a mixture, each radionuclide being identified by its emissions and their characteristic energies.

All measurements of radioactivity must be corrected for dead-time losses and by subtracting the background signal due to radiation in the environment and to spurious signals generated in the equipment itself.

The radioactivity of a drug product is stated on a given date. If the half-life of the radionuclide is less than 70 days, the time is also indicated with reference to a specified time zone. The radioactivity at other times may be calculated from the exponential decay equation or from tables.

In general, a correct measurement of radioactivity requires that consideration is given to some or all of the following:

Dead-time losses. Due to the finite resolving time (dead time) of the detector and its associated electronic equipment, it may be necessary to correct for losses by coincidence. The resolving time of a counter is the minimum time range required by the counter to resolve two single pulses. Incident radiation events at shorter intervals may not be detected or may be detected as a single event with the summed energy. These losses are sometimes referred to as "dead-time losses". For a counting system with a fixed dead time following each count, the true count rate, s^{-1}, is calculated using the following expression:

$$\frac{N_1}{1 - N_1 \cdot \tau},$$

where $N1$ is the observed count rate, per second;

τ is the dead time, in seconds.

With some equipment, this correction is made automatically. Corrections for losses by coincidence must be made before the correction for background radiation.

Correction for decay during measurement. If the time period of an individual measurement (t_m) is not negligibly short compared with the half-life of the radionuclide, $T_{1/2}$, the decay during this measurement time must be taken into account. For example, there is a 5% cumulative loss of counts due to decay during a counting period that is 15% of the half-life of the radionuclide.

After having corrected the instrument reading (count rate, ionisation current, etc.) for background signals and, if necessary, for losses due to electronic effects, the instrument reading corrected to the beginning of the individual measurement is calculated using the following expression:

$$\frac{R(\lambda t_m)}{1 - (e^{-\lambda t_m})},$$

where R is the instrument reading before decay correction, but already corrected for background signal, etc.;

λ is the radionuclide decay constant ($\ln 2 / T_{1/2}$);

e is the base of natural logarithm; t_m is the measurement duration.

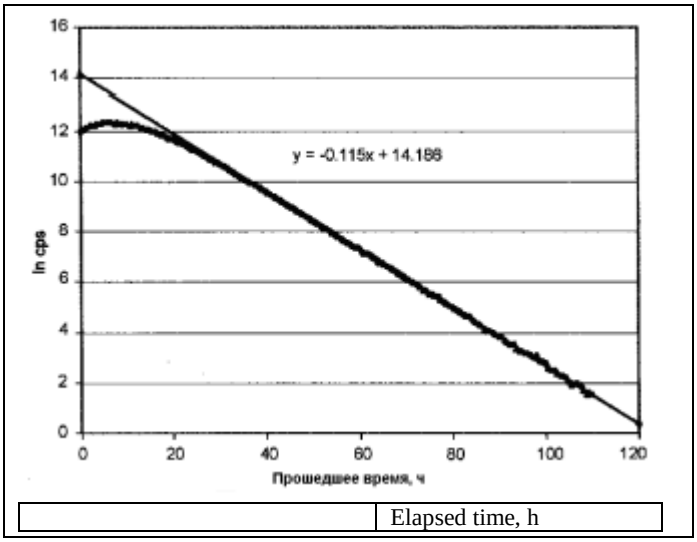


Figure 2.1.2.43.-1. – *Plot showing the measured and extrapolated count rate (natural logarithm of counts per second (*ln *cps)) from a technetium-99m source as a function of time, starting with a level of radioactivity above the linear range of the measuring equipment*

Statistics of radioactivity measurement. The results of determinations of radioactivity show variations that derive mainly from the random nature of nuclear transformations. Counting for any finite time can yield only an estimate of the true rate of nuclear transformations. A sufficient number of counts must be registered in order to compensate for variations in the number of pulses per time. In the case of measurement of radioactivity, the standard deviation of the recorded counts is the square root of the counts of these pulses. Thus, at least 10,000 counts are necessary to obtain a relative standard deviation of not greater than 1%.

Linearity. The linearity of an instrument is the range of radioactivity for a particular radionuclide over which its efficiency remains constant.

The linear range of a radioactivity measurement assembly can be determined by repeatedly counting a radioactive sample in a fixed geometry as it decays from an activity level that is above the linear range. After correction for the background signal, the natural logarithm of the count rate data is plotted against the elapsed time after the first measurement (Figure 2.1.2.43.-1).

Linear regression analysis of the central, linear portion of the data set yields a slope which is the decay constant λ, which has a characteristic value for each radionuclide:

$$\ln cps - \lambda t + c$$

where c is the natural logarithm of the count rate at $t = 0$ of a perfectly linear instrument.

The resulting regression equation is used to calculate the theoretical count rate at each time that the actual data were recorded. Where the deviation of the measured count rate from the theoretical count rate is unacceptably high, the linear range of the measuring equipment has been exceeded.

Alternatively, a series of dilutions of a radioactive substance solution with known radioactivity can be made. Equal volumes of each of the dilutions are then counted using standardised geometry and counter settings. The ratio of the count rate for each sample (after correction for background signals and decay) to the calculated radioactivity of the respective sample in Bq is the counting efficiency. The range over which this ratio is constant is the useable range of the measuring equipment for the radionuclide concerned.

The detection limit and quantitation limit for equipment and procedures used for radioactivity measurement must be established before their routine use.

Limit of detection. The detection limit (LOD) of an individual procedure is the lowest amount of radioactivity in a sample that can be detected but not necessarily quantified as an exact value. In practical terms, this requires an estimate of the background signal and its standard deviation. The LOD is usually considered to be three times the standard deviation of the background signal.

Quantitation limit. The quantitation limit (LOQ) of an individual procedure is the lowest amount of radioactivity in a sample that can be quantitatively determined with suitable precision and accuracy. The LOQ is used particularly for the determination of impurities and/or degradation products. In practical terms, the LOQ is usually considered to be 10 times the standard deviation of the background signal.

MEASUREMENT OF RADIOACTIVITY USING IONISATION CHAMBERS

Apparatus. Ionisation chambers (including dose calibrators) are the most common equipment for the measurement of radioactivity in the practice of radiopharmacy. It generally can measure activities from a few tens of kBq to hundreds of GBq and usually comprises a sealed well-type ionistion chamber and built-in electronics to convert the detector signal to a measure of radioactivity.

The chamber is filled with a gas across which an electrical field is applied. When the gas is ionised by the radiation emitted by the source, the resulting ionisation current is measured and related to the radioactivity present in the ionisation chamber. The ionisation current is influenced by the applied voltage, the energy and the intensity of the radiation, and the nature and pressure of the gas. The instrument settings (calibration factor) may be adjusted to keep a direct relationship between the ionisation produced by the radiation of a specific radionuclide and the radioactivity value obtained for each measurement geometry.

As an ionisation chamber measures only the current resulting from the overall ionisation produced within the chamber, it cannot discriminate between the emissions of different radionuclides.

For an accurate measurement of the radioactivity of a specific radionuclide, the measurement must be corrected for the contributions to the ionisation current caused by radionuclidic impurities present in the drug preparation. The measured activity levels depend on saturation considerations, the range of the amplifier, and the design of the chamber itself. The linearity range of the ionisation chamber is established as described above under "Linearity".

The ionisation chamber must be shielded to minimise background signals to an acceptable level.

Method. The sample is positioned inside the well of the ionisation chamber at a given position, using a holder. After putting the sample in the ionisation chamber, the activity reading is made once the response becomes stable. Measuring the sample under exactly the same geometrical conditions as the calibration source will yield the most accurate results. If necessary, dilute the drug preparation to be measured to the same volume as that of the calibration source.

Calibration. The ionisation chamber is calibrated taking into account the shape, dimensions, material of the container, volume, and composition of the solution, the position within the chamber, and the radionuclide being measured. Limits for uncertainty in calibration can be found in national and international regulations.

Calibrate the ionisation chamber at least once a year, by using sources of radionuclides traceable to national or international standards in the appropriate containers (vial, syringe) with regard to geometry. Establish and implement subsidiary correction factors to take account of the differing configurations of the radionuclides to be measured. Perform a linearity check of the instrument's response over the complete range of energies and activities for which the equipment is used.

For each setting and before each use (minimum once on each day of use) perform a constancy check of the ionisation chamber using standard sources of radionuclides with long half-lives to verify its calibrated state.

A check with a reference source, such as cesium-137, must be performed on each day of use to verify that the ionisation chamber is still in its calibrated state.

MEASUREMENT OF RADIOACTIVITY USING SOLID-STATE DETECTORS

Solid-state detectors include scintillating plastic fluorescent and crystal scintillator, and semiconductors. Further to their application in spectrometry (see section "Spectrometry"), solid-state detectors can be used for the measurement of radioactivity. In particular, due to their high sensitivity, plastic and crystal scintillation detectors are used in counting low levels of radioactivity. Dead-time losses must be carefully considered with these types of detectors. Semiconductor detectors are used when higher energy discrimination is required, for example in mixtures of radionuclides or when there are potential radionuclidic impurities with emissions of similar energy.

Apparatus. The equipment consists of a shielded detector comprising a plastic or crystal scintillator coupled to a photomultiplier, or a semiconductor, which are connected to an amplifier and counting electronics. The system may have an adjustable energy window, used for selecting a counting region of the radionuclide energy spectrum that may be adjusted by the operator. Instruments have different properties of energy resolution and detection efficiency depending on the type of detector and its volume and geometry. Lower efficiency requires a longer counting time.

Samples to be measured may be placed in front of the detector or into the well of a well-type detector. Measuring chambers may be enclosed in the detector shielding and single samples may be introduced using lids or other positioning systems to ensure correct measurement geometry.

A scintillation detector can be used for dynamic radioactivity measurement when, for example, the eluate of a liquid chromatograph is directed over or through a detector, as described in the section on detection and measurement of radioactivity in combination with a separation technique (determination of radiochemical purity).

Method. Ensure that the sample radioactivity gives a counting rate in the linearity range of the equipment. The measurement is started after any shielding is in place or the well cover is replaced and the counting time is selected to reach sufficient counts for a statistically significant value.

Calibration. The detector has to be calibrated by measuring its efficiency using a source of the radionuclide in question traceable to national or international standards. Calibration in terms of efficiency uses sources such as cesium-137, cobalt-60, barium-133, and others covering the desired energy range.

MEASUREMENT OF RADIOACTIVITY USING LIQUID SCINTILLATION DETECTORS

Liquid scintillation counting is commonly used for beta-particle emitting samples but is also used for alpha-particle emitting samples. For the principles of the detection of radioactivity using liquid scintillation detectors see under "Beta-particle spectrometry" below.

Calibration. In order to take into account the loss of counting efficiency due to quenching, the liquid scintillation counter may make use of an external source, typically barium-133 or europium-152, which is brought close to the sample vial to release Compton electrons. The shape of the resulting spectrum is analysed automatically to compute an absorption-indicating parameter. This parameter can then be related to the counting efficiency measuring sources of known activity at a determined level of the quenching agent. The obtained quench curve allows the determination of the activity of an unknown sample knowing the count rate and the value of the quenching parameter.

DETERMINATION OF HALF-LIFE

The half-life is a characteristic of the radionuclide that may be used for its identification.

The half-life is calculated by measuring the variation of the radioactivity of a sample to be tested as a function of time. Perform the measurements in the linearity range of a calibrated instrument.

Apparatus. The half-life can be measured by using any type of quantitative radioactivity detector provided it is used within a linearity range throughout the range of activities that are present during the measurement and the geometry is not changed during the measurement.

For drug preparations containing a radionuclide with a short half-life and when stated in a monograph, determination of the approximate half-life contributes to the identification (identification of the radionuclide).

Method

Half-life. The drug preparation to be examined is used as such or diluted or dried in a capsule after appropriate dilution. The radioactive sample is prepared in a manner that will avoid loss of substance during handling. If the radioactive sample is a liquid (solution), it is contained in a closed flask or a sealed tube. If it is a residue from drying in a capsule, it is protected by a cover consisting of a sheet of adhesive cellulose acetate or of some other material.

The radioactivity of the sample must be high enough to allow measurements over a period corresponding to 3 estimated half-lives but must be, for each measurement, within the linearity range of the equipment. Correction for dead-time losses is applied if necessary.

The same source is measured in the same geometrical conditions and at intervals usually corresponding to at least half of the estimated half-life. Each value is tabulated against the time interval from the initial measurement. To avoid influence of decay during measurement, the counting time is the same for all measurements.

A graph can be drawn with time as the abscissa and the logarithm of the relative instrument reading (e.g. count rate) as the ordinate. The half-life is calculated from the slope of the best linear fit of the measured values against the time corresponding to each measurement.

Approximate half-life. For this purpose, not fewer than three measurements are made over a period of not less than 1/4 of the estimated half-life.

The sample to be examined and the instrument to be used should meet the indications given above. The data are processed in the same way as above.

SPECTROMETRY

Radionuclides can be identified by their emission spectrum. Each type of emission (e.g., alpha particles, beta particles and electrons, gamma- and X-rays) requires specific equipment to acquire an emission spectrum. Spectrometers must be calibrated in order to work properly. A description of the various measuring equipment and a detailed description of the basic procedures for reliable measurement are provided in the following sections.

GAMMA-RAY SPECTROMETRY

General principles. In gamma-ray spectrometry using a scintillation detector, absorption of gamma- and X-rays results in production of light, which is converted into an electrical pulse by a photomultiplier. In gamma-ray spectrometry using a semiconductor detector, absorption of gamma- and X-rays results in the immediate production of an electrical pulse.

In both cases the pulse amplitude is proportional to the energy of the absorbed radiation. The most common detectors for gamma- and X-ray spectrometry are thallium-activated sodium iodide (NaI(Tl)) scintillation counters and high-purity germanium (HPGe) semiconductor detectors.

A gamma-ray spectrum can be produced by collecting and analysing a sufficient number of pulses.

Apparatus. A gamma-ray spectrometer usually comprises a shielded measuring chamber where the sample is positioned, a detector, an electronic chain and a multichannel analyser.

The shielding of the chamber must be able to reduce the background signal to a level that allows the registration of a correct gamma-ray spectrum.

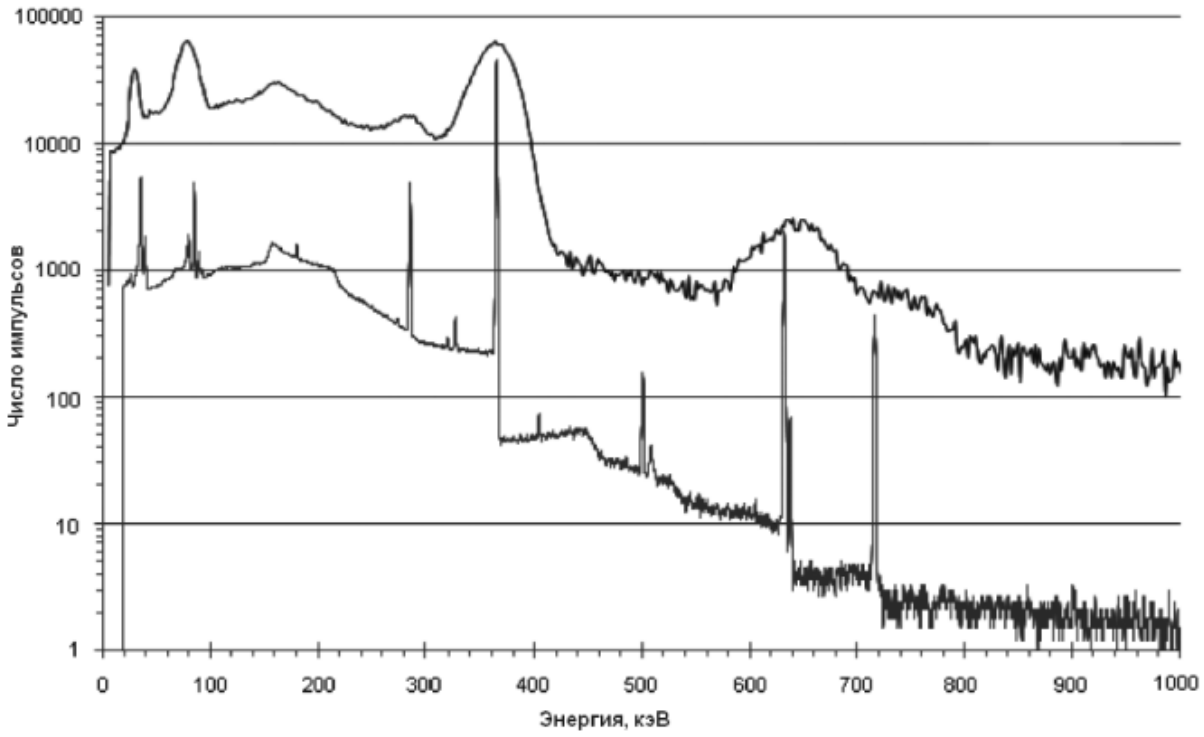
The measurement chamber has a movable cover or a drawer to allow the positioning of the sample. A sample holder may be present to ensure reproducible geometry between measurements.

The duration of measurement is related to the radioactivity of the target radionuclide and a long period of acquisition may be required to achieve the necessary counting statistics. Dead-time losses must be carefully considered with this type of detector.

The sensitivity of a NaI(Tl) detector is higher than that of a germanium detector of the same size. In general, peaks in an energy spectrum are identified with an uncertainty depending upon the full width of the peak at its half-maximum height. The energy resolution of a solid-state scintillation detector is much poorer than that of a semiconductor detector and hence peaks obtained with a semiconductor detector are much narrower than those obtained with a scintillation detector. Figure 2.1.2.43.-2 shows a comparison of the spectra obtained from the same source with the two types of detector.

The different performances of NaI(Tl) and HPGe detectors may limit their use in some spectrometric analyses.

For the identification of the radionuclide(s) in a radioactive pharmaceutical drug preparation (RPDP) and determination of radionuclidic purity, a risk assessment on the process of radionuclide production must assess the potential presence of other radionuclides with photon energies in the same range (± 10%) as that of the radionuclide(s) present in the RPDP.



	Number of pulses
	Energy, keV

Figure 2.1.2.43.-2. – *Comparative pulse-height spectra recorded using a thallium-activated sodium iodide scintillator (upper curve) and a high-purity germanium semiconductor detector (lower curve). The source was gamma- and X-ray radiation from the decay of iodine-131*

In case radionuclidic impurities can be present that emit gamma- or X-rays with an energy in the same range as that of the photons emitted by the radionuclide in the drug preparation, a measured peak energy within a maximum interval of ± 2 keV or ± 2% (the larger peak) with respect to the nominal peak energy (see Chapter *Physical characteristics of radionuclides indicates in the Pharmacopoeia*) is sufficient for peak identification.

In the case where such impurities are not expected to be present, a maximum range of ± 10 keV or ± 6% (the larger peak) with respect to the nominal peak energy is acceptable for peak identification.

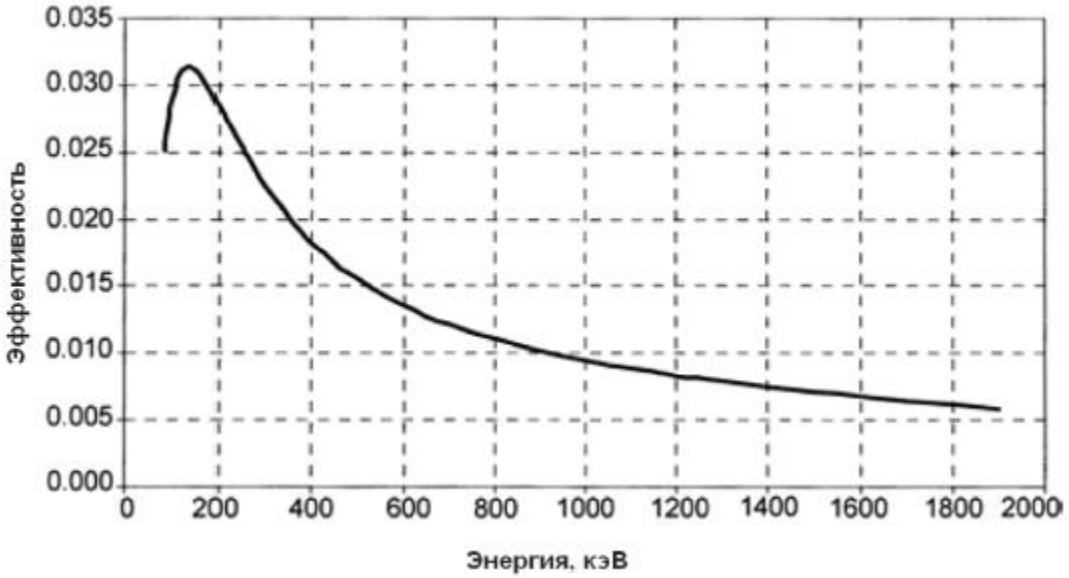
Method. Ensure that the count rate of the sample falls within the linearity range of the equipment. For liquid samples this may be achieved by appropriate dilution; for solid samples, by increasing the source-to-detector distance or by using a solvent. Introduce the drug preparation to be examined in a container into the instrument chamber and record the spectrum.

Ensure that the container used for quantitative measurements is of the same shape, dimensions, volume and material as that of the calibration standard.

Ensure that the composition of the solution and the position of the container in the measuring chamber is the same for the container for the quantitation as for the calibration standard.

Radionuclide identification. Calibrate the spectrometer in relation to energy. Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

Radionuclidic purity. Calibrate the spectrometer in relation to efficiency and energy. Determine the LOQ and resolution of the measurement equipment. Ensure that they meet the limits of the radionuclides to be determined. Record the spectrum of the drug preparation.



	Efficiency
	Energy, keV

Figure 2.1.2.43.-3 – *Typical HPGe efficiency curve measured using a dedicated container set on top of the detector*

- Record the spectrum of the drug preparation.

Identify the radionuclides present in the drug preparation to be examined and determine their radioactivity using chapter *Physical characteristics of radionuclides indicated in the Pharmacopoeia.* Because the level of radionuclidic impurities, expressed as a percentage of the total radioactivity, may increase or decrease with time, the measured activity of each impurity must be recalculated to the activity during the period of validity of the drug preparation. The activities of all radionuclidic impurities need to be summed up (taking into account the quantitation limit) and related to the total radioactivity of the drug preparation.

The sample is placed close to the detector or within a well-type detector. All the events within a pre-set energy range are collected and displayed on a ratemeter as pulses per second or accumulated over a pre-set period of time. If there is sufficient difference in photon energies emitted by the radionuclide(s), a sodium iodide detector can be suitable, given its high sensitivity. However, if there is a need to discriminate emissions of similar energy, a HPGe detector or another semiconductor detector is needed.

Calibration. Calibration in relation to energy is done by using the peaks of known sources traceable to national or international standards, such as cobalt-57, cesium-137, cobalt-60 and others covering the desired energy range. In parallel, a calibration in relation to efficiency can be performed, so that not only the energy spectrum but also the activity of the sample and the radionuclide impurities can be further determined. The calibration of efficiency can be performed with a standard radionuclide source with the desired energy range.

To obtain the efficiency curve, the detector response as a function of the energy has to be measured using each separate sample/detector geometry. For this reason, it is possible to carry out the measurement with the aid of a primary standard source. Primary standards may not be available for radionuclides with a short half-life, e.g. some positron emitters. When measuring, the sample will mostly have to be in a primary packaging and set at a defined position in relation to the detector. The sample/detector geometry is then defined by the position of the sample relative to the detector and the characteristics of the container and sample, e.g. shape, volume and density.

Figure 2.1.2.43.-3 shows a typical HPGe detector efficiency curve obtained for a cylindrical container placed on top of the detector.

BETA-PARTICLE SPECTROMETRY

In the case of a beta-particle emitter, a beta-particle spectrometer is necessary to determine the energy distribution of the emitted beta particles. It is analogous to a gamma-ray spectrometer but frequently uses liquid scintillators to convert the energy of the beta particles into detectable light, which can then be analysed. Beta-particle spectrometry is mostly achieved by dissolving or suspending the sample in a liquid scintillation cocktail in transparent or translucent (glass or plastic) containers and subsequent counting of the electrical pulses generated by a photomultiplier from the emitted light. The pulse amplitude is related to the energy of the absorbed radiation. A beta-particle spectrum can be produced by collecting a sufficient number of pulses. The liquid scintillation mixture is chosen in such a way that counting errors due to quenching, chemoluminescence, phosphorescence, etc., are minimised. Coincidence counting with two or more photomultipliers is also used to minimise counts from background radiation, electronics, etc. To differentiate between alpha- and beta-particle emissions, pulse-shape discrimination is commonly used.

Identification of the radionuclide. Determination of the correspondence of the mean and/or maximum energies in the energy spectrum from the sample to the energies prescribed by a monograph is a valid identification test.

Calibration. A common method of energy calibration is to use an unquenched reference sample to determine the maximum energy of the beta particles emitted by the radionuclide of interest.

ALPHA-PARTICLE SPECTROMETRY

For the identification and quantitation of alpha-particle emitters, spectrometry using liquid scintillation is mostly used. The principle is explained in the previous section on beta-particle spectrometry.

For the identification and determination of radionuclidic purity of alpha-particle emitters, spectrometry using a silicon-diode semiconductor detector can be used. Using this detector, the absorption of alpha particles results in the immediate production of an electrical pulse. The movement of electron-hole pairs created by the interaction of radiation induces an electrical charge, which is amplified and measured.

The sample preparation is of crucial importance. After a chemical separation of the radionuclide of interest, the sample is electro-deposited on a stainless steel disk in the form of a very thin layer of substance to minimise self-absorption. The yield of the whole procedure can be determined experimentally by adding a known amount of an indicator, which will take into account the chemical separation efficiency, the electro-deposition efficiency and the counting efficiency.

For both types of detectors, the pulse amplitude is related to the energy of the absorbed radiation. An alpha-particle spectrum can be produced by collecting a sufficient number of pulses.

Identification of the radionuclide. Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

Calibration. An alpha particle spectrometer has to be calibrated in relation to energy and efficiency. Calibration is performed by using the peaks from known sources covering the desired energy range, such as americium-241 and plutonium-242. Not all alpha-particles emitted by the source will produce a pulse in the system. The probability that an emitted alpha particle will interact with the detector material and produce a pulse is the efficiency of the detector, which depends on the geometry.

DETERMINATION OF RADIOCHEMICAL PURITY

A RPDP may contain the radionuclide in different chemical forms other than the required one. Therefore it is necessary to separate the different substances containing the radionuclide and determine their relative radioactivity. For this purpose, instruments for the detection and measurement of radioactivity are used in combination with a physico-chemical separation technique. In principle, any method of separation may be used.

Monographs for RPDPs may include the co-use of radioactivity measurement with paper chromatography (*2.1.2.25*), thin-layer chromatography (*2.1.2.26*), gas chromatography (*2.1.2.27*), liquid chromatography (*2.1.2.28*), size-exclusion chromatography (*2.1.2.29*), or electrophoresis (*2.1.2.30*).

In all cases the radioactivity of each analyte is measured after the separation has been achieved using the stated method.

Radioactivity measurement may be performed using detectors mounted in series with other detectors in analytical instruments, such as liquid chromatographs, making *in-line* detection of analytes, or performed *off-line*, i.e. after the analytical separation has been completed, by measuring the radioactivity of eluate fractions obtained after liquid chromatographic separation or as the distribution of radioactivity on paper chromatography or thin-layer chromatography supports (stripes/plates).

IN-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY IN COMBINATION WITH LIQUID CHROMATOGRAPHY

Apparatus. High-performance liquid chromatography (*2.1.2.28*) may be used to separate the principal radioactive substance of a radiopharmaceutical preparation from radiochemical impurities or degradation products. *In-line* detection is usually obtained by using a scintillation detector connected to a ratemeter and recording device.

The scintillating material of the detector is selected on the basis of the emission to be detected, e.g. plastic scintillator for beta-particle emissions or scintillation crystals for gamma- and X-ray radiations. The addition of a liquid scintillation cocktail before the eluate reaches the in-line radioactivity detector may also be used in the case of beta-particle-emitting radionuclides.

The simultaneous use of a radioactivity detector and other detectors (ultraviolet, refractive, conductimetric, etc.) connected in series may be used to identify the substance, e.g. in relation to the retention time of a known standard, to determine the amount of the substance using a suitable reference standard and to measure the radioactivity of the substance. When different detectors are coupled in series, correct the experimentally obtained retention times for the delay in time between the detectors.

In liquid chromatography some radiochemical impurities, such as colloidal impurities, may be retained on the column. In such cases a separate method is required for the determination of the content of the retained radiochemical impurities and the calculation formula for the expression of the total radiochemical purity takes into account the relative amount of the retained radiochemical impurities.

For evaluation of such retention problems during procedure validation, it is possible to determine the radioactivity recovery from the column by measuring the total radioactivity recovered from the chromatographic equipment with and without the column.

Method. Dilute the sample, if necessary, and then add the specified volume to the column under the specified conditions. In this respect it is important to demonstrate the LOD and LOQ, and the linearity of the detector throughout the range of activities to be measured.

Flow-through detector. A portion of the tubing where the eluate containing the radioactive species is flowing is placed in front of or within the detector.

Counting efficiency may be increased using a longer portion of the tube (e.g. making multiple turns in front of or within the detector); however, this will reduce the ability of the system to separate two closely eluting peaks of radioactivity.

When the radiochemical purity test prescribes determination of the total radiochemical impurities or there is a quantitation of an individual impurity, it is important to choose an appropriate threshold setting and appropriate conditions for integration of the peak areas. In such tests the disregard limit, i.e. the limit at or below which a peak is disregarded, is dependent on the procedure and is related to the detection limit and quantitation limit. Thus, the threshold setting of the data collection system corresponds to at least half of the disregard limit.

Record the signal of the detectors as a function of time.

Identification of peaks in the radiometric signal (radiochromatogram) is made on the basis of the retention time of the analytes. The profile from other detectors may be used for this purpose.

Quantitation of the different components of chromatogram and radiochromatogram profiles is made on the basis of peak areas. Peak areas are usually obtained by direct integration of the detector signal using commercially available software.

OFF-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY

High-performance liquid chromatography (*2.1.2.28*). Provided the retention times of the various radiochemical substances are reproducibly consistent, an alternative method of radioactivity quantitation is to collect the liquid chromatography effluent in a series of timed samples (fractions) for *off-line* analysis for radioactivity content. The radioactivity in the fractions corresponding to the peaks can be expressed as a percentage of the total of the radioactivity in all fractions, taking into account the quantitation limit.

Method. The sample is applied on the column in the prescribed volume and conditions. Fractions are collected at the end of the chromatographic line.

The volume between the detector used to identify the retention time of the peaks and the collection point is measured and a delay factor is calculated on the basis of the effluent flow rate and applied to each peak to estimate the time of elution of the peak at the point of collection. The fractions are collected on the basis of a fixed time interval or at the time of appearance estimated from the delay time so that any relevant peak is collected in one or more fractions.

The radioactivity of each fraction is counted using a calibrated instrument such as a dose calibrator or a scintillation detector, taking into account the quantitation limit and the linearity.

An elution profile is obtained tabulating the pulses per fraction against the elution time or volume. The activity of fractions belonging to the same peak may be summed up and the relative percentage calculated to define radiochemical purity.

Thin-layer chromatography (*2.1.2.26*) and paper chromatography (*2.1.2.25*). Provided a thin-layer chromatography or a paper chromatography analytical procedure has been validated for the separation of components of a radioactive preparation, the number and relative intensities of the separated spots can be detected and measured using a radioactivity detector.

The positions of the spots (peaks) may permit chemical identification by comparison with solutions of the same chemical substances (non-radioactive), using a suitable detection method.

Apparatus

Scanning device. The apparatus generally comprises a radioactivity detector, such as a position-sensitive proportional counter or a collimated scintillation detector placed at a fixed distance from a scanning platform where the chromatographic support (plate/strip) to be scanned is positioned.

The radioactivity of the sample applied to the chromatography support must result in a count rate in the linearity range of the equipment and the sample may be diluted if necessary. The area to be scanned is positioned at the reference position so that the desired lane is aligned with the detector scanning trip. Adjusting the scanning time is necessary to allow enough counting time during the single run.

The detector or the platform may be moved in-plane, along the *x*-axis or the *y*-axis, so that the entire surface can be scanned during a single run.

The detector is connected to a suitable counting device, so that the radioactivity revealed can be quantified and the count rate related spatially to the surface scanned.

The radioactivity is automatically reported against the development distance and the profile describes peaks having an area proportional to the number of counts per unit of distance.

Radioactivity counter. In the case where a maximum of only three radiochemical components needs to be identified and they are fully separated, the support can be cut into equal strips, each having a size not greater than half the length of the support corresponding to the difference between the retardation factors (R_f) of the two closest spots. Every single strip is numbered starting from the origin side and counted separately. Alternatively, for well-characterised systems, the strip may be cut into two or more unequal portions, the dimensions of which before the count can be adjusted if necessary. An ionisation chamber or a scintillation counter can be used for this purpose, provided they are used within the instrument's linearity range and above its LOQ.

Autoradiography. Autoradiography may also be used to acquire an image of the radioactivity distribution on the chromatographic support. In this case, the response of the system used for the acquisition of the image, such as a phosphor imager or a photographic film, must be shown to be linear with respect to the radioactivity in the chromatogram. Otherwise, the system must be pre-calibrated or exposed at the same time to a series of reference radioactive sources, obtained by dilution from a calibrated standard solution, covering the expected radioactivity range that may be present on the support.

Method. Deposit the required amount of sample at the origin of the chromatographic support, with drying if necessary to avoid spreading of the spot. Develop the chromatogram according to the specified method. A carrier may be added when directed in a monograph.

In paper and thin-layer chromatography, it is preferable not to dilute the drug preparation to be examined but it is important to avoid depositing a substance with such a quantity of radioactivity that counting losses by coincidence (dead-time losses) occur during measurement of the radioactivity.

After development, the support is dried and the positions of the radioactive areas are detected by measurement of radioactivity over the length of the chromatogram, using a suitable collimated counter, by autoradiography, or by cutting the strips into portions and counting each portion.

Radioactivity may be measured by integration using an automatic-plotting instrument or a digital counter.

The ratios of the areas under the peaks give the ratios of the percentages of radioactivity due to the respective radiochemical substances.

When the strips are cut into portions, the ratios of the quantities of radioactivity measured give the ratio of percentages of radioactivity due to the respective radiochemical substances.

Calibration. It is important to demonstrate the detection and quantitation limits, and the linearity of the detector throughout the range of activities to be measured and in all positions on the support of the chromatographic system. This may be done by applying samples covering a range of activities from 0.1% to 100% of the expected range. Prepare the samples by dilution and apply equal volumes of each, with drying if necessary. After examining the radioactivity profile using the equipment's standard settings, the peak areas are integrated for comparison with the calculated amount of radioactivity applied to each spot.

Verify that the response of the detector over the complete length and width of the detector path is the same, as the response may vary with the detector position.

The peak-resolving power is influenced by the size of the spot, the total radioactivity of the radionuclide, and the detector equipment. It can be checked by applying 5 µL spots separated by distances increasing from 4 mm to 20 mm in 2 mm increments. The approximate resolution of the detection system can be determined from the radioactivity profile as the distance between the two spots where the baseline is only just clearly separated.

2.1.3. IDENTIFICATION

This section presents procedures for performing identification reactions for ions, functional groups, and individual groups of substances.

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2.1.3.1. Identification Reactions

ALKALOIDS

a) Dissolve a weighing amount of the test sample specified in the monograph, in 5 mL of *water R*, add *dilute hydrochloric acid R* until an acid reaction occurs (*2.1.2.4*), then add 1 mL of *potassium iodobismuthate solution R*; an orange or orange-red precipitate is formed immediately.

b) Place 1.0 g of crushed plant raw materials in a 100 mL flask, add 25 mL of *1% (V/V) hydrochloric acid R*, and heat in a boiling water bath for 5 minutes, then cool down, and decant the extraction through a paper filter. Add 1 mL of a 10 mg/mL solution of *phosphomolybdic acid R* in *water R* to 1 mL of the filtrate; a yellowish precipitate is formed which after a while turns blue or green.

c) Add 1 mL of a 10 g/l solution of *phosphotungstic acid R* in *water R* to 1 mL of the filtrate obtained in test (b); an off-white precipitate is formed.

ALUMINUM

Dissolve about 15 mg of the test sample in 2 mL of *water R*. Add about 0.5 mL of *dilute hydrochloric acid R* and about 0.5 mL of *thioacetamide reagent R* to the obtained solution or to 2 mL of the solution prescribed in the monograph; no precipitate is formed. Then add dropwise *diluted sodium hydroxide solution R*; a gelatinous white precipitate is formed which dissolves on further addition of *dilute sodium hydroxide solution R*. Gradually add *ammonium chloride solution R* to the obtained solution; the gelatinous white precipitate is re-formed.

PRIMARY AROMATIC AMINES

Acidify the solution specified in the monograph with *dilute hydrochloric acid R* and add 0.2 mL of *sodium nitrite solution R*, and after 1-2 min, add 1 mL of *β-naphthol solution R*; an intense orange or red colour and usually a precipitate of the same colour are produced.

AMMONIUM SALTS

Add 0.2 g of *magnesium oxide R* to the solution prescribed in the monograph. Pass a current of air flow through the mixture and direct the gas that escapes just beneath the surface of a mixture of 1 mL of *0.1 M hydrochloric acid* and 0.05 mL of *methyl red solution R*; the colour of the indicator changes to yellow. Then add 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R*; a yellow precipitate is formed.

AMMONIUM SALTS AND SALTS OF VOLATILE BASES

Dissolve about 20 mg of the substance to be examined in 2 mL of *water R*. Add 2 mL of *dilute sodium hydroxide solution R* to the resulting solution or to 2 mL of the solution prescribed in the monograph. On heating, the solution gives off a vapour of ammonia and volatile bases that can be identified by its odor and by its alkaline reaction (*2.1.2.4*).

ACETATES

a) Heat the test sample with an equal quantity of *oxalic acid R*; acetic acid, detected by its odor and acid reaction, is liberated (*2.1.2.4*).

b) Dissolve about 30 mg of the test sample in 3 mL of *water R*. Add successively 0.25 mL of *lanthanum nitrate solution R*, 0.1 mL of 0.05 M *iodine*, and 0.05 mL of *dilute ammonia R2* to the resulting solution or to 3 mL of the solution prescribed in the monograph.

Heat the mixture carefully to boiling; within a few minutes a blue precipitate is formed or a dark blue colour develops.

c) Add 0.2 mL of a solution of 30 g/L of *iron (III) chloride R* to 2 mL of a neutral solution of the test sample which contains 20 to 60 mg of the acetate ion ($CH3COO^-$); red-brown colour develops, which disappears after adding dilute mineral acids.

d) Heat 2 mL of the solution of the test sample containing 20 mg to 60 mg of the acetate ion (CH_3COO^-) with an equal amount of *sulfuric acid R* and 0.5 mL of *96% ethanol R*; ethyl acetate detectable by smell is formed.

ACETYL

In a test-tube, about 180 mm long and 18 mm in external diameter, place about 15 mg of the test sample or the prescribed quantity, and 0.15 mL of *phosphoric acid R*. Close the test tube with a stopper through which passes a small test-tube about 100 mm long and 10 mm in external diameter containing *water R* to act as a condenser. On the outside of the smaller tube, hang a drop of *lanthanum nitrate solution R*. If the substance is relatively easily hydrolyzed, place the apparatus in a water-bath for 5 min, then take out the smaller tube. Remove the drop and mix it with 0.05 mL of *0.01 M iodine* on a porcelain tile. Add at the edge 0.05 mL of *dilute ammonia R2*; after 1-2 min, a blue colour develops at the junction of the two drops, the colour intensifies and persists for a short time.

For substances hydrolyzable only with difficulty, heat the mixture slowly to boiling over an open flame and then proceed as prescribed above.

BARBITURATES (NON-NITROGEN SUBSTITUTED)

Dissolve about 5 mg of the test sample in 3 mL of *methanol R*, add 0.1 mL of a solution containing 100 g/L of *cobalt nitrate R* and 100 g/L of *calcium chloride R*, mix and add, with shaking, 0.1 mL of *dilute sodium hydroxide solution R*; a violet-blue colour and precipitate are formed.

BENZOATES

a) To 1 mL of the solution specified in the monograph add 0.5 mL of *ferric (III) chloride solution R1*; a dull-yellow precipitate, soluble in *ether R*, is formed.

b) Place 0.2 g of the test sample, treated if necessary as specified in the monograph, in a test-tube, moisten with 0.2 mL to 0.3 mL of *sulfuric acid R*, and gently warm the bottom of the tube; a white sublimate is deposited on the inner wall of the tube.

c) Dissolve 0.5 g of the test sample in 10 mL of *water R*. To the resulting solution or to 10 mL of the solution specified in the monograph, add 0.5 mL of *hydrochloric acid R*; the precipitate obtained, after crystallisation from warm *water R* and drying in vacuo, has a melting point (*2.1.2.14*) of 120 °oC to 124 °C.

BROMIDES

a) Dissolve in 2 mL of *water R* a quantity of the test sample equivalent to about 3 mg of bromide (Br^-). Acidify the solution obtained or 2 mL of the solution, specified in the monograph, with *dilute nitric acid R*, add 0.4 mL of *silver nitrate solution R1*, mix and allow to stand; a curdled, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light. The supernatant solution may have opalescence. Suspend the precipitate obtained in 2 mL of *water R* and add 1.5 mL of *ammonia R*; the precipitate dissolves with difficulty.

b) Introduce into a small test-tube a quantity of the test sample equivalent to about 5 mg of bromide (Br^-) or the quantity specified in the monograph, add 0.25 mL of *water R*, about 75 mg of *lead dioxide R*, 0.25 mL of *acetic acid R* and shake gently. Dry the inside of the upper part of the test-tube with a piece of filter paper and allow to stand for 5 min.

Impregnate a strip of suitable filter paper of appropriate size immersing the tip in a drop of *decolourised fuchsin solution R* and introduce the impregnated part immediately into the tube. Starting from the tip, a violet colour appears within 10 s that is clearly distinguishable from the red colour of fuchsin, which may be visible on a small area at the top of the impregnated part of the paper strip.

c) To 1 mL of the solution of the test sample containing between 2 mg and 30 mg of the bromide ion (Br^-), add 1 mL of *dilute hydrochloric acid R*, 0.5 mL of a freshly prepared 50 g/L solution of *chloramine R*, 1 mL of *chloroform R* and shake; the chloroform layer turns yellow-brown.

BISMUTH

a) Add 10 mL of *dilute hydrochloric acid R* to 0.5 g of the test sample. For 1 min, heat to boiling the solution obtained or 10 mL of the solution prescribed in the monograph, cool, and filter if necessary. Add 20 mL of *water R* to 1 mL of the solution obtained; a white or slightly yellow precipitate is formed which on the addition of 0.05 mL to 0.1 mL of *sodium sulfide solution R* turns brown.

b) Add 10 mL of *dilute nitric acid R* to about 45 mg of the test sample. For 1 min, boil the solution obtained or 10 mL of the solution prescribed in the monograph, allow to cool and filter if necessary. To 5 mL of the solution obtained, add 2 mL of a 100 g/L solution of *thiourea R*; a yellowish-orange colour or an orange precipitate is formed. Add 4 mL of a 25 g/L solution of *sodium fluoride R*; the solution is not decolourised within 30 min.

IRON

a) Dissolve a quantity of the test sample examined equivalent to about 10 mg of iron (Fe^{2+}) in 1 mL of *water R*. To 1ml of this solution or to 1 mL of the solution prescribed in the monograph, add 1 mL of *potassium ferricyanide solution R*; a blue precipitate is formed that does not dissolve on the addition of 5 mL of *dilute hydrochloric acid R*.

b) Dissolve a quantity of the test sample equivalent to about 1 mg of iron (Fe^{3+}) in 30 mL of *water R*. To 3 mL of this solution or to 3 mL of the solution prescribed in the monograph, add 1 mL of *dilute hydrochloric acid R* and 1 mL of *potassium thiocyanate solution R*; the solution is coloured red. Take two portions, each of 1 mL, of the resulting solution. To one portion add 5 mL of *isoamyl alcohol R* or 5 mL of *ether R*, shake, and allow to stand; the organic layer is coloured pink. To the other portion add 2 mL of *mercuric chloride solution R*; the red colour disappears.

c) Dissolve a quantity of the test sample equivalent to not less than 1 mg of iron (Fe^{3+}) in 1 mL of *water R*. To 1 mL of this solution or to 1 mL of the solution prescribed in the monograph, add 1 mL of potassium *ferrocyanide solution R*; a blue precipitate is formed that does not dissolve on the addition of 5 mL of *dilute hydrochloric acid R.*

IODIDES

a) Dissolve a quantity of the test sample examined equivalent to about 4 mg of iodide (I^-) in 2 mL of *water R* . Acidify this solution or 2 mL of the solution specified in the monograph with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*, mix and allow to stand till a curdled, pale-yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light. The supernatant solution may have opalescence. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*; the precipitate does not dissolve.

b) To 0.2 mL of a solution of the test sample containing about 5 mg of iodide (I^-) per millilitre, or to 0.2 mL of the solution prescribed in the monograph, add 0.5 mL of *dilute sulfuric acid R*, 0.1 mL of *potassium dichromate solution R*, 2 mL of *water R* and 2 mL of *chloroform R*, shake for a few seconds, and allow the phases to separate; the chloroform layer is coloured violet or violet-red.

POTASSIUM

a) Dissolve 0.1 g of the test sample in 2 mL of *water R*. To the solution obtained or to 2 mL of the solution prescribed in the monograph, add 1 mL of *sodium carbonate solution R*, and heat; no precipitate is formed. Add to the hot solution 0.05 mL of *sodium sulfide solution R*; no precipitate is formed. Cool in iced water and add 2 mL of a 150 g/L solution of *tartaric acid R*, allow to stand; a white crystalline precipitate is formed.

b) Dissolve about 40 mg of the test sample in 1 mL of *water R*. To this solution or to 1 mL of the solution prescribed in the monograph, add 1 mL of *dilute acetic acid R* and 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R*; a yellow or orange-yellow precipitate is formed immediately.

c) Potassium salt introduced into a colorless flame turns it violet or, when viewed through blue glass, turns purple-red.

CALCIUM

a) To 0.2 mL of a neutral solution containing a quantity of the test sample equivalent to about 0.2 mg of calcium (Ca^{2+}) per millilitre or to 0.2 mL of the solution specified in the monograph, add 0.5 mL of a 2 g/L solution of *glyoxalhydroxyanil R* in *96% ethanol R*, 0.2 mL of *dilute sodium hydroxide solution R* and 0.2 mL of *sodium carbonate solution R*. Shake the mixture with 1 mL to 2 mL of *chloroform R* and add 1 mL to 2 mL of *water R*; the chloroform layer is coloured red.

b) Dissolve about 20 mg of the test sample or the quantity specified in the monograph in 5 mL of *acetic acid R*. Add 0.5 mL of *potassium ferrocyanide solution R* to the solution obtained; the solution remains clear. About 50 mg of *ammonium chloride R* is added to the solution; a white crystalline precipitate is formed.

c) To 1 mL of a solution of the test sample containing from 2 mg to 20 mg of calcium ion (Ca^{2+}), add 1 mL of a 40 g/L solution of *ammonium oxalate R*. A white precipitate is formed, insoluble in *acetic acid dilute R* and *ammonia solution R* but soluble in dilute mineral acids.

d) Moistened with *hydrochloric acid R*, calcium salt stains a nonluminous flame orange-red.

CARBONATES AND BICARBONATES

a) Introduce into a test-tube 0.1 g of the test sample and suspend in 2 mL of *water R*. To this suspension or to 2 mL of the solution specified in the monograph, add 3 mL of *dilute acetic acid R*. Close the tube immediately using a stopper fitted with a glass tube bent twice at right angles; the solution or the suspension becomes effervescent and gives off a colorless and odorless gas. Heat gently and collect the gas in 5 mL of *barium hydroxide solution R*; a white precipitate is formed that dissolves on the addition of an excess of *hydrochloric acid R1*.

b) Dissolve 0.2 g of the test sample in 2 ml of *water R*. To the resulting solution add 0.5 ml of a saturated solution of *magnesium sulfate R*; a white precipitate is formed (in contrast to hydrocarbonates, solutions of which form a precipitate only when the mixture is boiled).

NOTE. *To obtain a saturated solution of magnesium sulfate, add 100 ml of water R to 100 g of magnesium sulfate R and allow to stand for 24 h with frequent shaking. Filter the solution.*

c) Dissolve 0.2 g of the test sample in 2 mL of *water R.* To the resulting solution, add 0.05 mL of a solution of *phenolphthalein R*; a red colouration develops (in contrast to bicarbonates, solutions of which remain colourless).

XANTHINES

To a few milligrams of the test sample or the prescribed quantity add 0.1 mL of *strong hydrogen peroxide solution R* and 0.3 mL of *dilute hydrochloric acid R*, heat to dryness on a water-bath until a yellowish-red residue is obtained. Add 0.1 mL of *dilute ammonia R2*; the colour of the residue changes to violet-red.

LACTATES

Dissolve a quantity of the test sample equivalent to about 5 mg of lactic acid in 5 mL of *water R*. To this solution or to 5 mL of the solution specified in the monograph, add 1 mL of *bromine water R* and 0.5 mL of *dilute sulfuric acid R*, and heat on a water-bath until the colour is discharged, stirring occasionally with a glass rod. Add 4 g of *ammonium sulfate R* and mix, then add dropwise and without mixing 0.2 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R*, still without mixing add 1 mL of *concentrated ammonia R*, and allow to stand for 30 min; a dark green ring appears at the interface of the two liquids.

MAGNESIUM

Dissolve about 15 mg of the test sample in 2 mL of *water R*. To this solution or to 2 mL of the solution specified in the monograph, add 1 mL of *dilute ammonia R*; a white precipitate is formed that dissolves on the addition of 1 mL of *ammonium chloride solution R*. Then add 1 mL of *disodium hydrogen phosphate solution R*; a white crystalline precipitate is formed.

ARSENIC

a) Heat 5 mL of the prescribed solution on a water-bath with an equal volume of *hypophosphorous reagent R*; a brown precipitate is formed.

b) To 0.3 mL of the test sample solution containing about 30 mg of arsenite ion (AsO_3^{3-}), add 0.5 mL of *dilute hydrochloric acid R* and 0.1 mL of *sodium sulfide solution R;* a yellow precipitate is formed, insoluble in concentrated *hydrochloric acid R* and soluble in *ammonia solution R*.

c) To 0.3 mL of the test sample solution containing about 1 mg of arsenate ion (AsO_4^{3-}), add 1 mL of a 100 g/L *ammonium chloride R, ammonia solution R*, and a 100 g/L *magnesium sulfate R* solution; a white crystalline precipitate is formed, soluble in *dilute hydrochloric acid R* (distinction from arsenites).

SODIUM

a) Dissolve 0.1 g of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 2 mL of a 150 g/L solution of *potassium carbonate R* and heat to boiling; no precipitate is formed. Add 4 mL of *potassium pyroantimonate solution R* and heat to boiling, then allow to cool in an ice bath and if necessary rub the inside of the test-tube with a glass rod; a dense white precipitate is formed.

b) Dissolve a quantity of the test sample equivalent to about 2 mg of sodium (Na^+) in 0.5 mL of *water R*. To this solution or to 0.5 mL of the solution specified in the monograph, add 1.5 mL of *methoxyphenylacetic reagent R* and cool in an ice bath for 30 min; a voluminous, white, crystalline precipitate is formed. Place the mixture in the water at 20 °C and stir for 5 min; the precipitate does not disappear. Add 1 mL of *dilute ammonia R1*; the precipitate dissolves completely. Add 1 mL of ammonium *carbonate solution R*; no precipitate is formed.

c) Moistened with *hydrochloric acid R*, sodium salt stains a nonluminous flame yellow.

NITRATES

To a mixture of 0.1 mL of *nitrobenzene R* and 0.2 mL of *sulfuric acid R*, add a quantity of the test sample equivalent to about 1 mg of nitrate (NO3-) or the quantity specified in the monograph and allow to stand for 5 min. During cooling, add slowly and with mixing 5 mL of *water R*, then add 5 mL of *strong sodium hydroxide solution R*, 5 mL of *acetone R*, shake and allow to stand; the upper layer is coloured deep violet.

NITRITES

Dissolve several crystals of *phenazone R*in a porcelain dish with 0.1 mL of *dilute hydrochloric acid R*, add 0.1 mL of a solution containing about 1 mg of nitrite ion (NO2-); a green colour develops (distinction from nitrates).

MERCURY

a) Place about 0.1 mL of a test sample solution on well-scraped copper foil; a dark-grey spot that becomes shiny on rubbing is formed. Dry the foil and heat in a test-tube. The spot disappears.

b) To the solution prescribed in the monograph add *diluted sodium hydroxide solution R* until strongly alkaline (*2.1.2.4*); a dense yellow precipitate is formed (mercuric salts).

c) Carefully add *potassium iodide R* solution dropwise to 1 mL of the test sample solution of the test sample containing from 10 mg to 30 mg of mercury ion (Hg^{2+}); a red precipitate is formed that dissolves in excess of this reagent.

SALICYLATES

a) To 1 mL of the solution prescribed in the monograph, add 0.5 mL of a solution of *ferric (III) chloride R1*; a violet colouration develops, which does not disappear on the addition of 0.1 mL of *acetic acid R.*

b) Dissolve 0.5 g of the test sample in 10 mL of *water R*. To this solution or to 10 mL of the solution specified in the monograph, add 0.5 mL of *hydrochloric acid R*. The resulting precipitate after recrystallisation from hot *water R* and drying *in vacuo* has a melting point (*2.1.2.14.*) between 156 °C and 161 °C.

LEAD

a) Dissolve 0.1 g of the test sample in 1 mL of *acetic acid R*. To this solution or to1 mL of the solution specified in the monograph, add 2 mL of *potassium chromate solution R*; a yellow precipitate is formed that dissolves on the addition of 2 mL of *strong sodium hydroxide solution R*.

b) Dissolve 50 mg of the test sample in 1 mL of *acetic acid R*. To this solution or to1 mL of the solution specified in the monograph, add 10 mL of *water R* and 0.2 mL of *potassium iodide solution R*; a yellow precipitate is formed. Heat the mixture to boiling for 1-2 min; the precipitate dissolves. Cool the solution, and the precipitate is re-formed as glistening, yellow plates.

SILVER

Dissolve about 10 mg of the test sample in 10 mL of *water R*. To this solution or to 10 mL of the solution specified in the monograph, add 0.3 mL of *hydrochloric acid R1*; a curdled, white precipitate is formed that dissolves on the addition of 3 mL of *dilute ammonia R1*.

SILICATES

Mix the prescribed quantity of the substance to be examined in a lead or platinum crucible by means of copper wire with about 10 mg of *sodium fluoride R* and a few drops of *sulfuric acid R* to give a fine suspension. Cover the crucible with a thin, transparent plate of plastic under which a drop of *water R* is suspended and warm gently; within a short time, a white ring is rapidly formed around the drop of water.

ESTERS

To about 30 mg of the test sample or the quantity specified in the monograph, add 0.5 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* in *methanol R* and 0.5 mL of a 100 g/L solution of *potassium hydroxide R* in *96% ethanol R*, heat to boiling, cool, acidify with *dilute hydrochloric acid R*, and add 0.2 mL of *ferric (III) chloride solution R1* diluted ten times; a bluish-red or red colour is produced.

SULFATES

a) Dissolve about 45 mg of the test sample in 5 mL of *water R*. To this solution or to 5 mL of the solution specified in the monograph, add 1 mL of *dilute hydrochloric acid R* and 1 mL of *barium chloride solution R1*; a white precipitate is formed.

b) To the suspension obtained during reaction (a), add 0.1 mL of *0.05 M iodine*; the suspension remains yellow (distinction from sulfites and dithionites) but is decolourised by adding dropwise *stannous chloride solution R* (distinction from iodates). Boil the mixture; no colouration of the presipitate (distinction from selenates and tungstates).

SULFITES

To 2 mL of the solution of the test sample containing from 10 mg to 30 mg of sulfite ion (SO2-), add 2 mL of *dilute hydrochloric acid R* and shake; gradually, sulfur dioxide is liberated, detectable by a characteristic strong odor.

ANTIMONY

Dissolve with gentle heating about 10 mg of the test sample in a solution of 0.5 g of *sodium potassium tartrate R* in 10 mL of *water R* and allow it to cool. To 2 mL of this solution or to 2 mL of the solution specified in the monograph, add *sodium sulfide solution R* dropwise; an orange-red precipitate is formed which dissolves on the addition of *dilute sodium hydroxide solution R*.

TARTRATES

a) Dissolve about 15 mg of the substance to be examined in 5 mL of *water R*. To this solution or to 5 mL of the solution specified in the monograph, add 0.05 mL of a 10 g/L solution of *ferrous (II) sulfate R* and 0.05 mL of *dilute hydrogen peroxide solution R*; a transient yellow colour is produced. After the colour has disappeared, add diluted *sodium hydroxide solution R* dropwise; a violet or purple colour is produced.

b) To 0.1 mL of a solution of the test sample containing the equivalent of about 15 mg/mL of tartaric acid or to 0.1 mL of the solution specified in the monograph, add 0.1 mL of a 100 g/L solution of *potassium bromide R*, 0.1 mL of a 20 g/L solution of *resorcinol R* and 3 mL of *sulfuric acid R*, and heat on a water-bath for 5-10 min; a dark-blue colour develops. Allow the solution to cool and pour it into *water R*; the colour changes to red.

PHOSPHATES (ORTHOPHOSPHATES)

a) To 5 mL of the solution specified in the monograph, neutralised if necessary, add 5 mL of *silver nitrate solution R1*; a yellow precipitate is formed whose colour is not changed by boiling and which dissolves on the addition of *ammonia R*.

b) Mix 1 mL of the solution specified in the monograph with 2 mL of *molybdovanadic reagent R*; a yellow colour develops.

CHLORIDES

a) Dissolve in 2 mL of *water R* a quantity of the test sample equivalent to about 2 mg of chloride ion (Cl^-). Acidify this solution or 2 mL of the solution specified in the monograph with *dilute nitric acid R*, add 0.4 mL of *silver nitrate solution R1*, mix and allow to stand; a curdled, white precipitate is formed, which is centrifuged and washed with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light. The supernatant solution may have opalescence. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*; the precipitate dissolves easily. A few large slowly dissolving particles are allowed.

b) Introduce into a test-tube a quantity of the test sample equivalent to about 15 mg of chloride (Cl^-) or the prescribed quantity, add 0.2 g of *potassium dichromate R* and 1 mL of *sulfuric acid R*. Place a filter-paper strip impregnated with 0.1 mL of *diphenylcarbazide solution R* over the opening of the test-tube (the impregnated paper must not come into contact with the potassium dichromate); the paper turns violet-red.

ZINC

Dissolve 0.1 g of the test sample in 5 mL of *water R*. To this solution or to 5 mL of the solution specified in the monograph, add 0.2 mL of *strong sodium hydroxide solution R*; a white precipitate is formed. Add a further 2 mL of *strong sodium hydroxide solution R*; the precipitate dissolves. Add 10 mL of *ammonium chloride solution R*; the solution remains clear. Add 0.1 mL of *sodium sulfide solution R*; a flocculent white precipitate is formed.

b) To 2 mL of a solution of the test sample containing from 5 mg to 20 mg of zinc ion (Zn^{2+}), add 0.5 mL of a solution of *potassium ferrocyanide R*; a white precipitate is formed, insoluble in *dilute hydrochloric acid R*.

CITRATES

a) Dissolve in 5 mL of *water R* a quantity of the test sample equivalent to about 50 mg of citric acid. To this solution or to 5 mL of the solution specified in the monograph, add 0.5 mL of *sulfuric acid R* and 1 mL of *potassium permanganate solution R*. Heat the solution until discolouration, add 0.5 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R* and 4 g of *sulfamic acid R*. Make alkaline with *concentrated ammonia R*, added dropwise until all the sulfamic acid has dissolved. The addition of an excess of *concentrated ammonia R* produces a violet colour, turning to violet-blue.

b) To 1 mL of a neutral solution of the test sample containing from 2 mg to 10 mg of citrate ion, add 1 mL of a 200 g/L solution of *calcium chloride R*; the solution remains clear. When the solution is boiled, a white precipitate is formed, which dissolves in *dilute hydrochloric acid R*.

c) Add 0.5 mL of *acetic anhydride R* to a quantity of the test sample equivalent to 1 mg to 2 mg of citrate ion and heat; red staining appears 20-40 s later.

201030002-2019

2.1.3.2. Odour

The odor should be characterised by the terms such as "odorless", "characteristic odor", "weak characteristic odor".

The test is usually performed immediately after opening a container.

On a watch-glass 6 cm to 8 cm in diameter, spread in a thin layer 0.5 g to 2.0 g of the test sample; after 15 min, determine the odor at a distance of 4-6 cm, or verify the absence of odor.

To determine the odor of highly volatile liquid drugs, 0.5 mL of the test sample is applied to the filter paper and the odor is immediately determined unless otherwise specified.

2.1.4. IMPURITY LIMIT TESTS

This section provides procedures for determining the maximum content of impurities of various origins.

201040001-2019

2.1.4.1. Ammonium salts

Unless otherwise prescribed, use method A.

METHOD A

a) Introduce the solution specified in the monograph into a test-tube or dissolve the prescribed quantity of the test sample in 14 mL of *water R* in a test-tube. Make the solution alkaline if necessary by the addition of *dilute sodium hydroxide solution R*, dilute to 15 mL with *water R*, and add 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. As a reference solution, use the solution prepared by mixing 10 mL of *ammonium standard solution (1 ppm NH4) R*, 5 mL of *water R* and 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. Stopper the test-tubes.

After 5 min, any yellow colour in the test solution is not more intense than that in the reference solution.

METHOD B

In a 25 mL jar fitted with a cap, place a quantity of the finely powdered test sample and dissolve or suspend in 1 mL of *water R*. Add 0.30 g of *heavy magnesium oxide R*. Close immediately after placing a piece of *silver manganese paper R* 5 mm^2, wetted with a few drops of *water R*, under the cap. Mix the contents of the jar in a circular motion, avoiding projections of liquid on the paper, and allow to stand at 40 °C for 30 min.
Prepare a reference solution at the same time and in the same manner. To the specified volume of *ammonium standard solution (1 ppm NH_4^+) R*, add 1 mL of *water R* and 0.30 g of *heavy magnesium oxide R* and then proceed as with the test solution.

The grey colouration of the *silver manganese paper R* obtained in the experiment with the test solution should not be more intense than the colouration of the *silver manganese paper R* obtained in the experiment with the reference solution.

METHOD B

The method is intended for the determination of ammonium salts in the presence of alkaline earths and heavy metals.

Test solution. Dissolve the quantity of the sample specified in the monograph in as little *water R* as possible, add 2 mL of a 10% (*m/V*) solution of *sodium hydroxide R*, 2 mL of a 10% (*m/V*) solution of *sodium carbonate R* and dilute with *water R* to the required concentration, shake, and filter. Use 10 mL of the resulting filtrate.

Reference solution. 10 mL *of ammonium standard solution (2 ppm NH_4^+).*

To the test solution and the reference solution, add 0.15 mL of *alkaline potassium tetraiodomercurate solution R* and mix. After 5 minutes, compare the colouration of the solutions.

METHOD D

The method is intended for the determination of ammonium salts in the presence of no greater than 0.03% of iron impurities.

Test solution. To 10 mL of the solution prepared as specified in the monograph, add two drops of a 10% (*m/V*) solution of *sodium hydroxide R* and 3 mL of a 20% (*m/V*) solution of *potassium-sodium tartrate R*, then mix.

Reference solution. To 10 mL *of ammonium standard solution (2 ppm NH_4^+)*, add two drops of a 10% (*m/V*) solution of *sodium hydroxide R* and 3 mL of a 20% (*m/V*) *solution of potassium-sodium tartrate R.*

To the test solution and the reference solution, add 0.15 mL of a *solution of alkaline potassium tetraiodomercurate R* and mix. After 5 minutes, compare the colouration of the solutions.

201040002-2019

2.1.4.2. Arsenic

METHOD A

The apparatus (see Figure 2.1.4.2.-1) consists of a 100 mL conical flask closed with a ground-glass stopper through which passes a glass tube about 200 mm long and of internal diameter 5 mm. The lower part of the tube is drawn to an internal diameter of 1.0 mm, and 15 mm from its tip is a lateral orifice 2 mm to 3 mm in diameter. When the tube is in position in the stopper, the lateral orifice should be at least 3 mm below the lower surface of the stopper. The upper end of the tube has a perfectly flat, ground surface at right angles to the axis of the tube. A second glass tube of the same internal diameter and 30 mm long, with a similar flat ground surface, is placed in contact with the first and is held in position by two spiral springs.

Into the lower tube insert 50 mg to 60 mg of *lead acetate cotton R*, loosely packed, or a small plug of cotton and a rolled piece of *lead acetate paper R* weighing 50 mg to 60 mg. Between the flat surfaces of the tubes place a disc or a small square of *mercuric bromide paper R* large enough to cover the orifice of the tube (15 mm × 15 mm).

In the conical flask dissolve the quantity of the test sample specified in the monograph in 25 mL of *water R*, or in the case of a solution adjust the prescribed volume to 25 mL with *water R*. Add 15 mL of *hydrochloric acid R*, 0.1 mL of *stannous chloride solution R*, and 5 mL of *potassium iodide solution R*, allow to stand for 15 min, and then introduce 5 g of *activated zinc R*.

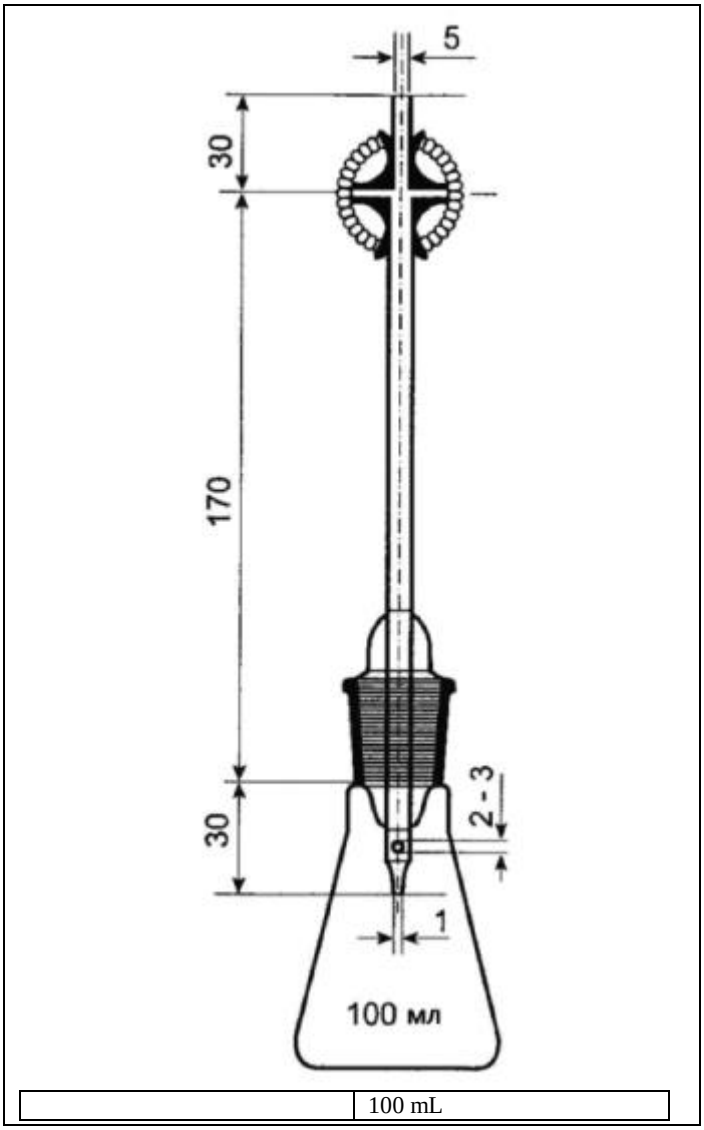


Figure 2.1.4.2.-1. – *Arsenic limit test apparatus (Method A) (dimensions in millimetres)*

Assemble the two parts of the apparatus immediately and immerse the flask in a water bath at a temperature such that a uniform evolution of gas is maintained.

At the same time and in the same manner, test the reference solution prepared using 1 mL of *arsenic standard solution (1 ppm As^{3+}) R*, diluted to 25 mL with *water R*.

After not less than 2 h the stain produced on the mercuric bromide paper in the experiment with the test solution is not more intense than that in the reference solution.

METHOD B

Introduce the quantity of the test sample specified in the monograph into a test-tube containing 4 mL of *hydrochloric acid R* and about 5 mg of *potassium iodide R* and add 3 mL of *hypophosphorous reagent R.*

Heat the mixture on a water bath for 15 min, shaking occasionally.

Prepare a reference solution, in the same manner, using 0.5 mL of *arsenic standard solution (10 ppm As^{3+}) R*.

After heating on the water bath, any colour in the test solution is not more intense than that in the reference solution.

201040003-2019

2.1.4.3. Calcium

All solutions used for this test are prepared with *distilled water R*.

To 0.2 mL of *alcoholic calcium standard solution (100 ppm Ca^{2+}) R*, add 1 mL of *ammonium oxalate solution R*. After 1 min, add a mixture of 1 mL of *dilute acetic acid R* and 15 mL of the solution specified in the monograph or of a solution containing the prescribed quantity of the test sample, and shake. Prepare a standard, in the same manner, using a mixture of 10 mL of aqueous *calcium standard solution (10 ppm Ca^{2+}) R*, 1 mL of *dilute acetic acid R*, and 5 mL of *distilled water R*.

After 15 min, any opalescence in the test solution is not more intense than that in the reference solution.

201040004-2019

2.1.4.4. Chlorides

To 15 mL of the solution specified in the monograph add 1 mL of *dilute nitric acid R* and pour the mixture as a single addition into a test-tube containing 1 mL of *silver nitrate solution R2*. Prepare a reference solution, in the same manner, using 10 mL of *chloride standard solution (5 ppm Cl^-) R* and 5 mL of *water R*. Place the tubes in a place protected from light.

After 5 minutes, examine the tubes against a black background in transmitted light (perpendicular to the axis of the test tubes). Any opalescence in the test solution is not more intense than that in the reference suspension.

201040005-2019

2.1.4.5. Fluorides

Introduce into the internal tube of the apparatus, depicted in Figure 2.1.4.5.-1, the quantity of the test sample prescribed in the monograph, 0.1 g of acid-washed *sand R*, and 20 mL of a mixture of equal volumes of *sulfuric acid R* and *water R*. Heat the jacket containing *tetrachloroethane R* maintained at its boiling point (146 °C). Connect the steam generator and distill the contents of the tube with superheated steam, collecting the distillate in a 100 mL volumetric flask containing 0.3 mL of *0.1 M sodium hydroxide R* and 0.1 mL of *phenolphthalein solution R*.

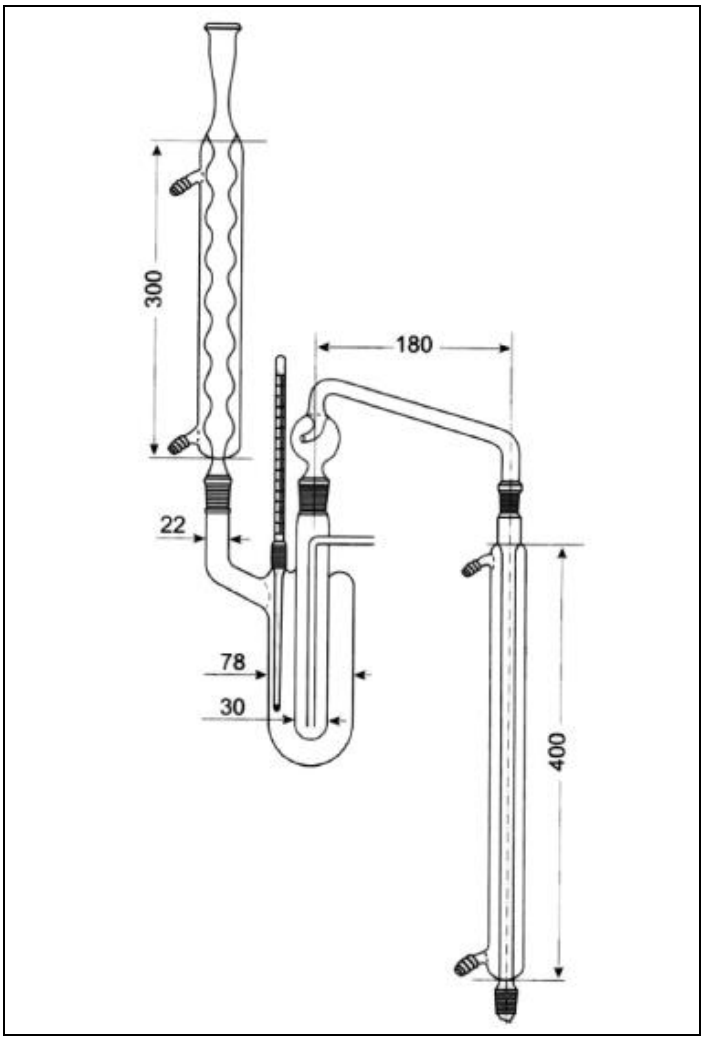


Figure 2.1.4.5.-1 – *Apparatus for limit test for fluorides (dimensions in millimetres)*

The volume of the solution in the tube during distillation should be constant (20 mL). Ensure that the contents of the volumetric flask remain alkaline, adding *0.1 M sodium hydroxide R* solution if necessary. Dilute the distillate to 100 mL with *water R* (test solution).

Prepare a standard in the same manner by distillation, using 5 mL of *fluoride standard solution (10 ppm F^-) R* instead of the substance to be examined.

Into one glass-stoppered cylinder introduce 20 mL of the test solution, and 20 mL of the reference solution is placed to another identical cylinder. In each cylinder, add 5 mL of *aminomethylalizarindiacetic acid reagent R*.

After 20 min, any blue colour in the test solution, which was originally red, is not more intense than that in the reference solution.

201040006-2019

2.1.4.6. Magnesium

To 10 mL of the solution specified in the monograph, add 0.1 g of *disodium tetraborate R*. Adjust the solution, if necessary, to pH 8.8 to pH 9.2 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Pour the solution into a separating funnel, shake with two quantities, each of 5 mL, of a 1 g/L solution of *hydroxyquinoline R* in *chloroform R*, for 1 min each time, allow to stand, and discard the organic layer. To the aqueous solution add 0.4 mL of *butylamine R* and 0.1 mL of *triethanolamine R*. Adjust the solution, if necessary, to pH 10.5 to pH 11.5. Add 4 mL of the 1 g/L solution of *hydroxyquinoline R* in *chloroform R*, shake for 1 min, allow the phases to separate; use the lower layer for comparison (test solution). Prepare a standard, in the same manner, using a mixture of 1 mL of *magnesium standard solution (10 ppm Mg^{2+}) R* and 9 mL of *water R*.

Any colour in the test solution is not more intense than that in the reference solution.

2010400007-2019

2.1.4.7. Magnesium and Alkaline-Earth Metals

To 200 mL of *water R* add 0.1 g of *hydroxylamine hydrochloride R*, 10 mL of *ammonium chloride buffer solution pH 10.0 R*, 1 mL of *0.1 M zinc sulfate R*, and about 15 mg of *mordant black 11 indicator R*. Heat to about 40 °C and titrate with *0.01 M sodium edetate R* until the violet colour changes to blue. To the resulting solution, add the quantity of the test sample specified in the monograph and dissolve in 100 mL of *water R* or use the prescribed solution. If the colour of the solution changes to violet, titrate until the full blue colour is again obtained.

The volume of *0.01 M sodium edetate R* used in the second titration does not exceed the quantity specified in the monograph.

201040008-2019

2.1.4.8. Heavy Metals

The methods described below require the use of *thioacetamide reagent R*. As an alternative, *sodium sulfide solution R1* (0.1 mL) is usually suitable. Since test procedures prescribed in monographs have been developed using *thioacetamide reagent R*, if *sodium sulfide solution R1* is used instead, it is necessary to include also for methods A, B, and H a monitor solution, prepared from the quantity of the substance to be examined prescribed for the test, to which has been added the volume of lead standard solution prescribed for preparation of the reference solution. The test results are valid if the colour of the monitor solution is equal or more intense than that in the reference solution.

METHOD A

Test solution. 12 mL of the solution of the test sample specified in the monograph.

Reference solution. A mixture of 10 mL of *lead standard solution (1 ppm Pb^{2+}) R* or *lead standard solution (2 ppm Pb^{2+}) R*, as specified in the monograph, and 2 mL of the prescribed solution of the test sample.

Blank solution. A mixture of 10 mL of *water R* and 2 mL of the solution of the test sample specified in the monograph.

To each solution, add 2 mL of *buffer solution pH 3.5 R*, mix, add 1.2 mL of *thioacetamide reagent R*, and mix immediately. Examine the solutions after 2 min.

The test results are considered reliable if the reference solution shows a slight brown colour compared to the blank solution.

The test sample passes the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the spots on the filters obtained with the different solutions.

METHOD B

Test solution. 12 mL of the solution of the test sample specified in the monograph, prepared using an organic solvent containing a minimum percentage of water (for example, dioxane containing 15% of water or acetone containing 15% of water).

*Reference solution.*A mixture of 10 mL of *lead standard solution*
(1 ppm or 2 ppm Pb^{2+}) *P,* as specified in the monograph, and 2 mL of the prescribed solution of the test sample in an organic solvent. Prepare the *lead standard solution (1 or 2 ppm Pb^{2+}) R* by dilution of *lead standard solution (100 ppm Pb^{2+}) R* with the solvent used for the dissolution of the test sample.

Blank solution. A mixture of 10 mL of the solvent used for the dissolution of the test sample and 2 mL of the prescribed solution of the test sample in an organic solvent.

To each solution, add 2 mL of *buffer solution pH 3.5 R*, mix, add 1.2 mL of *thioacetamide reagent R*, and mix immediately. Examine the solutions after 2 min.

The test results are considered reliable if the reference solution shows a slight brown colour compared to the blank solution.

The test sample passes the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the colour of the spots on the filters obtained with the different filtering solutions.

METHOD B

Test solution. Place the quantity (not greater than 2 g) of the test sample, specified in the monograph, into a silica crucible with 4 mL of a 250 g/L solution of *magnesium sulfate R* in *dilute sulfuric acid R*. Mix using a fine glass rod and heat cautiously. If the mixture is liquid, evaporate gently to dryness on a water bath, then progressively heat to ignition and continue heating until an off-white or at most greyish residue is obtained. Carry out the ignition at a temperature not exceeding 800 °C. Allow to cool, then moisten the residue with a few drops of *dilute sulfuric acid R*. Evaporate to dryness, ignite again, and allow to cool. The total period of ignition must not exceed 2 h. Take up the residue in two quantities, each of 5 mL, of *dilute hydrochloric acid R*.

Add 0.1 mL of *phenolphthalein solution R*, then make more alkaline with *concentrated ammonia R* until pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolourised, and add 0.5 mL of *glacial acetic acid R*. Filter if necessary and wash the filter with *water R*. Dilute to 20 mL with *water R*.

Reference solution. Prepare as described for the test solution, using the specified volume of *lead standard solution (10 ppm Pb^{2+}) R* instead of the test sample. To 10 mL of the solution obtained add 2 mL of the test solution.

Monitor solution. Prepare as described for the test solution, adding to the test sample the volume of *lead standard solution (10 ppm Pb^{2+}) R* specified in the monograph for the preparation of the reference solution. Add 2 mL of the test solution to 10 mL of the solution obtained.

Blank solution. A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of a *buffer solution R* with a pH of 3.5, mix, add 1.2 mL of *thioacetamide reagent R*, and mix immediately. Examine the solutions after 2 min.

The test results are considered reliable if:

- the reference solution shows a slight brown colour compared to the blank solution;
- the monitor solution is at least as intense as the reference solution.

The test sample passes the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the colour of the spots on the filters obtained with the different filtering solutions.

METHOD D

Test solution. In a silica crucible, mix thoroughly the quantity of the test sample specified in the monograph with 0.5 g of *magnesium oxide R1*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained. If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod, and repeat the ignition. If necessary repeat the operation. Heat at 800 °C for about 1 h. Take up the residue in two quantities, each of 5 mL, of a mixture of equal volumes of *hydrochloric acid R1* and *water R*. Add 0.1 mL of *phenolphthalein solution R* and then *concentrated ammonia R* until pink colour is obtained. Cool, acidify with *glacial acetic acid R* until the solution is decolourised, and add 0.5 mL of *glacial acetic acid R*. Filter if necessary and wash the filter with *water R*. Dilute to 20 mL with *water R*.

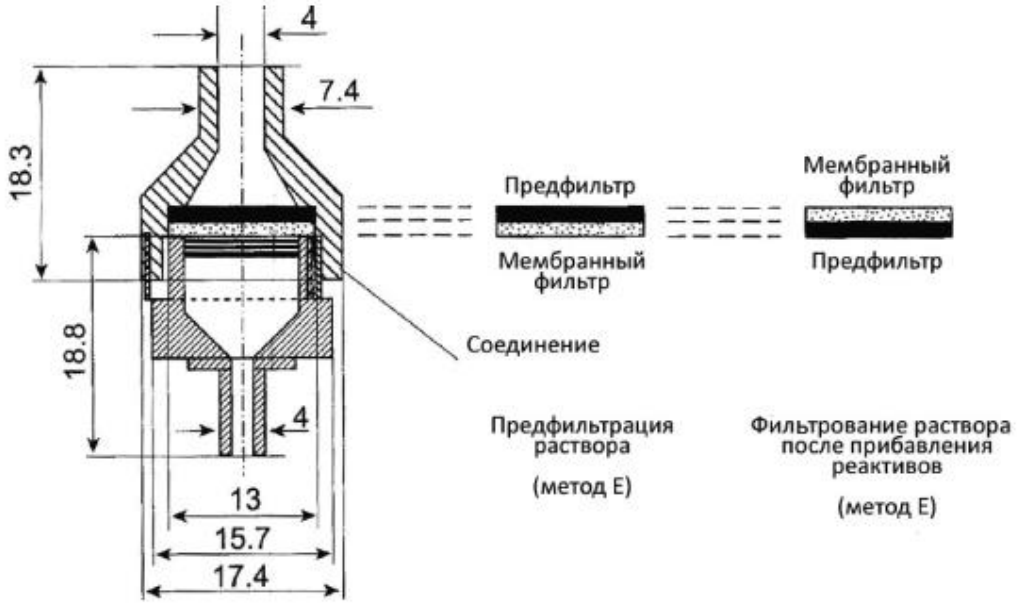
Reference solution. Prepare as described for the test solution using the specified volume of *lead standard solution (10 ppm Pb^{2+}) R* instead of the test sample and drying in an oven at 100-105 °C. To 10 mL of the solution obtained, add 2 mL of the test solution.

Monitor solution. Prepare as described for the test solution, adding to the test sample the volume of *lead standard solution (10 ppm Pb^{2+}) R* specified for preparation of the reference solution and drying in an oven at 100-105 °C. To 10 mL of the solution obtained add 2 mL of the test solution.

Blank solution. A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of a *buffer solution R* with a pH of 3.5, mix, add 1.2 mL of *thioacetamide reagent R*, and mix immediately. Examine the solutions after 2 min.

The test results are considered reliable if:



	Prefilter
	Membrane filter
	Joint
	Prefiltration of the solution (Method E)
	Filtration of the solution after addition of the reagents (Method E)

Figure 2.1.4.8.-1. – *Apparatus for the test for heavy metals (dimensions in millimetres)*

- the reference solution shows a slight brown colour compared to the blank solution;

- the monitor solution is at least as intense as the reference solution.

The test sample passes the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the spots on the filters obtained with the different solutions.

METHOD E

Test solution. Dissolve the quantity of the test sample specified in the monograph in 30 mL of *water R* or the prescribed volume of *water R*.

Reference solution. Unless otherwise specified in the monograph, dilute the prescribed volume of *lead standard solution (1 ppm Pb^{2+}) R* to the same volume as the test solution.

Prepare the filtration apparatus by setting a 50 mL syringe without its piston to a support, which contains, on the plate, a membrane filter (pore size of 3 µm) and above it a prefilter (Figure 2.1.4.8.-1).

Transfer the test solution into the syringe, put the piston in place, and then apply such even pressure on it sufficient to the whole of the liquid has been filtered. Open the support, remove the prefilter, and check that the membrane filter remains uncontaminated with impurities. Otherwise, replace it with another membrane filter and repeat the operation under the same conditions.

To the prefiltrate or to the prescribed volume of the prefiltrate, add 2 mL of *buffer solution pH 3.5 R* and mix, add 1.2 mL of *thioacetamide reagent R*, then mix immediately and allow to stand for 10 min. Filter again as described above, but inverting the order of the filters, the liquid passing first through the membrane filter before passing through the prefilter (Figure 2.1.4.8.-1).

Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. After complete filtration, open the support, remove the membrane filter, and dry using filter paper.

In parallel, treat the reference solution in the same manner as the test solution.

The test sample passes the test if the colour of the spot obtained with the test solution is not more intense than that obtained with the reference solution.

METHOD F

Test solution. Place the prescribed quantity or volume of the test sample in a clean, dry, 100 mL long-

Adjust the solution to pH 3.0 - 4.0, using short-range pH indicator paper as an external indicator, with *concentrated ammonia R1* (*diluted ammonia solution R1* may be used, as the specified range is approached), dilute with *water R* to 40 mL, and mix. Add 2 mL of *buffer solution pH 3.5 R*. To this mixture, add 1.2 mL of *thioacetamide reagent R* and mix immediately. Dilute to

necked Kjeldahl flask (a 300 mL flask should be used if the reaction foams excessively). Clamp the flask at an angle of 45° and, if the substance is a solid, add a sufficient volume of a mixture of 8 mL of *sulfuric acid R* and 10 mL of *nitric acid R* to moisten the substance thoroughly; if the substance is a liquid, add a few millilitres of the above-mentioned mixture. Warm gently until the reaction commences. After the reaction stops, add supplementary portions of the same acid mixture, heating after each addition. The operation is repeated until a total of 18 mL of the acid mixture has been added. Increase the amount of heat and boil gently until the solution darkens. Cool, add 2 mL of *nitric acid R*, and heat again until the solution darkens. Continue the heating, followed by the addition of *nitric acid R* until no further darkening occurs, then heat strongly until dense, white fumes are produced. Cool, cautiously add 5 mL of *water R*, boil gently until dense, white fumes are produced and continue heating to reduce to 2-3 mL. Cool, cautiously add 5 mL of *water R* and examine the colour of the solution. If the colour is yellow, cautiously add 1 mL of *strong hydrogen peroxide solution R* dropwise and again evaporate until dense, white fumes are produced, and reduce to a volume of 2-3 mL. If the solution is still yellow in colour, repeat the addition of 5 mL of *water R* and 1 mL of *strong hydrogen peroxide solution R* until the solution is colourless. Cool, dilute cautiously with water R, and rinse into a 50 mL colour comparison tube, ensuring that the total volume does not exceed 25 mL.

50 mL with *water R* and mix.

Reference solution. Prepare at the same time and in the same manner as the test solution, using the specified volume of *lead standard solution (10 ppm Pb^{2+}) R.*

Monitor solution. Prepare as described for the test solution, adding to the test sample the volume of *lead standard solution (10 ppm Pb^{2+}) R* specified in the monograph for the preparation of the reference solution.

Blank solution. Prepare similarly to the test solution but without adding the test sample.

After 2 minutes, compare the solutions by viewing them perpendicular to the vertical axis of the test tubes on a white background. The test results are considered reliable if: - the comparison solution has a brown colour compared to the control solution;

- the monitor solution is at least as intense as the reference solution.

The test sample passes the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the colour of the spots on the filters obtained with the different filtering solutions.

METHOD G

CAUTION. *When using high-pressure digestion vessels the safety precautions and operating instructions given by the manufacturer must be followed.*

The digestion cycles have to be elaborated depending on the type of microwave oven to be used (for example, energy-controlled microwave ovens, temperature-controlled microwave ovens or high-pressure ovens). The digestion cycle must meet the manufacturer's instructions. The digestion cycle is suitable if a clear solution is obtained.

Test solution. Place the amount of the test sample specified in the monograph (not greater than 0.5 g) in a suitable, clean beaker with a magnetic stirrer. Add successively 2.7 mL of *sulfuric acid R*, 3.3 mL of *nitric acid R*, and 2.0 mL of *strong hydrogen peroxide solution R*, allow the test sample to react with a reagent before adding the next one. Transfer the mixture to a dry high-pressure-resistant digestion vessel (e.g., fluoropolymer or quartz glass).

Reference solution. Prepare as described for the test solution, using the specified volume of *lead standard solution (10 ppm Pb^{2+}) R* instead of the test sample.

Monitor solution. Prepare as described for the test solution, adding to the test sample the volume of *lead standard solution (10 ppm Pb^{2+}) R* specified in the monograph for the preparation of the reference solution.

Blank solution. Prepare similarly to the test solution but without adding the test sample.

Close the vessels and place in a laboratory microwave oven. Digest using a sequence of two separate suitable programs. Design the programs in several steps in order to control the reaction, monitoring pressure, temperature, or energy depending on the type of microwave oven available. After the first program, allow the digestion vessels to cool before opening. Add to each vessel 2.0 mL of *strong hydrogen peroxide solution R* and digest using the second program. After the second program, allow the digestion vessels to cool before opening.

If necessary to obtain a clear solution, repeat the addition of *strong hydrogen peroxide solution R* and the second digestion program.

Cool, dilute cautiously with *water R*, and rinse into a flask with *water R*, ensuring that the total volume does not exceed 25 mL.

Using short-range pH indicator paper as an external indicator, adjust the solutions to pH 3.0-4.0 with *concentrated ammonia solution R1* (*diluted ammonia solution R1* may be used as the specified range is approached). To avoid heating of the solutions, use an ice-bath and a magnetic stirrer. Dilute to 40 mL with *water R* and mix. Add 2 mL of *buffer solution pH 3.5 R*, mix, add 1.2 mL of *thioacetamide reagent R*, and mix immediately. Dilute to 50 mL with *water R*, mix and allow to stand for 2 min.

Filter the solution through a suitable 0.45 µm membrane filter. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston of the syringe. Compare the spots on the filters obtained with the different solutions.

The test results are considered reliable if:

- the spot obtained with the reference solution shows a brown colour compared to the spot obtained with the blank solution;
- the spot obtained with the monitor solution is at least as intense as the spot obtained with the reference solution.

The test sample passes the test if the brown colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

METHOD 3

Test solution. Dissolve the quantity of the test sample specified in the monograph in 20 mL of the solvent or solvent mixture prescribed.

Reference solution. Dilute the specified volume of *lead standard solution (10 ppm Pb^{2+}) R* to 20 mL with the solvent or solvent mixture specified.

Blank solution. 20 mL of the solvent or solvent mixture prescribed.

To each solution, add 2 mL of *buffer solution pH 3.5 R* and mix. (*In some cases* precipitation occurs, *in which case the specific individual monograph would describe re-dissolution in a defined volume of a given solvent*). Each of the solutions obtained, add to 1.2 mL of *thioacetamide reagent R*, mix immediately, and allow to stand for 2 min. Filter the solution through a suitable 0.45 µm membrane filter. Compare the spots on the filters obtained with the different solutions.

The test results are considered reliable if the spot obtained with the reference solution shows a brownish-black colour compared to the spot obtained with the blank solution.

The test sample passes the test if the brownish-black colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

201040009-2019

2.1.4.9. Iron

Dissolve the quantity of the test sample specified in the monograph in *water R*, dilute to 10 mL with *water R* or use 10 mL of the prescribed solution. Add 2 mL of a 200 g/L solution of *citric acid R* and 0.1 mL of *thioglycolic acid R*. Mix, make alkaline with *ammonia R*, and dilute to 20 mL with *water R*. Prepare a reference solution, in the same manner, using 10 mL of *iron standard solution (2 ppm Fe^{3+}) R* diluted to 10 mL with *water R*.

After 5 min, any pink colour in the test solution is not more intense than that in the standard.

201040010-2019

2.1.4.10. Lead in Sugars

Determine the lead by atomic absorption spectrometry (*2.1.2.22, Method II*).

Test solution. Dissolve 20.0 g of the test sample in a mixture of equal volumes of *dilute acetic acid R* and *water R*, dilute to 100.0 mL with the same mixture of solvents. Add 2.0 mL of a clear 10 g/L solution of *ammonium pyrrolidinedithiocarbamate R* and 10.0 mL of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions. Prepare three reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *lead standard solution (10 ppm Pb^{2+}) R* in addition to the 20.0 g of the test sample.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for the test solution without the test sample. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as a source of radiation and an air-acetylene flame.

The test sample contains not greater than 0.5 ppm of lead unless otherwise prescribed in the monograph.

201040011-2019

2.1.4.11. Phosphates

Add 4 mL of *sulfomolybdic reagent R3* to 100 mL of the solution prepared and, if necessary, neutralised as prescribed in the monograph. Shake and add 0.1 mL of *stannous chloride solution R1*. Prepare a standard, in the same manner, using the mixture of 2 mL of *phosphate standard solution (5 ppm $PO4_4^{3-}$) R* and 98 mL of *water R*.

After 10 min, compare the colours using 20 mL of each solution.

Any colour in the test solution is not more intense than that in the reference solution.

201040012-2019

2.1.4.12. Potassium

To 10 mL of the prescribed solution, add 2 mL of a freshly prepared 10 g/L solution of *sodium tetraphenylborate R*.

Prepare a reference solution, in the same manner, using a mixture of 5 mL of *potassium standard solution (20 ppm K+) R* and 5 mL of *water R*.

After 5 min, any opalescence in the test solution is not more intense than that in the reference solution.

201040013-2019

2.1.4.13. Sulfates

All solutions used for this test must be prepared with *distilled water R*.

Add 3 mL of a 250 g/L solution of *barium chloride R* to 4.5 mL of *sulfate standard solution (10ppm SO_4^{2-}) R* . Shake and allow to stand for 1 min. To 2.5 mL of this suspension, add 15 mL of the solution specified in the monograph and 0.5 mL of *acetic acid R*.

Prepare a reference solution, in the same manner, using 15 mL of *sulfate standard solution (10 ppm $SO2_4^{2-}$) R* instead of the prescribed solution.

After 5 min, any opalescence in the test solution is not more intense than that in the reference solution.

201040014-2019

2.1.4.14. Sulfated Ash

Ignite a suitable crucible (for example, porcelain quartz, or platinum) at (600 ± 50) °C for 30 min, allow to cool in a desiccator over silica gel or other suitable desiccant and weigh accurately. Place the amount of the test sample specified in the monograph in the pre-ignited crucible and weigh accurately. Moisten the test sample with 1 mL of *sulfuric acid R* and heat gently at as low a temperature as practicable until the sample is thoroughly charred. After cooling, moisten the residue with 1 mL of *sulfuric acid R*, heat gently until white fumes are no longer evolved, and ignite at 600 ± 50 °C until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel or another suitable desiccant, weigh it again accurately, and calculate the percentage of residue.

If the amount of the residue so obtained exceeds the specified limit, repeat the moistening with *sulfuric acid R* and ignition, as previously, for 30 min. The incineration is repeated until two consecutive weighings do not differ by greater than 0.5 mg or until the percentage of residue meets the prescribed limit.

The amount of substance used for the test (usually 1-2 g) is chosen so that at the specified limit the mass of the residue (usually about 1 mg) can be measured with sufficient accuracy.

201040015-2019

2.1.4.15. Nickel in Polyols

Determine the nickel by atomic absorption spectrometry *(2.1.2.22, Method II).*

Test solution. Dissolve 20.0 g of the test solution in a mixture of equal volumes of *dilute acetic acid R* and *water R*, dilute to 100.0 mL with the same mixture of solvents, and mix. Add 2.0 mL of a saturated solution of *ammonium pyrrolidinedithiocarbamate R* (about 10 g/L) and 10.0 mL of *methyl isobutyl ketone R*, shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions. Prepare three reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *nickel standard solution (10 ppm Ni^{2+}) R* in addition to the 20.0 g of the test sample.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for the test solution without the test sample. Measure the absorbance at 232.0 nm wavelength using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

The test sample contains not greater than 1 ppm of nickel unless otherwise prescribed in the monograph.

201040016-2019

2.1.4.16. Total Ash

Heat a silica or platinum crucible to redness (600 ± 50) °C for 30 min, allow to cool in a desiccator, and weigh. Unless otherwise prescribed in the monograph, evenly distribute 1.00 g of the test sample or the powdered herbal drug in the crucible. Dry at 100 °C to 105 °C for 1 h and ignite to constant mass in a muffle furnace at (600 °C ± 25) °C. Allow the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, transfer the contents of the crucible quantitatively with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant mass.

201040017-2019

2.1.4.17. Aluminum

METHOD A

Test solution. Place the test solution prepared as specified in the monograph in a separating funnel and shake with two quantities, each of 20 mL, and then with one 10 mL quantity of a 5 g/L solution of *hydroxyquinoline R* in *chloroform R*. Dilute the combined chloroform solutions to 50.0 mL with *chloroform R*.

Reference solution. Prepare in the same manner using the specified reference solution.

Blank solution. Prepare in the same manner using the specified blank solution.

Measure the intensity of the fluorescence (2.1.2.20) of the test solution ($I1$), of the reference solution ($I2$), and of the blank solution (I_3) using an excitant beam at 392 nm and a secondary filter with a transmission band centered on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence ($I1 - I_3$) of the test solution is not greater than that of the reference solution ($I2 - I3$).

METHOD B

Use this method for substances employed in hemodialysis.

Determine by atomic absorption spectrometry (*2.1.2.22*).

Test solution. Unless otherwise specified in the monograph, place a quantity of the test sample containing from 1.2 µg to 3.8 µg of aluminum ion in a 100 mL polymer measuring flask, add 50 mL of *water R*, and dissolve in an ultrasonic bath for 30 min. Add 4 mL of *nitric acid R* and dilute to 100.0 mL with *water R*.

Reference solutions. For a few minutes, immerse an aluminum wire into *6 M hydrochloric acid R* heated to 80 °C. Dissolve about 0.1 g of the treated wire in a mixture of 10 mL of 25% (*V/V*) hydrochloric acid and 2 mL of concentrated nitric acid at a temperature of about 80 °C and continue heating until a mixture volume of about 4 mL is obtained. Cool the mixture to room temperature and add 4 mL of *water R*, then evaporate to a volume of about 2 mL, cool, transfer to a 100 mL volumetric flask, dilute to 100.0 mL with *water R*, and mix. Dilute 10 mL of the resulting solution to 100.0 mL with *water R* and mix. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

Then place 1.0 mL, 2.0 mL, and 4.0 mL of the resulting solution into individual 100 mL volumetric flasks and dilute to 100.0 mL with *diluted nitric acid R1* (aluminum ion concentration 0.01 µg/mL, 0.02 µg/mL, and 0.04 µg/mL, respectively).

Blank solution. Nitric acid, diluted R1.

Measure the absorption of the test solution and reference solutions at 309.3 nm using a lamp with a hollow aluminum cathode and a flameless electric furnace as a radiation source.

The concentration of aluminum in the test solution is determined by a calibration curve plotted based on the reference solutions. Calculate the content of aluminum in the substance.

201040018-2019

2.1.4.18. Free Formaldehyde

Use method A, unless otherwise prescribed in the monograph. Method B is suitable for vaccines where sodium metabisulfite has been used to neutralise excess formaldehyde.

METHOD A

Vaccines for human use are diluted 10 times. Bacterial toxoids for veterinary use are diluted 25 times.

To 1 mL of the dilution, add 4 mL of *water R* and 5 mL of *acetylacetone reagent R1*. Place the tube in a water-bath at 40 °C for 40 min. Examine the tubes down their vertical axes. The solution is not more intensely coloured than a standard, prepared at the same time and in the same manner, using 1 mL of dilution of *formaldehyde solution R* containing 20 µg of formaldehyde (CH_2O) per millilitre, instead of the diluted vaccine.

METHOD B

Test solution. Prepare a 1 in 200 dilution of the vaccine to be examined with *water R*. If the vaccine to be examined is an emulsion, prepare an equivalent dilution using the aqueous phase separated by a suitable procedure (see below). If one of the procedures described below is used for separation of the aqueous phase, a 1 in 20 dilution of the latter is used.

Reference solutions. Prepare solutions containing 0.25 g/L,

0.50 g/L, 1.00 g/L, and 2.00 g/L of CH_2O by dilution of *formaldehyde solution R* with water R. Prepare a 1 in 200 dilution of each solution with *water R*.

To 0.5 mL of the test solution and of each of the reference solutions in test-tubes, add 5.0 mL of a freshly prepared 0.5 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R.* Close the tubes, shake and allow to stand for 60 min. Add 1 mL of *ferric (III) chloride - sulfamic acid reagent R* and allow it to stand for 15 min.

Measure the absorbance (*2.1.2.24*) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine to be examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient (*r*) of the calibration curve is less than 0.97.

Emulsions. If the vaccine to be examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. Use the following procedures.

(a) Add 1.0 mL of the vaccine to be examined to 1.0 mL of *isopropyl myristate R* and mix. Add 1.3 mL of *1 M hydrochloric acid*, 2.0 mL of *chloroform R*, and 2.7 mL of a 9 g/L solution of *sodium chloride R*, mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*. If this procedure fails to separate the aqueous phase, add 100 g/L of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuge *at* 22 500 *g*.

(b) Add 1.0 mL of the vaccine to be examined to 1.0 mL of a 100 g/L solution of *sodium chloride R* and mix. Centrifuge at 1000 *g* for 15 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

(c) Add 1.0 mL of the vaccine to be examined to 2.0 mL of a 100 g/L solution of *sodium chloride R* and 3.0 mL of *chloroform R* and mix. Centrifuge at 1000 *g* for 5 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

201040019-2019

2.1.4.19. Identification and Control of Residual Solvents

The test procedures described in this general method may be used:

1) for the identification of the majority of Class 1 and Class 2 residual solvents in an active substance, excipient, or drug product when the residual solvents are unknown;

2) as a limit test for Class 1 and Class 2 solvents when present in an active substance, excipient, or drug product;

3) for the quantitation of Class 2 solvents when the limits are greater than 1000 ppm (0.1%) or for the quantification of Class 3 solvents when required.

Class 1, Class 2, and Class 3 residual solvents are listed in general chapter *2.3.2.0. Residual solvents*.

Three diluents are described for test sample preparation and the conditions to be applied for headspace injection of the gaseous sample onto the chromatographic system. Two chromatographic systems are prescribed but System A is preferred. System B is employed normally for identification. The choice of test sample preparation procedure depends on the solubility of the substance to be examined and in certain cases the residual solvents to be controlled.

Such residual solvents as formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, N-methylpyrrolidone, and sulfolane are not readily detected by the headspace injection conditions described. Other appropriate procedures should be employed for the control of these residual solvents.

When the test procedure is applied quantitatively to control residual solvents in a substance, then it must be validated.

PROCEDURE

Examine by gas chromatography with static headspace injection (*2.1.2.27*).

Sample preparation 1. This is intended for the control of residual solvents in water-soluble substances.

Sample solution (1). Dissolve 0.200 g of the test samples in *water R* and dilute to 20.0 mL with the same solvent.

Sample preparation 2. This is intended for the control of residual solvents in water-insoluble substances.

Sample solution (2). Dissolve 0.200 g of the substance to be examined in *dimethylformamide R* (DMF) and dilute to 20.0 mL with the same solvent.

Sample preparation 3. The test sample is intended for the control of N,N-dimethylacetamide and/or N,N-dimethylformamide, when it is known or suspected that one or both of these solvents are present in the substance to be examined.

Sample solution (3). Dissolve 0.200 g of the test sample in *1,3-dimethyl-2-imidazolidinone R* (DMI) and dilute to 20.0 mL with the same solvent.

In some cases, none of the above sample preparation procedures are appropriate, in which case the diluent to be used for the preparation of the sample solution and the static headspace conditions to be employed must be demonstrated to be suitable.

Solvent solution (a). To 1.0 mL of *Class 1 residual solvent solution CRS*, add 9 mL of *dimethyl sulfoxide R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

The reference solutions meet the following residual solvent limits:

- benzene: 2 ppm;
- carbon tetrachloride: 4 ppm;
- 1,2-dichloroethane: 5 ppm;

- 1,1-dichloroethene: 8 ppm;
- 1,1,1-trichloroethane: 10 ppm.

Solvent solution (b). Dissolve appropriate quantities of Class 2 residual solvents in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute the resulting solution with *water R* to give a concentration of 1/20 of the limits stated in Table 2 in chapter *2.3.2.0. Residual solvents*.

Solvent solution (c). Dissolve 1.00 g of the solvent or solvents present in the substance in *dimethyl sulfoxide R* or *water R*, if appropriate, and dilute to 100.0 mL *with water R*. Dilute to give a concentration of 1/20 of the limit(s) stated in Table 1 or 2 in chapter *2.3.2.0. Residual solvents*.

Blank solution. Prepare as described for solvent solution (c) but without the addition of solvent(s) (used to verify the absence of interfering peaks).

Test solution. Introduce 5.0 mL of the sample solution and 1.0 mL of the blank solution into a headspace vial.

Reference solution (a) (Class 1). Introduce 1.0 mL of solvent solution (a) and 5.0 mL of the appropriate diluent into a headspace vial.

Reference solution (a1) (Class 1). Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (a) into a headspace vial.

Reference solution (b) (Class 2). Introduce 1.0 mL of solvent solution (b) and 5.0 mL of the appropriate diluent into a headspace vial.

Reference solution (c). Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (c) into a headspace vial.

Reference solution (d). Introduce 1.0 mL of the blank solution and 5.0 mL of the appropriate diluent into a headspace vial.

Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminum crimped cap. Shake to obtain a homogeneous solution.

The following static head-space injection conditions given in Table 2.1.4.19.-1 may be used.

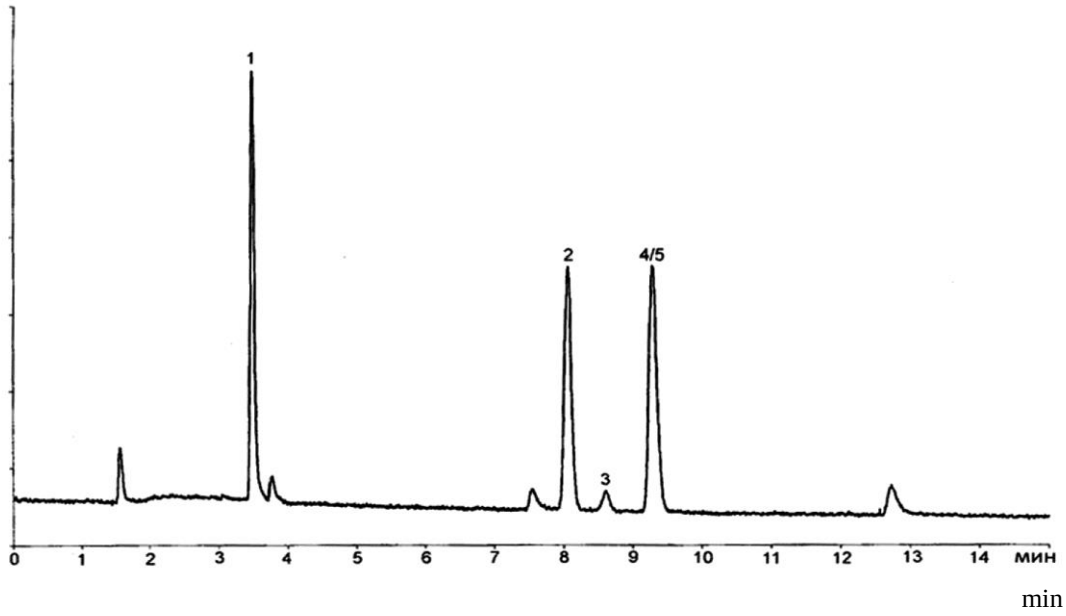
The chromatographic procedure may be carried out using:

SYSTEM A

- a fused-silica capillary column, 30 m x 0.32 mm or 30 m x 0.53 mm, coated with cross-linked 6% polycyanopropylphenylsiloxane and 94% polydimethylsiloxane film 1.8 µm or 3 µm thick;
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas;
- split ratio 1:5;
- a linear velocity of the carrier gas about 35 cm/s;
– a flame-ionisation detector (a mass spectrometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1);
- the temperature of the column at 40 °C for 20 min, then raising the temperature at a rate of 10 °C per min to 240 °C and maintaining it at 240 °C for 20 min;

Table 2.1.4.19.-1. – *Conditions for static headspace analysis*

Operating parameters	Sample preparation procedure		
	1	2	3
Equilibration temperature (°C)	80	105	80
Equilibration time (min)	60	45	45
Gas sample transfer-line temperature (°C)	85	110	105
Carrier gas: *Nitrogen for chromatography R* or *Helium for chromatography R* at an appropriate pressure			
Pressurisation time (s)	30	30	30
Injection volume (mL)	1	1	1



1) 1,1-dichloroethene 2) 1,1,1-trichloroethane 3) carbon tetrachloride 4) benzene 5) 1,2-dichloroethane

Figure 2.1.4.19.-1 – *Typical chromatogram of Class 1 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector*

- the temperature of the injection port at 140 °C;
- the detector temperature at 250 °C.

In cases where there is interference from the matrix, use System B.

SYSTEM B

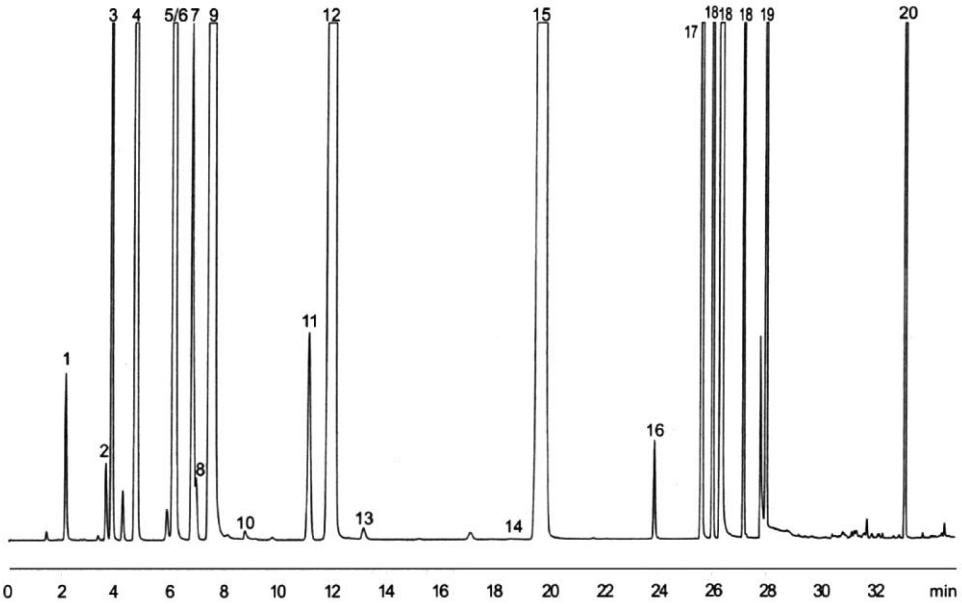
- a fused-silica capillary column, 30 m x 0.32 mm or 30 m x 0.53 mm, coated with *macrogol 20 000 R* (film thickness: 0.25 µm);
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas;
- split ratio 1:5;
- a linear velocity of the carrier gas about 35 cm/s;
- a flame-ionisation detector (a mass spectrometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1);
- the temperature of the column at 50 °C for 20 min, then raising the temperature at a rate of 6 °C per min to 165 °C and maintaining it at 165 °C for 20 min;
- the temperature of the injection port at 140 °C;
- the detector temperature at 250 °C.

Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System A and record the chromatogram under such conditions that the signal-to-noise ratio for 1,1,1-trichloroethane can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.1.4.19.-1.

Inject 1 mL of the gaseous phase of reference solution (a_1) onto the column described in System A. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System A and record the chromatogram under such conditions that the resolution between acetonitrile and methylene chloride can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.1.4.19.-2 and the resolution between acetonitrile and methylene chloride is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System A. If in the chromatogram obtained, there is no peak which corresponds to one of the residual solvent peaks in the chromatograms obtained with reference solution (a) or (b), then the test sample meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) then System B is to be employed.



1) methanol
2) acetonitrile
3) dichloromethane
4) hexane
5) 1,2-dichloroethene
6) nitromethane
7) tetrahydrofuran
8) chloroform
9) cyclohexane
10) 1,2-dimethoxyethane
11) 1,1,2-trichloroethene
12) Methylcyclohexane
13) 1,4-dioxane
14) pyridine (t_r = 18.7 min)
15) toluene
16) methylbutyl ketone
17) chlorobenzene
18) xylene *ortho*, *meta*, and *para*
19) cumene
20) tetralin

Figure 2.1.4.19.-2. – *Chromatogram of Class 2 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector*

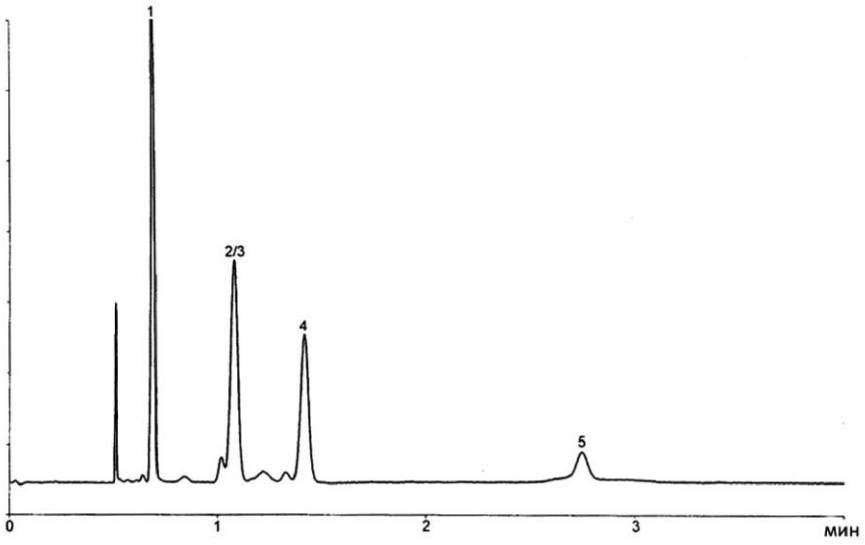
Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System B and record the chromatogram under such conditions that the signal-to-noise ratio for benzene can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.1.4.19.-3.

Inject 1 mL of the gaseous phase of reference solution (a1) onto the column described in System B. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System B and record the chromatogram under such conditions that the resolution between acetonitrile and trichloroethene can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.1.4.19.-4 and the resolution between acetonitrile and trichloroethene is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System B. If in the chromatogram obtained, there is no peak which corresponds to any of the residual solvent peaks in the chromatogram obtained with the reference solution (a) or (b), then the test sample meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) and confirms the correspondence obtained when using System A, then proceed as follows.

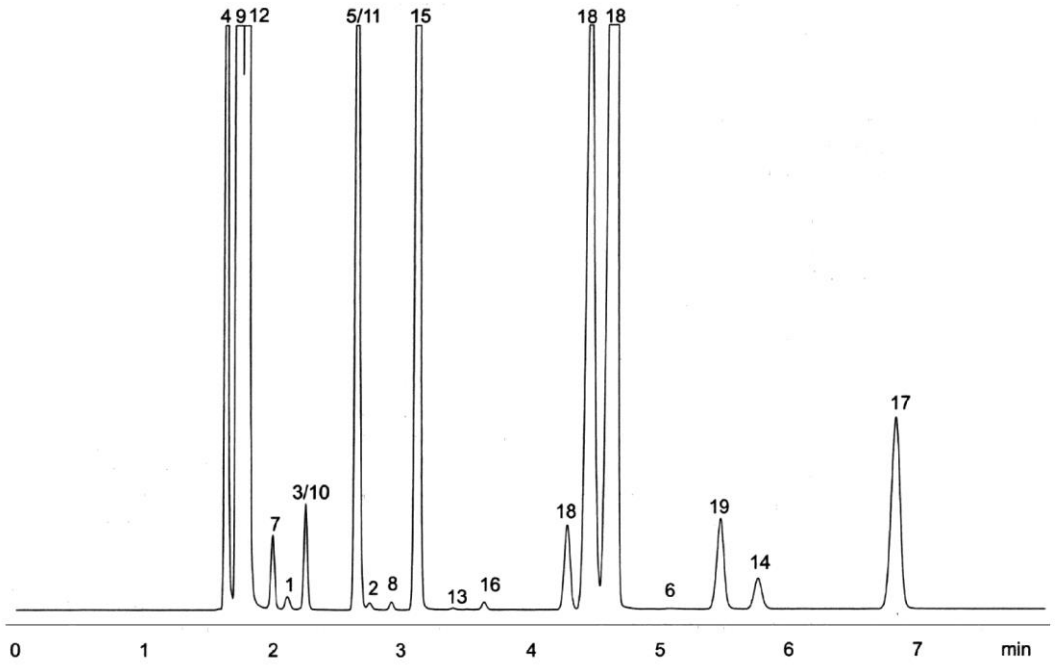
Inject 1 mL of the gaseous phase of reference solution (c) onto the column described for System A or System B. If necessary, adjust the sensitivity of the system so that the height of the peak corresponding to the identified residual solvent(s) is at least 50% of the full scale of the recorder.



min

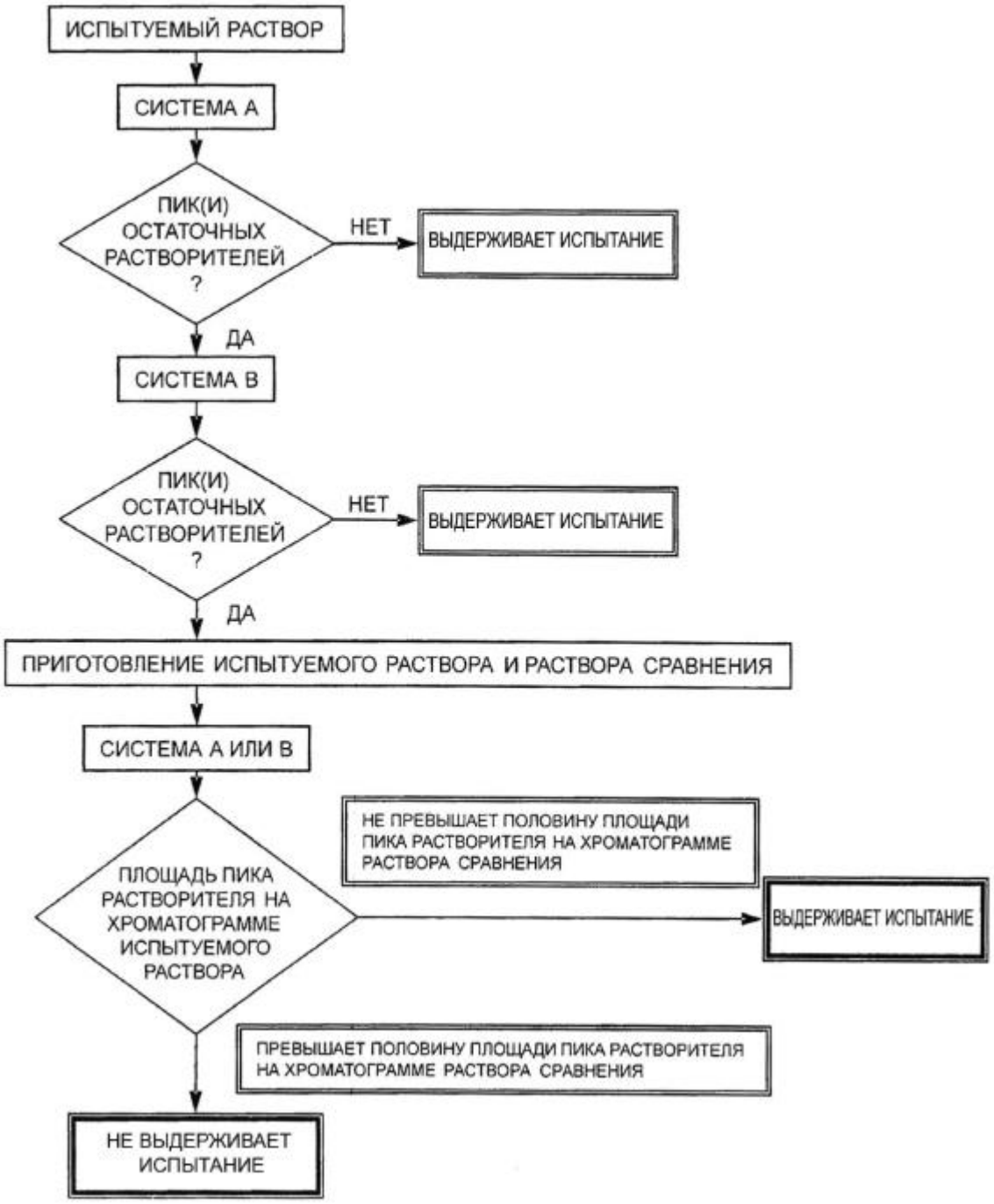
1) 1,1-dichloroethene 2) 1,1,1-trichloroethane 3) carbon tetrachloride 4) benzene 5) 1,2-dichloroethane

Figure 2.1.4.19.-3. – *Chromatogram of Class 1 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector*



1) methanol
2) acetonitrile
3) dichloromethane
4) hexane
5) 1,2-dichloroethene
6) nitromethane
7) tetrahydrofuran
8) chloroform
9) cyclohexane
10) 1,2-dimethoxyethane
11) 1,1,2-trichloroethene
12) Methylcyclohexane
13) 1,4-dioxane
14) pyridine
15) toluene
16) methylbutyl ketone
17) chlorobenzene
18) xylene *ortho*, *meta*, and *para*
19) cumene
20) tetralin (t_r = 28 min)

Figure 2.1.4.19.-4. – *Typical chromatogram of Class 2 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector*



	TEST SOLUTION
	SYSTEM A
	PEAK(S) CORRESPONDING TO A RESIDUAL SOLVENT?
	NO
	PASSES THE TEST
	YES
	SYSTEM B
	PREPARATION OF TEST AND REFERENCE SOLUTIONS
	SYSTEM A OR B
	AREA OF PEAK OBTAINED WITH THE TEST SOLUTION
	LESS THAN HALF THE AREA OF THE PEAK OBTAINED WITH THE REFERENCE SOLUTION
	GREATER THAN HALF THE AREA OF THE PEAK OBTAINED WITH THE REFERENCE SOLUTION
	FAILES THE TEST

Figure 2.1.4.19.-5. – *Diagram relating to the identification of residual solvents and the application of limit tests*

Inject 1 mL of the gaseous phase of reference solution (d). No interfering peaks should be observed.

Chromatograph 1 mL of the gaseous phase of the test solution and 1 mL of the gaseous phase of reference solution (c). Repeat these injections not less than thrice.

The mean area of the peak of the residual solvent(s) in the chromatograms obtained with the test solution is not greater than half the mean area of the peak of the corresponding residual solvent(s) in the chromatograms obtained with reference solution (c). The test is not valid unless the relative standard deviation of the differences in areas between the analyte peaks obtained from three replicate paired injections of reference solution (c) and the test solution, is at most 15%.

A flow diagram of the procedure is shown in Figure 2.1.4.19.-5.

When a residual solvent (Class 2 or Class 3) is present at a level of 0.1% or greater then the content may be quantified by the method of standard additions.

2.1.4.20. N,N-Dimethylaniline

METHOD A

Examine by gas chromatography (*2.1.2.27*), using *N,N-diethylaniline R* as the internal standard.

Internal standard solution. Dissolve 50 mg of *N,N-diethylaniline R* in 4 mL of *0.1 M hydrochloric acid* and dilute to 50 mL with *water R*. Dilute 1 mL of this solution to 100 mL with *water R*.

Test solution. Dissolve in a ground-glass-stoppered tube 0.50 g of the test sample in 30.0 mL of *water R*, then add 1.0 mL of the internal standard solution, and heat the solution to a temperature of 26-28 °C. Add 1.0 mL of *strong sodium hydroxide solution R* and mix until completely dissolved. Add 2.0 mL of *trimethylpentane R*, shake for 2 min, and allow the phases to separate. Use the upper layer.

Reference solution. Dissolve 50.0 mg of *N,N-dimethylaniline R* in 4.0 mL of *0.1 M hydrochloric acid* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 30.0 mL with *water R*. Add 1.0 mL of the internal standard solution and 1.0 mL of *strong sodium hydroxide solution R*, add 2.0 mL of *trimethylpentane R*, shake for 2 min, and allow the phases to separate. Use the upper layer.

The determination can be performed on a gas chromatograph with a flame-ionisation detector under the following conditions:

- a fused-silica capillary column, 25 m x 0.32 mm, coated with cross-linked *polymethylphenylsiloxane R* (film thickness 0.52 µm);
- *helium for chromatography R* as the carrier gas;
- split ratio 1:20;
- a column head pressure of 50 kPa;
- a volumetric velocity of the vented carrier gas of 20 mL/min;
- a fused-silica split-liner about 1 cm long, packed with *diatomaceous earth for gas chromatography R* impregnated with 10% (*m/m*) of *poly(dimethyl)siloxane R*;
- the temperature of the column at 150 °C for 5 min, then raising the temperature at a rate of 20 °C per min to 275 °C and retention it at 275 °C for 3 min;
- the temperature of the injection port at 220 °C;
- the temperature of the detector at 300 °C.

The retention times are: *N,N*-dimethylaniline about 3.6 min, *N,N*-diethylaniline about 5.0 min.

Inject 1 µL of the test solution and 1 µL of the reference solution.

METHOD B

Examined by gas chromatography (*2.1.2.27*), using *naphthalene R* as the internal standard.

Internal standard solution. Dissolve 50 mg of *naphthalene R* in *cyclohexane R* and dilute to 50 mL with the same solvent. Dilute 5 mL of this solution to 100 mL with *cyclohexane R*.

Test solution. To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

Reference solution. To 50.0 mg of *N,N-dimethylaniline R*, add 2 mL of *hydrochloric acid R* and 20 mL of *water R*, shake to dissolve and dilute to 50.0 mL with *water R*. Dilute 5.0 mL of this solution to 250.0 mL with *water R*. To 1.0 mL of the latter solution in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

Chromatography can be performed on a gas chromatograph with a flame-ionisation detector under the following conditions:

- a glass column, 2 m x 2 mm, packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 3% (*m/m*) of *polymethylphenylsiloxane R*;
- *nitrogen for chromatography R as* carrier gas;

- carrier gas velocity of 30 mL/min,
- the temperature of the column at 120 °C;
- the temperature of the injection port at 150 °C;
- the temperature of the detector at 150 °C.

Inject 1 µL of the test solution and 1 µL of the reference solution.

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2.1.4.21. Heavy Metals and Arsenic in Herbal Drugs and Herbal Drug Preparations

CAUTION. *When using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions required by the manufacturer.*

APPARATUS

The apparatus typically consists of the following:

- as digestion flasks made of polytetrafluoroethylene, polytetrafluoroethylene, silica, or glass, with a volume of 20 mL to 150 mL, fitted with an airtight closure, a valve to adjust the pressure inside the container, and a polytetrafluoroethylene tube to allow the release of gas;
- a system to make flasks airtight, using the same torsional force for each of them;
- a programmable microwave oven (e.g., with a magnetron frequency of 2450 MHz and a selectable output from 0 to (1500 ± 70) W in 1% increments), a programmable digital computer, a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system and exhaust tubing to vent fumes;
- an atomic absorption spectrometer (*2.1.2.21*), an inductively coupled plasma atomic emission spectrometer (*2.1.2.41*), or an inductively coupled plasma mass spectrometer.

PROCEDURE

Perform the tests using atomic absorption spectrometry (AAS) (*2.1.2.22*), inductively coupled plasma atomic emission spectrometry (ICP-AES) (*2.1.2.41*), or inductively coupled plasma mass spectrometry (ICP-MS). Deviations from the experimental parameters of the sample preparation process and the procedure described below are acceptable, provided that the validation requirements are met and the system suitability test is performed on the day of analysis.

Sample preparation 1

Clean all the glassware and laboratory equipment with a 10 g/L solution of *nitric acid R* before use.

Test solution. In a digestion flask place the prescribed quantity of the test sample (about 0.50 g of a powdered herbal drug), add 4 mL of *heavy metal-free hydrochloric acid R* and 6 mL of *heavy metal-free nitric acid R*, and mix. Make the flask airtight.

Place the digestion flasks in the microwave oven and program heating in 3 steps according to the following program: 80% power for 15 min, 100% power for 5 min, 80% power for 20 min. For the test, use 7 flasks each containing the test solution.

At the end of the cycle allow the flasks to cool in air or water. After cooling, open each digestion flask and introduce the clear, colorless solution obtained into a 50 mL volumetric flask. Rinse each digestion flask with two quantities, each of 15 mL, of *heavy metal-free dilute nitric acid R*. Collect the rinsings in the same volumetric flask and dilute to 50 mL with *water R*. If necessary, it is allowed to use modifiers (for example, when determined by electrothermal atomisation (ETA) atomic absorption spectrometry (AAS) of 1.0 mL of a 10 g/L solution of *magnesium nitrate R* and 1.0 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R*) and stabilisers.

Blank solution. Mix 4 mL of *heavy metal-free hydrochloric acid R* and 6 *mL of heavy metal-free nitric acid R* in a digestion flask and keep in the microwave oven using the same program as for the test solution.

Sample preparation 2

Mineralisation is carried out in a microwave digestion system. Digestion in a microwave system may be implemented in a variety of instruments and using different acids and reagents. When using such systems, one should follow the manufacturer's recommendations. It is necessary to validate the digestion procedure for herbal drugs and herbal drug preparations.

Test solution. Weigh accurately about 0.5 g of powdered herbal drugs/herbal drug preparation and place it in a digestion vessel, add 4 mL of *water R* and 6 mL of *nitric acid R*, mix cautiously until completely wetted and allow to stand for 10-15 minutes. The vessel is hermetically sealed, placed in a protective cover, and then placed in a microwave digestion rotor. Next, treat according to the program shown in Table 2.1.4.21.-1.

At the end of the cycle, cool the vessel in air, open carefully, and transfer quantitatively the resulting clear or slightly precipitated solution to a 50 mL volumetric flask, filter through an ash-free filter washed with *0.1 M hydrochloric acid*, dilute to 25.0 mL with *water R*, and mix.

Table 2.1.4.21.-1. – *Sample processing program for medicinal plant raw materials/preparations in microwave digestion*

Stage	Time (min)	Temperature (°C)	Radiation power (W)
1	9	80	up to 550
2	7	160	up to 1500
3	10	200	up to 1800
4	14	200	up to 1500

Blank solution. Mix 4 mL of water R and 6 mL of nitric acid R in a digestion vessel and keep in the microwave digestion rotor according to the same program as for the test solution.

DETERMINATION OF ARSENIC, CADMIUM, COPPER, NICKEL, AND LEAD BY ELECTROTHERMAL ATOMISATION AAS (2.1.2.22)

METHOD A

Measure the content of arsenic, mercury, cadmium, copper, nickel, and lead by direct calibration curve (*2.1.2.22, Method I*) or by the standard additions method (*2.1.2.22, Method II*) using the reference solutions of arsenic or each heavy metal and instrumental parameters described in Table 2.1.4.21.-1.

The absorbance value of the blank solution is automatically subtracted from the absorbance value obtained with the test solution prepared in accordance with Sample preparation 1.

METHOD B

Measure the content of arsenic, cadmium and lead by the calibration curve (*2.1.2.22, Method I*) under the conditions given in Table 2.1.4.21.-3.

The test solution and the blank solution are prepared in accordance with sample preparation procedure 2.

Measure the content of elements in the tested solutions by the calibration curve. Solutions for plotting the curve are prepared from standard solutions of the corresponding ions.

Table 2.1.4.21.-2. – *Instrumental parameters for electrothermal atomisation AAS*

Instrumental parameters		As	Cd	Cu	Ni	Pb
Wavelength	nm	193.7	228.8	324.8	232	283.5
Slit width	nm	0.5	0.5	0.5	0.2	0.5
Lamp current	mA	10	6	7	10	5
Ignition temperature	°C	1400	800	800	800	800
Atomisation temperature	°C	2600	1800	2300	2500	2200
Gas flow rate	L/min	3	3	3	3	3

Table 2.1.4.21.-3. – *Instrumental parameters for electrothermal atomisation AAS and analytical conditions*

Parameter		Pb	Cd	As
Instrumental parameters				
Wavelength	nm	283.3	228.8	193.7
Slit width	nm	0.5	0.5	0.5R
Lamp current	mA	10	4	11
Ignition temperature	°C	800	600	1400
Atomisation temperature	°C	2000	1700	2600
Type of integration		by peak area	by peak area	by peak area
Background correction system (based on the Zeeman effect)		on	on	on
Injection volume of the test solution, mL		20	10	30 (for 2 times)
Dosage of the modifier, µL		10	10	10 (for 2 times)

To determine the lead content, plot the graph using solutions with the following concentrations of lead ions: 0.005 µg/mL, 0.01 µg/mL, 0.02 µg/mL, 0.04 µg/mL; for the determination of cadmium, solutions with the next cadmium concentrations: 0.0005 mg/mL, 0.001 µg/mL, 0.002 µg/mL, 0.003 µg/mL; for the determination of arsenic, solutions with the next arsenic concentrations: 0.002 µg/mL, 0,004 µg/mL, 0,006 µg/mL, 0.008 µg/mL.

DETERMINATION OF ARSENIC AND MERCURY BY COLD-VAPOUR OR HYDRIDE ATOMISATION AAS (2.1.2.22)

METHOD A

The content of arsenic and mercury is determined by the calibration curve *(2.1.2.22, Method I)* or by the method of standard additives *2.1.2.22, Method II),* using comparison solutions of arsenic and mercury and an automated system for generating a continuous stream of hydride vapours of the analyte.

The absorbance of the blank solution is automatically subtracted from the resulting absorbance of the test solution.

Arsenic

Sample solution. To 19.0 mL of the test solution or of the blank solution as prescribed above, add 1 mL of a 200 g/L solution of *potassium iodide R*. Allow the test solution to stand at room temperature for about 50 min or at 70 °C for about 4 min.

Acid reagent. *Heavy metal-free hydrochloric acid R.*

Reducing reagent. A 6 g/L solution of *sodium tetrahydroborate R* in a 5 g/L solution of *sodium hydroxide R.*

The instrumental parameters in Table 2.1.4.21.-2. may be used.

Mercury

Sample solution. Prepare test and blank solutions, as prescribed above.

Acid reagent. A 515 g/L solution of *heavy metal-free hydrochloric acid R.*

Reducing reagent. A 10 g/L solution of *stannous chloride R* in *heavy metal-free dilute hydrochloric acid R.*

The instrumental parameters in Table 2.1.4.21.-4. may be used.

METHOD B

Measure the mercury content by the calibration curve (*2.1.2.22, Method I*) under the conditions given in Table 2.1.4.21.-5.

Prepare the test solution and the blank solution in accordance with Sample preparation 2.

DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL, AND LEAD BY ICP-AES (2.1.2.41)

Determine the content of arsenic, cadmium, copper, mercury, nickel, and lead by the calibration curve (*2.1.2.22, Method I*) using the reference solutions for each element or a mixture of all the elements to be determined, as well as instrumental parameters shown in Table 2.1.4.21.-6.

The value of the emission rate of the blank solution is automatically subtracted from the obtained value of the emission rate of the test solution.

Table 2.1.4.21.-4. – *Instrumental parameters for cold-vapour or hydride atomisation AAS*

Instrumental parameters		As	Hg
Wavelength	nm	193.7	253.7
Slit width	nm	0.2	0.5
Lamp current	mA	10	4
Acid reagent flow rate	mL/min	1.0	1.0
Reducing reagent flow rate	mL/min	1.0	1.0
Sample solution flow rate	mL/min	7.0	7.0
Absorption cell		quartz (heated)	quartz (unheated)
Nitrogen flow rate	L/min	0.1	0.1

Table 2.1.4.21.-5. – *Instrumental parameters for the hydride atomisation AAS*

Instrumental parameters		Hg
Wavelength	nm	253.7
Slit width, nm	nm	0.5R
Lamp current	mA	3

DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL, AND LEAD BY ICP-MS

Determine the content of arsenic, cadmium, copper, mercury, nickel, and lead by the calibration curve (*2.1.2.22, Method I*), using the reference solutions of each element, analytical isotopes, and additional masses shown in Table 2.1.4.21.-7.

The signal intensity of the blank solution is automatically subtracted from the value obtained with the test solution.

The limit of heavy metals and arsenic in herbal drugs and herbal drug preparations should not exceed the values given in Table 2.1.4.21.-8.

SYSTEM SUITABILITY

The system suitability test should be performed on the day of analysis to ensure that the sample preparation and measurement system are acceptable.

Acceptance criteria for the preparation of the sample solution: clear solution.

Acceptance criteria for the measurement system: the measured concentration of the standard element solution within the concentration range of the calibration curve used must not differ from the actual concentration by greater than 20%.

VALIDATION REQUIREMENTS

Analytical procedures should be confirmed according to the requirements of the general methods of AAS (*2.1.2.2 2*), ICP-AES (*2.1.2.41*), and ICP-MS. In addition, the following criteria must be met. SPECIFICITY Specificity is the ability of analytical procedures for sample preparation and measurement to provide a reliable determination of the element(s) in the presence of the intended components (e.g., carrier gas, impurities, matrix).

Table 2.1.4.21.-6. –*Instrumental parameters for ICP-AES*

Instrumental parameters		As	Cd	Cu	Hg	Ni	Pb
Wavelength	nm	193.696/ 197.197/ 189.042	214.438/ 226.502/ 228.802	324.754/ 327.396/ 224.700	189.950/ 253.652/ 435.835	231.604/ 231.997/ 352.454	220.351/ 283.306/ 168.215
Argon, monitor line	nm	430.010	430.010	430.010	430.010	430.010	430.010
Plasma energy	W	1200	1200	1200	1200	1200	1200
Algorithm peak with background correction		yes	yes	yes	yes	yes	yes

Table 2.1.4.21.-7. – *Recommended analytical isotopes and additional masses for ICP-MS*

Isotope	Element to be determined
75	Arsenic
106, 108, 111, 114	Cadmium
63, 65	Copper
202	Mercury
60, 62	Nickel
206, 207, 208	Lead

Table 2.1.4.21.-8 – *Limits of heavy metals and arsenic in herbal drugs and herbal drug preparations*

Element	Limit, ppm
Lead	6.0
Cadmium	1.0
Mercury	0.1
Arsenic	0.5

Acceptance criteria: The procedure must be able to uniquely evaluate each element to be determined in the presence of the intended components, including other heavy metals, matrix components, and other sources of interference; specificity is confirmed by meeting the requirement for the correct determination of the element(s).

RANGE

The calibration range for each metal should be within the linear range of the procedure; test solutions containing residual amounts of metal at concentrations outside the detection range can be diluted to concentrations within the calibration range.

Acceptance criteria: The range must meet the requirements for recoverability.

ACCURACY

Accuracy is confirmed using a certified reference standard or by meeting the requirements for recoverability.

Recovery. Recoverability may be determined on a test sample of a substance that contains a known amount of the reference standard element (three concentration values in the range from 50% to 150% of the limit set by the specification, even if the true concentration of the reference standard corresponds to the specified value) in three parallel experiments.

Acceptance criteria: Recoverability should be between 70% and 150% for the average of the three definitions of each concentration.

REPEATABILITY

Test samples. Prepare six individual test samples with the addition of a suitable reference standard with a concentration corresponding to the regulated level, or prepare samples of three concentrations for three parallel experiments.

Acceptance criteria: The relative standard deviation in both cases should not exceed the values shown in Table 2.4.27.-9.

INTERMEDIATE PRECISION

The influence of random factors (intermediate variations) on the analytical precision of the procedure should be established. Acceptable tests for establishing intermediate precision are repeated analysis on different days, or on different instruments, or by different analysts. Only one of the three tests is required to confirm intermediate precision.

Acceptance criteria: The relative standard deviation should not exceed the values specified in Table 2.1.4.21.-9.

QUANTITATION LIMIT

Determine the lowest concentration that meets the acceptance criteria. Use the results of the determination of accuracy.

Acceptance criteria: The quantitation limit must be lower than the specification limit.

Table 2.1.4.21.-9. – *Repeatability and intermediate precision depending on the concentration range of a metal*

Metal concentration range (mg/kg)	Repeatability (RSD) (%)	Intermediate precision (RSD) (%)
0.01 - 1	20	32
>1	10	16

DETECTION LIMIT (APPLICABLE ONLY FOR TESTS ON THE IMPURITY LIMIT)

Determine the lowest concentration, the signal of which clearly differs from the signal of the blank solution.

Acceptance criteria: The detection limit must not exceed greater than 0.1 times the concentration corresponding to the specification limit value.

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2.1.4.22. 2-Ethylhexanoic Acid

Examine by gas chromatography (*2.1.2.27*), using *3-cyclohexylpropionic acid R* as the internal standard.

Internal standard solution. Dissolve 100 mg of *3-cyclohexylpropionic acid R* in *cyclohexane R* and dilute to 100 mL with the same solvent.

Test solution. To 0.300 g of the test sample, add 4.0 mL of a 33% *(V/V) solution of hydrochloric acid R*. Shake vigorously for 1 min with 1.0 mL of the internal standard solution. Allow the phases to separate, if necessary, centrifuge. Use the upper layer for testing.

Reference solution. Dissolve 75.0 mg of *2-ethylhexanoic acid R* in the internal standard solution and dilute to 50.0 mL with the same solution. To 1.0 mL of the solution add 4.0 mL of a 33% (*V/V*) solution of *hydrochloric acid R* and shake vigorously for 1 min. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer for testing.

The chromatographic procedure may be carried out using a flame-ionisation detector under the following conditions:

– a wide-bore fused-silica column, 10 m x 0.53 mm, coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 1.0 µm);

- *helium for chromatography R* as the carrier gas;
- the carrier gas velocity of 10 mL/min;
- the temperature program mode:

	Time (min)	Temperature (°C)	Temperature rise rate (°C/min)	Notes
Column	0-2	40	—	Isothermal mode
	2-7.3	40 → 200	30	Linear gradient
	7.3-10.3	200	—	Isothermal mode
Injection port		200		
Detector		300		

Inject 1 µL of the test solution and 1 µL of the reference solution.

The chromatographic system is considered suitable if the resolution between the peaks due to 2-ethylhexanoic acid (first peak) and the internal standard is at least 2.0.

Calculate the percentage content of 2-ethylhexanoic acid from the expression:

$$\frac{S_T \times I_S \times m_S \times 2}{S_S \times I_T \times m_T},$$

where S_T is the area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the test solution;

S_s is the area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the reference solution;

I_T is the area of the peak due to the internal standard in the chromatogram obtained with the test solution;

I_S is the area of the peak due to the internal standard in the chromatogram obtained with the reference solution;

m_T is the weighed amount of the test sample, in grams;

m_S is the weighed amount of 2-ethylhexanoic acid, in grams.

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2.1.5.1. Acid Value

The acid value I_A is the number that expresses, in milligrams, the quantity of potassium hydroxide required to neutralise the free acids present in 1 g of the substance.

Dissolve 10.00 g of the test sample, or the quantity prescribed in the monograph, in 50 mL of a mixture of equal volumes of *96% ethanol R* and *light petroleum R3*, previously neutralised with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide*, unless otherwise specified, using 0.5 mL of *phenolphthalein solution R1* as indicator. To improve the dissolution of the test sample, if necessary, heat the resulting mixture to about 90 °C and maintain this temperature during the titration. When the substance to be examined has dissolved, titrate with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide* until the pink colour persists for at least 15 s.

Calculate the acid value using the formula:

$$I_A = \frac{5{,}61 \cdot V}{m},$$

where V is the volume of *0.1 M potassium hydroxide solution* (*or 0.1 M sodium hydroxide solution*) spent on titration of the test sample, in millilitres;

m is the weighed amount of the test sample in grams.

5.610 is the quantity of potassium hydroxide corresponding to 1 mL of *0.1 M potassium hydroxide solution* (*or 0.1 M sodium hydroxide solution*), in millilitres.

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2.1.5.2. Ester Value

The ester value I_E is the number that expresses in milligrams the quantity of potassium hydroxide required to saponify the esters present in 1 g of the substance.

It is calculated using the equation:

$$I_E = I_S — I_A,$$

where I_S is the saponification value;

I_A is the acid value.

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2.1.5.3. Hydroxyl Value

The hydroxyl value *I*OH is the number that expresses, in milligrams, the quantity of potassium hydroxide required to neutralise the acid combined by acylation in 1 g of the substance.

METHOD A

Introduce the quantity of the test sample shown in Table 2.1.5.3.-1 into a 150 mL acylation flask fitted with an air condenser, unless another quantity is prescribed in the monograph. Add the quantity of *acetic anhydride solution R1* stated in Table 2.1.5.3.-1 and attach the air condenser.

Table 2.1.5.3.-1. – *Estimated values of the hydroxyl number depending on the weighed amount of the test sample and the volume of the acylating reagent*

Estimated value of I_{OH}	Weighed test sample (g)	Acylating reagent volume (mL)
10-100	2.0	5.0
100-150	1.5	5.0
150-200	1.0	5.0
200-250	0.75	5.0
250-300	0.60 or 1.20	5.0 or 10.0
300-350	1.0	10.0
350 - 700	0.75	15.0
700 - 950	0.5	15.0

Heat the flask in a water-bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask and allow to cool. Add 5 mL of *water R* through the upper end of the condenser. If cloudiness appears add sufficient *pyridine R* to clear it, noting the volume added. Shake the flask and replace in the water-bath for 10 min, withdraw the flask, and allow to cool. Rinse the condenser and the walls of the flask with 5 mL of *alcohol R*, previously neutralised with *phenolphthalein solution R1*. Titrate with *0.5 M alcoholic potassium hydroxide* using 0.2 mL of *phenolphthalein solution R1 as indicator.* Carry out a blank test under the same conditions.

Calculate the hydroxyl value by the formula:

$$I_{OH} = \frac{28{,}05 \cdot (V_2 - V_1)}{m} + I_A,$$

where V_1 is the volume of *0.5 M alcoholic potassium hydroxide* spent on titration of the test sample, in millilitres;

V_2 is the volume of *0.5 M alcoholic potassium hydroxide* spent on titration of the blank, in millilitres;

m is the weighed amount of the test sample in grams.

28.05 is the quantity of potassium hydroxide equivalent to 1 mL of *0.5 M alcoholic potassium hydroxide*, in milligrams;

I_A is the acid value.

METHOD B

Introduce the prescribed quantity of the test sample into a perfectly dry 5 mL conical flask fitted with a ground-glass or suitable plastic stopper and add 2.0 mL of *propionic anhydride reagent R.* Close the flask and shake gently to dissolve the substance and allow to stand for 2 h unless otherwise prescribed in the monograph.

Remove the stopper and transfer the flask and its contents into a wide-mouthed 500 mL conical flask containing 25.0 mL of a 9 g/L solution of *aniline R* in *cyclohexane R* and 30 mL of *glacial acetic acid R*. Swirl the contents of the flask, allow to stand for 5 min, add 0.05 mL of *crystal violet solution R* and titrate with *0.1 M perchloric acid* until an emerald-green colour is obtained. Carry out a blank test under the same conditions.

Calculate the hydroxyl value by the formula:

$$I_{OH} = \frac{5{,}610 \cdot (V_1 - V_2)}{m},$$

where V_1 is the volume of *0.1 M perchloric acid solution* used for titration of the test sample, in millilitres

V_2 is the volume of *0.1 M perchloric acid solution* used for titration of the blank, in millilitres

m is the weighed amount of the test sample in grams

5.610 is the quantity of potassium hydroxide equivalent to 1 mL of *0.1 M perchloric acid solution*, in milligrams.

The water content in the substance is determined by the semi-micro method (*2.1.5.12*).

The hydroxyl value is recalculated by the equation:

I_{OH} = (hydroxyl value as determined) - 31.1 · *y*,

where *y* is the water content of the substance as a percentage.

METHOD B

Place the weighed amount of the test sample specified in Table 2.1.5.3.-2 in a dry 250 mL conical glass-stoppered flask and add 5 mL of the mixture of freshly distilled *pyridine R* – freshly distilled *acetic anhydride R* (3:1, *V/V*), heat under reflux in a boiling water bath for 1 h. Then, through the upper end of the condenser, add 10 mL of *water R* and heat for 10 min and cool.

Add 25 mL of *butanol R*, previously neutralised with *0.5 M alcoholic potassium hydroxide solution* using phenolphthalein: first, 15 mL of *butanol R* is added through the upper end of the condenser, then the condenser is disconnected and the walls of the flask are washed with 10 mL of *butanol R*. Add 1 mL of *phenolphthalein solution R1* and titrate with *0.5 M alcoholic potassium hydroxide solution.* Carry out a blank test under the same conditions.

Determination of free acids. Place about 10.0 g of the test sample in a 125 mL conical flask, add 10 mL of freshly distilled *pyridine R*, previously neutralised to phenolphthalein, 1 mL of *phenolphthalein solution R1*, and titrate with *0.5 M alcoholic potassium hydroxide solution.*

Calculate the hydroxyl value by the formula:

$$I_{OH} = \frac{28{,}05}{m_1} \cdot \left[V_1 + \frac{m_1 \cdot V_2}{m_2} - V \right],$$

where V is the volume of *0.5 M alcoholic potassium hydroxide solution* used for titration of the test sample, in millilitres;

V_1 is the volume of *0.5 M alcoholic potassium hydroxide solution* used in the blank titration, in millilitres;

V_2 is the volume of *0.5 M alcoholic potassium hydroxide solution* used for titration of free acids, in millilitres;

$m1$ is the weighted amount of the test sample in grams.

Table 2.1.5.3.-2. – *Estimated values of the hydroxyl number depending on the weighed amount of the test sample*

Estimated value of I_{OH}	Weighed test sample (g)
less than 20	10
20-50	5
50-100	3
100-150	2
150-200	1.5
200-250	1.25
250-300	1.0
300-350	0.75

m_2 is the weighed amount of the test sample for the determination of free acids in grams;

28.05 is the quantity of potassium hydroxide equivalent to 1 mL of *0.5 M alcoholic potassium hydroxide*, in milligrams.

When analysing coloured oils, the endpoint of the titration is set potentiometrically (*2.1.2.19*).

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2.1.5.4. Iodine Value

The iodine value I_I is the number that expresses in grams the quantity of halogen, calculated as iodine, that can be fixed in the prescribed conditions by 100 g of the substance.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Unless otherwise prescribed in the monograph, use the following quantities of the test sample as shown in Table 2.1.5.4.-1.

Introduce the weighed amount of the test sample into a 250 mL flask fitted with a ground-glass stopper and previously dried or rinsed with *glacial acetic acid R*, and dissolve it in 15 mL of *chloroform R* unless otherwise prescribed in the monograph. Add very slowly 25.0 mL of *iodine bromide solution R*. Close the flask and keep it in the dark for 30 min unless otherwise prescribed in the monograph, mixing frequently.

Table 2.1.5.4.-1. – *Presumed iodine values depending on quantities of sample*

Presumed value *II*	Weighed test sample (g)
less than 20	1.0
20-60	0.5-0.25
60-100	0.25-0.15
greater than 100	0.15-0.10

Add 10 mL of a 100 g/L solution of *potassium iodide R*,100 mL of *water R*, and titrate with *0.1 M sodium thiosulfate*, shaking vigorously until the yellow colour is almost discharged. Then add 5 mL of *starch solution R* and continue the titration adding *0.1 M sodium thiosulfate* dropwise until the colour is discharged. Carry out a blank test under the same conditions.

Calculate the iodine value using the formula:

$$I_1 = \frac{1{,}269 \cdot (V_2 - V_1)}{m},$$

where V_1 is the volume of *0.1 M sodium thiosulfate solution* used for titration of the test sample, in millilitres;

V_2 is the volume of *0.1 M sodium thiosulfate solution* used for titration of the blank, in millilitres;

m is the weighed amount of the test sample in grams.

METHOD B

Unless otherwise prescribed in the monograph, use the following quantities of the test sample as shown in Table 2.1.5.4.-2.

The mass of the sample is such that there will be an excess of *iodine chloride solution R* of 50-60% of the total amount added, i.e. 100-150% of the amount absorbed.

Introduce the prescribed quantity of the test sample into a 250 mL flask fitted with a ground-glass stopper and previously dried or rinsed with *glacial acetic acid R*, and dissolve it in 15 mL of a mixture of equal volumes of *cyclohexane R* and *glacial acetic acid R*, unless otherwise specified in the monograph.

If necessary, melt the substance before dissolution (melting point greater than 50 °C). Add very slowly the volume of *iodine chloride solution R* specified in Table 2.1.5.4.-2. Close the flask and keep it in the dark for 30 min, unless otherwise prescribed in the monograph, mixing frequently.

Add 10 mL of a 100 g/L solution of *potassium iodide R*, 100 mL of *water R*, and titrate with *0.1 M sodium thiosulfate*, mixing vigorously until the yellow colour is almost discharged. Then add 5 mL of *starch solution R* and continue the titration adding the *0.1 M sodium thiosulfate* dropwise until the colour is discharged. Carry out a blank test under the same conditions.

Calculate the iodine value using the formula:

$$I_1 = \frac{1{,}269 \cdot (V_2 - V_1)}{m}$$

where: V_1 is the volume of *0.1 M sodium thiosulfate solution* used for titration of the test sample, in millilitres

V_2 is the volume of *0.1 M sodium thiosulfate solution* used for titration of the blank, in millilitres

m is the weighed amount of the test sample in grams.

Table 2.1.5.4.-2. – *Presumed iodine values depending on masses of the test sample and volumes of iodine chloride (ICl) solution*

Presumed value I_1	Mass (g) of the test sample (corresponding to an excess of 150% ICl)	Mass (g) of the test sample (corresponding to an excess of 100% ICl)	ICl solution volume (mL)
<3	10	10	25
3	8.4613	10.5760	25
5	5.0770	6.3460	25
10	2.5384	3.1730	20
20	0.8461	1.5865	20
40	0.6346	0.7935	20
60	0.4321	0.5288	20
80	0.3173	0.3966	20
100	0.2538	0.3173	20
120	0.2115	0.2644	20
140	0.1813	0.2266	20
160	0.1587	0.1983	20
180	0.1410	0.1762	20
200	0.1269	0.1586	20

2.1.5.5. Peroxide Value

The peroxide value I_p is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance.

The peroxide value is determined by the methods described below.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Place 5.00 g of the test sample in a 250 mL conical flask fitted with a ground-glass stopper, add 30 mL of a mixture of *chloroform R – glacial acetic acid R* (2:3, *V/V*). Shake to dissolve the substance and add 0.5 mL of *saturated potassium iodide solution R*, mix for accurately 1 min, then add 30 mL of *water R*. Titrate the resulting solution with *0.01 M sodium thiosulfate*, adding the titrant slowly with continuous vigorous mixing, until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration, mixing vigorously, until the colour is discharged. Carry out a blank test under the same conditions. The volume of *0.01 M sodium thiosulfate* used in the blank titration must not exceed 0.1 mL.

Calculate the peroxide value using the formula:

$$I_P = \frac{10 \cdot (V_1 - V_2)}{m}$$

where V_1 is the volume of *0.01 M sodium thiosulfate* used for titration of the test sample, in millilitres.

V_2 is the volume of *0.01 M of sodium thiosulfate* used on the blank titration, in millilitres;

m is the weighed amount of the test sample in grams.

METHOD B

Carry out tests in a dark place.

Place 50 mL of a mixture of *trimethylpentane R – glacial acetic acid R* (2:3, *V/V*) in a conical flask and replace the stopper. Place the weighed amount of the test sample (see Table 2.1.5.5.-1) and swirl the flask until the test sample has dissolved. Using a suitable volumetric pipette, add 0.5 mL of *saturated potassium iodide solution R* and replace the stopper. Allow the solution to stand for 60 ± 1 s, shaking the solution continuously, then add 30 mL of *water R*.

Titrate the solution with *0.01 M sodium thiosulfate*, adding the titrant gradually and with constant, vigorous mixing, until the yellow iodine colour has almost disappeared. Add about 0.5 mL of *starch solution R1* and continue the titration, with constant, vigorous mixing, especially near the endpoint, to liberate all of the iodine from the organic solvent layer. Add the sodium thiosulfate solution dropwise until the blue colour just disappears.

Depending on the volume of *0.01 M sodium thiosulfate* used for titration, it may be necessary to titrate with *0.1 M sodium thiosulfate*.

NOTE. There is a 15 s to 30 s delay in neutralising the starch indicator for peroxide values of 70 and greater, due to the tendency of trimethylpentane to float on the surface of the aqueous medium. Therefore, some time is necessary to adequately mix the solvent and the aqueous titrant, thus liberating the last traces of iodine. It is recommended to use *0.1 M sodium thiosulfate* for peroxide values greater than 150.

Table 2.1.5.5.-1. – *Estimated values of the peroxide number depending on the weighed amount of the test sample*

Estimated I_P value	Weighed test sample (g)
0 to 12	5.00 to 2.00
12 to 20	2.00 to 1.20
20 to 30	1.20 to 0.80
30 to 50	0,800 to 0.500
50 to 90	0.500 to 0.300

A small amount (0.5% to 1.0%, *m/m*) of an emulsifier with high hydrophilic-lipophilic balance (e.g., polysorbate 60) may be added to the mixture to retard the phase separation and decrease the time lag in the liberation of iodine.

Carry out a blank determination. If a volume of the titrant used for the blank determination exceeds 0.1 mL, repeat the determination using freshly prepared reagents.

Calculate the peroxide value using the formula:

$$I_P = \frac{1000 \cdot (V_1 - V_0) \cdot c}{m},$$

where V_1 is the volume of *0.01 M sodium thiosulfate* used for titration of the test sample, in millilitres.

V_0 is the volume of *0.01 M of sodium thiosulfate* used for the blank titration, in millilitres;

m is the weighed amount of the test sample in grams.

c is the concentration of the sodium thiosulfate solution, in mol per litre.

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2.1.5.6. Saponification Value

The saponification value I_S is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids and to saponify the esters present in 1 g of the substance.

Unless otherwise prescribed in the monograph, use the quantities of the test sample indicated in Table 2.1.5.6.-1.

Table 2.1.5.6.-1. – *Presumed saponification values depending on quantities of sample*

Presumed values I_s	Weighed test sample (g)
<3	20
3 to 10	12 to 15
10 to 40	8 to 12
40 to 60	5 to 8
60 to 100	3 to 5
100 to 200	2.5 to 3
200 to 300	1 to 2
300 to 400	0.5 to 1

Place weighed test sample into a 250 mL borosilicate glass flask fitted with a ground-glass stopper with a reflux condenser. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Attach the condenser and heat for 30 min, unless otherwise prescribed in the monograph.

Add 1 mL of *phenolphthalein solution R1*, the hot solution is titrated immediately with *0.5 M hydrochloric acid*. Carry out a blank test under the same conditions.

The saponification value is calculated by the formula:

$$I_S = \frac{28{,}05 \cdot (V_2 - V_1)}{m}$$

where V_1 is the volume of *0.5 M hydrochloric acid* used for the test titration, in millilitres;

V_2 is the volume of *0.5 M hydrochloric acid* used for the blank titration, in millilitres;

m is the weighed amount of the test sample in grams.

28.05 is the quantity of potassium hydroxide corresponding to 1 mL of *0.5 M hydrochloric acid*, in milligrams.

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2.1.5.7. Unsaponifiable matters

The term "unsaponifiable matter" applies to the substances non-volatile at 100-105 °C and obtained by extraction with an organic solvent from the substance after it has been saponified. The content of unsaponifiable matter is calculated as a percent (*m/m*).

Use ungreased ground-glass glassware.

Place weighed test sample specified in the monograph into a 250 mL flask fitted with a reflux condenser.

Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water bath for 1 h, swirling frequently, then cool to a temperature below 25 °C. Transfer the contents of the flask to a separating funnel using 100 mL of *water R*. Shake the resulting solution carefully with three quantities, each of 100 mL, of *peroxide-free ether R*. Collect all the ether extractions in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes (no greater than 5 min), and allow to separate and reject the aqueous layer. Wash the ether layer with 2 quantities, each of 40 mL, of *water R*, then wash successively with 40 mL of a 30 g/L solution of *potassium hydroxide R* and 40 mL of *water R*; repeat this procedure three times. Wash the ether layer several times, each with 40 mL of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether layer quantitatively to a tared flask, washing the separating funnel with *peroxide-free ether R*.

Distill off the ether with suitable precautions and add 6 mL of *acetone R* to the residue. Carefully remove the solvent in a current of air. Dry to constant mass at 100-105 °C, allow to cool in a desiccator and weigh.

Calculate the content of unsaponifiable substances by the formula:

$$\text{Unsaponifiable substances} = \frac{100 \cdot a}{m},$$

where *a* is the mass of the residue in grams;

m is the weighed amount of the test sample in grams.

Dissolve the residue in 20 mL of *alcohol R*, previously neutralised to *phenolphthalein solution R*, and titrate with *0.1 M alcoholic sodium hydroxide*. If the volume of *0.1 M alcoholic sodium hydroxide* used is greater than 0.2 mL, the separation of the layers has been incomplete; the residue weighed cannot be considered as "unsaponifiable matter". In this case, the test must be repeated.

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2.1.5.8. Determination of Primary Aromatic Amino-Nitrogen

Dissolve the prescribed quantity of the test sample in 50 mL of dilute *hydrochloric acid R* or in another prescribed solvent and add 3 g of *potassium bromide R*. Cool in ice water and titrate by slowly adding *0.1 M sodium nitrite* with constant stirring.

Determine the end-point electrometrically or by the use of the indicator specified in the monograph.

201050009-2019

2.1.5.9. Determination of Nitrogen by Sulfuric Acid Digestion

SEMI-MICRO METHOD

Place weighed sample containing about 2 mg of nitrogen in a combustion flask, add 4 g of a powdered mixture of 100 g of *dipotassium sulfate R*, 5 g of *copper sulfate R*, and 2.5 g of *selenium R*, and three glass beads. Wash any adhering particles from the neck into the flask with 5 mL of *sulfuric acid R*, allowing it to run down the sides of the flask. Mix the contents of the flask by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with a condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed in the monograph. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of *water R*, cool again, and place in a steam-distillation apparatus. Add 30 mL of *strong sodium hydroxide solution R* and distill immediately by passing steam through the mixture. Collect about 40 mL of distillate in 20.0 mL of *0.01 M hydrochloric acid* and enough *water R* to cover the tip of the condenser.

Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with *0.01 M sodium hydroxide*, using *methyl red mixed solution R* as indicator.

Repeat the test using about 50 mg of *glucose R* in place of the test sample.

Calculate the content of nitrogen in percent using the formula:

$$\text{Content of nitrogen} = \frac{0{,}01401 \cdot (V_2 - V_1)}{m},$$

where V_1 is the volume of *0.01 M sodium hydroxide solution* used for titration of the solution obtained after the sample ignition, in millilitres;

V_2 is the volume of *0.01 M of sodium hydroxide solution* used for titration of the solution obtained after the glucose ignition, in millilitres;

m is the weighed amount of the test sample in grams.

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2.1.5.10. Oxygen-Flask Method

The method is based on the destruction of organic substances by ignition in oxygen, dissolving the resulting combustion products in an absorbing liquid for the subsequent determination of elements that are in the form of ions in the solution. Use the method for the determination of halogens (fluorine, chlorine, bromine, and iodine), sulfur, and phosphorus.

The combustion flask is a conical flask of at least 500 mL capacity of borosilicate glass with a ground-glass stopper into which a platinum, nichrome, or platinum-iridium wire with a diameter from 0,7 mm to 0,8 mm is soldered, ending with a sample holder in the form of a basket or a spiral made of the same material, at a distance of 1.5 cm to 2.0 cm from the bottom of the flask (Figure 2.1.5.10.-1).

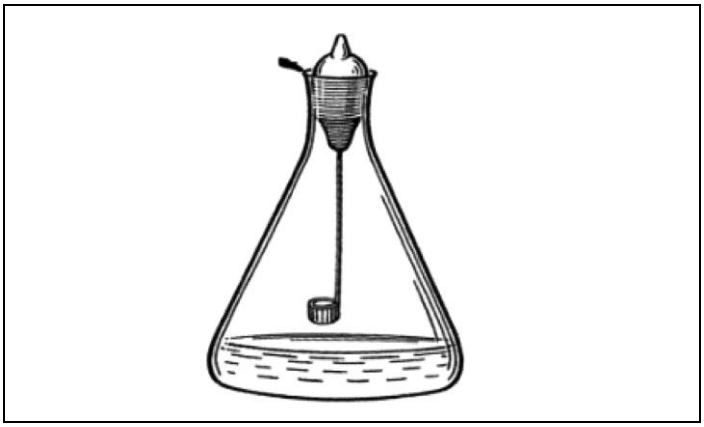


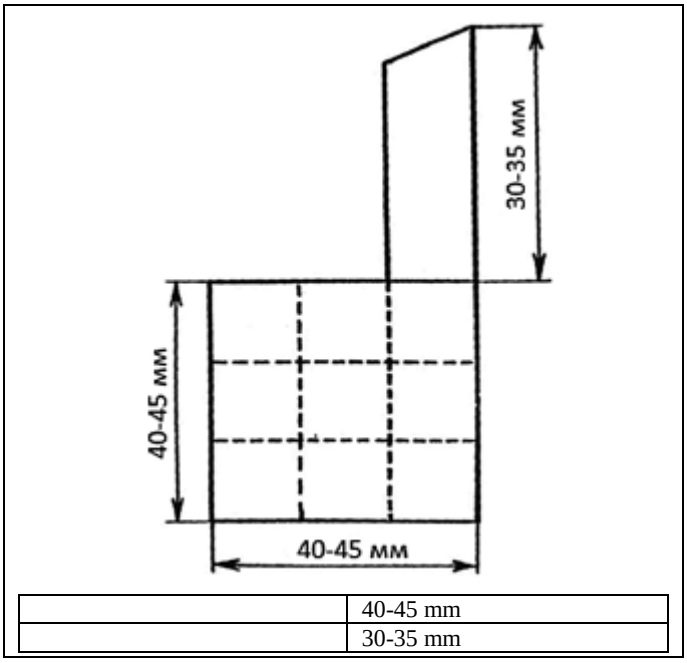
Figure 2.1.5.10.-1 – *Oxygen-combustion flask*

Finely grind the substance to be examined and place the quantity specified in the monograph in the center of a piece of filter paper measuring about 40 mm by 45 mm and envelope in the form of a bag, provided with a small strip about 10 mm wide and 30-35 mm long, according to the diagram in Figure 2.5.10.-2.

If paper impregnated with lithium carbonate is prescribed in the monograph, moisten the center of the paper with a saturated solution of *lithium carbonate R* and dry in an oven at 100-105 °C before use.

When testing a liquid, place a weighed sample in a wax-fused capillary or in a capsule made of polyethylene, nitrofilm, or methylcellulose. For non-volatile liquids, a double paper bag is allowed. For the analysis of ointment-like substances, a capsule made of nitrofilm or a package made of wax paper is used. The capsules and capillaries are enveloped in a filter paper as a bag as shown below. In the case of solid and ointment-like substances that burn with a flash, from 3 mg to 5 mg of wax is added to the suspension.

The prepared sample bag is placed in the holder. Introduce into the flask *water R* or the solution specified in the monograph which is intended to absorb the combustion products. Displace the air with oxygen by means of a tube having its end just above the liquid. Ignite the narrow end of the paper strip by suitable means with the usual precautions, close the flask firmly with a stopper wetted in *water R*.



	40-45 mm
	30-35 mm

Figure 2.1.5.10.-2. – *Filter paper used to form a "bag"*

Keep the flask firmly closed during the whole combustion process. Shake the flask vigorously to completely dissolve the combustion products. Cool and after about 5 min, unless otherwise prescribed in the monograph, carefully unstopper the flask. Wash the ground parts and the walls of the flask, as well as the sample carrier, with *water R*. Combine the combustion products and the washings and determine the element as prescribed in the monograph.

In parallel, conduct a blank determination.

NOTE. During the test, you must take precautions (use safety glasses, place the flask in a safety cover, install a protective screen). The combustion flask must be thoroughly washed and free of traces of organic substances and solvents.

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2.1.5.11. Complexometric Titration

ALUMINUM

Introduce 20.0 mL of the solution specified in the monograph into a 500 mL conical flask, add 25.0 mL of *0.1 M sodium edetate* and 10 mL of a mixture of equal volumes of a 155 g/L solution of *ammonium acetate R* and *dilute acetic acid R*, boil for 2 min and cool. Add 50 mL of *ethanol R* and 3 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *ethanol R*.

LEAD

Introduce the solution specified in the monograph into a 500 mL conical flask. Dilute to 200 mL with *water R*, add about 50 mg of *xylenol orange indicator R*, then *hexamethylenetetramine R* until the solution becomes violet-pink. Titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

Titrate the excess of sodium edetate with *0.1 M zinc sulfate* until the colour changes from greenish-blue to reddish-violet.

1 mL of *0.1 M sodium edetate* is equivalent to 2.698 mg of Al.

BISMUTH

Introduce the solution specified in the monograph into a 500 mL conical flask. Dilute to 250 mL with *water R* and then, unless otherwise prescribed in the monograph, add dropwise, with mixing, *concentrated ammonia R* until the mixture becomes cloudy. Add 0.5 mL of *nitric acid R*, heat to about 70 °C until the cloudiness disappears completely, add about 50 mg of *xylenol orange indicator R* and titrate with *0.1 M sodium edetate* until the colour changes from pinkish-violet to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

CALCIUM

Introduce the solution specified in the monograph into a 500 mL conical flask. Dilute the solution to 300 mL with *water R*, add 6.0 mL of *strong sodium hydroxide solution R* and about 200 mg of *calconcarboxylic acid indicator R*, and titrate with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

MAGNESIUM

Introduce the solution specified in the monograph into a 500 mL conical flask. Dilute to 300 mL with *water R*, add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 indicator R*. Heat to about 40 °C then titrate at this temperature with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

K. Fisher reagent. A solution of sulfur dioxide, iodine, and pyridine (or imidazole) in methanol.

When determining water in solid substances that are insoluble in methanol, shake the finely ground suspension with *methanol R*, and then titrate with K. Fisher reagent. Some substances or mixtures of substances can be dissolved in *acetic acid anhydrous R*, *chloroform R*, *pyridine R*, or other

1 mL of *0.1 M sodium edetate* is equivalent to 20.72 mg of Pb.

ZINC

Introduce the solution specified in the monograph into a 500 mL conical flask. Dilute to 200 mL with *water R*, add about 50 mg of *xylenol orange indicator R*, then *hexamethylenetetramine R* until the solution becomes violet-pink. Add 2 g of *hexamethylenetetramine R* in excess and titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 6.54 mg of Zn.

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2.1.5.12. Water: Semi-Micro Determination

The semi-micro determination of water (K. Fisher's method) is based upon the quantitative reaction of water with sulfur dioxide and iodine in a suitable anhydrous medium in the presence of a base with sufficient buffering capacity.

The reaction proceeds in two stages stoichiometrically according to the equations:

$$R_1OH + SO_2 + R_2N = [R_2NH]SO_3R_1,$$
$$I_2 + H_2O + [R_2NH]SO_3R1 + 2R_2N =$$
$$= [R_2NH]SO_4R_1 + 2[R_2NH]I,$$

where R_1OH is the aliphatic alcohol;
R_2N is the base (pyridine or imidazole).

solvents as prescribed in monographs. Using reagent K. Fischer reagent, hygroscopic and crystallisation water may be defined.

K. Fisher reagent is not applicable to the analysis of substances that react with one or more reagent components.

APPARATUS

The apparatus consists of a titration vessel with:

- two identical platinum electrodes;
- tight inlets for the introduction of solvent and titrant;
- an inlet for air delivery via a desiccant;
- a sample inlet fitted with a stopper or, for liquids, a septum.

Inlet systems for the introduction of dry nitrogen or for aspiration of solvents may also be fitted.

The titration is carried out according to the instrument supplier’s instructions. Care is taken throughout the determination to avoid exposure of reagents and solvents to atmospheric moisture. The endpoint is determined using two identical indicator electrodes connected to an electrical source that maintains between the electrodes either a constant current (*Voltamperometric titration*) or a constant voltage (*2.1.2.18. Amperometric titration*). Where direct titration is used (Method A), the addition of titrant causes either a decrease in voltage where a constant current is maintained or an increase in a current where constant voltage is maintained, until the end-point is reached. Instruments with automatic endpoint detection are commonly used. The instrument is qualified according to the methods established within the quality system, for example, using a suitable certified reference standard.

Standardisation. To the titration vessel, add *methanol R*, dried if necessary, or the solvent recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce a suitable amount of water in an appropriate form (*water R* or certified reference material) and carry out the titration, stirring for the necessary time. The water equivalent is not less than 80% of that indicated by the manufacturer. Standardise the titrant before the first use and at suitable intervals thereafter.

Unless otherwise prescribed, use Method A.

Method A. Introduce into the titration vessel *methanol R*, or the solvent specified in the monograph or recommended by the manufacturer of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the test sample rapidly and carry out the titration, stirring for the necessary extraction time.

Method B. Introduce into the titration vessel *methanol R*, or the solvent specified in the monograph or recommended by the manufacturer of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Rapidly introduce the test sample powdered to the desired degree into a titration flask. Add an accurately measured volume of the titrant, sufficient to give an excess of about 1 mL or the volume specified in the monograph. Allow to stand protected from light for 1 min or the time specified in the monograph, with stirring. Titrate the excess of reagent using *methanol R* or the solvent specified in the monograph, containing an accurately known quantity of water.

System Suitability. The suitability of the determination with the chosen titrant must be verified for each combination of substance, titrant, and solvent. The following procedure, given as an example, is suitable for samples containing 2.5-25 mg of water.

The water content of the substance to be examined is determined using the reagent/solvent system chosen. Thereafter, in the same titration vessel, sequential known amounts of *water R*, corresponding to about 50-100% of the amount found in the substance, are added in an appropriate form (at least 5 additions) and the water content is determined after each addition. Calculate the percentage recovery (*r*) after each addition using the following expression:

$$r = 100 \cdot \frac{W_2}{W_1},$$

where W_1 is the quantity of water added, in milligrams;

W_2 is the quantity of water found, in milligrams.

Calculate the mean percentage recovery (*r*). The reagent/solvent system is considered to be acceptable if *r* is between 97.5% and 102.5%.

Calculate the regression line. The *x*-axis represents the cumulative water added whereas the *y*-axis represents the sum of the initial water content determined for the substance (M) and the cumulative water determined after each addition. Calculate the slope *(b)*, the intercept with the *y-axis (a)*, and the intercept of the extrapolated calibration line with the *x-axis (d).*

Calculate the percentage errors (e_1 и e_2) using the following expressions:

$$e_1 = 100 \frac{a - M}{M},$$

$$e_2 = 100 \frac{|d| - M}{M},$$

where: *a* is the *y*-axis intercept, in milligrams of water;

d is the *x*-axis intercept, in milligrams of water;

M is the water content of the substance, in milligrams of water.

The reagent/solvent system is considered to be acceptable if:

- $|e_1|$ and $|e_2|$ are not greater than 2.5%;
- *b* value is between 0.975 and 1.025.

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2.1.5.13. Water: Micro-Detection

PRINCIPLE

The coulometric titration of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in an anhydrous medium in the presence of a base with sufficient buffering capacity. In contrast to the volumetric method described under *2.1.5.12. Water: Semi-Micro Determination*, iodine is produced at the anode reacts immediately with the water and the sulfur dioxide contained in the reaction cell. The iodine produced at the anode immediately reacts with water and sulfur dioxide contained in the reaction cell. The quantity of water in the test sample is directly proportional to the quantity of electricity (in coulombs) expressed as electric current intensity (in amperes) multiplied by time and used to produce iodine up until the titration endpoint. When all of the water in the cell has been consumed, the endpoint is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, a quantity of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the cell by pre-titration, i.e. titrate the electrolytic reagent to dryness before analysing the test sample.

Individual determinations may be carried out successively in the same reagent solution, under the following conditions:

- each component of the test mixture is compatible with the other components;
- no other reactions take place;
– the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is restricted to the quantitation of small amounts of water (from 10 µg), however, a range of 100 µg up to 10 mg of water is recommended.

Accuracy and precision of the method are predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

APPARATUS

The apparatus consists of a reaction cell, electrodes, and a magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in a tube (oven) and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

The apparatus is qualified in accordance with established quality system methods, such as using suitable certified reference materials. *Reference standard of amoxicillin trihydrate for performance check of apparatus* can be used for an apparatus with an oven.

PROCEDURE

Fill the compartments of the reaction cell with *electrolyte reagent for the micro determination of water R* according to the manufacturer’s instructions and perform the coulometric titration to a stable endpoint.

Introduce the prescribed amount of the test sample into the reaction cell, stir for 30 s, if not otherwise indicated in the monograph, and titrate again to a stable endpoint. If a furnace is used, the specified amount of the test sample is placed in the furnace and heated. After water is evaporated from the sample, titration is performed in the reaction cell. Alternatively, to prevent the loss of water already collected in the reaction solution during prolonged heating, the evaporated water is titrated immediately, simultaneously with heating the test sample in the furnace. In case an oven is used, the prescribed sample amount is introduced into the tube and heated. When appropriate to the type of sample and the sample preparation, perform a blank titration.

VERIFICATION OF THE ACCURACY

At regular intervals, at least at the beginning and end of the titration of a series of samples, place an exact water sample of the same order as the quantity of water in the sample, using a suitable reference standard, and perform coulometric titration. The recovery should be in the range of 97.5% to 102.5% for a 1000 µg H_2O and in the range of 90.0% to 110.0% for a 100 µg of H_2O.

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2.1.5.14. Total Protein

Many of the quantitation methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for quantitation purposes. If the buffer solution used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid. However, if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may significantly reduce the content in the solution. This can be prevented by preparing samples at a higher concentration or by using a non-ionic detergent in the preparation.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/mL and 2 mg/mL.

Reference solution. Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

Procedure. Keep the test solution, the reference solution, and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (*2.1.2.24*) of the test solution and the reference solution in quartz cells at 280 nm, using the specified buffer solution as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

Light scattering. The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm, and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression.

Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

Calculations. Use corrected absorbance values for the calculations. Calculate the concentration of protein in the test solution (*C*U) from the following equation:

$$C_U = C_S (A_U / A_S)$$

where C_S is the concentration of protein in the reference solution, in milligrams per millilitre;

A_U is the corrected absorbance of the test solution;

A_S is the corrected absorbance of the reference solution.

METHOD 2

This method (commonly referred to as the Lowry protein quantitation method) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm. The phosphomolybdotungstic reagent reacts primarily with tyrosine residues in the protein. Colour development reaches a maximum in 20-30 min at room temperature, after which there is a gradual loss of colour. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test sample may be used. Most interfering substances cause a lower colour yield; however, some detergents cause a slight increase in colour. A high salt concentration may cause a precipitate to form. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same. Where separation of interfering substances from the protein in the test sample is necessary, proceed as directed below for interfering substances prior to preparation of the test solution. The effect of interfering substances may be minimised by dilution, provided the concentration of the test protein remains sufficient for accurate measurement.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a test solution having a concentration within the range of the standard curve. A suitable buffer solution will produce a solution of pH 10.0 to 10.5.

Reference solutions. Dissolve the reference substance for the protein to be determined in the buffer specified in the monograph. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 5 µg/mL and 100 µg/mL.

Blank solution. Buffer solution used to prepare the test solution and the reference solutions.

Copper sulfate reagent. Dissolve 100 mg of *copper (II) sulfate R* and 0.2 g of *sodium tartrate R* in distilled *water R* and dilute to 50 mL with the same solvent. Dissolve 10 g of *anhydrous sodium carbonate R* in distilled *water R* and dilute to 50 mL with the same solvent. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Use the solution within 24 h.

Alkaline copper reagent. A mixture of copper sulfate reagent – 50 g/L solution of *sodium dodecyl sulfate R*– 32 g/L solution of *sodium hydroxide R* (1:2:1, *V/V).* Store at room temperature and use within two weeks.

*Diluted phosphomolybdotungstic reagent.*Mix 5 mL of *phosphomolybdotungstic reagent R* with 55 mL of *distilled water R.* Store in an amber bottle, at room temperature.

Procedure. To 1.0 mL of each reference solution, of the test solution and of the blank, add 1.0 mL of alkaline copper reagent and mix. Allow to stand for 10 min. Add 0.5 mL of the diluted phosphomolybdotungstic reagent, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.1.2.24*) of the solutions at 750 nm, using the solution from the blank as compensation liquid.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the calibration curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to build the calibration curve. From the calibration curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. In the following procedure, deoxycholate-trichloroacetic acid is added to a test sample to remove interfering substances by precipitation of proteins before determination. This procedure can also be used to concentrate proteins from a dilute solution.

Add 0.1 mL of a 1.5 g/L solution of *sodium deoxycholate R* to 1 mL of a solution of the test sample, mix using a vortex mixer and allow to stand at room temperature for 10 min. Add 0.1 mL of a 720 g/L solution of *trichloroacetic acid R* and mix using a vortex mixer. Centrifuge at 3000 *g* for 30 min, decant the liquid, and remove any residual liquid with a pipette. Dissolve the protein precipitate obtained in 1 mL of alkaline copper reagent.

METHOD 3

This method (commonly referred to as the Bradford quantitation method) is based on the absorption shift from 470 nm to 595 nm observed when the acid blue 90 dye binds to protein. The acid blue 90 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the quantitation to different proteins. The protein used as a reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Prepare a solution of the test sample in a buffer solution specified in the monograph, with a protein concentration within the concentration range of the calibration curve.

Reference solutions. Dissolve the reference substance for the protein to be determined in the buffer specified in the monograph. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg/mL and 1 mg/mL.

Blank solution. Buffer solution used for the preparation of the test solution and reference solutions.

Acid blue 90 reagent. Dissolve 0.10 g of *acid blue 90 R* in 50 mL of *96% alcohol R*. Add 100 mL of *phosphoric acid R*, dilute to 1000 mL with *distilled water R* and mix. Filter the solution and store in an amber bottle at room temperature. Slow precipitation of the dye occurs during storage, therefore, filter the reagent before using.

Procedure. Add 5 mL of acid blue 90 reagent to 0.100 mL of each reference solution, of the test solution and of the blank, mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances (*2.1.2.24*) of the standard solutions and of the test solution at 595 nm, using the blank solution as compensation liquid. Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the calibration curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to build the calibration curve. From the calibration curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 4

This method (commonly referred to as the bicinchoninic acid or BCA quantitation method) is based on the reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion (monovalent copper). Few substances interfere with the reaction, however, their effect may be minimised by dilution, provided that the concentration of the protein to be determined remains sufficient for accurate measurement. Alternatively, the protein precipitation procedure given in Method 2 may be used to remove interfering substances. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Prepare a solution of the test sample in a buffer solution specified in the monograph, with a protein concentration within the concentration range of the calibration curve.

Reference solutions. Dissolve the reference substance for the protein to be determined in the buffer specified in the monograph. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 1200 µg/mL.

Blank solution. Buffer solution used for the preparation of the test solution and reference solutions.

BCA reagent. Dissolve 10 g of *disodium bicinchoninate R*, 20 g of *sodium carbonate monohydrate R*, 1.6 g of *sodium tartrate R*, 4 g of *sodium hydroxide R*, and 9.5 g of *sodium hydrogen carbonate R* in *distilled water R*. Adjust, if necessary, to pH 11.25 with a solution of *sodium hydroxide R* or a solution of *sodium hydrogen carbonate R*. Dilute to 1000 mL with *distilled water R* and mix.

Copper-BCA reagent. Mix 1 mL of a 40 g/L solution of *copper sulfate pentahydrate R* and 50 mL of BCA reagent.

Procedure. Mix 0.1 mL of each reference solution, of the test solution and of the blank with 2 mL of the copper-BCA reagent. Incubate the resulting solutions at 37 °C for 30 min, note the time, and allow the mixtures to cool to room temperature. Within 60 min of the end of incubation, determine the absorbances (*2.1.2.24*) of the reference solutions and of the test solution in quartz cells at 562 nm, using the blank as compensation liquid. It should be taken into account that after the solutions have cooled to room temperature, the colour intensity continues to increase gradually.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the calibration curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to build the calibration curve. From the calibration curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 5

This method (commonly referred to as the biuret quantitation method) is based on the interaction of cupric (Cu^{2+}) ion with protein in alkaline solution and the resultant development of absorbance at 545 nm. This test shows the minimal difference between equivalent IgG and albumin samples.

The addition of the sodium hydroxide and the biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimise the effects of interfering substances also can be used to determine the protein content in test samples at concentrations below 500 μg/mL.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Prepare a solution of the test sample in a 9 g/l *sodium chloride R* solution with a protein concentration within the concentration range of the calibration curve.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than three reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.5 mg/mL and 10 mg/mL.

Blank solution. 9 g/L solution of *sodium chloride R.*

Biuret reagent. Dissolve 3.46 g of *copper (II) sulfate R* in 10 mL of hot *distilled water R*, and allow to cool (Solution A). Dissolve 34.6 g of *sodium citrate R* and 20.0 g of *anhydrous sodium carbonate R* in 80 mL of hot *distilled water R*, and allow to cool (Solution B). Mix solutions A and B and dilute to 200 mL with *distilled water R.* Use the solution obtained within 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure. To one volume of the test solution add an equal volume of a 60 g/L solution of *sodium hydroxide R* and mix. Immediately add biuret reagent equivalent to 0.4 volumes of the test solution and mix rapidly. Allow to stand at a temperature between 15 °C and 25 °C for not less than 15 min. Within 90 min of the addition of the biuret reagent, determine the absorbances (*2.1.2.24*) of the reference solutions and of the test solution at the maximum at 545 nm, using the blank as compensation liquid.

Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

Calculations. The relationship of absorbance to protein concentration is approximately linear within the indicated range of protein concentrations for the reference solutions. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to build the calibration curve. Calculate the correlation coefficient for the calibration curve. A suitable system is one that yields a line having a correlation coefficient not less than 0.99. From the calibration curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. To minimise the effect of interfering substances, the protein can be precipitated from the test sample as follows: add 0.1 volumes of a 500 g/L solution of *trichloroacetic acid R* to 1 volume of a solution of the test sample, withdraw the supernatant layer and dissolve the precipitate in a small volume of *0.5 M sodium hydroxide.* Use the solution obtained to prepare the test solution.

METHOD 6

This fluorimetric method is based on the derivatisation of the protein with *o*-phthalaldehyde, which reacts with the primary amines of the protein (*N*-terminal amino acid and the ε-amino group of lysine residues). The sensitivity of the quantitation can be increased by hydrolysing the protein before adding *o*-phthalaldehyde. Hydrolysis makes the α-amino group of the constituent amino acids available for reaction with the phthalaldehyde reagent. The method requires very small quantities of the protein. Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with phthalaldehyde, and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this method may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Prepare the test sample solution in a 9 g/l *sodium chloride R* solution with a protein concentration within the concentrations of the reference solutions. Adjust the solutions to pH 8 to 10.5 before the addition of the phthalaldehyde reagent.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R.* Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 200 µg/mL. Adjust the solutions to pH 8 to 10.5 before the addition of the phthalaldehyde reagent.

Blank solution. 9 g/L solution of *sodium chloride R.*

Borate buffer solution. Dissolve 61.83 g of *boric acid R* in distilled *water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 mL with *distilled water R*, and mix.

Phthalaldehyde stock solution. Dissolve 1.20 g of *phthalaldehyde R* in 1.5 mL of *methanol R*, add 100 mL of borate buffer solution, and mix. Add 0.6 mL of a 300 g/L solution of *macrogol 23 lauryl ether R*, and mix. Store at room temperature and use within three weeks.

Phthalaldehyde reagent. To 5 mL of phthalaldehyde stock solution add 15 µL of *2-mercaptoethanol R.* Prepare at least 30 min before use. Use within 24 h.

Procedure. Mix 10 µL of the test solution and of each of the reference solutions with 0.1 mL of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 mL of *0.5 M sodium hydroxide* and mix. Determine the fluorescent intensities (*2.1.2.20*) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

Calculations. The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to build the calibration curve. From the calibration curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis procedure is based on the destruction of the test sample during the analysis but is not limited to protein presentation in an aqueous environment.

Procedure A. Proceed as prescribed for the determination of nitrogen by sulfuric acid digestion (*2.1.5.9*) or use commercial instrumentation for Kjeldahl nitrogen quantitation.

Procedure B. Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C). This produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the test samples. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2*), which emits light when it decays and can be quantified with a chemiluminescence detector.

A protein reference standard that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations. The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference standard.

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2.1.5.15. Anisidine Value

The anisidine value (I_{AH}) is the number determining the content of secondary oxidation products (aldehydes, ketones) in the substance to be examined (oil, solid fats, lipids) defined as 100 times the absorbance measured in a 1 cm cell of a solution containing 1 g of the test sample in 100 mL of a mixture of

solvents after reaction with *p*-anisidine, according to the following procedure.

Carry out the operations as rapidly as possible, avoiding exposure to sunlight.

Test solution (a). Dissolve 0.500 g of the substance to be examined in *trimethylpentane R* and dilute to 25.0 mL with the same solvent.

Test solution (b). To 5.0 mL of test solution (a) add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Reference solution. To 5.0 mL of *trimethylpentane R* add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake, and store protected from light. Use the solution of *p-anisidine R* freshly prepared.

Measure the absorbance (*2.1.2.24*) of test solution (a) at the maximum at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$I_{AH} = \frac{25 \cdot (1{,}2A_1 - A_2)}{m},$$

where A_1 is the absorbance of test solution (b) at 350 nm;

A_2 is the absorbance of test solution (a) at 350 nm;

m is the mass of the test sample taken to prepare the test solution (a), in grams;

1.2 is the coefficient that takes into account the volume of the tested solutions.

2.1.6.1. Sterility

This general chapter is applied to sterility test procedures for various drugs – preparations for injection/infusion, eye drops/inserts, pharmaceutical substances and excipients, including biological drug products and their solvents, etc., which are required to be sterile.

TEST CONDITIONS

The test for sterility is carried out under aseptic conditions in laminar airflow units, clean rooms, or Class A isolators. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the samples of drugs to be examined, including biological drug products (BDP). The test conditions are monitored regularly in accordance with good manufacturing and laboratory practices.

STERILITY TEST METHODS

Carry out the test for sterility using two methods: direct inoculation or membrane filtration. The membrane filtration method is used in all cases when the nature of the drug, its physicochemical properties make it possible to filter it through membrane filters.

The direct inoculation method is used for testing the sterility of drugs that do not have an antimicrobial effect or whose antimicrobial effect can be eliminated by dilution or inactivation, as well as for samples that cannot be tested by membrane filtration.

When testing for sterility, the corresponding negative control tests are carried out in parallel.

1. Verification of the suitability of the test procedure (determination of antimicrobial action)

The suitability of the sterility test procedure should be verified in the following cases:

a) when testing the sterility of a new drug;

b) when making any changes to the experimental test conditions;

c) in case of changes in the composition of the drug or changes in the manufacturing processes.

To check the antimicrobial activity, the same test strains are used as when evaluating the growth properties of culture media (Table 2.1.6.1.-3).

Antimicrobial activity is determined using the same methods and under the same conditions as the sterility test.

Membrane filtration. The suitability test (determination of antimicrobial activity) can be performed simultaneously with the test for sterility of the test sample (para. 2.2.). After transferring the required quantity of the test sample to the filter, add not greater than 100 colony forming units (CFU) of test strains of microorganisms to the final portion of liquid used to rinse the membrane (para. 2.2.8).

Direct inoculation. When checking the suitability (determination of antimicrobial activity), prepare suspensions of test strains with a final concentration of no greater than 100 CFU per millilitre. The test is performed with each type of micro-organism.

Use 4 test tubes for each test strain with 10 mL of an appropriate culture medium. In the first two tubes with the culture of the microorganism, add 1 mL (g) of the test sample and the solvent in the other two tubes, 1 mL to each (positive control). In all four test tubes, add 1 mL of the corresponding test strain.

Inoculations on a thioglycol medium are incubated at a temperature of 32.5 ± 2.5 °C for 3 days. Inoculations on a fluid soya-bean casein medium or a fluid Sabouraud medium are incubated at a temperature of 22.5 ± 2.5 °C for 5 days.

The results are recorded visually in transmitted light, comparing the growth of test strains of microorganisms in the experimental and control inoculations. If the detected growth in test tubes is visually comparable to the growth in control inoculations that do not contain the test sample, it is concluded that the drug does not have an antimicrobial effect under test conditions. In this case, the sterility test is performed using standard methods.

If the growth of the test strain is observed in the control, but there is no growth in the experiment, it is considered that the test sample has an antimicrobial effect that should be eliminated.

1.1. Elimination of the antimicrobial action of a drug

To eliminate the antimicrobial effect of a drug, use the following:

A) Increase the dilution of the test sample by taking a larger volume of solvent/diluent/culture medium (but not greater than 200 mL per 1 mL (g) of the test sample). For BDP, only dilution with a culture medium is allowed.

The experimentally obtained ratio of the volume of the culture medium and the seed material, which ensures the neutralisation of the antimicrobial action of the drug, must be observed when testing the product for sterility.

B) Apply the membrane filtration method with subsequent washing of filters, if the product is soluble in aqueous diluents or in isopropyl myristate (IPM).

C) It is allowed to use a sterile neutralising liquid, manufactured or prepared in the laboratory, of the following composition:

• Tween 80	30.0 g
• Egg lecithin	3.0 g
• L-histidine hydrochloride	1.0 g
• Peptone (meat or casein)	1.0 g
• Sodium chloride	4.3 g
• Potassium phosphate monobasic	3.6 g
• Disodium hydrogen phosphate	7.2 g
• Purified water pH 7.0 ± 0.2.	1000 mL

D) Use non-specific inactivators. To inactivate preservatives that are part of a number of drug products, the following non-specific inactivators are added to the diluent and/or to culture media before sterilisation: 3% of tween 80 or 0.3% of lecithin (egg or soya-bean) from the volume of the medium.

If the preparation contains greater than two preservatives of different chemical structures, 3% of tween 80, 0.3% of lecithin, 0.1% of L-histidine, and 0.5% of sodium thiosulfate are added to the medium simultaneously. If dilution in the above solution does not inactivate the antimicrobial action of the drug, increase the concentration of tween 80 or lecithin.

Some antimicrobial inactivators are listed in the chapter "Microbiological purity".

Given that the composition of the thioglycollate medium includes sodium thioglycolate, an inactivator of mercury compounds, before testing biological drug products containing mercury preservatives by direct inoculation, determine the neutralising properties of this medium to confirm inactivation.

Inactivators are not used to neutralise the action of other preservatives that are part of BDP, and the main way to eliminate their action is to dilute the nutrient medium. Inoculation of the test sample in the culture medium can be carried out in a ratio of 1:20, taking into account the results of determining the antimicrobial action of the drug.

E) Use specific inactivators that neutralise the antimicrobial effect of drugs but do not inhibit the growth of microorganisms.

To inactivate penicillins and cephalosporins, regardless of their dosage form, a sterile solution of β-lactamase is aseptically added to the buffer solution used for dilution, suspension or emulsification of the sample, as well as to the culture media before their use, in the amount specified in the monograph or regulatory documentation.

The inactivating effect of β-lactamase on penicillins and cephalosporins should be determined by introducing 50 to 100 CFU of *S. aureus* into media with the enzyme and antibiotic. The typical growth of the test strain in the culture medium confirms that the concentration of the β-lactamase enzyme is sufficient.

To inactivate sulfanilamide preparations, regardless of their dosage form, paraamine-benzoic acid (PABA) is added to the buffer solution used for dilution, suspension or emulsification of the sample, as well as to culture media, if necessary, before sterilisation, at the rate of 0.05 - 0.1 g/L of the medium.

When developing new drugs, the monograph and regulatory quality document should include information about the presence/absence of antimicrobial action of the drug with recommendations for its elimination and information about the test method for its sterility. In case of changes in the manufacturing process or composition of the drug, it is necessary to confirm the absence of antimicrobial action.

2. Test for sterility

2.1. Sampling for the test

During the test for sterility, the number of controlled primary packages is determined based on the total number of units in the batch. Sampling is carried out as indicated in Table 1.

The in-process sterility test of BDP is carried out in accordance with the manufacturing specification.

If necessary, special requirements for the required number of controlled containers can be regulated to ensure reliable control of the sterility of the drug.

For inoculation on the appropriate culture medium, use a sample in the amount shown in Table 2.

2.2. Membrane filtration method

When determining the sterility of drugs with a pronounced antimicrobial effect, and drugs in containers with a capacity of greater than 100 mL, the method of membrane filtration is preferred. The exception is antimicrobial drugs that are insoluble in water diluents or IPM.

Table 1. – *Number of units of the drug for sterility testing depending on the size of the batch*

Number of units (ampoules, vials, etc.) in the batch*	The minimum number of units (ampoules, vials, etc.) for the inoculation of each culture medium**
Drugs	
1. Parenterals:	
NMT 100	10% or 4
100 to 500	10
greater than 500	2% or 20
Large-volume parenterals (greater than 100 mL)	2% or 10
2. Non-injection drugs (including ophthalmic dosage forms):	
Maximum 200	5% or 2
greater than 200	10
Preparations in single-dose containers	See the column "Parenterals"
3. Solid forms, in bulk:	
No greater than 4 containers	Every one
greater than 4, but NMT 50	20% or 4
Over 50	2% or 10

* If the number of units in the batch is unknown, use the maximum number specified in the column.
** if the contents of one drug container (other than biological drug products) are sufficient to inoculate two culture media, this column shows the number of samples required for sterility testing on two culture media.

The sterility test method by membrane filtration consists of the following main stages: wetting the membranes, preparing samples and filtering the contents of all containers through membrane filters, washing the membrane filters with an appropriate sterile solution, adding a culture medium, and incubating the inoculations.

The test is performed using open- or closed-type filtration systems that allow the test sample to be transferred and filtered under aseptic conditions through membrane filters (external diameter 47 mm; pore diameter 0.45 μm) capable of trapping microorganisms. The open filtration system must be installed in such a way that the test sample can be introduced and filtered under aseptic conditions. After filtration, the membrane is aseptically transferred to the culture medium. When using a closed sterility testing system with a membrane mounted in a canister, after filtration, the culture medium is introduced directly into the canister on the membrane. Nitrate cellulose filters are used for water, oil and weak alcohol solutions, and acetate cellulose filters are used for concentrated alcohol solutions and acids. The hydrophobic filter edge and low sorption capacity ensure effective washing of the membrane and minimise the adsorption of the drug having antimicrobial action.

For products that do not have an antimicrobial effect, you can use filters without a hydrophobic edge, wetting them before filtering with the diluent used.

If the test sample does not have an antimicrobial effect, it is possible to exclude the procedure of washing the filter during the test.

2.2.1. Testing of aqueous solutions of drug products

Mix a certain quantity of the drug, aseptically selected from all samples, and aseptically transferred to one or more previously wetted filters. Remove the filters aseptically from the filter holder and immerse in media or place in containers with filter holders and fill them with media. When using a closed system, fill the canisters with an equal volume of media. At the same time, aeration of the thioglycollate medium should be avoided.

2.2.2. Testing of water-immiscible liquid preparations

The test is carried out in the same way as for aqueous solutions of drugs. When testing viscous liquids, aseptically add a sufficient amount of suitable sterile solvent to the total sample before filtration to increase the filtration rate.

Table 2. – *Minimum sample amount for inoculation on culture media*

Quantity of the preparation in the primary package	Minimum amount of preparation for inoculating on each culture medium, unless otherwise justified and permitted
Liquid:	
Less than 1 mL	the entire volume of primary packages combined up to 1 mL
1 - 40 mL	1/2 of the contents, but not less than 1 mL
40 - 100 mL	20 mL
greater than 100 mL	10% of the contents, but not less than 20 mL
Antibiotics (liquids)	1 mL
Other drugs that are soluble in water or IPM	package contents, but NLT 200 mg
Insoluble preparations, ointments, and creams that can be emulsified or suspended	package contents, but NLT 200 mg
Solid:	
Less than 50 mg	entire contents
50 - 300 mg	1/2 of the contents, but not less than 50 mg
300 mg to 5 g	150 mg
greater than 5 g	500 mg

If the test sample contains lecithin, oil or preservative, and the drug itself has an antimicrobial effect, liquid No. 2 is used to wash the filters.

2.2.3. Sample preparation and testing of ointments and creams soluble in IPM, and oil-soluble solutions

Fat-based ointments and emulsions of the "water in oil" type are dissolved in IPM, previously sterilised by filtration (a membrane with a pore diameter of 0.22 μm). Heat the sterile diluent/solvent and, if necessary, the test sample to a temperature of no greater than 44 °C immediately before filtration. Filter the solution of the test sample in the IPM and wash the membrane with three portions of liquid No. 2, each of 100 mL. The test is carried out on culture media with the addition of 1 g/L of tween 80.

If the test sample contains petroleum jelly, liquid No. 3 is used to wash the membrane filters.

If the product is a solution in oil, the filter and filtration unit must be thoroughly dried before use.

2.2.4. Testing of preparations in syringe tubes

Transfer the contents of each syringe tube to a membrane filtration unit or collect a total sample in a sterile tube for subsequent transfer to the filter.

2.2.5. Testing of solid dosage forms for injection (other than antibiotics)

Dilute the test sample as indicated in the instructions for use and test according to the method given in Sections 2.2.1 and 2.2.2.

2.2.6. Testing of sterile aerosol dosage forms

Transfer aseptically the required amount of the test sample in an aerosol package to a sterile flask by pressing the spray valve stem. If possible, remove the propellant by evaporation. Add liquid No. 2 to the flask and mix gently. The test is performed as specified in Sections 2.2.1 and 2.2.2.

2.2.7. Washing liquids for membrane filters in the test of samples with antimicrobial action

To wash the filters, you can use any sterile liquid that does not inhibit the growth of microorganisms:

• 0.9% sodium chloride solution pH 7.0 ± 0.2 (after sterilisation).

• Liquid No. 1: dissolve 1 g of the peptic digest in 1000 mL of water, filter or centrifuge for clarification, pour into vessels and sterilise; pH 7.0 ± 0.2.

When filtering penicillin or cephalosporin samples (if necessary), add a validated amount of β-lactamase specified in the monograph and the quality regulatory document to Liquid No. 1, sufficient to inactivate the residual antimicrobial action of the antibiotic on the filter.

• Liquid No. 2: add 1 mL of tween 80 to 1000 mL of Liquid No. 1, pour into vessels and sterilise; pH 7.0 ± 0.2

• Liquid No. 3: dissolve 5 g of the peptic digest, 3 g of meat extract, and 10 g of tween 80 in 1000 mL of water, pour into vials, and sterilise; pH 7.0 ± 0.2.

When testing BDP, membrane filters can be washed with any sterile solution that does not inhibit the growth of microorganisms used in determining the antimicrobial effect of the preparation, for example, 0.9% sodium chloride solution (pH 7.0 ± 0.2) or Liquid No. 1.

2.2.8. Verification of the suitability of the membrane filtration method for testing samples with antimicrobial action

Filter the volume of the test sample using the same number of units (ampoules, vials, etc.) for one filter as in the sterility test (Table 2). Wash the filter with at least three portions of the corresponding liquid, each of 100 mL. In the last portion of the washing liquid, add 1 mL of prepared suspensions of the test strains of microorganisms (each separately) with a concentration of 100 CFU/mL (Table 3).

Place the filter in a container with 100 mL of the corresponding culture medium or add the medium to the closed canister. Incubate inoculations at the appropriate temperature for not greater than 3 days for bacteria and not greater than 5 days for fungi.

During recording of the results, determine visually whether there is any growth of indicator microorganisms in the transmitted light.

If growth is detected, it is considered that the antimicrobial effect is completely inactivated and a test for sterility is performed using the same amount of the preparation, the same volume of washing liquid, and the same culture media.

If there is no growth of test strains, it is concluded that the antimicrobial effect is not inactivated.

Table 3. – *Test strains of microorganisms used to determine the growth properties of culture media and test the antimicrobial action of the preparation**

Culture media	Test strains of microorganisms	Conditions of incubation	
		Temperature	Time
Fluid thioglycollate medium	**Aerobic bacteria:**	32.5 ± 2.5 °C	3 days
	Bacillus subtilis GKPM 010011, ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134 or *Bacillus cereus* GKPM 010014, ATCC 10702		
	Staphylococcus aureus GKPM 201108, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276		
	Pseudomonas aeruginosa GKPM 190155, ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275		
	Alcaligenes faecalis 415** GKPM 300205		2 days
	Anaerobic bacteria:		3 days
	Clostridium sporogenes 272 GKPM 300524, ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437, NBRC 14293		
	Clostridium novyi 198** GKPM 242484		2 days
	Fungi:**	22.5 ± 2.5 °C	5 days
	Candida albicans NCTC 885-653, ATCC 10231, IP 48.72, NCPF3179, NBRC 1594		
Tryptic soy broth	**Aerobic bacteria:**	22.5 ± 2.5 °C	5 days
	Bacillus subtilis GKPM 010011, ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134 or *Bacillus cereus* GKPM 010014, ATCC 10702		
	Fungi:		
	Candida albicans NCTC 885-653, ATCC 10231, IP 48.72, NCPF3179, NBRC 1594		
	Aspergillus brasiliensis ATCC 9642, ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455		
Fluid Sabouraud Medium	**Fungi:**	22.5 ± 2.5 °C	5 days
	Candida albicans NCTC 885-653, ATCC 10231, IP 48.72, NCPF3179, NBRC 1594		
	Aspergillus brasiliensis ATCC 9642, ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455		

* other test strains from different collections that are typical in terms of cultural-morphological, tinctorial, and biochemical properties can also be used. The set of test strains can be changed depending on the method of application or the composition of the test preparation.

** test strains are indicated for cases where the thioglycollate medium is used as a universal medium for testing BDP. Perform the cultivation under two temperature conditions: 32.5 ± 2.5 °C and 22.5 ± 2.5 °C.

Repeat the test, increasing the volume of liquid for washing the filter (but not greater than 500 mL) or using other methods of neutralisation (para. 1.1).

2.3. Direct inoculation

Use the direct inoculation method for testing the sterility of drugs that do not have an antimicrobial effect or those samples that cannot be tested by membrane filtration.

If an antimicrobial action is detected under test conditions, it is neutralised by adding suitable inactivators or increasing the volume of the culture medium (para. 1.1). The added inactivator at a given concentration should not inhibit the growth of test strains. If necessary, the inactivator can be added to the culture medium.

Inoculate the test samples directly into the culture media in a ratio typically of 1:10. The ratio of the amount of the material to be examined to the culture medium used should be determined when checking the antimicrobial effect of the preparation.

2.3.1. Testing of unfiltered liquids

From a certain number of vials, ampoules, etc. (Table 1), aseptically thieve a sample volume sufficient for inoculation on culture media in a ratio of 1:10. After inoculation, gently mix the medium with the exception of aeration.

2.3.2. Testing of ointments, creams, and solutions in oils

Select the required number of units from each test series (Table 1).

Solutions in oils. Prepare an emulsion of the drug in a dilution of 1:10, placing in a sterile flask containing the appropriate sterile diluent, glass beads with a diameter of 5-6 mm, and, if necessary, a certain amount of tween-80.

Carefully mix the inoculations of solutions in oils every day.

Ointments and creams. Before testing, disinfect the tubes (vials), open aseptically, and remove the first portion of the drug without examination.

Ointments and creams, easily emulsified in water. Prepare a 1:10 dilution of the drug by placing the sample in a sterile flask with an appropriate sterile diluent (for example, 0.9% sodium chloride solution or liquid No. 1) and glass beads with a diameter of 5-6 mm. Heat the mixture in a water bath to 40 °C and shake vigorously for 5-15 min until a homogeneous emulsion is obtained, which is inoculated in fluid media — thioglycollate, soya-bean casein, or Sabouraud.

Difficult water-miscible ointments and creams. Prepare a dilution of the test sample 1:10, placing in a sterile flask with the appropriate sterile diluent (for example, a 0.9% sodium chloride solution or liquid No. 3), tween-80 in an amount of 50% of the weighed amount of the sample, and glass beads with a diameter of 5-6 mm. Heat the mixture in a water bath to 40 °C (in exceptional cases, to 45 °C), vigorously shake for 5-15 min (maximum 30 min), until a homogeneous emulsion is obtained, which is then inoculated in fluid media — thioglycollate, soya-bean casein, or Sabouraud.

2.3.3. Test for solid dosage forms

Transfer the powdered test sample in the amount indicated in Table 2 to liquid media — thioglycol, casein soybean, or Sabouraud and carefully stir. If a sterile solvent is added to the sample, test the resulting suspension for sterility.

2.4. Conditions of incubation

Incubate the inoculations regardless of the inoculation method for at least 14 days at 32.5 ± 2.5 °C in a fluid thioglycollate medium and at 22.5 ± 2.5 °C in a fluid soya-bean casein digest medium or Sabouraud-dextrose broth.

When testing BDP, it is possible to use only a thioglycollate medium and incubate the inoculations at two temperature conditions of 32.5 ± 2.5 °C and 22.5 ± 2.5 °C.

2.5. Accounting and interpretation of test results

During incubation, review the inoculations periodically. Determine visually in transmitted light whether there is any microbial growth. If the test sample causes opalescence of the culture medium and it is not possible to visually determine whether or not there is any microbial growth, transfer at least 1 mL of the cloudy medium to test tubes with a similar sterile medium 14 days after the start of the test. Incubate the initial and repeated inoculations.

The total incubation time should be at least 14 + 4 days from the start of the test.

If there is no growth of microorganisms, it is considered that the test sample meets the requirements of the sterility test.

When the growth of microorganisms is detected, visually determined by the presence of turbidity, sediment, flakes, and other changes in the environment and confirmed by microscopic examination, it is considered that the test sample does not meet the requirements of the sterility test. In this case, investigate the reasons for the noncompliance.

The results of the test for sterility may be considered unreliable if one or more of the following conditions are met:

1) unsatisfactory results of microbiological control of the environment (air, surfaces, and hands of personnel, etc.) were obtained during the test for sterility;

2) errors made during the test were detected;

3) microbial growth was detected in the negative control (control of a sterile solvent/diluent or culture medium);

4) the culture medium is non-sterile and/or its growth properties are unsatisfactory;

5) errors made during the material sterilisation process were detected.

If the test results are found to be unreliable (errors are detected during the analysis), repeat the test on the same number of samples as initially, excluding biological drug products (BDP), which are retested on twice the number of samples.

If the repeated test does not detect the growth of microorganisms, it is considered that the test sample is sterile. If, as a result of repeated testing, the growth of microorganisms is detected, it is considered that the test sample does not meet the requirements of the regulatory documentation for "Sterility".

If the investigation proves that the sterility test was performed correctly, it is considered that the test sample does not meet the requirements of the regulatory documentation for "Sterility".

3. Culture media

To test sterility, use fluid media — thioglycollate, soya-bean casein, or Sabouraud. Thioglycollate medium is used to detect aerobic and anaerobic bacteria. Fluid soya-bean casein medium for the detection of fungi and aerobic bacteria. Sabouraud-dextrose broth is used to detect fungi.

When testing the sterility of BDP, it is not recommended to use Sabouraud-dextrose broth.

When testing BDP for sterility, including products containing mercury preservatives, it is permissible to use only a thioglycollate medium as a universal medium for detecting aerobic and anaerobic bacteria and fungi (provided that pre-determination of its growth and neutralising properties was performed using test microorganisms in accordance with Table 3). Incubate the inoculations under two temperature conditions.

3.1. Preparation of culture media

Culture media are prepared in the laboratory using commercial dry culture media or individual components. Ready-to-use media with a manufacturer's certificate are allowed. Check the sterility of the culture media prepared in the laboratory and determine their growth properties.

Sterilise culture media and washing liquids for filters in an autoclave at 121 °C for 15 min, unless otherwise specified in the monograph or regulatory documentation.

Thioglycol medium

• L-cystine	0.5 g
• Sodium chloride	2.5 g
• Glucose monohydrate	5.5 g
• Microbiological agar (humidity no greater than 15%)	0.75 g
• Yeast extract (water-soluble)	5.0 g
• Pancreatic digest of casein	15.0 g
• Sodium thioglycolate	0.5 g
• or thioglycolic acid	0.3 mL
• Sodium resazurin solution (1 g/L), freshly prepared	1.0 mL
• Purified water	1000.0 mL

pH 7.1 ± 0.2 after sterilisation.

Add L-cystine, microbiological agar, sodium chloride, glucose, water-soluble yeast extract, and pancreatic hydrolysate of casein to purified water and heat until completely dissolved. After that, add sodium thioglycolate or thioglycolic acid and, if necessary, adjust the pH of the medium to the required value with 1M sodium hydroxide. Add resazurin to the solution, stir, pour into vials of the appropriate volume, and sterilise.

Tryptic soy broth

• Pancreatic digest of casein	17.0 g
• Papaic digest of soya-bean meal	3.0 g
• Sodium chloride	5.0 g
• Dipotassium phosphate	2.5 g
• Glucose	2.5 g
• Purified water pH 7.3 ± 0.2 after sterilisation.	1000.0 mL

Dissolve the components in water (if necessary, with heating). Cool at room temperature. If necessary, add 1 M sodium hydroxide so that the pH of the medium is 7.3 ± 0.2 after sterilisation. Filter to obtain a clear medium, pour into test tubes and sterilise.

Fluid Sabouraud Medium

• Peptic digest (peptone)	10.0 g
• Glucose monohydrate	40.0 g
• Purified water	1000.0 mL

the pH after sterilisation is 5.6 ± 0.2.

Add peptone and glucose to purified water and completely dissolve under gentle heating. Cool to room temperature and adjust the pH to the desired value. If necessary, filter, pour into test tubes, and sterilise.

Differences in the composition of dry and ready-to-use media are allowed, provided that they meet the requirements for growth properties.

3.2. Sterility of culture media

After sterilisation, place NLT 5% of the containers from each batch of culture medium in a thermostat and incubate for at least 14 days. Exercise control before testing a drug product or in parallel with the inoculation of the sample in the test for sterility. There should be no growth of microorganisms.

3.3. Growth promotion test for culture media

Determine the growth properties of the media for each batch of commercial culture medium having a batch number, and for each batch of medium prepared in the laboratory.

Add each type of microorganism in the amount of 10-100 CFU to a separate portion of the test medium (in 2 test tubes). Incubate in accordance with the conditions specified in Table 3. If the growth of microorganisms is visually observed during the required incubation period in inoculated media, the medium is considered suitable for use.

3.3.1. Preparation of test strains of microorganisms

The used test strains of bacteria and fungi from specialised collections should be typical in terms of cultural, morphological, and biochemical properties.

The number of passages of working cultures should not exceed five.

Before testing, inoculate cultures of aerobic bacteria on slant soya-bean casein agar, Medium No. 1 or another adequate solid culture medium; cultures of fungi *C. albicans* and *A. brasiliensis* – on slant Sabouraud agar (or medium No. 2); cultures of anaerobes *Clostridium novyi* and *C. sporogenes** — on media for anaerobic microorganisms (for example, fluid thioglycollate medium), and incubate at the appropriate temperature.

* It is possible to inoculate on aerobic media under anaerobic conditions of incubation in an anaerostat.

3.3.2. Inoculate preparation

Wash off the grown cultures of bacterial test strains (including *C. sporogenes* grown under anaerobic conditions) and *C. albicans* from the surface of the slant agar with a sterile 0.9% sodium chloride solution. Prepare a suspension of each test strain corresponding to 10 IU according to the opalescence reference standard.

Adjust the concentration of *B. subtilis*, *B. cereus*, *C. albicans*, and *A. brasiliensis* cells to 1 x 10^7 CFU/mL; *S. aureus*, *P. aeruginosa*, *C. sporogenes*, and *A. faecalis* — to 1 x 10^9 CFU/mL.

After centrifugation at 3000 rpm for 20 min, dilute *C. novyi* culture, grown on a liquid growth medium for anaerobic microorganisms (2 reinoculations), with a sterile liquid of the following composition:

• sodium chloride	8.5 g
* thioglycolic acid	0.3 mL
• purified water	1000 mL

pH 7.2 ± 0.2 after sterilisation.

To wash off *A. brasiliensis* conidia, use a sterile 0.9% sodium chloride solution containing 0.05% of tween 80. Determine the number of conidia in 1 mL of washings using a Goryaev chamber or by inoculating a suitable dilution on Sabouraud-dextrose agar or Medium No. 2.

Adjust the concentration of standardised suspensions of bacteria and fungi to 10-100 CFU/mL with a sterile 0.9% sodium chloride solution by 10-fold dilution series for inoculating in liquid and semi-liquid culture media to determine their growth properties.

To confirm the obtained concentration, inoculate bacteria, including *C. sporogenes* (if the latter is incubated in an anaerostat), on soya-bean casein agar (Medium No. 1 or specialised medium for Clostridia, respectively) by 0.1 mL from a suspension with a concentration of 10^3 CFU/mL, *C. novyi* — on a special medium for Clostridia. Inoculate fungi on Sabouraud-dextrose agar (or Medium No. 2).

3.4. Determination of neutralising properties of the thioglycollate medium

When testing BDP containing merthiolate (thiomersal), to determine the neutralising properties of the thioglycollate medium, use the test strain *Alcaligenes faecalis* 415 (see para. 3.3.2 for preparation of the inoculate). Before inoculating the culture, add 0.5 mL of a freshly prepared 0.01% thiomersal solution diluted with a sterile 0.9% sodium chloride solution to each test tube in the middle of the column with the thioglycollate medium.

The thioglycollate medium is considered to be suitable for neutralising properties if the growth of the test strain *A. faecalis* 415 is visually detected no later than 5 days of incubation at 32.5 ± 2.5 °C.

3.5. Storage of culture media

Store the media prepared in the laboratory at 2-25 °C protected from light for no greater than 1 month or for another period confirmed during validation tests.

If the top layer of the medium (greater than 1/3 of the volume) turns pink when storing the thioglycollate medium containing resazurin, the medium can be regenerated by heating in a boiling water bath for 10-15 min until the pink colour disappears, followed by rapid cooling. If the colour does not disappear after heating, the medium is considered unsuitable for use. Media regeneration can only be performed once.

Store commercial ready-to-use culture media in airtight containers, provided that their sterility and growth properties are preserved during the shelf life.

Store commercial dry culture media in accordance with the instructions for use and dispose of them after the expiration date specified by the manufacturer.

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2.1.6.2. Pyrogenicity

This chapter applies to testing the pyrogenicity of injectable solutions and the pharmaceutical substances from which they are made. The test is based on the measurement of body temperature in rabbits before and after injection.

ANIMAL CARE AND PREPARATION OF ANIMALS FOR TESTING

Keep each rabbit in an individual cage fed a complete and balanced diet, protected from irritating effects (acoustic, optical, and others). In the rooms where animals are kept and tests are carried out, maintain a constant temperature of about 20 °C. The room temperature should not differ by greater than 3 °C. Before the test, examine the animals and select healthy rabbits of the same sex, not albinos, with a body weight of at least 1.5 kg, not showing loss of weight during the week preceding the test.

18 hours before the test, deprive the rabbits of food without water restriction. During the experiment, the animals receive neither food nor water. Rabbits that are intended for the experiment for the first time or have not participated in the experiment for greater than four weeks are pre-prepared for the test procedure, performing all working operations (examination, weighing, body temperature measurement) with the exception of injection.

Rabbits that participated in the experiment previously can be reused after three days, provided the drug administered to them was non-pyrogenic. If the animal's body temperature increases by 0.6 °C or more, the rabbit can be used for further experiments not earlier than two weeks later.

If the drug to be examined has antigenic properties, then the monograph prescribes the method for re-using animals for testing.

MATERIALS AND EQUIPMENT

Dilution glassware, syringes and needles for injections must be sterile and non-pyrogenic, which is provided by heating at 250 °C for 30 min or at 200 °C for 60 min.

A 0.9% sodium chloride solution is used to dilute the test sample of the drug unless another solvent is specified in the monograph. All solvents must be sterile and non-pyrogenic.

Measure rectal temperature in rabbits with an accuracy of 0.1 °C with a medical mercury thermometer or electronic thermometer with a temperature sensor. A thermometer or sensor is inserted into the rabbit's rectum to a depth of 5 to 7.5 cm, depending on the animal's body weight.

INTRODUCTION OF THE TEST DRUG

The test drug is injected into the rabbit's ear vein unless a different route of administration is specified in the monograph. The volume of the injected solution of the test sample should be NLT 0.2 mL and NMT 10 mL per 1.0 kg of the bodyweight of the animal. Before administration, heat the solution to 37.0 ± 2 °C. Administer the entire volume of the drug for a period of NMT 2 minutes.

A test dose of the drug to be examined, the volume of the solution administered and, if necessary, the rate of administration are indicated in the monograph.

TESTING

The test drug is tested on a group of three rabbits with an initial temperature of 38.5-39.5 °C.

Before the experiment, with an interval of at least 30 minutes, measure each rabbit's body temperature twice. Differences in temperature readings for the same animal should not exceed 0.2 °C. Otherwise, exclude the rabbit from the test. The value of the last measurement result is taken as the initial temperature.

Administer the solution of the test sample to the animals immediately after the second temperature measurement.

Carry out temperature measurements after intravenous administration of the test sample at intervals of NMT 30 minutes for three hours. For other routes of parenteral administration — for five hours.

RECORD OF THE RESULTS

The test drug can be tested in stages. Use three rabbits for each stage. The maximum number of stages must not exceed four.

At the end of each stage of the test, determine the maximum change in temperature (Δt) of the body of each rabbit compared to the initial value. The change in the animal's body temperature below the initial value is taken as zero and is not taken into account.

For three rabbits, determine the sum of individual maximum temperature increases ($\Sigma\Delta t$). Sum up successively the values of $\Sigma\Delta t$ obtained at different stages of the test and compare the results with the levels indicated in Table 2.1.6.2.-1.

After the first stage of the test, the test drug is recognised as non-pyrogenic if the result obtained is less than or equal to 1.2 °C (Table 1, column 3), and the individual temperature increase in none of the three rabbits exceeds 0.5 °C (column 4).

Table 2.1.6.2.-1. – *Evaluation of test results*

Stage	Total number of animals	Evaluation of test results (ΣΔt)				
		The drug is recognised as non-pyrogenic		Re-testing (rearrangement) is carried out		The drug is recognised as pyrogenic if ΣΔt
		if ΣΔt	at the number of animals with increment Δt > 0.5 °C not more than	if ΣΔt	at the number of animals with increment Δt >0,5 °C	
1	*2*	*3*	*4*	*5*	*6*	*7*
I	3	<1.2	—	>1.2	>1	—
II	6	<2.8	1	>2.8 but <4.3	>1	>4.3
III	9	<4.5	2	>4.5 but <6.0	>2	>6.0
IV	12	<6.6	3	—	—	>6.6*

* If the individual temperature rises above 0.5 °C in greater than three rabbits out of twelve, the test drug is considered to be pyrogenic.

If the result obtained in the first stage exceeds 1.2 °C (column 5) or an individual temperature increase of greater than 0.5 °C is registered in at least one of the three rabbits (column 6), then the next stage of the test must be carried out.

After the second stage of the test, the drug to be examined is recognised as non-pyrogenic if the result obtained is less than or equal to 2.8 °C (column 3), and an individual temperature increase above 0.5 °C is observed in no greater than one of the six rabbits (column 4).

If the result obtained in the second stage of the test is greater than 2.8 °C but less than 4.3 °C (column 5), or greater than one animal has an individual temperature increase higher than 0.5 °C (column 6), then it is necessary to proceed to the next stage of the test.

After the third stage of the test, the drug to be examined is recognised as non-pyrogenic if the result is less than or equal to 4.5 °C (column 3), and an individual temperature increase above 0.5 °C is observed in no greater than two of the nine rabbits (column 4).

If the result obtained in the third stage of the test is greater than 4.5 °C but less than 6.0 °C (column 5), or an individual temperature increase of greater than 0.5 °C is registered in greater than two animals (column 6), then the next stage of the test must be carried out.

After the fourth stage of the test, the test drug is recognised as non-pyrogenic if the result obtained is less than or equal to 6.6 °C (column 3), and an individual temperature increase above 0.5 °C is observed in no greater than three of the twelve rabbits (column 4).

The test drug is considered pyrogenic if the result at the second or subsequent stages of the test is higher than the values indicated in column 7.

The test drug is also considered pyrogenic if, as a result of four stages of the test, an individual temperature increase of greater than 0.5 °C is registered in greater than three rabbits out of twelve.

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2.1.6.3. Abnormal toxicity

This chapter applies to the method of determination of abnormal toxicity of drugs.

GENERAL TEST PROCEDURE

Perform the test on 5 healthy outbred mice (both males and females) weighing 19 g to 21 g, which were not tested before. Conditions of keeping and feeding must ensure the normal functioning of animals. Dissolve or dilute the test sample (if necessary) with 0.9% solution of sodium chloride or water for injection.

The test dose should be contained in a volume of 0.5 mL of the test solution, which is injected into the tail vein of the animal at a rate of 0.1 mL per second. The test dose is indicated in the individual monograph. The observation period for animals is 48 h.

In the case when a monograph gives other instructions, follow them.

A sample passes the test if none of the animals die during the specified observation period.

If one of the animals dies, repeat the test with 5 mice weighing 20.0 ± 0.5 g. The substance passes the test if none of the animals in the second group die.

If greater than one animal dies during the specified observation period, the preparation fails the test.

TESTING OF IMMUNOBIOLOGICAL DRUGS

Carry out tests on two species of animals: 5 white mice weighing 18 g to 20 g and/or two Guinea pigs weighing 250 g to 300 g. The animals are weighed on the first day of the test. Healthy animals that have not previously been used in experiments are taken for the test. Conditions of keeping and feeding must ensure the normal functioning of animals.

TEST ON WHITE MICE

Introduce the test sample to each of the 5 animals intraperitoneally in one maximum single human dose or a dose for an animal for which the drug is intended (but not greater than 1.0 mL), unless otherwise specified in the regulatory documentation. Restore the lyophilised test sample with the attached solvent in accordance with the instructions on the label. If the test sample is intended for intravenous administration, its abnormal toxicity is determined by intravenous injection, however, the test dose should not exceed 0.5 mL. The test sample injected intravenously must have a temperature of 36 ± 1 °C.

The observation period for animals is 7 days. In the case when a monograph gives other instructions, follow them.

The sample passes the test if throughout the entire observation period:

- none of the experimental animals die;
- none of the animals show signs of intoxication;
- there is no reduction in body weight of the animals compared to the initial value. In the case when a monograph gives other instructions, follow them.

If greater than one animal dies, the preparation fails the test. If one animal dies, or signs of intoxication appear, or a decrease in body weight is noted, repeat the test on twice the number of animals. The sample passes the test if none of the animals from the second group die, or shows signs of intoxication, or exhibits a decrease in body weight throughout the observation period.

TEST ON GUINEA PIGS

Inject the test sample subcutaneously into 2 animals in a dose equal to one maximum single human dose or a dose for an animal for which the drug is intended, but not greater than 5 mL (unless otherwise specified in the regulatory documentation).

Restore the lyophilised test sample with the attached solvent in accordance with the instructions on the label. If the test sample is intended for intravenous administration, its abnormal toxicity is determined by intraperitoneal administration, however, the dose administered should not exceed 5 mL.

Observe the animals for 7 days, unless otherwise specified in the regulatory document.

The sample passes the test if throughout the entire observation period:

- none of the experimental animals die or shows notable signs of intoxication;
- none of the animals exhibits a decrease in body weight on the final day of observations compared to the initial weight;
- none of the animals that received the test sample subcutaneously has necrosis or abscess developed at the injection site (the possibility of developing other injection site reactions related to the test sample is indicated in the regulatory documentation).

In the case when a monograph gives other instructions, follow them.

The test sample passes the test if none of the animals shows signs of intoxication or a decrease in body weight.

In the case when both animals die, the test is invalid.

In the case when one animal dies, or a disease, weight loss, development of necrosis or abscess at the test sample injection site are registered in at least one animal, the test should be repeated on twice the number of animals of the same species. Repeated test is considered satisfactory if the test sample meets the above requirements.

The test sample is recognised as having passed the test if none of the animals from the second group die or shows signs of intoxication or a decrease in body weight during the observation period.

If both animals die, the preparation fails the test.

If, during the observation period, the death of one animal, disease, weight loss, development of necrosis or abscess at the site of administration of the test preparation in at least one animal is recorded, the test should be repeated on twice the number of animals of the same species. Re-testing is considered satisfactory if the preparation meets the above requirements.

The drug preparation passes the test if none of the animals from the second group die or shows signs of intoxication and there is no decrease in body weight during the observation period.

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2.1.6.4. Test on histamine

This monograph applies to the determination of histamine content *in vitro* in parenteral drugs.

PREPARATION OF AN ISOLATED ORGAN

For the experiment, take a male Guinea pig weighing 200-350 g. 24 h before the experiment, deprive the animal of food but allow free access to water. After euthanasia, open the pig's abdominal cavity from the pubic symphysis to the sternum and find the cecum. The place of its transition to the colon is a reference point when searching for the ileum, which departs from the cecum 1-2 cm before this site.

In order to remove the ileum, use a blunt clamp or tweezers to tightly grasp its base and cut it off with scissors. Slightly raise the cut end of the intestine, and then without tension and without intercepting it, cut off the mesentery tissue with small incisions using blunt scissors. The remains of the mesentery should not be removed. All manipulations with the ileum should be carried out carefully, without stretching it. The distal part of the ileum is suitable for the experiment, excluding the 10-15 cm closest to the cecum.

Cut the ileum into equal parts (about 6 cm each) and place them in a Petri dish with a *hypocalcic Tyrodes solution* (see Note 1). Carefully wash the segments with this solution using a syringe or a rubber bulb syringe with a blunt-ended Pasteur pipette until the contents of the intestine are completely removed. Place the washed segments of the ileum in a pure *hypocalcic Tyrodes solution.* They can be used immediately or stored for 24 h at temperatures between 2 °C and 4 °C (see Note 2).

Immediately before the experiment, cut the washed segment of the intestine to the length required by the experimental conditions (10 mm when using an electronic sensor or 20 mm when using a mechanical lever and a kymograph).

PREPARATION OF REFERENCE SOLUTIONS AND DILUTIONS OF THE TEST SAMPLE

1. Reference solutions

Use solutions of histamine dihydrochloride R or AR grade in three concentrations as *reference solutions*: *solution 1* ($1.30 \cdot 10^{-6}$ g/mL); *solution 2* ($2.5 \cdot 10^{-6}$ g/mL), and *solution 3* ($5.00 \cdot 10^{-6}$ g/mL), causing 50, 75, and 100% intestinal contraction, respectively.

Use a 0.9% sodium chloride solution as a solvent. The volume of introduction of *reference solutions* is 1/100 of the bath volume.

2. Dilution of the test sample

Test an undiluted test sample when the maximum permissible concentration of histamine in an undilute preparation is in the range from $1.30 \cdot 10^{-6}$ g/mL to $2.50 \cdot 10^{-6}$ g/mL equivalent to histamine dihydrochloride. The volume of injection of the test sample should be 1/100 of the bath volume.

If the value of the maximum allowable histamine concentration equivalent to histamine dihydrochloride in an undiluted test sample is less than the specified range or close to its lower limit, it is permissible to increase the volume of administration of the undiluted test sample to 1/20 of the bath volume.

If the maximum permissible concentration of histamine equivalent to histamine dihydrochloride in an undiluted test sample is above the specified range, the test sample is diluted with 0.9% sodium chloride solution to the estimated concentration of histamine dihydrochloride $2.50 \cdot 10^{-6}$ g/mL (the volume of administration is 1/100 of the bath volume).

RECORDING SYSTEM

To register contractions of an isolated segment of ileum of a Guinea pig under isotonic conditions in response to the introduction of *reference solutions* and the test sample, use a recording system consisting of a temperature-controlled bath with *hypocalcic Tyrodes solution* at 34-36 °C, as well as an electronic sensor with a recording device or a mechanical lever with a kymograph. The bath is aerated with carbogen (95% O_2 and 5% CO_2) or air. The load is usually 500-800 mg. If a mechanical lever is used to calculate the load, the balance rule should be applied:

force x moment arm = load x load arm.

CONDUCTING AN EXPERIMENT

Place the isolated segment of ileum in the bath and attach to the recording system using a ligature diagonally at opposite ends: one to the hook at the bottom of the bath, and the other to the sensor or lever. Apply a load to the segment and allow to rest for 30 min. During this time, at least 3 times, change the *hypocalcic Tyrodes solution* in the bath.

1. Adaptation of the isolated segment of Guinea pig ileum to submaximal dose of histamine

In a temperature-controlled bath, introduce *Solution 3* at the ratio of 1/100 of the bath volume. After 30 seconds (exposure time), wash the bath with a triple volume of *hypocalcic Tyrodes solution.* After the first washing, the second one is carried out with the same volume of solution. At least 4 minutes after the first introduction, repeat the "introduction - exposure - two washings" cycle. These cycles are repeated until at least two identical peaks are obtained. Their height is taken as 100% (see Note 3). The time intervals between injections of the test substance and between two washings should be constant.

2. Test of the sample for histamine

2.0 . Preliminary test

After reaching a constant value of the response of the intestinal segment to the introduction of *Solution 3*, test the sample for histamine. To do this, randomly administer *Solution 1* and *Solution 3* and an undiluted test sample once at intervals of at least 4 min. The "introduction - exposure - two washings" cycles are the same as when adapting the organ to the submaximal dose.

If the peak obtained in response to the introduction of the test sample is not less in height than the peak of *Solution 1,* carry out a quantitation of the histamine content in the test sample (see para. 2.1). If the peak obtained in response to the introduction of the test sample is less than the peak of *Solution 1* or does not exist at all, perform a control test (see para. 2.2).

2.1 . Quantitation of the test sample for histamine

In random order, introduce *Solution 1* and *Solution 3* (1/100 of the bath volume) and the diluted or undiluted test sample (the same volume as in the preliminary test) until at least three peaks are obtained in response to the introduction of each solution.

Find the average value of the response of the intestine segment for each solution. Using regression analysis, calculate the parameters of the linear dependence of the average intestine response to the introduction of *reference solutions* on the logarithm of their concentration. Then, substituting the obtained values of these parameters into the regression equation, calculate the concentration of histamine in the dilution of the test sample, which corresponds to the average height of its peak, and based on this, calculate the content of histamine in the undiluted test sample.

The test sample passes the test if the found histamine content does not exceed the maximum permissible value specified in the quality standard document (the conversion coefficient of histamine dihydrochloride to histamine base is 0.6038).

2.2. Blank test

The scheme of the blank test is the same as for the quantitation of the histamine content in the test sample, only instead of the test sample, a *solution 2* (1/100 of the bath volume) is used. If the average height of its peak corresponds to the introduced concentration of histamine dihydrochloride in this solution ($2.50 \cdot 10^{-6}$ g/mL), then the results of the experiment should be recognised as reliable.

The results of the experiment should be considered unreliable in each of the following cases:

If the average peak height of *solution 2* does not correspond to the introduced concentration of histamine dihydrochloride in this solution ($2.50 \cdot 10^{-6}$ g/mL).

If the quantitation of the histamine content in the test sample does not reproduce the responses of the segment of the intestine to the introduction of the test sample.

If during the experiment there is a significant decrease in the height of the peaks.

In each of these 3 cases, the test sample should be tested for depressor substances as prescribed in the chapter "Test for depressor substances".

Notes

1. Hypocalcium Tyrodes solution.

Composition:

• sodium chloride	80.00 g
• Sodium bicarbonate	10.00 g
• D-glucose	11.00 g
• Potassium chloride	2.00 g
• Calcium chloride dihydrate	1.30 g
• Magnesium chloride hexahydrate	2.10 g
• Sodium dihydrogen phosphate monohydrate	0.58 g
• purified water	to 10 L

Preparation

In a 1 L measuring cylinder, dissolve weighing amounts of sodium chloride, sodium bicarbonate, and D-glucose in purified water in any order. Dilute the solution to volume with the same solvent, stir and transfer the contents of the cylinder into a 10-litre glass-stoppered container or a screw-cap polyethylene vessel of the same volume.

In the same way but separately, dissolve each of the remaining amounts in 1 L of purified water and transfer them in turn to the same 10-L vessel, strictly following the order:

1) potassium chloride
2) calcium chloride
3) magnesium chloride
4) sodium dihydrogen phosphate.

Then dilute to the volume with purified water and mix thoroughly again.

The resulting solution can be stored at 3-5 °C for no longer than 24 h. Cloudiness is unacceptable.

The cloudy solution should be poured out, the vessel should be thoroughly washed in running water and rinsed with purified water. Surfactants should not be used.

As an additional measure to prevent the spontaneous activity of an isolated organ, atropine sulfate can be added to the solution at a concentration of 0.5 mg/L.

2. The vessel in which the segments of the ileum are stored is not tightly closed but is tightened with a double layer of gauze to ensure air access. Before using in the experiment, the segments should be prepared. To do this, keep the vessel at room temperature for 10 min, and then for 20 min at 34-36 °C in a thermostat. After heating, the mucus should be removed from the segment. This is achieved by light stroking movements in the longitudinal direction.

3. The jet of the injected solution should not be directed straight to the isolated segment of the intestine, but towards the wall of the bath, and the direction of the jet should not change.

The rate of administration should be as high and constant as possible.

Recording of contractions is carried out continuously (tape speed 2 mm/min). In the case of using a mechanical lever and kymograph, it is possible to draw the recorder aside and stop the recording during washing.

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2.1.6.5. Test for depressor substances

This chapter applies to the determination of depressor substances *in vivo* in injectable drugs for intravascular administration and pharmaceutical substances from which they are produced.

Carry out the test on anesthetised healthy cats of either sex weighing NLT 2 kg. Females should not be pregnant or lactating. 24 h before the test, deprive the animal of food but allow free access to water. Anesthesia is performed using any anesthetic that allows maintaining a stable level of blood pressure, such as a mixture of chloralose and urethane. The animal's body temperature is maintained within physiological limits.

Fix the cat in the machine in the position on the back. Dissect the carotid artery. A cannula filled with a solution that prevents blood clotting is inserted into the prepared carotid artery. For example, 50 U of heparin in 1 mL of a 9 g/L solution of sodium chloride or a 250 g/L solution of magnesium sulfate. The cannula is attached to a system that provides a constant recording of blood pressure. A second cannula or needle filled with one of the above anticoagulants is inserted into the femoral vein, through which a solution of histamine dihydrochloride (*histamine solution R* in Option 1, reference solution in Option 2) and a drug are administered.

To prepare a solution of histamine R or a r*eference solution*, use histamine dihydrochloride equivalent to histamine base (the conversion factor of histamine dihydrochloride to histamine base is 0.6038).

Qualification of histamine dihydrochloride — pure or analytical pure (AR grade).

The determination of depressor substances is possible in one of two ways.

OPTION 1

Before the test, prepare a test sample solution, dissolve or dilute it in a 9 g/L solution of sodium chloride or in another solvent specified in the individual monograph and taken in an amount sufficient to obtain the required concentration.

To determine the cat's sensitivity to histamine, prepare *histamine solution R* with a concentration of 0.1 μg/mL. Then inject to the animal, at regular intervals, 1.0 mL and 1.5 mL per kg of body mass *histamine solution R*.

Injection of 1 mL/kg of *histamine solution R* is repeated at least three times. Carry out the second and subsequent injections of this solution no earlier than one minute after the blood pressure returns to the level observed immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the 1 mL/kg of *histamine solution R* dose is obtained and if the higher dose of *histamine solution R (1.5 mL/kg*) causes greater responses.

Perform two cycles of administration of the test sample and histamine solution R. Each cycle includes the injection per kilogram of body mass of 1.0 mL of histamine solution R, followed by 2 successive injections of the prescribed amount of the solution and, finally, 1.0 mL of histamine solution R. The volume and concentration of the drug solution to be administered is specified in the monograph. Conclude the test by giving 1.5 mL of histamine solution R per kilogram of body mass.

If the response to *1.5 mL* of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 mL, the test is invalid. The substance to be examined fails the test if:

- the mean of the series of responses to the substance is greater than the mean of the responses to *1.0 mL of histamine solution R per kilogram of body mass*;

- any one dose of the substance causes a greater depressor response than the concluding dose of 1.5 mL/kg of *histamine solution R*.

The test animal must not be used in another test for depressor substances if:

- any one dose of *histamine solution R* (1.0 mL/kg) causes a greater depressor response than the concluding dose of *histamine solution R* (1.5 mL/kg);

- the response to the higher dose of *histamine solution R* (1.5 mL/kg) given after the administration of the test substance is less than the mean response to the low doses of *histamine solution R* previously injected (1.0 mL/kg).

OPTION 2

For the preparation of *reference solutions* and drug solutions, mainly 0.9% sodium chloride solution for injection or water for injection is used. The concentration of *reference solutions* equivalent to histamine base should be 0.5 μg/mL *(Solution 1*) and 1.0 μg/mL (*Solution 2*).

The administration of solutions throughout the test is carried out at a rate of 0.1 mL per second and an interval between injections of at least 5 min.

At the beginning of the experiment, check the animal's sensitivity to histamine. To do this, inject intravenously at regular intervals, *Solution 1* and *Solution 2* (0.2 μg per kilogram of the cat's body mass. Animals whose blood pressure decreases by less than 20 mm Hg after administration of *Solution 2* are excluded from the experiment. *Solution 1* is administered twice to confirm the stability of the cat's blood pressure response to histamine.

Next, the cat is once injected with the drug solution, the volume and concentration of which are specified in the monograph.

When analysing two or more test samples on one animal before each injection of the drug, it is necessary to check the amount of blood pressure response to the administration of *Solution 1*. If there is a significant decrease in blood pressure after the administration of *Solution 1* compared to the blood pressure value obtained after its administration at the beginning of the test, it is necessary to re-test the sensitivity of the animal to *Solution 2*. If the decrease in blood pressure is not less than 20 mm Hg, continue the test in accordance with the above requirements.

The drug product passes the test if within 60 s after the introduction in the test dose, the decrease in blood pressure does not exceed the response to the administration of *Solution 1*.

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2.1.6.6. Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests

1. INTRODUCTION

The test of the microbiological purity of drugs is carried out under aseptic conditions using the following methods and culture media.

The tests described below include the selection of test samples for analysis, methods for preparing various dosage forms, methods for determining the antimicrobial action of drugs, and a quantitation of viable microorganisms.

For incubation of the inoculations on culture media, the standard temperature for bacteria is (32.5 ± 2.5) °C, for fungi – (22.5 ± 2.5) °C.

2. WORKING WITH TEST STRAINS OF MICROORGANISMS

To conduct tests (to determine the antimicrobial action of drugs, the quality of culture media, and biochemical testing of isolated microorganisms), it is necessary to use test strains of microorganisms (Table 2.1.6.6.-1) deposited in official collections, for example:

- State Collection of Pathogenic Microorganisms (GKPM), Russia;

- Russian Collection of Pathogenic Fungi (RKPG), Russia;

- All-Russian Collection of Microorganisms (VKM), Russian Academy of Sciences, Russia;

- American Type Culture Collection (ATCC), USA;

- National Collection of Type Crops (NCTC), UK;

- Collection des bactéries de l’Institut Pasteur (CIP), France;

- Culture Collections of the Institute of Hygiene and Epidemiology (IHE), Czech Republic.

In addition to the test strains of microorganisms listed in Table 2.6.6.6.-1, it is possible to use other cultures that are typical in morphological, tinctorial, and biochemical properties.

The set of test microorganisms can be reduced or increased if necessary.

Test microorganisms in lyophilised form in ampoules, in test tubes on semi-liquid agar are stored at a temperature of 2 to 8 °C. Cultures of microorganisms on disks are stored at a temperature not higher than minus 20 °C.

No greater than 5 passages from the original culture are allowed.

2-1. ACTIVATION OF LYOPHILISED TEST STRAINS OF MICROORGANISMS

Lyophilised test strains of microorganisms in ampoules are obtained from official collections with a certificate of conformity (strain certificate).

Ampoules are opened under aseptic conditions as prescribed by the manufacturer.

To restore the viability of the culture, at least 2 re-inoculations are necessary on a culture medium that corresponds to the biological properties of the strain when incubating in optimal temperature conditions for this strain.

To obtain isolated colonies of the test strain, they are re-inoculated to the appropriate dense culture medium.

After incubation of cultures, investigate the morphology of grown colonies, examine Gram-stained smears under a microscope, and study biochemical properties using approved test systems. The test strain of the microorganism must have typical morphological, tinctorial, and biochemical properties in accordance with the submitted collection certificates.

After confirming the properties of the test strain, reinoculate the culture to the appropriate growth medium (first passage) and incubate under standard conditions.

To obtain *A. brasiliensis* conidia, the culture is grown on Sabouraud glucose agar (or medium No. 2) for 5-7 days under standard conditions.

2-2. ACTIVATION OF TEST STRAINS OF MICRO-ORGANISMS STORED ON DISCS

Place the disc in a fluid culture medium that meets the needs of this microorganism. After incubation, the same operations are performed under the appropriate conditions and in the same sequence as when activating the lyophilised culture.

Table 2.1.6.6.-1. – *Test strains of microorganisms used in tests*

Microorganism	Strain number
Bacillus subtilis	GKPM 010011, ATCC 6633, NCTC 10400, DSM 347, CIP 52.62, NCIMB 8054, NBRC 3134
Bacillus cereus	GKPM 010014, ATCC 10702, NCTC 8035, DSM 487
Escherichia coli	GKPM 240533, ATCC 25922, ATCC 8739, NCTC 12923, NCTC 12241, DSM1103, NCIMB 8545, CIP 53.126, NBRC 3972
Salmonella enterica subsp. enterica serovar abony (former name *Salmonella abony*)	GKPM 100329, ATCC 14028, IHE* 103/39, NCTC 6017, CIP 80.39, NBRC 100797
Pseudomonas aeruginosa	GKPM 190155, AGSS 9027, NCTC 12924, NCIMB 8626, CIP 82.118, NBRC 13275, GISK 453
Staphylococcus aureus	GKPM 201108, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276, ATCC 6538 R (FDA209-P)
Staphylococcus epidermidis	GKPM 202001, ATCC 14990, GKPM 202004, ATCC 12228
Candida albicans	GKPM 303903, GKPM 303901, RKPG Y401/NCTC 885-653, NCPF 3179, ATCC10231, IP 48.72, NBRC 1594
Aspergillus brasiliensis (former name *Aspergillus niger*)	VKM F-1119, ATCC 9642, ATCC 16404, NCPF2275, IP 1431.83, IMI 149007, NBRC 9455, RKPGF106

2-3. STORAGE OF TEST STRAINS IN THE DEEP FREEZE

Storage of test strains of microorganisms in deep freezing conditions is carried out at a temperature of minus (70 ± 5) °C (cryosystem). The cryosystem consists of a set of tightly closed test tubes containing ceramic beads immersed in a specific cryofluid, and a lead cryoblock with cells. Test strains are handled in full compliance with the recommendations of the cryosystem manufacturer.

3. DETERMINATION OF ANTIMICROBIAL ACTIVITY

In order to avoid incorrect evaluation of the results obtained, before testing for microbiological purity, it is necessary to determine the possibility of antimicrobial activity of the drug against certain types of microorganisms.

The method for determining antimicrobial activity is based on comparing the growth rate of test strains of microorganisms with and without the test sample.

3-1. INOCULATE PREPARATION

Depending on the chosen method for determining the antimicrobial action, prepare the inoculate using one of the following methods:

Dilute the 24-h bacterial broth cultures grown on soy-casein broth (Medium No. 8) and 24-48-h *C. albicans* culture grown on Sabouraud liquid medium (soya-bean casein broth or Medium No. 8) with a sterile 0.9% sodium chloride solution 1:1000 (*B. cereus, C. albicans*) and 1:100,000 (*E. coli*, S. abony, *P. aeruginosa, S. aureus*) to a concentration of about 10^4 CFU/mL.

Wash off the 24-h bacterial cultures grown on slant soya-bean casein agar (Medium No. 1) and 24-48-h *C. albicans* culture grown on slant Sabouraud agar (Medium No. 2) using sterile 0.9% sodium chloride solution, standardise and make successive dilution series until a suspension with a certain concentration is obtained.

Use the prepared cultures for 2 h or for 24 h when stored at 2-8 °C.

A suspension of *B. subtilis* spores is also diluted to the required concentration, depending on the method.

Wash off the culture of *A. brasiliensis* grown on slant Sabouraud agar (Medium No. 2) using 0.9% sodium chloride solution with a 0.05% solution of polysorbate 80. Determine the number of conidia in 1 mL of washings using a Goryaev chamber or a dish agar method, and dilute to the required concentration.

It is allowed to use ready-to-use commercial systems that represent substrates containing a certain number of microbial cells.

3-2. PREPARATION OF THE TEST SAMPLE TO DETERMINE ANTIMICROBIAL ACTIVITY

Add a suitable diluent to the test sample to obtain a dilution of 1:10. Phosphate buffer solution with sodium chloride and peptone (pH 7.0), soya-bean casein broth, a neutralising liquid or a buffer solution containing NMT 5% polysorbate 80 are typically used as a diluent. From a 1:10 dilution, prepare successive dilutions of 1:50, 1:100, 1:500, 1:1000, etc.

3-3. METHODS FOR DETERMINING ANTIMICROBIAL ACTIVITY

Determination of the antimicrobial action of drugs is carried out using one of the methods described below.

3-3-1. Determination of antimicrobial activity under microbiological purity testing conditions

Each dilution of the test sample in an amount of 1 mL is introduced into 6 Petri dishes with a diameter of 90 mm, in two of which 0.2 mL of a suspension of *B. cereus* broth culture (or *B. subtilis* spores) is added, in the other two — 0.2 mL of a working suspension of *C. albicans* broth culture, and in the last two — 0.2 mL of a suspension of *A. brasiliensis conidia.* Pour 10 - 15 mL of soya-bean casein agar (Medium No. 1), melted and cooled to 42.5 ± 2.5 °C, into dishes with bacteria, and pour the same quantity of Sabouraud agar (Medium No. 2) into dishes with fungal cultures.

Transfer 1.0 mL of each dilution of the test sample into test tubes with 10 mL of liquid media – Mossel broth and soya-bean casein broth (or similar – Medium No. 3 and Medium No. 8). Then transfer 1 mL of a suspension of test strains grown on a liquid culture medium, *E. coli*, *S. abony*, *P. aeruginosa*, *S. aureus* (each strain separately), into a test tube with a medium corresponding to the needs of the microorganism.

Instead of dilutions of the test sample, add the same amount of solvent to the control dishes and test tubes.

Incubate inoculations under standard conditions for 48 h for bacteria and 5 days for fungi.

3-3-2. Replication method

The replication method is recommended for determining the antimicrobial activity of water-insoluble (suspensions, emulsions, etc.) or coloured drugs.

Transfer 1 mL of each dilution of the test sample to sterile Petri dishes. Transfer 1 mL of the diluent used to obtain dilutions into control dishes. In Petri dishes, both in the experiment and in the control, add 10 - 15 mL each of soya-bean casein agar (Medium No. 1), melted and cooled to 42.5 ± 2.5 °C , in other dishes — the same quantity of Sabouraud agar (Medium No. 2), and mix thoroughly. After agar solidification, dry the dishes in a thermostat or laminar cabinet to remove condensate from the surface of the medium, to which then a working suspension of each test strain of bacteria and fungi, prepared from the broth culture, is applied in the form of plaques, using a bacteriological loop, pipette, or replicator. Incubate the inoculations on media under standard conditions during 48 h for bacteria and 5 days for fungi.

3-3-3. Quantitation method

To the test sample prepared as described above, as well as to the control solution (diluent used to obtain dilutions), add working suspensions of each test strain of the microorganism so that the cell concentration in the final solution is not greater than 100 CFU. The volume of the inoculate should not exceed 1% of the sample dilution volume.

To assess the suitability of methods for determining the total aerobic microbial count, use *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans*, *A. brasiliensis*, and for the total yeast and mold count — *C. albicans*, *A. brasiliensis*.

To evaluate procedures for determining individual types of pathogenic bacteria and fungi, use test strains corresponding to their intended purpose.

Transfer 1 mL of each sample of the test sample or control solution to sterile Petri dishes. Add 10 - 15 mL each of soya-bean casein agar (Medium No. 1), melted and cooled to 42.5 ± 2.5 °C, and to other dishes – the same amount of Sabouraud agar (Medium No. 2), and mix thoroughly.

Incubate the inoculations under standard conditions for NMT 5 days, after which compare the quantitative results obtained for the test sample and the control solution.

When evaluating the applicability of the procedure for determining individual types of microorganisms, reproduce the corresponding procedure using the test sample and the control solution.

3-4. RECORD AND INTERPRETATION OF RESULTS OF ANTIMICROBIAL ACTION

When using methods 3-3-1 and 3-3-2, after the end of the incubation time, examine the inoculations and note how the test microorganisms start to grow in control dishes and tubes (without the test sample) and experiment ones (with different dilutions of the test sample). In cases that make it difficult to record the results (turbidity or discolouration of the liquid medium as a result of the interaction of the drug with the culture medium), re-inoculate on agar media.

If the growth of *E. coli*, *S. abony*, *P. aeruginosa*, and *S. aureus* on culture media is detected, it is concluded that the test sample does not demonstrate any antimicrobial activity.

The growth of test microorganisms in test dishes and test tubes similar to the control ones is indicated by the "+" symbol, and the absence of growth is indicated by the "-" symbol. If the media with the test sample shows a decrease in the number of colonies on the dishes or the absence of growth of test microorganisms, a conclusion is made that the test sample demonstrates antimicrobial activity. The first of the successive dilutions of the test sample, in which there is no antimicrobial effect, is used for inoculating on the appropriate culture medium.

When taking into account the results obtained by method 3-3-3, the number of colonies on the dishes is compared with the test sample and the control solution. A greater than two-fold difference in the average values indicates an antimicrobial activity of the test sample in the dilution used.

The results of determining individual types of microorganisms in the test sample should meet the results obtained for the control solution.

3-5. METHODS FOR ELIMINATING THE ANTIMICROBIAL ACTIVITY OF DRUGS

To eliminate the antimicrobial effect of drugs, the following methods are recommended:

• increase the dilution of the test sample by applying a larger volume of diluent or culture medium within the limits of permissible microbial contamination (as a diluent, instead of a standard phosphate buffer solution, use a neutralising liquid (para. 9) prepared in the laboratory or industrially manufactured);

• use specific inactivators (for example, the β-lactamase for some β-lactam antibiotics and *para*-aminobenzoic acid (PABA) for sulfonamides) that neutralise the antimicrobial activity of the sample but do not inhibit the growth of microorganisms that contaminate the drug;

• use non-specific inactivators for samples with preservatives.

After validation, Tween 80, soya-bean or egg lecithin, etc. can be added to the buffer solution and/or to the culture media.

• for samples that are soluble in water or in isopropyl myristate (IPM), use the membrane filtration method followed by filter washing.

3-5-1. Inactivation of certain antibiotics. To inactivate penicillins and cephalosporins, regardless of their dosage form, aseptically introduce a sterile solution of β-lactamase in the amount specified in the quality regulations into the buffer solution used for dissolving, suspending, or emulsifying the sample, as well as into the culture media before their use.

3-5-2. Inactivation of sulfonamides. To inactivate sulfonamide preparations, regardless of their dosage form, add PABA to the buffer solution used for dissolving, suspending, or emulsifying the sample, as well as to culture media, if necessary, at the rate of 0.05 g/L of the medium before sterilisation, if the antimicrobial effect cannot be eliminated by dilution.

3-5-3. Inactivation of preservatives that are part of a drug. To inactivate preservatives that are part of a number of drugs, add the following non-specific inactivators to the buffer solution in which the test sample is emulsified, as well as to the culture media before sterilisation: 3% Tween 80 or 0.3% lecithin (egg or soya-bean) of the medium volume.

Table 2.1.6.6.-2 – *Inactivators of antimicrobial action of drugs*

Chemicals	Inactivating substances or method
Glutaraldehyde, mercury-containing compounds	sodium hydrosulfite (sodium bisulfite)
Phenols, alcohols, aldehydes, sorbates	dilution
Aldehydes	glycine
Quaternary ammonium compounds (QAC), bisbiguanides, *para*-hydroxybenzoates (parabens)	lecithin
QAC, iodine-containing compounds, parabens	Polysorbate, Tween 80
Mercury-containing compounds	thioglycolate
Mercury-containing compounds, halogens, aldehydes	thiosulfate
Ethylenediaminetetraacetate (EDTA) salts	Mg(II) or Ca(II) ions

If the preparation contains greater than 2 preservatives of different chemical structures, 3% of Tween 80, 0.3% of lecithin, 0.1% of L-histidine, and 0.5% of sodium sulfuric acid are added to the medium simultaneously. Inactivators of antimicrobial action of drugs are indicated in Table 2.1.6.6.-2.

If the method of membrane filtration cannot be used when analysing the drug quality, and all the above methods of eliminating its antimicrobial action against a specific test strain of microorganism are ineffective, this type of test is carried out in the maximum allowable dilution of the sample.

4. SAMPLING OF DRUG PRODUCTS

For testing, select the required number of test samples from each test batch of drugs in accordance with the drug category from a sufficient number of different containers (at least 3-10).

For aerosols based on liquid or solid substances, select 10 containers, for transdermal patches — 10 patches.

In some cases (high cost of the drug and/or a small batch volume), the test sample may be reduced to 2 - 3 g (mL). The reduction in the quantity of the test sample with the indication of the test method must be properly justified and approved in the regulatory quality document.

4-1. SOLID DOSAGE FORMS

- 10.0 g of the test sample — to determine the total aerobic microbial count, the total yeast and mold count in 1 g of the preparation, and test for the absence of *P. aeruginosa*, *S. aureus*, *E. coli*, *Candida albicans* in 1 g of the preparation.

- 25.0 g or 10.0 g of the test sample — to test for the absence of *Salmonella* bacteria;

- 10.0 g of the test sample — for quantitation of bile-tolerant enterobacteria.

4-1-1. Tablets, dragees, granules, powders, etc.

10.0 g of the test sample is powdered (if necessary) and transferred to 90 mL of buffer solution. Further, carry out quantitation and identification tests for microorganisms.

4-1-2. Capsules

Transfer 10.0 g of the test sample to 90 mL of a buffer solution containing NMT 5% of Tween 80 (if necessary) and heat to a temperature not higher than 40 °C. After suspending capsules in a buffer solution, carry out quantitation and identification tests for microorganisms.

4-2. SEMI-SOLID DOSAGE FORMS

- 10.0 g of the test sample — to determine the total aerobic microbial count, the total yeast and mold count in 1 g of the preparation, and test for the absence of *P. aeruginosa*, *S. aureus*, *E. coli*, *Candida albicans* in 1 g of the preparation.

- 10.0 g of the test sample — for quantitation or test for the absence of bile-tolerant enterobacteria in 1 g of the preparation.

4-2-1. Ointments, **liniments**, **creams**, **suppositories**, **readily miscible with water.** Place 10.0 g of the test sample in a sterile flask containing 90 mL of buffer solution. If necessary, add sterile glass beads with a diameter of 5-6 mm and surfactants, for example, 1 g/L of Tween 80. Heat the mixture in a water bath to a temperature not exceeding 40 °C and vigorously shake until a homogeneous emulsion is obtained, which is used for quantitation and identification of microorganisms.

4-2-2. Ointments, **liniments**, **creams**, **suppositories ,difficult miscible with water.** Mix 10.0 g of the test sample with the minimum required amount of sterile surfactant, for example, Tween 80. Heat the mixture in a water bath or in a thermostat to a temperature not higher than 40 °C (in exceptional cases, to 45 °C) and carefully mix. Add sterile phosphate buffer solution, preheated to an appropriate temperature, with glass beads in such an amount that the total volume of sterile surfactant and sterile phosphate buffer solution (excluding beads) should be 90 mL. Stir the mixture carefully to obtain a homogeneous emulsion, which is used for quantitation and identification tests for microorganisms.

It is possible to use other technical means, procedures of homogenisation in compliance with the aseptic regulations and temperature control modes.

4-3. LIQUID DOSAGE FORMS

- 10.0 mL of the test sample are examinated to determine the total aerobic microbial count, the total yeast and mold count in 1 g of the preparation, and test for the absence of *P. aeruginosa*, *S. aureus*, *E. coli*, *Candida albicans* in 1 mL of the preparation.

- 25.0 mL or 10.0 mL of the test sample — for testing for the absence of *Salmonella* bacteria;

- 10.0 mL of the test sample — for quantitation or test for the absence of bile-tolerant enterobacteria in 1 mL of the preparation.

4-3-1. Solutions, **suspensions**, **syrups**, **mixtures.** Transfer 10.0 mL of the test sample to 90 mL of a buffer solution, mix and conduct quantitation and identification tests for microorganisms.

4-3-2. Solutions in oils, **emulsions.** Place 10.0 mL of the test sample in a sterile flask containing 90 mL of a buffer solution with NMT 5% of Tween 80, and glass beads. Heat the mixture in a water bath to a temperature not exceeding 40 °C and vigorously shake until a homogeneous emulsion is obtained, which is used for quantitation and identification of microorganisms.

4-4. AEROSOLS

4-4-1. Aerosols on the basis of alcohols and solids. Transfer 3.0 g of the test sample (after evaporation of the propellant) in 30 mL of a buffer solution, mix and conduct quantitation and identification tests for microorganisms. Use at least 1.0 g of the test sample for respiratory purposes to check the absence of bile-tolerant bacteria.

4-4-2. Oil-based aerosols. Transfer 3.0 g of the test sample (after evaporation of the propellant) to a sterile container with 30 mL of a buffer solution with NMT 5% of Tween 80, and sterile glass beads. Heat the mixture in a water bath to a temperature not exceeding 40 °C and vigorously shake until a homogeneous emulsion is obtained, which is used for quantitation and identification of microorganisms.

Use at least 1.0 g of the test sample for respiratory purposes to check the absence of bile-tolerant bacteria.

4-5. TRANSDERMAL PATCHES

When selecting transdermal patches, use a test sample consisting of 10 units. Remove the protective film from each of the 10 patches using sterile instruments. If necessary, the patches are cut with sterile scissors into smaller fragments, which are transferred to a 1000 mL flask containing 500 mL of sterile buffer solution and glass beads (conditional dilution 1:50). Heat the flask in a water bath to a temperature not higher than 40 °C, vigorously shake for 30 min.

Use 50 mL (or another volume corresponding to one patch) of the resulting washings for quantitation of microorganisms by membrane filtration and testing for the absence of *P. aeruginosa and S. aureus.*

If it is known that the patch has an antimicrobial effect, add a suitable inactivator (Tween 80 and/or lecithin) to the diluent.

If washings from transdermal patches cannot be used for determination by membrane filtration, use the method of direct inoculation on culture media, using a 1:50 dilution**.**

5. QUANTITATION METHODS FOR AEROBIC MICROORGANISMS

Depending of the drug nature and its physical and chemical properties, use one of the variants of the agar dish method (poured plate, two-layer, spread plate, modified poured plate), membrane filtration method or a test tube method of the most probable numbers.

5-1. AGAR DISH METHODS

To culture microorganisms, use agar culture media: soya-bean casein digest agar or dry Medium No. 1 for the control of microbial contamination – to grow bacteria, Sabouraud glucose agar or dry Medium No. 2 for the control of microbial contamination – to grow yeasts and moulds.

For each dilution of the test sample, use at least 2 Petri dishes with a specific medium.

5-1-1. Poured plate method. Add 1 mL of dilution of the test sample prepared for analysis to a sterile Petri dish with a diameter of 90 mm. Add 15-20 mL of sterile agar culture medium, molten and cooled to a temperature of 42.5 ± 2.5 ℃, and quickly swirl. If Petri dishes of a bigger diameter are used, increase the medium volume to 20–25 mL. After agar solidification, turn over the dishes and incubate the inoculations.

5-1-2. Two-layer method. Add 15 to 20 mL of molten agar sterile culture medium to a sterile Petri dish with a diameter of 90 mm and allow to solidify. If Petri dishes of a bigger diameter are used, increase the amount of medium accordingly. Dry the agar surface in the dish.

In a test tube with 4 mL of the corresponding culture medium, melted and cooled to a temperature of 42.5 ± 2.5 °C, add 1 mL of dilution of the test sample prepared for analysis, and quickly mix the contents of the test tube. Then pour the content on the surface of the solidified and dried out agar in the Petri dish, by spreading uniformly the upper layer of the medium with circular movements. After solidification, turn over the dish and place it in a thermostat for incubation.

5-1-3. Spread plate method. Add 15-20 sterile culture media, melted and cooled to a temperature of 42.5 ± 2.5 °C, to each sterile Petri dish with a diameter of 90 mm, and allow to solidify. With a larger diameter of Petri dishes, the amount of medium is increased accordingly. Dry the surface of the agar in the dishes in a thermostat or laminar flow cabinet.

Apply the dilution of the test sample prepared for analysis with a sterile pipette to agar in an amount of 0.1 mL and evenly spread with a spatula over the surface of the medium.

Turn over the dishes and place them in a thermostat for incubation.

5-1-4. Modified pour plate method. Transfer 1.0 mL of the test sample dilution prepared for analysis into a sterile 90 mm Petri dish. Add 7-10 mL of culture medium, melted and cooled to a temperature of 42.5 ± 2.5 ℃, and quickly swirl. After agar solidification, turn over the dishes and incubate.

5-1-5. Recording and interpretation of results obtained by dish agar methods. The result is recorded after 5 days.

To obtain reliable results, select dishes in which the number of bacterial colonies does not exceed 250, and fungal colonies — 50. If the number of colonies on the dishes is within the above limits when taking into account the results of 2 subsequent dilutions, calculate the results from the smaller dilution.

If the average of greater than 250 colonies of bacteria or greater than 50 colonies of fungi have grown on the dishes, make a series of further successive dilutions of the test sample, choosing an acceptable value for inoculating.

If additional fungal colonies are found on the soya-bean casein agar (Medium No. 1), add them to the number of bacteria and determine the total aerobic microbial count, which is established for each category of drugs.

If additional bacterial colonies are found on Sabouraud medium (Medium No. 2), add them to the number of fungi and determine the total yeast and mold count, which is established for each category of drugs.

If there is no growth of microorganisms on the culture medium, record the results in the test report as follows: when inoculating the test sample in a 1:10 dilution — "1 g (or 1 mL) of the drug contains less than 10 microorganisms (or fungi)"; when seeding the test sample in a 1:100 dilution — "1 g (or 1 mL) of the drug contains less than 100 microorganisms (or fungi)", etc.

Calculate the number of microorganisms (*N*) in 1 g or 1 mL using the formula:

$$N = \frac{\sum c}{v \cdot n} \cdot d,$$

where *c* is the number of colonies in all Petri dishes;

n is the number of Petri dishes;

d is the dilution coefficient of the test sample;

v is the volume of the sample inoculated per dish (mL).

Example. When inoculating 1.0 mL of the test sample from a 10^{-2} dilution on 2 dishes, 168 and 215 colonies grew:

$$N = \frac{168 + 215}{2} \cdot 1 \cdot 10^2 = 191{,}50 \cdot 10^2 = 1{,}9 \cdot 10^4.$$

The result is rounded to 2 significant digits – 19000 and written as $1.9 \cdot 10^4$ colony-forming units (CFU).

Variants of the dish agar method (pour plate, double-layer, and modified pour plate) can be used for testing various dosage forms, regardless of the level of microbial contamination. The spread plate agar method is preferable for testing drugs with a high level of microbial contamination. To reduce the time for obtaining results of the quantitation of bacteria and fungi whose colonies are inclined to crowding, a modified agar inoculation method is used.

5-2. MEMBRANE FILTRATION METHOD

The method of membrane filtration is used for quantitative and qualitative determination of microorganisms in drugs that have or do not have antimicrobial action, in particular for solutions and water-soluble drugs, as well as for fat-containing drugs that are soluble in isopropyl myristate (IPM).

5-2-1. Test conditions. The membrane filtration unit must have a structure from which the filter can be easily removed and then transferred to culture media. Use membrane filters with a pore diameter of not greater than 0.45 μm, capable of effectively catch microorganisms, which must be confirmed by validation. The membrane material should be selected in such a way that the components of the test drug would not affect the effectiveness of its work. Cellulose nitrate filters are used for water, oil, and diluted alcohol solutions (less than 30%), cellulose acetate filters are used for alcohol solutions (greater than 30%), acids, and alkalis. Membrane filtration is carried out under aseptic conditions using a vacuum.

5-2-2. Testing. The test sample is usually dissolved in a buffer solution in a ratio of 1:10. First, a washing liquid (approximately 5 mL) is introduced into the funnel of the filter unit to wet the filter. Add the amount of the drug solution corresponding to 1 g of the test sample and immediately filter. If the test sample shows an antimicrobial activity, a 0.9% sodium chloride solution or the following liquids are used to wash the membrane (No. 1, No. 2, No. 3), for this purpose, at least 3 portions, each of 100 mL, of suitable sterile washing liquid are passed through the filter. If necessary, surfactants (for example, Tween 80) or antimicrobial inactivators can be added to the washing liquid. Not greater than 500 mL of washing liquid can be passed through 1 membrane.

It is allowed to use less than 3 portions of washing liquid for washing the membranes, provided that the method is validated.

In order to determine whether the membranes are completely washed from the filtered test sample that has an antimicrobial effect, after filtering the solution, 1 mL of a suspension of test strains of microorganisms (for each separately) corresponding to the type of test is added to the last portion of the washing liquid. The concentration of cells of the introduced suspension of the test strain should not exceed 100 CFU in 1 mL.

The growth of test strains on the filters confirms the absence of antimicrobial action. If the antimicrobial effect persists, use specific or non-specific inactivators or increase the volume of the washing liquid.

Washing from transdermal patches is passed through membrane filters in portions of 50 mL (corresponding to 1 patch) through each membrane.

At the end of the filtration process, the membranes are transferred to the appropriate culture media, poured into Petri dishes or vials with fluid culture media. Turn over the dishes with filters. Incubate the inoculations on dishes and in vials under standard conditions.

5-2-3. Accounting and interpretation of results. A preview of the inoculations is made after 24-72 h and the final result is recorded after 5-7 days.

Select dishes in which the number of bacterial colonies on the filters does not exceed 100, and fungi – 50, and calculate the number of microorganisms per 1.0 g (1.0 mL) of the test sample or per 1 patch. If the filter has a larger number of microorganisms, then make a consecutive dilution series of the test sample and select the appropriate one.

Record the results on fluid culture media as prescribed in chapter 2.1.6.6.

5-2-4. Washing liquids for the filters. To wash the filters, you can use any sterile liquid that does not inhibit the growth of microorganisms:

• 0.9% sodium chloride solution pH (7.0 ± 0.2) (after sterilisation);

• Liquid No. 1: dissolve 1 g of the peptic digest in 1000 mL of purified water, filter or centrifuge for clarification, pour into vessels, and sterilise; the pH after sterilisation is 7.0 ± 0.2;

• Liquid No. 2: add 1 mL of Tween 80 to 1000 mL of Liquid No. 1, pour into vials, and sterilise. pH value after sterilisation is 6.9 ± 0.2. Liquid No. 2 is used if the drug contains oil;

• Liquid No. 3: dissolve 5 g of meat peptone, 3 g of meat extract, and 10 g of polysorbate 80 in 1000 mL of purified water. Pour into vials and sterilise; pH after sterilisation is 6.9 ±0.2.

5-3. MOST PROBABLE NUMBER METHOD (MPN)

The MPN method is used when testing drugs with a low level of microbial contamination, as well as in cases where other methods cannot be used. The MPN method is less sensitive and accurate than the dish agar method or the membrane filtration method, and it is only used to determine the total number of bacteria, since the results obtained in determining the total number of fungi, especially mould, are considered unreliable.

5-3-1. Testing. The test sample is prepared as a solution, suspension, or emulsion in dilutions 1:10, 1:100, 1:1000, using a suitable solvent. The fluid culture medium is poured into 12 sterile tubes, each of 9 mL. Test tubes are placed in a tripod in 4 rows of 3 test tubes in a row.

In the first row of test tubes, 1 mL of the test sample is introduced in a dilution of 1:10, in the second row — 1 mL in a dilution of 1:100, in the third row – 1 mL in a dilution of 1:1000. In the fourth row of test tubes, 1 mL of diluent is added, which is used for dissolving, suspending, or emulsifying the sample. Incubate the inoculations under standard conditions for no greater than 3 days.

5-3-2. Accounting and interpretation of results. Record the number of test tubes in the first, second and third rows, in which the growth of microorganisms is visually observed. The medium in the fourth row of test tubes (diluent control) must remain sterile. The resulting three-digit number corresponds to the most likely number of viable microorganisms in 1.0 g or 1.0 mL of the test sample (Table 2.1.6.6.-3).

Example. In the first row, the growth of microorganisms is observed in 3 test tubes, in the second row – in 2 test tubes, in the third row – in 1 test tube. The resulting number "321" according to Table 5 corresponds to the number "150".

Therefore, the most likely number of bacteria in 1 g or 1 mL of the test sample is 150. If the results cannot be determined accurately due to the nature of the test sample (turbidity of the medium, change in its colour, etc.), re-inoculate to the appropriate liquid or agar medium to make sure that the growth of microorganisms develops.

5-4. RE-TESTING AND EVALUATION OF RESULTS

If necessary, repeat the section of the test, the results of which do not meet the requirements of the regulatory quality document. Perform the analysis using twice the number of test samples of the preparation.

For drug products (with the exception of herbal drug products), the microbial contamination limits are interpreted as follows:

- if the number of microorganisms in 1 g or 1 mL is not greater than 10 CFU – the limit is 20 CFU/g or mL;

Table 2.1.6.6.-3 – *The most probable count of microorganisms*

The number of test tubes in each row, in which the growth of MPN			of microorganisms is observed in 1 g (mL) of the preparation
Amount of the preparation in a test tube, g (mL)			
0.1	0.01	0.001	
0	0	0	less than 3
0	0	1	3
0	1	0	3
0	1	1	6.1
0	2	0	6.2
0	3	0	9.4
1	0	0	3.6
1	0	1	7.2
1	0	2	11
1	1	0	7.4
1	1	1	11
1	2	0	11
1	2	1	15
1	3	0	16
2	0	0	9.2
2	0	1	14
2	0	2	20
2	1	0	15
2	1	1	20
2	1	2	27
2	2	0	21
2	2	1	28
2	2	2	35
2	3	0	29
2	3	1	36
3	0	0	23
3	0	1	38
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	1	3	160
3	2	0	93
3	2	1	150
3	2	2	210
3	2	3	290
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	greater than 1100

- if the number of microorganisms in 1 g or 1 mL is not greater than 10^2 CFU – the limit is 200 CFU/g or mL;

- if the number of microorganisms in 1 g or 1 mL is not greater than 10^3 CFU, the limit is 2000 CFU, etc.

6. CHECKING THE GROWTH PROPERTIES AND STERILITY OF CULTURE MEDIA.

For each batch of commercial media (dry and ready-to-use), as well as for each batch of media prepared in the laboratory, determine growth properties using microorganisms and certified culture media. As certified media, use ready-to-use media with a manufacturer's certificate, as well as high-quality media previously certified in the laboratory.

Growth properties of a culture medium is its ability to provide effective and typical growth of the relevant test strains of microorganisms.

6-1. TEST STRAINS OF MICROORGANISMS

Test microorganisms and incubation conditions for determining the growth properties of culture media are presented in Table 2.1.6.6.-4.

6-1-1. Preparation of a working suspension of test microorganisms. Wash cultures of bacteria and *C. albicans* fungi off the surface of the slant agar with a sterile 0.9% sodium chloride solution. Prepare standard suspensions of each test strain corresponding to 10 IU according to the opalescence reference standard. For *B. subtilis*, *B. cereus*, and *C. albicans* cultures, the concentration is 10^7 CFU/mL, and for *E. coli*, *S. abony*, *P. aeruginosa*, and *S. aureus*, it is 10^9 CFU/mL. Adjust the concentration of standardised suspensions using 10-fold dilution series to 10^3 CFU/mL with a sterile 0.9% sodium chloride solution. To determine the actual concentration of working suspensions of bacteria and *C. albicans*, using the spread plate method, inoculate cultures with a concentration of 10^3 CFU/mL, each of 0.1 mL, on Petri dishes with the appropriate certified agar medium.

To wash off *A. brasiliensis* conidia from Sabouraud glucose agar, use a sterile 0.9% sodium chloride solution containing 0.05% Polysorbate-80. The number of conidia in 1 mL of the suspension is determined using a Goryaev chamber or by seeding a suitable dilution on certified Sabouraud glucose agar or Medium No. 2.

Table 2.1.6.6.-4. – *Test strains of microorganisms and incubation conditions for determining growth properties of culture media*

Culture media	Test strains of microorganisms	Conditions of incubation
Soya-bean casein (tryptic soya-bean) agar Growth medium No. 1 for bacteria	*Bacillus subtilis* or *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus brasiliensis* or *A. niger*	3 days (32.5 ± 2.5) °C
Sabouraud glucose agar Growth Medium No. 2 for fungi	*Candida albicans*, *Aspergillus brasiliensis* or *A. niger*	5 days, 22.5 ± 2.5 °C
Soya-bean casein (tryptic soya-bean) broth Growth Medium No. 8 for bacteria	*Bacillus cereus* or *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*	24 h, 32.5 ± 2.5 °C
Sabouraud Broth	*Candida albicans*	5 days, 22.5 ± 2.5 °C

For inoculation, prepare a working suspension of *A. brasiliensis* with a concentration of conidia about $0.5 \cdot 10^3$ in 1 mL, using the spread plate method, inoculate 0.1 mL on plates with Sabouraud glucose agar (or Medium No. 2).

Use the prepared working suspensions of test microorganisms to determine the growth properties of culture media. The number of test strain cells to be added to liquid or agar culture media should not exceed 10^2 CFU.

6-2. GROWTH PROMOTION TEST FOR CULTURE MEDIA

The tested agar medium is considered suitable for use if the germination coefficient is from 0.5 to 2 when compared with the certified culture medium.

The test liquid medium is considered suitable for use if the test and certified media show visually the same growth of the test strain.

6-2-1. Testing of agar media. Pour the tested and certified agar media into Petri dishes with a diameter of 90 mm, each portion of 15-20 mL, drying the agar after solidification. Inoculate 0.1 mL of the working suspension of the test microorganism with a concentration of 10^3 CFU/mL by the spread plate method on Petri dishes with the tested and certified media in duplicate.

On agar media after incubation, count colonies of test bacterial strains and determine the germination coefficient K_g by the formula:

$$K_g = \frac{N}{N_o},$$

where N is the arithmetic mean of the number of colonies on the Petri dish with the test medium;

N_o is the arithmetic mean of the number of colonies on a Petri dish with a certified medium.

6-2-2. Testing of fluid media. Liquid test subjects and certified culture media are poured into sterile 10 mL tubes measuring 15 x 150 mm. Inoculate 0.1 mL of the working suspension of the test strain of microorganism with a concentration of 10^3 CFU/mL in test tubes with the test and standard medium (3 tubes for each type of medium). Incubate at the appropriate temperature within the minimum time for this test. Determine the growth of microorganisms visually.

6-3. STERILITY OF CULTURE MEDIA

At least 5% of the containers (vials, test tubes) from each batch of the prepared culture medium are monitored for sterility, keeping them at the appropriate temperature for 2-3 days. If microbial growth is detected in at least one of the containers, the test batch of the culture medium must be disposed of.

7. STORAGE OF CULTURE MEDIA

Dry culture media should be stored in airtight containers in a dry place protected from light at 2-30 °C. Prepared from dry mixtures and bottled culture media are stored for 1 month at room temperature or 3 months at 2-8 °C. The shelf life of media poured into Petri dishes is 7 days at 2-8 °C. Other storage periods for culture media may be established based on experimental data.

8. RECOMMENDED CULTURE MEDIA AND SOLUTIONS

Phosphate buffer solution with sodium chloride and peptone (pH 7.0):

• Monopotassium phosphate	3.6 g
• Disodium hydrogen phosphate	7.2 g
• sodium chloride	4.3 g
• Peptone (meat or casein)	1.0 g
• purified water	1000.0 mL

Neutralising liquid

• Polysorbate-80	30.0 g
• Lecithin (egg or soy)	3.0 g
• Histidine hydrochloride	1.0 g
• Peptone (meat or casein)	1.0 g
• sodium chloride	4.3 g
• Monopotassium phosphate	3.6 g
• Disodium hydrogen phosphate	7.2 g
• purified water	1000.0 mL

the pH after sterilisation is 7.6 ± 0.2.

Casein soybean digest agar

• Pancreatic digest of casein	15.0 g
• Papaic digest of soybean meal	5.0 g
• sodium chloride	5.0 g
• Microbiological agar	15.0 g
• purified water	1000.0 mL

the pH after sterilisation is 7.3 ± 0.2.

Alternative growth medium for aerobic bacteria is Medium No. 1 for controlling microbial contamination, dry; meat-peptone agar (MPA); fish hydrolysate agar media.

Sabouraud 4% Glucose Agar

• Peptone (meat or casein)	10.0 g
• Glucose monohydrate	40.0 g
• Bacteriological agar	15.0 g
• purified water	1000.0 mL

the pH after sterilisation is 5.6 ± 0.2.

An alternative medium for growing yeast and mold fungi is medium No. 2 (Sabouraud glucose agar) for controlling microbial contamination, dry, from various manufacturers.

To increase the selectivity of the medium in order to prevent bacterial growth, 50 mg of chloramphenicol (laevomycetin) is added to 1 L of the medium before sterilisation, or 0.1 g of benzylpenicillin sodium salt and 0.1 g of tetracycline per 1 L of the medium in the form of sterile solutions are added to the melted medium before pouring to Petri dishes.

Casein Soya Bean Digest Broth

• Pancreatic digest of casein	17.0 g
• Papaic digest of soybean meal	3.0 g
• sodium chloride	5.0 g
• Disodium hydrogen phosphate	2.5 g
• Glucose monohydrate	2.5 g
• purified water	1000.0 mL

the pH after sterilisation is 7.3 ± 0.2.

An alternative growth medium is medium No. 8 for controlling microbial contamination, dry, from various manufacturers.

Sabouraud Broth

• Peptone (meat)	5.0 g
• Peptone (casein)	5.0 g
• Glucose monohydrate	20.0 g
• purified water	1000.0 mL

the pH after sterilisation is 5.6 ± 0.2.

Semisolid agar for storage of test microorganisms

- Pancreatic digest of casein 8.0 g
- sodium chloride 5.0 g
- Microbiological agar 5.0 g
- purified water 1000.0 mL

the pH after sterilisation is 7.0 ± 0.2.

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2.1.6.7. Microbiological tests of non-sterile drug products for the presence of certain types of microorganisms

1. INTRODUCTION

The tests described below allow determining the absence or limits of certain types of microorganisms using selective and diagnostic culture media to confirm that the drug meets the requirements for microbiological purity.

2. GENERAL PROCEDURES

Samples are prepared as indicated in chapter 2.1.6.6.

If the test sample has antimicrobial action, it must be eliminated or neutralised as prescribed in chapter 2.1.6.6.

If surfactants are used for the preparation of the sample, their non-toxicity to microorganisms and compatibility with inactivators must be confirmed as prescribed in chapter 2.1.6.6.

3. IDENTIFICATION OF INDIVIDUAL TYPES OF MICROORGANISMS

3-1. BILE-TOLERANT ENTEROBACTERIA

3-1-1 Test for the absence of bile-tolerant enterobacteria (assay method). To restore the viability of microorganisms, pre-incubation of the test sample in a fluid culture medium is used. For this purpose, transfer 10.0 g or 10.0 mL of the test sample to 90 mL of soya-bean casein digest broth (or Medium No. 8), mix, and incubate at a temperature of 22.5 ± 2.5 °C for 2 h but not greater than 5 h.

After incubation, stir again the contents of the vial in which the viability of microorganisms was restored (homogenate A) and transfer 10 mL (the amount corresponding to 1 g or 1 mL of the test sample) to 100 mL of the enrichment medium (Mossel broth). Incubate the inoculations for 24-48 h under standard conditions. Using a bacteriological loop, re-inoculate to Mossel agar or Medium No. 4, which is incubated for 18-24 h.

If typical colonies of enterobacteria are found on Mossel agar, which are gram-negative non-spore-forming rods that do not have cytochrome oxidase (para. 4-1), it is considered that the test sample is contaminated with bile-tolerant enterobacteria.

3-1-2. Quantitation of bile-tolerant enterobacteria. For inoculating, use 3 test tubes with 9 mL of Mossel broth in each. Introduce 1 mL of the homogenate A (equivalent to 0.1 g or 0.1 mL of the test sample) into the first tube, mix thoroughly, and transfer 1 mL (equivalent to 0.01 g or 0.01 mL of the test sample) to the second tube, mix again, and transfer 1 mL (equivalent to 0.001 g or 0.001 mL of the test sample) to the third test tube, changing the pipette after each step. Incubate inoculates for 24-48 hours.

To confirm the absence of bile-tolerant enterobacteria, using a bacteriological loop, re-inoculate from each test tube with visible growth to Mossel agar (Medium No. 4) and incubate Petri dishes for 18-24 h. The microscopic examination of colonies found on a dense medium is carried out. Detection of gram-negative rod-shaped non-spore-forming bacteria indicates the presence of bile-tolerant enterobacteria in the drug. The most probable number of bile-tolerant enterobacteria in 1 g (mL) of the test sample is determined using Table 2.1.6.7.-1.

3-2. ESCHERICHIA COLI BACTERIA

3-2-1. Testing for the absence of *E. coli* bacteria (assay method). Transfer 10 g (mL) of the test sample, dissolved or diluted with a sterile phosphate buffer solution (1:10), in an amount of 10 mL (corresponding to 1 g or 1 mL of the test sample) in 100 mL of soya-bean casein broth (or Medium No. 8). Stir and incubate for 18-24 h. Transfer 1 mL of the contents of the vial to 100 mL of McConkey broth (or Medium No. 3) and incubate for 24-48 h at a temperature of 43 ± 1 °C.

Using a bacteriological loop, re-inoculate from the fluid culture medium to McConkey agar or Medium No. 4. Incubate the inoculations for 18-72 h (McConkey agar) or 18-24 h (Medium No. 4). If, after incubation on dense culture media, colonies typical for *E. coli* are detected (see Table 2.1.6.7.-2), examine them under a microscope. When small gram-negative rods are detected in smears, re-inoculate individual typical colonies to test tubes on slant soya-bean casein agar or Medium No. 1 and incubate for 18-24 h to accumulate a pure culture of the microorganism.

To identify the isolated bacteria, biochemical tests for cytochrome oxidase (para. 4-1), indole (para. 4-2), and the ability to utilise sodium citrate are used. To do this, transfer the inoculume from test tubes with pure culture to Simmons agar (Medium No. 14) and soya-bean casein broth (Medium No. 15). After 18-24 h of incubation, check whether there is any bacterial growth on Simmons agar (Medium No. 14). Citrate utilisation is determined by adjusting the pH of the medium towards the alkaline side (the medium colour changes from green to blue).

Table 2.1.6.7.-1. – *Interpretation of results*

Quantity of test sample			MPN of bacteria in 1 g (mL) of the sample
0.1 g (mL) 1 mL of homogenate A	0.01 g (mL) 1 mL of the homogenate A in 1:10 dilution	0.001 g (mL) 1 mL of homogenate A in 1:100 dilution	
+	+	+	greater than 10^3
+	+	—	10^2 to 10^3
+	—	—	10^1 to 10^2
—	—	—	less than 10^1

Symbols: "+" means that the culture is positive; "–" means that the culture is negative.

The presence of indole is determined by the appearance of a red ring on the surface of soya-bean casein broth (Medium No. 15) when adding Kovac's reagent.

If the study finds typical gram-negative rods that do not contain enzyme cytochrome oxidase, do not utilise sodium citrate and form indole, it is considered that the sample is contaminated with *E. coli* bacteria.

3-2-2. Quantitation of *E. coli* bacteria.

Method 1. Carry out a quantitation of *E. coli* in the same way as the assay of bile-tolerant enterobacteria (para. 3-1-2), re-inoculating from homogenate A to test tubes with McConkey broth (Medium No. 3), incubate at 43 ± 1 °C for 24-48 h. From each test tube, reinoculate with a loop to McConkey agar (Medium No. 4). Incubate the inoculations for 18-48 h (McConkey agar) or 18-24 h (Medium No. 4).

Method 2. Prepare a sample using a 1:10 dilution of at least 1 g (mL) of the test product as specified in monograph 2.1.6.6. For inoculating, use 3 test tubes with 9 mL of soya-bean casein broth (Medium No. 8), in which 1 mL of dilution of the sample is added, corresponding to 0.1, 0.01, and 0.001 g (mL). Stir and incubate under standard conditions for 18-24 h. At the end of the incubation period, transfer 1 mL of the contents of the test tubes to 100 mL of McConkey broth and incubate at a temperature of 43 ± 1 °C for 24-48 h. Re-inoculate to McConkey agar. Incubate the inoculations under standard conditions for 18-72 h.

When typical bacterial colonies are detected on these media (see Table 2.1.6.7.-2), according to the morphological and tinctorial properties of gram-negative rods that do not contain enzyme cytochrome oxidase, do not utilise sodium citrate and form indole, a conclusion is made that the drug is contaminated with *E. coli* bacteria. The most probable number of *E. coli* cells in 1 g or 1 mL of the test sample is determined using Table 2.1.6.7.-1.

3-3. BACTERIA OF THE GENUS SALMONELLA

Transfer 10 g (mL) or 25 g (mL) of the test sample to 100 or 225 mL of soya-bean casein broth (or Medium No. 8), stir, and incubate for 18-24 h.

After mixing, transfer 0.1 mL to 10 mL of enrichment broth for *Salmonella* bacteria – Rappoport-Vassiliadis medium, and incubate under standard conditions for 18-24 h. At the end of incubation, reinoculate with a loop to one of the two diagnostic solid media: xylose-lysine deoxycholate agar or bismuth sulfite agar (Medium No. 5), which are then incubated for 48 hours.

When colonies typical of *Salmonella* bacteria are detected on these media (see Table 2.1.6.7.-2), a microscopic examination is performed. If gram-negative rods are detected in smears, the characteristic colonies are transferred to a slant triple sugar iron agar (or Medium No. 13), applying a large amount of culture using a bacteriological loop first to the slanted part of the agar, and then by pricking the column without touching the bottom of the test tube. After 24 h of incubation under standard conditions, a change in the colour of the medium from red to yellow is recorded at the base of the column of the culture medium (glucose fermentation). In the slanted part of the agar, the colour of the medium does not change (no fermentation of sucrose and lactose). The blackening of the medium indicates the formation of hydrogen sulfide – a typical feature of most *Salmonella* bacteria. In parallel, the presence of the cytochrome oxidase enzyme is determined (para. 4-1), as well as other biochemical and serological tests, if additional confirmation is necessary.

If the test sample contains bacteria that are typical in their cultural, morphological, and tinctorial properties (Table 2.1.6.7.-2), and that do not contain cytochrome oxidase enzyme, do not ferment sucrose and lactose, and release hydrogen sulfide, it is considered that the sample is contaminated with *Salmonella* bacteria.

3-4. THE BACTERIA PSEUDOMONAS AERUGINOSA

Transfer 10 mL (equivalent to 1 g or 1 mL) of the test sample, dissolved or diluted with a sterile buffer solution (1:10), to 100 mL of fluid culture medium (soya-bean casein digest broth or Medium No. 8). Stir and incubate under standard conditions for 24-48 h. After the end of incubation, reinoculate with a loop to a selective culture medium to isolate Pseudomonas aeruginosa (cetrimide agar or cetylpyridinium chloride (CPC) agar – Medium No. 16). Incubate the inoculations under standard conditions for 24-48 h. Isolated colonies of microorganisms that are gram-negative by their tinctorial morphological properties are transferred to agar to detect the blue-green pigment pyocyanin (or Medium No. 9). Incubate inoculates for 24-48 hours.

To confirm the species belonging of the isolated bacteria to *P. aeruginosa*, the presence of the cytochrome oxidase enzyme (para. 4 - 1) and the ability of the isolated microorganisms to grow in soya-bean casein broth (or Medium No. 8) at a temperature of (42 ± 1) °C for 18-24 h are determined.

The sample preparation of transdermal patches is performed as specified in 2.1.6.6.

The resulting 50 mL of liquid is passed through a sterile membrane filter made of nitrate cellulose with a pore diameter of 0.45 μm, which is then transferred to 100 mL of soya-bean casein broth (or Medium No. 8). Incubate inoculates for 24-48 hours. After incubation, provided there is any growth, re-inoculate to selective media – cetrimide agar or CPC agar - using a bacteriological loop. Further identification is carried out as indicated above.

If the test sample contains bacteria typical for Pseudomonas in their morphological and tinctorial properties (Table 2.1.6.7.-2), forming the blue-green pigment pyocyanin, containing the enzyme cytochrome oxidase and growing at 42 ±1 °C, it is considered that the sample is contaminated with *P. aeruginosa* bacteria.

3-5. THE BACTERIA STAPHYLOCOCCUS AUREUS

Transfer 10 mL (which corresponds to 1 g or 1 mL of the sample) of the test sample, dissolved or diluted with a sterile buffer solution (1:10), to 100 mL of soya-bean casein broth or Medium No. 8. Stir and incubate for 24-48 h. Using a loop, re-inoculate to mannitol-salt agar (or Medium No. 10) and incubate under standard conditions for 24-48 h.

After the incubation, the emergence of typical golden-yellow colonies (Table 2.1.6.7.-2), surrounded by yellow zones on the medium with mannitol, indicates the growth of *S. aureus* fermenting mannitol. A microscopic examination of typical colonies is performed. If gram-positive cocci located in the form of grapes are detected in smears, they are re-inoculated to soya-bean casein agar (or Medium No. 1). Incubate under standard conditions for 18-24 h. For identification, perform a test for the presence of coagulase (para. 4-3).

Sample preparation of transdermal patches is carried out as specified in chapter 2.1.6.6.

Pass the resulting liquid in a volume of 50 mL through a sterile membrane filter made of cellulose nitrate with a pore diameter of 0.45 μm, which is then transferred to 100 mL of soya-bean casein broth (or Medium No. 8) and incubated for 24-48 h. After incubation, if there is growth, reinoculate with a loop to mannitol salt agar (or medium No. 10) to isolate *S. aureus.* Incubate the inoculations for 48 h.

If the test sample contains bacteria with typical cultural, morphological, and tinctorial properties (Table 2.1.6.7.-2), containing coagulase, and utilising mannitol, it is considered that the sample is contaminated with *S. aureus.*

3-6. YEAST FUNGI CANDIDA ALBICANS

Transfer 10 mL (which is equivalent to 1 g or 1 mL of the sample) of the test sample, dissolved or diluted with a sterile buffer solution (1:10), to Sabouraud broth, mix, and incubate for 3-5 days at a temperature of 32.5 ± 2.5 °C. Reinoculate with a loop to Sabouraud-dextrose agar (or Medium No. 2) and incubate for 24 - 48 h at the same temperature.

The growth of white round, convex, smooth, and shiny colonies may indicate the presence of *Candida albicans*, which is confirmed during further identification, one of the stages of which is microscopic examination (Gram staining), revealing gram-positive yeast-like budding oval or rounded cells of 4-8 μm in size.

For identification, it is possible to use a special medium designed to differentiate *C. albicans* and other species of fungi of the genus *Candida.*

If the test sample contains yeast-like fungi with typical morphological and tinctorial properties (Table 2.1.6.7. -2) identified as *C. albicans*, it is considered that the sample is contaminated with the specified type of fungi.

3-7. CULTURAL, MORPHOLOGICAL, AND TINCTORIAL PROPERTIES OF MICROORGANISMS

The characteristic cultural, morphological, and tinctorial properties of some microorganisms (possible drug contaminants) are presented in Table 2.1.6.7.-2.

3-8. RE-TESTING

If necessary, when detecting drug contamination, repeat the section of the test, the results of which do not meet the requirements of the quality regulatory document. Perform the analysis using twice the number of test samples of the preparation.

4. BIOCHEMICAL TESTS FOR IDENTIFICATION OF MICROORGANISMS

4-1. TEST FOR THE PRESENCE OF THE ENZYME CYTOCHROME OXIDASE (OXIDASE TEST)

The reagent is a 1% solution of N,N-dimethyl-*para*-phenylenediamine dihydrochloride. The solution is stored at a temperature of 2-8 °C in vials made of neutral light-proof glass within the established validated shelf life. The solution should be colourless.

A strip of filter paper is wetted with a reagent. A 24-hour pure culture of the studied bacteria grown on soya-bean casein agar (or Medium No. 1) is applied with a platinum loop or a glass stick. Dark red staining that appears within 1 min indicates a positive oxidase reaction. The positive control is the *P. aeruginosa* test microorganism, the negative control is the E. coli test microorganism (no colour).

Table 2.1.6.7.-2. – *Cultural, morphological and tinctorial properties of microorganisms*

Culture media	Colony morphology	Gram staining
	Escherichia coli	
McConkey broth	Discolouration of the medium, turbidity, gas formation	gram-negative bacilli without spores
Medium No. 3	Change in the colour of the medium, gas formation	
McConkey agar	Brick-red colonies may be surrounded by areas of precipitated bile	
Medium No. 4	Crimson or pink colonies with a metallic lustre surrounded by zones of crimson colour	
Mossel agar	Red colonies surrounded by red precipitation zones	
	Salmonella spp.	
Rappaport Vassiliadis broth	Turbidity of the medium while maintaining colour or absence of visible growth	gram-negative bacilli without spores
Xylose-lysine-deoxycholate agar	Red colonies with or without a black center	
Bismuth sulfite agar (or Medium No. 5)	Black colonies with anthracite lustre, the medium under the colonies is coloured black	
Mossel agar	Red colonies surrounded by red precipitation zones	
	Pseudomonas aeruginosa	
Soybean-casein digest broth (Medium No. 8)	Turbidity, surface growth in the form of a film	gram-negative bacilli without spores
Cetrimide agar	Greenish colonies, green in UV light	
Medium No. 16 (CPC agar)	Greenish colonies, green in UV light	
Agar for the detection of pyocyanin, Medium No. 9	Blue-green colonies, blue-green in UV light	
	Staphylococcus aureus	
Soybean-casein digest broth (Medium No. 8)	Uniform cloudiness	gram-positive cocci in clusters
Mannitol salt agar (or Medium No. 10)	Golden-yellow colonies surrounded by yellow zones	
	Staphylococcus epidermidis	
Mannitol salt agar (or Medium No. 10)	White colonies, no yellow zones around colonies	gram-positive cocci in clusters
	Candida albicans	
Sabouraud Broth	Bottom growth	gram-positive yeast-like budding oval or round cells 4-8 μm in size
Sabouraud agar (Medium No. 2)	White, round, convex, smooth and shiny colonies	

4-2. TEST FOR THE PRESENCE OF INDOLE

Kovac's reagent:

- Amyl or isoamyl alcohol 75 mL
- Para - dimethylaminobenzaldehyde 5 g
- Concentrated hydrochloric acid 20 mL

Dissolve the corresponding amount of *para*-dimethylamino benzaldehyde in isoamyl or amyl alcohol with heating in a water bath at 52.5 ± 2.5 °C, cool, and add hydrochloric acid dropwise. Store the solution protected from light at 2-8 °C. The colour of the reagent should be yellow. If stored incorrectly, the colour of the reagent turns brown and the reagent becomes unusable.

In a test tube with soya-bean casein broth, in which the daily culture under study has grown, 0.5 mL of Kovac's reagent is added and slightly shaken. After 3-5 min, in the presence of indole, the appearance of a red ring on the surface of the medium in the test tube is observed. The positive control is the E. *coli* test microorganism, the negative control is the *S. abony* test strain (no colour).

4-3. TEST FOR THE PRESENCE OF THE ENZYME COAGULASE (PLASMA COAGULATION REACTION)

Dry citrated rabbit plasma is diluted according to the attached instructions with 0.9% sterile sodium chloride solution and poured 0.5 mL into sterile test tubes. Add 1 loop of a daily pure culture of isolated bacteria grown on soya-bean casein agar (or Medium No. 1) to a test tube with reconstituted rabbit plasma. The second tube is not inoculated (negative control). The positive control is the *S. aureus* test strain, and the negative control is the *S. epidermidis* test strain. All test tubes are incubated under standard conditions. The reaction of plasma coagulation is checked every hour for 4-6 h, slightly tilting the test tube without shaking it.

In the absence of a positive plasma coagulation reaction, the incubation time is extended to 24 h to obtain final results. A coagulase test is considered positive when a plasma clot is detected.

5. QUALITY ASSESSMENT OF CULTURE MEDIA

For each batch of commercial media (dry and ready-to-use), as well as for each batch of media prepared in the laboratory, determine growth, selective and diagnostic properties.

The main biological criteria for the quality of culture media are their growth and selective properties, determined using microorganisms and certified culture media, which are used as ready-to-use media with manufacturer's certificates or previously certified high-quality media prepared in the laboratory.

Growth properties of a culture medium is its ability to provide effective and typical growth of the relevant test strains of microorganisms.

Selective properties means the ability of the culture medium to inhibit the growth of concomitant microorganisms from the microbial association.

Test microorganisms, associated strains, and incubation conditions for determining the growth and selective properties of culture media are presented in Table 2.1.6.7.-3.

5-1. GROWTH PROPERTIES OF CULTURE MEDIA

5-1-1. Preparation of a working suspension of test microorganisms. Wash cultures of bacteria and *C. albicans* fungi off the surface of the slant agar with a sterile 0.9% sodium chloride solution. Prepare standard suspensions of each test strain corresponding to 10 IU according to the opalescence reference standard. For *B. subtilis, B. cereus,* and *C. albicans* cultures, the concentration is 10^7 CFU/mL, and for *E. coli*, *S. abony, P. aeruginosa,* and *S. aureus,* it is 10^9 CFU/mL. Adjust the concentration of standardised suspensions using 10-fold dilution series to 10^3 CFU/mL with a sterile 0.9% sodium chloride solution. To determine the actual concentration of working suspensions of bacteria and *C. albicans,* using the spread plate method, inoculate cultures with a concentration of 10^3 CFU/mL, each of 0.1 mL, on Petri dishes with the appropriate certified agar medium.

To wash off *A. brasiliensis* conidia from Sabouraud glucose agar, use a sterile 0.9% sodium chloride solution containing 0.05% Polysorbate-80. The number of conidia in 1 mL of the suspension is determined using a Goryaev chamber or by seeding a suitable dilution on certified Sabouraud glucose agar or Medium No. 2.

For inoculation, prepare a working suspension of *A. brasiliensis* with a concentration of conidia about $0.5 \cdot 10^3$ in 1 mL, using the spread plate method, inoculate 0.1 mL on plates with Sabouraud glucose agar (or Medium No. 2).

Use the prepared working suspensions of test microorganisms to determine the growth properties of culture media. The number of test strain cells to be added to liquid or agar culture media should not exceed 102 CFU.

Table 2.1.6.7.-3. – *Test strains of microorganisms and incubation conditions for determining the growth and selective properties of culture media*

Culture media	Application	Test strains of microorganisms	Conditions of incubation
1	2	3	4
Soya-bean casein agar Growth medium No. 1 for bacteria	Isolation of aerobic microorganisms	*Bacillus subtilis* or *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus brasiliensis* or *A. niger*	3 days (32.5 ± 2.5) °C
Sabouraud Broth	Isolation of yeast fungi	*Candida albicans*, 5 days, 22.5 ± 2.5 °C	
Sabouraud glucose agar Growth Medium No. 2 for fungi	Isolation of yeast and mold fungi	*Candida albicans*, *Aspergillus brasiliensis* or *A. niger*	5 days, 22.5 ± 2.5 °C
Mossel Broth Medium No. 3	The enrichment of enterobacteria	*Escherichia coli*, *Salmonella enterica subsp. enterica* serovar *abony* Associated strain for determining selective properties: *Staphylococcus aureus*	24-48 h, 32.5 ± 2.5 °C
McConkey broth	Isolation of *E. coli*	*Escherichia coli* Associated strain for determining selective properties: *Staphylococcus aureus*, 24-48 h, 43.0 ± 1.0 °C	
McConkey agar Mossel agar Medium No. 4 for isolation of enterobacteria	Isolation of enterobacteria	*Escherichia coli*, *Salmonella enterica subsp. enterica* serovar *abony* Associated strain for determining selective properties: *Staphylococcus aureus*	24-48 h, 32.5 ± 2.5 °C
Xylose-lysine-deoxycholate agar Bismuth sulfite agar Medium No. 5 for identification of bacteria of the genus *Salmonella*	Isolation of bacteria of the genus *Salmonella*	*Salmonella enterica ssp. enterica serovar Abony* Associated strain for determining selective properties: *Escherichia coli*	24-8 h, 32.5 ± 2.5 °C
Soya-bean casein digest broth Growth Medium No. 8 for bacteria	Accumulation of aerobic bacteria	*Bacilluscereus* or *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*	24 h, 32.5 ± 2.5 °C
Agar for the detection of pyocyanin *P. aeruginosa* Medium No. 9 for identification of *P. aeruginosa*	Isolating *P. aeruginosa*	*Pseudomonas aeruginosa*	24-48 h, 32.5 ± 2.5 °C
Cetrimide agar CPC-agar for isolation of *P. aeruginosa*	Identification *P. aeruginosa*	*Pseudomonas aeruginosa* Associated strain for determining selective properties: *Escherichia coli*	24-48 h, 32.5 ± 2.5 °C
Mannitol salt agar Medium No. 10 for *S. aureus* identification	Identification *S. aureus*	*Staphylococcus aureus* Associated strain for determining selective properties: *Pseudomonas aeruginosa*	48 h, 32.5 ± 2.5 °C

Table 2.1.6.7.-3. – *(cont.)*

Culture media	Application	Test strains of microorganisms	Conditions of incubation
1	2	3	4
Rappaport Vassiliadis broth	Enrichment of *Salmonella* bacteria	*Salmonella enterica subsp. enterica* serovar *abony*	24 h, 32.5 ± 2.5 °C
Triple sugar iron agar Medium No. 13 for identification of *Salmonella* bacteria	Identification of *Salmonella* bacteria	*Salmonella enterica subsp. Enterica* serovar *abony*, *Escherichia coli*	24 h, 32.5 ± 2.5 °C
Simmons citrate agar Medium No. 14 for *E. coli* identification	Identification *E. coli*	*Escherichia coli*, *Salmonella enterica subsp. Enterica* serovar *abony* (a change in the colour of the medium from green to blue is observed)	24 h, 32.5 ± 2.5 °C

5-1-2. Testing of agar media. Pour the tested and certified agar media into Petri dishes with a diameter of 90 mm, each portion of 15-20 mL, drying the agar after solidification. Inoculate 0.1 mL of the working suspension of the test microorganism with a concentration of 10^3 CFU/mL by the spread plate method on Petri dishes with the tested and certified media in duplicate.

On agar media after incubation, count colonies of test bacterial strains and determine the germination coefficient K_g by the formula:

$$K_g = \frac{N}{N_o},$$

where N is the arithmetic mean of the number of colonies on the Petri dish with the test medium;

N_o is the arithmetic mean of the number of colonies on a Petri dish with a certified medium.

5-1-3. Testing of fluid media. Liquid test subjects and certified culture media are poured into 10 mL sterile test tubes 15 x 150 mm in size. Inoculate 0.1 mL of the working suspension of the test strain of microorganism with a concentration of 10^3 CFU/mL in test tubes with the test and standard medium (3 tubes for each type of medium). Incubate at the appropriate temperature within the minimum time for this test. Determine the growth of microorganisms visually.

5-1-4. The requirement for the growth properties of culture mediums. The agar medium is considered suitable for use if the germination coefficient is from 0.5 to 2 when compared with the certified culture medium.

The test liquid medium is considered suitable for use if the test and certified media show visually the same growth of the test strain.

5-2. SELECTIVE PROPERTIES OF CULTURE MEDIA

5-2-1. Testing. To determine the selective properties of culture media, the tested and certified media are contaminated with associated strains, each separately, with an inoculation dose of 100 CFU.

For inoculating on fluid culture media, add 0.1 mL of working suspension with a concentration of 10^3 CFU/mL of the associated strain to 3 test tubes with each medium. On all inoculated culture media in test tubes after the longest incubation period for this test at the appropriate temperature, the absence of growth of the associated strain is checked.

5-2-2. The requirement for selective properties of culture media. The test selective medium is considered suitable for use if there is a complete lack of growth of the associated strains during inoculation.

5-3. DIAGNOSTIC PROPERTIES OF CULTURE MEDIA

5-3-1. Testing. Diagnostic properties are tested for such culture media as Mossel agar (or Medium No. 4), McConkey agar, xylose-lysine-desoxycholate agar (or Medium No. 5), cetrimide agar (or CPC-agar), agar for detection of pyocyanin (or Medium No. 9), mannitol salt agar (or Medium No. 10), triple sugar iron agar (or Medium No. 13), Simmons citrate agar (or Medium No. 14).

To confirm the diagnostic properties of the culture medium, a bacteriological loop is used to inoculate a broth culture of test microorganisms (each individually) on 2 Petri dishes or in 2 test tubes with the test medium. After incubation at standard conditions, determine the characteristics of test-strains of a particular species of microorganisms: the appearance of colonies, colour, presence of pigment halo around the colonies, change of the medium colour, etc. (Table 2.1.6.7.-2).

To confirm the selective properties of diagnostic culture media, inoculate a broth culture of associated strains (each separately) on the test medium. After incubation under standard conditions, there should be no growth of associated strains.

5-3-2. Requirements for diagnostic properties of culture media. The test medium is considered suitable for use if the morphological and diagnostic features of the test microorganisms correspond to the description given in Table 2.1.6.7.-3, while the growth of the associated strains is completely absent.

5-4. STERILITY OF CULTURE MEDIA

At least 5% of the containers (vials, test tubes) from each batch of the prepared culture medium are monitored for sterility by keeping them at the appropriate temperature for 48-72 h. If microbial growth is detected in at least one of the containers, the test batch of the culture medium must be disposed of.

5-5. STORAGE OF CULTURE MEDIA

Dry culture media should be stored in an airtight container in a dry place, protected from light, at a temperature of 2-30 °C.

Culture media prepared from dry mixtures and poured into vials and dishes are stored under the conditions and for the period established during validation tests.

6. RECOMMENDED CULTURE MEDIA AND SOLUTIONS

Ready-to-use or manufactured media or media prepared in the laboratory are used for testing.

When preparing culture media in the laboratory, strictly follow the given recipe, and when using commercial dry culture media, follow the manufacturer's instructions. Indicators and dyes being part of the culture media are added in the form of solutions of a defined concentration. The required pH value of the culture medium is set at a temperature of 22.5 ± 2.5 °C.

Unless otherwise prescribed in the standard quality document, the media is sterilised in an autoclave at a temperature of 121 °C for 15 min, provided that the sterilisation process is validated.

Phosphate buffer solution with sodium chloride and peptone (pH 7.0):

• Monopotassium phosphate	3.6 g
• Disodium hydrogen phosphate	7.2 g
• sodium chloride	4.3 g
• Peptone (meat or casein)	1.0 g
• purified water	1000.0 mL

Neutralising liquid

• Polysorbate-80	30.0 g
• Lecithin (egg or soy)	3.0 g
• Histidine hydrochloride	1.0 g
• Peptone (meat or casein)	1.0 g
• sodium chloride	4.3 g
• Monopotassium phosphate	3.6 g
• Disodium hydrogen phosphate	7.2 g
• purified water	1000.0 mL

the pH after sterilisation is 7.6 ± 0.2.

Semisolid agar for storage of test microorganisms

• Pancreatic digest of casein	8.0 g
• sodium chloride	5.0 g

• Microbiological agar	5.0 g
• purified water	1000.0 mL

the pH after sterilisation is 7.0 ± 0.2.

Casein soybean digest agar

• Pancreatic digest of casein	15.0 g
• Papaic digest of soybean meal	5.0 g
• sodium chloride	5.0 g

Microbiological agar 15.0 g

• purified water	1000.0 mL

the pH after sterilisation is 7.3 ± 0.2.

Alternative growth medium for aerobic bacteria is Medium No. 1 for controlling microbial contamination, dry; meat-peptone agar (MPA); fish hydrolysate agar media.

Sabouraud Broth

• Peptone (meat)	5.0 g
• Peptone (casein)	5.0 g
• Glucose monohydrate	20.0 g
• purified water	1000.0 mL

the pH after sterilisation is 5.6 ± 0.2.

Sabouraud 4% glucose agar

• Peptone (meat or casein)	10.0 g
• Glucose monohydrate	40.0 g
• Bacteriological agar	15.0 g
• purified water	1000.0 mL

the pH after sterilisation is 5.6 ± 0.2.

An alternative medium for growing yeast and mold fungi is medium No. 2 (Sabouraud glucose agar) for controlling microbial contamination, dry, from various manufacturers.

To increase the selectivity of the medium in order to prevent bacterial growth, 50 mg of chloramphenicol (laevomycetin) is added to 1 L of the medium before sterilisation, or 0.1 g of benzylpenicillin sodium salt and 0.1 g of tetracycline per 1 L of the medium in the form of sterile solutions are added to the melted medium before pouring to Petri dishes.

Enterobacteria Enrichment Broth – Mossel

• Pancreatic digest of gelatin	10.0 g
• Glucose monohydrate	5.0 g
• Dried ox-gall	20.0 g
• Monopotassium phosphate	2.0 g
• Disodium hydrogen phosphate	8.0 g
• Brilliant green	0.015 g
• Purified water pH 7. ± 0.2.	1000.0 mL

The medium is heated at a temperature of 100 °C for 30 min, followed by rapid cooling.

Alternative medium for growing aerobic bacteria – Medium No. 3 for controlling microbial contamination, dry; from different manufacturers.

The Mossel Agar (Crystal Violet, Neutral Red, Bile Agar with Glucose)

• Yeast extract	3.0 g
• Pancreatic digest of casein	7.0 g
• Bile salts	1.5 g
• Lactose monohydrate	10.0 g
• Sodium chloride	5.0 g
• Glucose monohydrate	10.0 g
• Microbiological agar	15.0 g
• Neutral red	0.03 g
• Crystal violet	0.002 g
• Purified water pH 7.4 ± 0.2.	1000.0 mL

Heat to boiling. Do not autoclave the medium.

An alternative medium for isolating enterobacteria - Medium No. 4 (Endo) for controlling microbial contamination, dry, from different manufacturers.

MacConkey Broth

• Pancreatic digest of gelatin	20.0 g
• Lactose monohydrate	10.0 g
• Dried ox-gall	5.0 g
• Bromocresol purple	0.01 g
• purified water	1000.0 mL

the pH after sterilisation is 7.3 ± 0.2.

Alternative enrichment medium for enterobacteria – Medium No. 3 for controlling microbial contamination, dry, from different manufacturers.

MacConkey Agar

• Pancreatic digest of gelatin	17.0 g
• Peptone (meat or casein)	3.0 g
• Lactose monohydrate	10.0 g
• sodium chloride	5.0 g
• Bile salts	1.5 g
• Microbiological agar	13.5 g
• Neutral red	0.03 g
• Crystal violet	0.001 g
• purified water	1000.0 mL

the pH after sterilisation is 7.1 ± 0.2.

Before sterilisation, boil for 1 min, constantly shaking.

An alternative medium for isolating enterobacteria - Medium No. 4 (Endo) for controlling microbial contamination, dry, from different manufacturers.

Enrichment Medium for Salmonella Bacteria (Rappaport - Vassiliadis Broth)

• Soya-bean peptone	4.5 g
• Magnesium chloride hexahydrate	29.0 g
• sodium chloride	7.2 g
• Dipotassium hydrogen phosphate	0.18 g
• Potassium digidrogen phosphate	1.26 g
• Malachite green	0.036 g
• purified water	1000.0 mL

the pH after sterilisation is 5.2 ± 0.2.

The medium is autoclaved for 15 min at 115 °C.

Xylose-Lysine-Deoxycholate Agar

• Xylose	3.5 g
• L-lysine	5.0 g
• Lactose monohydrate	7.5 g
• Sucrose	7.5 g
• sodium chloride	5.0 g
• Yeast extract	3.0 g
* Phenol red	0.08 g
• Microbiological agar	13.5 g
• Sodium deoxycholate	2.5 g
• Sodium thiosulfate	6.8 g
• Iron ammonium citrate	0.8 g
• Purified water pH 7.4 ± 0.2.	1000.0 mL

Heat to boiling, cool to a temperature of 50 °C, and pour into Petri dishes. Do not autoclave the medium.

Bismuth Sulfite Agar

• Meat extract	5.0 g
• Meat peptone	10.0 g
• Glucose monohydrate	5.0 g
• Disodium hydrogen phosphate	4.0 g
• Iron sulfate	0.3 g
• Brilliant green	0.025 g
• Bismuth sulfite	8.0 g
• Microbiological agar	15.0 g
• Purified water pH 7.6 ± 0.2.	1000.0 mL

Do not autoclave the medium. The prepared medium is cloudy and green.

An alternative medium for Salmonella isolation - Medium No. 5 for controlling microbial contamination, dry, from different manufacturers.

Casein Soya Bean Digest Broth

• Pancreatic digest of casein	17.0 g
• Papaic digest of soybean meal	3.0 g
• sodium chloride	5.0 g
• Disodium hydrogen phosphate	2.5 g
• Glucose monohydrate	2.5 g
• purified water	1000.0 mL

the pH after sterilisation is 7.3 ± 0.2.

An alternative growth medium is medium No. 8 for controlling microbial contamination, dry, from various manufacturers.

Cetrimide Agar

• Pancreatic digest of gelatin	20.0 g
• Magnesium chloride	1.4 g
• Potassium sulfate, double-substituted	10.0 g
• Cetrimide (cetylpyridinium bromide)	0.3 g
• Microbiological agar	13.6 g
• Glycerin	10.0 mL
• purified water	1000.0 mL

the pH after sterilisation is 7.2 ± 0.2.

Alternative medium for Pseudomonas aeruginosa isolation – CPC (Medium No. 16) – agar for Pseudomonas aeruginosa isolation, dry.

CPC Agar (Medium No. 16)

• Dried peptic digest	20.0 g
• Potassium sulfate	7.6 g
• Magnesium sulphate heptahydrate	2.4 g
• Soda ash	1.0 g
• Fenozan acid	0.2 g
• CPC (N-cetylpyridinium chloride monohydrate)	0.3 g
• Microbiological agar	8.0 g
• Purified water pH 7. ± 0.2.	1000.0 mL

Do not autoclave the medium.

Pseudomonas Agar Medium for Detection of Pyocyanin

• Pancreatic digest of gelatin	20.0 g
• Magnesium chloride anhydrous	1.4 g
• Potassium sulfate anhydrous	10.0 g
• Microbiological agar	15.0 g
• Glycerin	10.0 mL
• purified water	1000.0 mL

the pH after sterilisation is 7.2 ± 0.2.

All components, except glycerol, are dissolved in water. Heat with stirring and boil for 1 min. Add glycerin and sterilise.

An alternative medium for identifying Pseudomonas aeruginosa - Medium No. 9 for controlling microbial contamination, dry, from different manufacturers.

Mannitol salt agar

• Dried peptic digest	10.0 g
• D-Mannitol	10.0 g
• sodium chloride	75.0 g
• Microbiological agar	15.0 g
* Phenol red	0.025 g
• purified water	1000.0 mL

the pH after sterilisation is 7.4 ± 0.2.

Alternative medium for isolation and identification of Staphylococcus aureus – Medium No. 10 for control of microbial contamination, dry, from different manufacturers.

Triple Sugar Iron Agar

• Meat extract	3.0 g
• Yeast extract	3.0 g
• Peptone (casein or meat)	20.0 g
• sodium chloride	5.0 g
• Lactose monohydrate	10.0 g
• Sucrose	10.0 g
• Glucose monohydrate	1.0 g
• Iron-ammonium citrate	0.3 g
• Sodium thiosulfate	0.3 g
* Phenol red	0.025 g
• Microbiological agar	12.0 g
• purified water	1000.0 mL

the pH after sterilisation is 7.4 ± 0.2.

The medium is poured into test tubes, filling them by 1/3 of the volume. After sterilisation, allow the medium to solidify in such a way that a column and a slanted part above it are formed.

An alternative medium for Salmonella identification - Medium No. 13 for microbial contamination control, dry, from different manufacturers.

Simmons citrate agar

• sodium chloride	5.0 g
• Magnesium sulfate	0.2 g
• Ammonium dihydrogen phosphate	1.0 g
• Potassium hydrogen phosphate	1.0 g
• Sodium citrate	3.0 g
• Bromothymol blue	0.08 g
• Microbiological agar	20.0 g
• purified water	1000.0 mL

the pH after sterilisation is 7.2 ± 0.2.

Alternative medium for *E. coli* identification – Medium No. 14 for microbial contamination control, dry, from different manufacturers.

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2.1.6.8. Bacterial endotoxins

This monograph describes methods for determining bacterial endotoxins in drug preparations intended for parenteral use and pharmaceutical substances used for their production.

Determination of the content of bacterial endotoxins is carried out using a reagent, which is a lysate of amoebocytes from the blood of the horseshoe crab *Limulus polyphemus* or *Tachypleus tridentatus* (LAL reagent or TAL reagent). Amoebocyte lysate reacts specifically with bacterial endotoxins. As a result of the enzymatic reaction, the reaction mixture changes in proportion to the endotoxin concentration.

There are three main methodological approaches for this test: the gel-clot method, based on gel formation; the turbidimetric method, based on the turbidity of the reaction mixture after cleavage of the substrate contained in the amoebocyte lysate; and the chromogenic method, based on the appearance of staining after cleavage of a synthetic peptide-chromogenic complex.

This monograph describes the following six tests based on the principles described above:

- High-quality gel-clot test (Method A);
- Quantitative gel-clot test (Method B);
- Turbidimetric kinetic test (Method C);
- Chromogenic kinetic test (Method D);
- Chromogenic end point test (Method E);
- Turbidimetric end point test (Method F).

The test is performed by any of the six methods listed. In case of doubt or disagreement, the final conclusion is made on the basis of the results obtained during the test Method A.

LABWARE AND ITS PREPARATION

Glass and plastic labware used in the test should not contain bacterial endotoxins in the amounts determined in the test, and should not affect the course of the reaction.

The recommended depyrogenation mode is heating at 250 °C for at least 30 min according to a validated method.

STANDARDS OF ENDOTOXIN

The content of bacterial endotoxins is expressed in units of endotoxin (UE) of the International endotoxin standard. One international unit (IU) of endotoxin corresponds to one UE.

The analysis can be performed using the Control Standard Endotoxin (CSE), the activity of which is established according to the International endotoxin standard. The CSE should be designed for analysis with the given batch of amoebocyte lysate. Dissolution and storage of CSE is carried out as prescribed by the manufacturer.

Amoebocyte lysate

It is necessary to use amoebocyte lysate from the blood of the horseshoe crab *Limulus polyphemus* or *Tachypleus tridentatus* (LAL reagent or TAL reagent), intended for the selected method for determining bacterial endotoxins.

The sensitivity of amoebocyte lysate (λ) is expressed in endotoxin units [UE/mL] and corresponds to the minimum concentration of the International standard endotoxin, which causes the formation of a dense gel when reacting with this amoebocyte lysate (Methods A and B), or corresponds to the point with the minimum value on the standard curve (Methods C, D, E, and F).

Dilution of lyophilised amoebocyte lysate and its storage is carried out as prescribed by the manufacturer.

Note: *Amoebocyte lysate, in addition to endotoxins, can also react with some β-glucans, so it is possible to use a specific amoebocyte lysate with the removed G factor that reacts with glucans. It is also allowed to use auxiliary solutions that block the reaction system of the G factor. Such reagents can be used to detect endotoxins in the presence of glucans.*

Water for the test bacterial endotoxins (water for BET)

To prepare solutions of reagents and dilutions of the test drug, water for BET is used.

Water for BET must meet the requirements for water for injection, and at the same time must not contain bacterial endotoxins in the amounts determined in the test.

PREPARATION OF THE TEST SAMPLE

Each selected sample is tested individually.

To dissolve and/or dilute the test drug, water for BET is used, unless a different solvent is specified in the monograph. The test solution should have a pH within the limits specified by the manufacturer of amoebocyte lysate, usually 6.0-8.0. If necessary, adjust the pH to the desired value with an acid, base, or buffer solution. The solutions used should not contain bacterial endotoxins in the amounts determined in the test, and should not affect the course of the reaction.

Maximum allowable dilution of the test drug

The maximum valid dilution (MVD) is the largest dilution of the test drug, in which it is possible to determine the endotoxin concentration corresponding to the value of the limit content of bacterial endotoxins established for this drug.

The test drug can be tested in a single dilution or in a dilution series, provided that the final degree of dilution does not exceed the MVD value, which is calculated by the formula:

To calculate the maximum content of bacterial endotoxins, use the following formula:

$$MVD = \frac{\text{Bacterial endotoxin limit} \cdot \text{Concentration of the test solution}}{\lambda}$$

where "bacterial endotoxin limit" is the permissible content of bacterial endotoxins in the test drug specified in the monograph;

"concentration of the test solution" — concentration of the drug product or active substance for which the maximum content of bacterial endotoxins is indicated;

λ — sensitivity of the lysate of amebocytes in UE/mL.

$$\text{Bacterial endotoxin limit} = \frac{K}{M},$$

where K — threshold pyrogenic dose equal to 5 UE/kg per 1 hour for the test drug (if it is administered to the patient by any parenteral route other than intrathecal). With the intrathecal route of drug administration, K is equal to 0.2 UE/kg;

M is the maximum therapeutic dose of the test drug administered within one hour (expressed in mg, mL, or units per 1 kg of body weight).

For radiopharmaceutical drugs administered intravenously, the limit of bacterial endotoxins is calculated as $175/V$, where V is the maximum recommended dose in mL. For radiopharmaceutical drugs administered intrathecally, the limit of bacterial endotoxins is $14/V$.

For drug preparations whose dose is calculated per 1 m^2 of body surface (for example, antitumor preparations), the threshold pyrogenic dose (K) is 100 UE/m^2.

THE GEL-CLOT TEST (METHODS A AND B)

The gel-clot method allows to determine the presence or quantify the concentration of endotoxins in the sample. As a result of the reaction of amoebocyte lysate with endotoxin, the viscosity of the reaction mixture increases until a dense gel is formed.

To ensure the accuracy and reliability of the tests, the claimed sensitivity of amoebocyte lysate should be confirmed, as well as tested for the presence of interfering factors, as described in the section *"Preliminary analyses"*.

Test procedure. Equal volumes of the test solution and amoebocyte lysate (0.1 mL each) are added to round-bottomed tubes with a diameter of 10 mm. Mix the reaction mixtures carefully and incubate at 37 ± 1 °C for 60 ± 2 min. During incubation, vibration and shock should be avoided. After the specified period, the results are visually registered as positive or negative. A positive reaction (+) is characterised by the formation of a dense gel that does not break down when the test tube is carefully turned 180° once. A negative reaction (-) is characterised by the absence of such a gel.

PRELIMINARY ANALYSES

Confirmation of the declared sensitivity of amoebocyte lysate

The analysis is performed for each new batch of amoebocyte lysate used, as well as for changes in the experimental conditions, materials used, and reagents that can affect the test results.

Test procedure. Solutions C and D are prepared for analysis according to the scheme shown in Table 2.1.6.8.-1.

Solutions C – a series of dilutions of KSE in water for the test (testing the sensitivity of amoebocyte lysate);

Solution D is water for BET(negative control).

Results and interpretation. The analysis is considered reliable if:

- for *Solution D* (negative control), negative results were obtained in all replicates;

- *for Solution C* with a concentration of 2λ, positive results were obtained;

- *negative results were obtained* for Solution C with a concentration of 0.25 λ.

The endpoint of the reaction for each of the repetitions of *Solution C* is the positive result obtained for the solution with the lowest concentration of CSE. Based on these results, the geometric mean value of amoebocyte lysate sensitivity is calculated using the following formula:

$$\text{Geometric mean value of CSE concentrations at the end point of the reaction} = antilog\left(\frac{\sum e}{f}\right)$$

where Σe is the sum of the logarithms of the CSE concentrations at the endpoint of the reaction in each of the replications;

f is the number of replicates.

The declared sensitivity of amoebocyte lysate is considered confirmed and is used in further calculations if the value of the sensitivity of amoebocyte lysate obtained in the experiment is not less than 0.5 λ and not greater than 2λ.

Interfering factors

The test drug may contain interfering factors that enhance and/or inhibit the reaction of amoebocyte lysate with bacterial endotoxins. These phenomena can be detected by comparing the ability of the amoebocyte lysate used to react with a solution of CSE in water for BET and in a solution of the test drug under standard experimental conditions.

The drug may be tested in any dilution not exceeding the MVD value. The samples of the test drug (or its dilution) used in this analysis must not contain bacterial endotoxins in the amounts determined in the test.

Table 2.1.6.8.-1. – *Schematic diagram of the experiment "Confirmation of the declared sensitivity of amoebocyte lysate"*

Solution	Stock solution	Solvent	Dilution factor	Final concentration of CSE in the test solution	Number of replicates
S	CSE solution in water for BET with a concentration of 2λ	Water for BET	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
D	Water for BET	—	—	—	2

Test procedure. Solutions A - D are prepared for analysis according to the scheme shown in Table 2.1.6.8.-2.

Solution A is the test drug in the selected dilution (control of the absence of bacterial endotoxins);

Solutions B is the dilution series of CSE in the solution of the test drug (identification of the possibility of inhibiting or enhancing the reaction);

Solutions C is the dilution series of CSE in water for BET (positive control);

Solution D is water for BET (negative control).

Results and interpretation. The results of the experiment are considered reliable if:

- for *Solutions A* and *D*, negative results were obtained in all replicates;

- for *Solutions C* (positive control), the average geometric value of the concentration of bacterial endotoxins is not less than 0.5 λ and not greater than 2λ.

Based on the results obtained for each of the replicates of *Solutions B*, the geometric mean value of the sensitivity of amoebocyte lysate is calculated. The calculation is performed as described in the section *"Confirmation of the declared sensitivity of amoebocyte lysate"*. If the average value obtained is not less than 0.5 λ and not greater than 2λ, it is considered proof that the test drug in the selected dilution does not contain interfering factors that can inhibit and/or enhance the reaction of amoebocyte lysate with bacterial endotoxins and it can be analysed for the content of bacterial endotoxins.

If the presence of interfering factors is detected for the test drug that was tested in a dilution smaller than MVD, the analysis is repeated in a larger dilution, up to a dilution equal to MVD. In most cases, additional dilution of the test drug can eliminate the effect of interfering factors. The use of amoebocyte lysate of greater sensitivity allows increasing the degree of dilution.

The effect of interfering factors can be overcome by appropriate sample preparation, such as filtration, neutralisation, dialysis, or temperature treatment. The chosen method of removing interfering factors should not change the concentration of bacterial endotoxins in the test drug, so a known concentration of CSE is added to the solution of the test drug before such treatment, after which the "*Interfering factors*" analysis is performed. If the results of the analysis are satisfactory after treatment with the chosen method, the test drug can be analysed for the content of bacterial endotoxins.

If the test drug cannot be released from interfering factors, it cannot be tested using the test that determines the content of bacterial endotoxins.

Table 2.1.6.8.-2. – *Schematic diagram of the experiment "Interfering factors"*

Solution	Stock solution	Solvent	Dilution factor	Final concentration of the endotoxin in the test solution	Number of replicates
A	Test drug	—	—	—	4
B	The test drug containing a CSE at a concentration of 2λ	Test drug	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
S	CSE solution in water for BET with a concentration of 2λ	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	Water for BET	—	—	—	2

IDENTIFICATION (Method A)

The purpose of this analysis is to confirm that the content of bacterial endotoxins in the test sample does not exceed the limit value of bacterial endotoxins specified in the monograph.

Test procedure. *Solutions A - D* are prepared for the analysis according to the scheme shown in Table 2.1.6.8.-3.

Solution A is the test drug in a dilution in which there are no interfering factors, or in a larger dilution not exceeding MVD;

Solution B is the test drug in the selected dilution, to which CSE is added. The final concentration of endotoxin in the analysed solution should be 2λ (positive product control).

Solution C is the solution of CSE in water for BET with a final concentration of 2λ (positive control).

Solution D is water for BET (negative control).

Results and interpretation. The analysis is considered reliable if:

- for *Solution D* (negative control), negative results were obtained in both replicates;

- for *Solution C* (positive control), positive results were obtained in all replicates;

- for *Solution B* (positive control of the test sample), positive results were obtained in both replicates.

If negative results are obtained for *Solution A* in two replicates, the drug is deemed to have passed the test.

If positive results are obtained in two replicates for the test drug in a dilution smaller than MVD, the analysis should be repeated in a larger dilution or in a dilution equal to MVD.

If positive results are obtained for the test drug in a dilution equal to MVD in two replicates, then the drug does not meet the requirements of the section "Bacterial endotoxins" in the monograph on the drug.

If a positive result is obtained in one of the replicates for *Solution A*, then repeat the analysis. A drug product passes the test if negative results are obtained in the repeated analysis for two replicates.

QUANTITATIVE ANALYSIS (Method B)

This method determines the content of bacterial endotoxins using a series of consecutive dilutions of the test drug.

Test procedure. *Solutions A - D* are prepared for analysis according to the scheme shown in Table 2.1.6.8.-4.

Solutions A are dilutions of the test drug, starting from the dilution in which there are no interfering factors to the maximum dilution not exceeding MVD.

Solution B is the smallest dilution of the dilution series of Solution A, to which the CSE solution is added. The final concentration of endotoxin in the analysed solution should be 2λ (positive product control).

*Solutions C*is the dilution series of CSE in water for BET (positive control).

Table 2.1.6.8.-3. – *Scheme of the experiment "Identification"*

Solution	Stock solution	Final concentration of the endotoxin (CSE) in the test solution	Number of replicates
A	Test drug	—	2
B	The test drug containing a CSE at a concentration of 2λ	2λ	2
S	CSE solution in water for BET with a concentration of 2λ	2λ	2
D	Water for BET	—	2

Solution D is water for BET (negative control).

Results and interpretation. The analysis is considered reliable if:

- for *Solution D* (negative control), negative results were obtained in two replicates;

- for *Solutions C* (positive control), the average geometric value of the concentration of bacterial endotoxins is not less than 0.5 λ and not greater than 2λ;

- for *Solution B* (positive control of the test sample), positive results were obtained in two replicates.

For *Solutions A*, the endpoint of the reaction is the positive result obtained for the greatest dilution of the test drug.

The value of the product of this dilution factor by the sensitivity of amoebocyte lysate (λ) is equal to the concentration of the endotoxin in *Solution A* obtained for this replicate. The geometric mean value of the endotoxin concentration is calculated as described in the section *"Confirmation of the claimed sensitivity of amoebocyte lysate"*.

If negative results are obtained in all replicates of the series of *Solutions A*, then the concentration of bacterial endotoxins in the test drug is less than the product of the sensitivity of amoebocyte lysate and the lowest dilution factor.

If positive results are obtained in all replicates of the series of *Solutions A*, then the concentration of bacterial endotoxins in the test drug is greater than the product of the sensitivity of amoebocyte lysate and the largest dilution factor.

The drug is deemed to have passed the test if the average content of bacterial endotoxins defined in the experiment is less than the limit specified in the monograph on the drug.

PHOTOMETRIC METHOD (METHODS C, D, E, AND F) TURBIDIMETRIC METHODS (C AND F)

Turbidimetric methods refer to photometric methods based on measuring the turbidity of a reaction mixture. Depending on the principle underlying the test, the specified method can be performed as a turbidimetric end-point test, or as a turbidimetric kinetic analysis.

The end-point turbidimetric test (Method F) is based on measuring the degree of turbidity of the reaction mixture at the end of the incubation period, which depends on the endotoxin concentration.

Table 2.1.6.8.-4. – *Schematic diagram of the "Quantitation" experiment*

Solution	Stock solution	Solvent	Dilution factor	Final concentration of CSE in the test solution	Number of replicates
A	Test drug	Water for BET	1	—	2
			2	—	2
			4	—	2
			8	—	2
			etc. up to MVD		
B	The test drug containing a CSE at a concentration of 2λ	Test drug	1	2λ	2
S	CSE solution in water for BET with a concentration of 2λ	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	Water for BET	—	—	—	2

The turbidimetric kinetic test (Method C) is based on determining the rate of turbidity development of the reaction mixture, measured by the time required to achieve a given value of absorbance.

Perform the test at the incubation temperature recommended by the manufacturer of amoebocyte lysate (usually 37 ± 1 °C).

CHROMOGENIC METHODS (D AND E)

Chromogenic methods are used to measure the amount of chromophore released from a chromogenic substrate as a result of the reaction of endotoxins with amoebocyte lysate. Depending on the principle underlying the test, this method can be performed as a chromogenic endpoint test or as a chromogenic kinetic analysis.

The chromogenic endpoint test (Method *E*) is based on measuring the colour intensity of the reaction mixture, depending on the amount of chromophore released at the end of the incubation period. The amount of chromophore released depends on the endotoxin concentration.

In the process of testing by the chromogenic kinetic method (Method *D*), the rate of colour development of the reaction mixture is determined, measured by the time required to achieve a given value of the absorbance of the reaction mixture.

Perform the test at the incubation temperature recommended by the manufacturer of amoebocyte lysate (usually 37 ± 1 °C).

PRELIMINARY ANALYSES

To confirm the reliability and accuracy of the turbidimetric or chromogenic test, preliminary analyses are performed to ensure that the criteria for the standard curve are reliable and that the test solution does not contain factors that interfere with the reaction.

If any changes are made that may affect the results of the experiment, additional confirmation of the reliability and accuracy of the test is required.

Verification of the standard curve criteria

The analysis is performed for each new batch of amoebocyte lysate.

To plot a standard curve, at least three different endotoxin concentrations are prepared from the CSE stock solution in accordance with the recommendations of the amoebocyte lysate manufacturer. The analysis is performed in at least three replicates under the conditions provided by the manufacturer of amoebocyte lysate (volume ratios, incubation time, temperature, pH, etc.).

In kinetic methods, if it is necessary to construct a standard curve with a CSE range exceeding 2 lg of the endotoxin concentration value for each change in the measurement range by lg of the endotoxin concentration value, a CSE solution of the corresponding concentration should be included in the experiment scheme.

For the tested range of endotoxin concentrations, the absolute value of the correlation coefficient $|r|$ should be equal to or greater than 0.980.

Interfering factors

The drug may be tested in any dilution not exceeding the MVD value.

Test procedure. Prepare solutions *A* - *D* as indicated in Table 2.1.6.8. -5. The Solutions *A*, *B*, *C*, and *D* are tested in at least two replicates, in accordance with the recommendations of the manufacturer of amoebocyte lysate (volumes and volume ratios of the test drug and amoebocyte lysate, incubation time, temperature, pH, etc.).

Table 2.1.6.8.-5. – *Schematic diagram of the "Interfering factors" experiment*

Solution	The concentration of endotoxin	Solution to which endotoxin is added	Number of replicates
A	—	Test solution	NLT 2
B	Average concentration of the standard curve	Test solution	NLT 2
C	NLT 3 concentrations (the lowest concentration is denoted by λ)	Water for BET	NLT 2 for each of the concentrations
D	—	Water for BET	NLT 2

Solution A is the solution of the test drug in a dilution not exceeding the MVD value;

Solution B is the test drug in the selected dilution, to which CSE is added. The final concentration of endotoxin in the analyzed solution should meet or be close to the average value of the CSE concentrations used to construct the standard curve (positive control of the test sample);

Solutions C are CSE solutions used to plot the standard curve at the same concentrations that were used in the "Verification of the standard curve criteria" analysis (positive control);

Solution D is water for BET (negative control).

The test is considered reliable if the following conditions are met:

- the results obtained for the standard curve (Solution *C*) meet the reliability requirements established in the section "Verification of the reliability of the standard curve criteria";

- the result obtained for Solution *D* (negative control) does not exceed the value specified in the instructions for the amoebocyte lysate used or less than the endotoxin concentration determined by the method used.

The average value of the added endotoxin concentration obtained in the experiment is calculated by subtracting from the average value of the endotoxin concentration in Solution *B* (containing the added endotoxin) the average value of the endotoxin concentration in solution *A* (if present).

It must be taken as proved that the test solution does not contain interfering factors if, under test conditions, the measured concentration of endotoxin added to the test solution is 50-200% of the known concentration of added endotoxin.

If the concentration of endotoxin determined in the experiment does not fit into the specified framework, it is concluded that the test drug contains factors that interfere with the reaction. In this case, the experiment can be repeated in a larger dilution, up to a dilution equal to MVD. In addition to increasing the dilution of the test drug, the influence of interfering factors can be overcome by appropriate treatment, such as filtration, neutralisation, dialysis, or temperature exposure.

The chosen method of removing interfering factors should not lead to a decrease in the concentration of bacterial endotoxins in the test drug, so before performing such treatment, a solution of CSE of a known concentration should first be added to the test solution, and then the "Interfering factors" analysis should be repeated. If the results of the analysis are satisfactory after treatment with the chosen method, the test drug can be analysed for the content of bacterial endotoxins.

If the test drug cannot be cleared of interfering factors, it cannot be tested for bacterial endotoxins using these methods.

Testing

Test procedure. The test is carried out in accordance with the procedure given in the section "Interfering factors".

Results. For *Solution A*, the concentration of endotoxins in each replicate is determined using a standard curve obtained from a series of dilutions of CSE (*Solution C*).

The test is considered reliable if the following conditions are met:

1) the results obtained for the standard curve (*Solutions C*) meet the reliability requirements established in the section *"Verification of the reliability of the standard curve criteria"*;

2) the concentration of the endotoxin added to *Solution B* determined in the experiment after subtracting the value of the endotoxin concentration determined in *Solution A* is in the range from 50 to 200% of the known value;

3) the result obtained for *Solution D* (negative control) does not exceed the value specified in the instructions for the amoebocyte lysate used or less than the endotoxin concentration determined by the method used.

Interpretation of results. The drug is deemed satisfactory if the average content of bacterial endotoxins in the replicates of *Solution A* defined in the experiment (taking into account dilution and concentration of the test drug) is less than the limit specified in the monograph.

2.1.6.9. Microbiological tests of oral drug products of natural origin and raw materials used for their production

1. INTRODUCTION

Herbal drug preparations include preparations made from one type or several types of herbal drugs and sold in pre-packaged form in secondary (consumer) packaging (boxes, packs, briquettes, etc.).

Requirements for the microbiological purity of drug products are prescribed in chapter 2.3.1.2 Requirements for the microbiological purity of drug products, pharmaceutical substances, and excipients for their production

2. SAMPLING AND SAMPLE PREPARATION FOR ANALYSIS

A bulk sample is taken from each controlled batch of herbal drug preparation, from which the test sample is isolated to determine microbiological purity (minimum 5 unopened consumer packages with a total weight of at least 50 g).

Before testing, open consumer packages using sterile instruments, select samples in equal amounts, mix and transfer to a sterile container.

For quantitation of aerobic microorganisms and fungi, transfer the test sample of 10.0 g (fruits, bark, roots, rhizomes, buds, etc.) or 2.0 g (grass, leaves, flowers, and others with a high coefficient of water absorption) into a sterile flask. When the mass of the test sample is 10.0 g, 100 mL of a sterile 0.9% sodium chloride solution is placed in the flask. Shake the flask with the test sample in a nutator or shaking apparatus for at least 15 min. The resulting washing is considered a 1:10 dilution. When the mass of the test sample is 2.0 g, 200 mL of a sterile 0.9% sodium chloride solution is added to the flask. The resulting washing is considered a 1:100 dilution.

If the test sample is poorly wetted, add a surfactant to the flask – sterile Tween 80 (0.1% of the solution volume).

3. QUANTITATION OF AEROBIC MICROORGANISMS

Conduct the quantitation of the total aerobic microbial count, the total yeast and mold count in 1 g (mL) of the product by dish agar method as specified in chapter 2.1.6.6. Given the high source of contamination of these products, obtained from washings of the herbal drug preparation corresponding to dilutions of 1:10 or 1:100, prepare serial 10-fold dilutions in the same diluent.

4. DETERMINATION OF INDIVIDUAL TYPES OF MICROORGANISMS

Quantitation and testing for the absence of *E. coli*, bile-tolerant enterobacteria, *P. aeruginosa,* and *S. aureus* are performed using the methods given in chapter 2.1.6.7.

To isolate *Salmonella* bacteria, 25 g (mL) of the test product is used for inoculating in 225 mL of the corresponding diluent. Further tests are carried out as specified in chapter 2.1.6.7.

5. RECORD AND INTERPRETATION OF RESULTS

The quantitation results are recorded as specified in chapter 2.1.6.6.

Due to the fact that herbal drug preparations representing herbals or their parts (leaves, flowers, grass, fruits, seeds, bark, roots, rhizomes, etc.) are heterogeneous in terms of the number of aerobic bacteria and fungi, the standards of permissible microbial contamination of herbal drugs are interpreted taking into account the coefficient "5" as follows:

- if the number of microorganisms in 1 g is not greater than 10^5 CFU, the limit is $5 \cdot 10^5$ CFU/g;
- if the number of microorganisms in 1 g is not greater than 10^7 CFU, the limit is $5 \cdot 10^7$ CFU/g, etc.

2.1.6.10. Enzyme-linked immunosorbent assay technique

This chapter applies to the enzyme-linked immunosorbent assay (ELISA) technique. ELISA is a highly sensitive and highly specific immunodiagnostic technique, which is used for the identification and quantitation of various substances that have the properties of an antigen, hapten (incomplete antigen) or antibody. The ELISA technique is used to determine the quality of biological drug preparations (BDP).

The ELISA principle is based on the specific interaction of an antigen and an antibody to form an immune complex, which, in the presence of a conjugate (an enzyme-labelled component) and the corresponding substrate, generates a signal. Signal detection can be either direct (when the test substance itself has enzymatic activity or enzyme labelled) or indirect (when the test substance bound to antibodies immobilised on the solid phase is incubated with proteins (antibodies against immunoglobulins, Staphylococcus protein A, etc.) labelled with enzyme. An identification test is used to get information about the content of an antigen or antibody in the substance to be examined on the "Yes/No" principle. When performing a quantitation, the concentration of an antigen or antibody in the test substance is determined using a calibration graph.

GENERAL PROVISIONS

The ELISA technique includes 3 major stages: 1) formation of an immune complex "antigen (test substance) - specific antibody" or vice versa; 2) formation of a conjugate bond with the immune complex formed at the previous stage or with free binding sites (determinants); 3) transformation of the substrate under the action of an enzyme label into a registered signal as a result of a biochemical reaction.

All techniques of performing enzyme immunoassay are classified as homogeneous or heterogeneous.

The techniques in which all 3 stages of ELISA take place in a solution, and between the main stages there are no additional stages of separation of the formed immune complexes from unreacted components, belong to the group of homogeneous ELISA techniques. Homogeneous ELISA technique, which is usually used for the determination of low-molecular substances, is based on the process of inhibiting the activity of the enzyme when it is combined with an antigen or antibody. As a result of the antigen-antibody reaction, the activity of the enzyme is restored. When an antigen-antibody immune complex containing an enzyme label is formed, the activity of the enzyme is inhibited by 95% with respect to the high-molecular substrate, which is due to the steric exclusion of the substrate from the active center of the enzyme. As the antigen concentration increases, more and more antibodies bind, and more free "antigen - enzyme" conjugates are retained that can hydrolyze the high-molecular substrate. The homogeneous ELISA is an express technique.

Heterogeneous techniques are characterised by conducting analysis in a two-phase system with the participation of a solid carrier phase and the stage of separation of immune complexes from unreacted components (washing), which are in different phases (formed immune complexes are in the solid phase, and unreacted complexes – in solution). Heterogeneous techniques, in which the formation of immune complexes at the first stage occurs in the solid phase, are called solid-phase techniques.

The techniques are homogeneous-heterogeneous if stage 1 – the formation of specific complexes – occurs in solution, and then a solid phase with an immobilised reagent is used to separate the components.

The heterogeneous ELISA technique consists of 3 major stages:

1) immobilisation of the antigen or antibody in the solid phase (the resulting complex is called an immunosorbent) and removal of the unbound reagent, as well as blocking of binding sites in the solid phase using blocking proteins (albumin, casein);

2) incubation of the drug preparation with an immunosorbent;

3) detection of the analyte by its enzymatic activity. In the direct variant, the analyte either has enzymatic activity or acquires it as a result of binding to the enzymatic label. In the indirect variant, an additional incubation of the "immunosorbent - test substance" complex with secondary antibodies conjugated with an enzymatic label is performed.

Quantitation of the test substance is carried out by adding a suitable substrate for the detector used and comparing the signal of the test substance with a reference standard.

The heterogeneous ELISA technique is divided into non-competitive ELISA and competitive ELISA. The analysis patterns can be modified in the course of the drug development in accordance with the appropriate requirements. Changes should be specified in the monograph or a quality regulatory document. The choice of the ELISA formulation method depends on the nature of the test substance and its quantity since different types of ELISA have different sensitivity. To assess the quality of substances containing antibodies, it is possible to use specific anti-idiotypic antibodies.

Non-competitive ELISA technique

The non-competitive ELISA technique is divided into several types according to the type of detection (direct non-competitive, indirect non-competitive) and the type of substance immobilised on the solid phase (antigen or antibody).

Direct non-competitive ELISA

It can be performed in 2 ways. In the first case, the test substance (antigen) is directly immobilised on the solid phase; then the labelled antibody bound to the antigen will act as a detector. When performing the test in a different way, antibodies immobilised on the solid phase are used. In this case, the detector is the test substance labelled with the enzyme.

Indirect non-competitive ELISA

When performing the indirect version of the ELISA, the antigen is immobilised in the solid phase. After blocking, add a solution of specific antibodies to the antigen. After incubation, the resulting antigen-antibody complex is washed from unbound antibodies, and an enzyme-labelled anti-immunoglobulin (anti-Ig) is added, acting as a detector. Anti-Ig detectors are commercially available for specific Ig classes and subclasses, making this analysis format convenient for antibody isotyping. In addition, the use of labelled anti-Ig increases the signal compared to the direct method of enzyme immunoassay, thereby increasing the sensitivity of the assay.

Sandwich ELISA

Sandwich ELISA is the most common non-competitive technique. When it is performed on the solid phase, primary antibodies are immobilised with their subsequent blocking. Then a substance to be examined containing the antigen is added to them, and the complex is incubated. After incubation, the antigen-antibody complex is washed from the unbound antigen and secondary antibodies labeled with the enzyme are added, and detection is performed.

Competitive ELISA technique

The competitive ELISA technique is divided into several types: by the type of detection (direct competitive, indirect competitive) and by the type of substance immobilised on the solid phase (antigen or antibody).

Direct competitive ELISA

To detect or quantify soluble antigens, a direct competitive ELISA with an antigen immobilised on the solid phase is used. To do this, employ antigen-specific antibodies conjugated with the appropriate detector (e.g., horseradish peroxidase, alkaline phosphatase, ruthenium, or fluorescein). A standard antigen is immobilised on the solid phase, followed by blocking. The antibody conjugated with the enzyme label is incubated with the test substance (soluble antigen). Then this mixture is added to the immobilised antigen, incubated, and then washed from the unbound antigen-antibody complex. The next step is to add a suitable substrate for the enzyme used as a label.

The inhibition of the reaction due to the presence of 2 antigens in the system, compared with the control sample without a competitive soluble antigen, is inversely proportional to the amount of the test substance.

Performing a direct competitive ELISA with a solid-phase immobilised antibody is similar to a direct competitive ELISA with a solid-phase immobilised antigen, but is used for detection or quantitation of antibodies.

Indirect competitive ELISA This ELISA technique is similar to the direct competitive variant, but instead of a labelled antibody or antigen, a labelled anti-Ig reagent or labelled secondary antibodies are used for detection, respectively.

General conditions for conducting ELISA technique

Various materials are used as the solid phase for enzyme immunoassay: silicone, nitrocellulose, polyamides, polystyrene, polyvinyl chloride, polypropylene, acrylic, and others. The solid phase can be the walls of a test tube, 96-well and other plates, balls, beads, as well as nitrocellulose and other membranes actively sorbing proteins. The choice of the solid phase determines the principle of immobilisation (hydrophobic, hydrophilic, covalent interaction). More often than others, 96-well plastic microtiter plates are used as the solid phase. The number of wells in the tablet may vary. The plate can be clear (colourimetric detection) and opaque (chemiluminescent detection, fluorimetry).

Immobilisation should be performed without air bubbles in the well since their presence changes the absorbance reading. It is possible to use biotinylated immobilised reagents. In this case, the reaction uses streptavidin and a biotinylated enzyme label. This technique is used to amplify the signal. The time and temperature of immobilisation, depending on the kinetic nature, stability, and concentration of the reagent, should be specified in the monograph and the quality regulatory document.

All stages of enzyme immunoassay, washing and blocking solutions, time intervals and temperature conditions for each stage, the number of revolutions per minute for incubation on a shaker, detection conditions must also be specified in the monograph or a quality regulatory document.

Detection

Enzyme-labelled antibodies are used for detection. Horseradish peroxidase, alkaline phosphatase, or galactosidase are most commonly used as enzyme labels. Substrates for enzymes can be chromogenic, chemiluminescent, or fluorescent. Spectrophotometry, luminometry, or fluorometry can be used as detection methods based on the choice of substrate.

Results of quantitative ELISA technique

The results of the quantitative ELISA technique are calculated using a linear calibration curve with reverse regression or using a complex method that employs a nonlinear calibration curve with reverse regression. The procedure of interpreting the results depends on the ELISA technique. For example, the test results can be used to estimate the concentration of an unknown sample, estimate the half-maximum inhibition concentration, or estimate the effective concentration using a calibration curve. This helps determine the amount of the test substance or its activity in comparison with the reference/calibration reference standard (RS). Usually, the type of calibration curve that characterises the concentration of the analyte when performing the quantitative ELISA technique depends on the calculated average value non-linearly. In this regard, it is recommended to use various mathematical models to analyse the resulting curve. If the ELISA is performed using automatic plate spectrophotometers, luminometers, or fluorimeters, the results are processed using the software for devices. In other cases, ELISA is used as a qualitative technique to assess the presence of a particular test substance in the sample within the sensitivity of the procedure.

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2.1.7.1. Sampling

This chapter establishes general requirements for the sampling of manufactured (produced) drugs, as well as materials for determining their quality compliance with the requirements specified in general or individual monographs.

This chapter does not apply to the sampling of herbal drugs.

GENERAL TERMS AND DEFINITIONS

Sample is one or more sample units selected in accordance with the established sampling method from the general population.

Sample unit is a certain quantity of drugs or materials that forms a unit and is taken from one place at one time to form part of the sample.

General population is the controlled series (batch).

Finished product (end-product, final product) is a drug product that has passed all stages of the manufacturing process, including the final packaging.

Sample splitting is the process of selecting one or more samples from a sample of bulk non-packaged products in such a way as slicing, mechanical division, or quartering.

Contamination is the undesirable introduction of chemical or microbiological impurities or foreign substances into raw materials, intermediate products, or pharmaceutical substances during the technological process, sampling, packaging or repackaging, storage, or transportation.

Quality control is the testing for compliance with the requirements of the monograph or a quality regulatory document.

Materials is a general concept that refers to raw materials (starting materials, reagents, solvents), excipients, intermediate products, pharmaceutical substances, and materials for packaging and labeling.

Quality regulatory document is a document that establishes requirements for quality control of the drug product (containing specification and description of analytical procedures and tests, or references to them, as well as the corresponding acceptance criteria for these quality indicators, etc., on the basis of the examination of a drug, approved by the competent authority when obtaining marketing authorisation on the territory of the Eurasian Economic Union and is designed to control the quality of the drug in the territory of the Eurasian Economic Union in the post-marketing period.

Sample (for testing) (final sample) is a certain amount of a specific drug or material used as a representative of these objects during testing.

Representative sample is a sample obtained using a sampling method that ensures that different parts of the series or different properties of non-uniform products are represented proportionally.

Bulk sample is a sample of a drug product or materials obtained by combining several spot samples taken from the same drug product or materials, intended for testing for compliance with the requirements of regulatory documentation.

Sample size is the number of sample units in the sample.

Sampling is a complex of actions to withdraw (select) samples of drugs and materials for testing them for compliance with the requirements of the monograph or a quality regulatory document or other purposes.

Sampling plan is a plan that sets out the number of sample units that are required for testing and meet this acceptance criterion.

Sample is a certain amount of drugs and materials selected from a controlled series (batch).

In-bulk product is a drug in a large container, including a certain dosage form, that has passed all stages of the manufacturing process, except for packaging, and is intended for subsequent packaging or production of drugs.

Intermediate product is a material that is obtained during the production process of a pharmaceutical substance and which undergoes further molecular transformations or undergoes purification before becoming a pharmaceutical substance. Intermediate products may or may not be separated during the process.

Sampling method is all sampling operations that must be performed with a specific drug or material to achieve a specific goal.

Series (batch) is a specific quantity of materials obtained as a result of a production process or a series of processes in such a way that its uniformity within the established limits can be trusted. In the case of continuous production, the series may meet a specific part of the product. The batch size, in this case, can be determined either by a fixed quantity or by the quantity produced over a certain period of time.

Container is the key element of packaging intended for placing finished products and materials.

Transport container is a container intended for packaging, storage, and transportation of finished products and materials, forming an independent transport unit. For drugs, transport containers provide for the transportation of a certain amount of drugs in consumer or group packaging (box, bag, barrel, bottle).

Spot sample is an amount of in-bulk products or materials taken at a time in a single action, from one place, from a larger volume of the same objects.

Packaging is a material or device that guarantees the preservation of the quality of a medicinal product during the established shelf life (storage), protects the medicinal product from damage and loss, and protects the environment from contamination.

Secondary (consumer) packaging is a packaging in which a medicinal product is placed in primary or intermediate packages for sale to consumers.

Multiple packaging is a consumer packaging containing a number of similar or identical products secured using packaging or binding materials.

Primary (internal) packaging is a packaging that is directly in contact with the medicinal product.

Intermediate packaging is a packaging in which the primary packaging can be placed for the purpose of additional protection of the medicinal product or based on the features of the use of the medicinal product.

Packaging unit is a package containing a certain amount of finished products.

Note. Definitions of the key terms used in this chapter are specified in the General Notices.

GENERAL PROVISIONS

Sampling of manufactured medicinal products and materials used in the process of their production (manufacturing) or characterising the stages of the production (manufacturing) process must be carried out in accordance with the approved sampling method unless otherwise specified in the quality regulatory document.

The sampling method must meet the specific purposes of sampling, the type of testing, and the specifics of the selected samples.

When conducting the sampling procedure, the following aspects shall be provided for and considered:

- sampling plan or scheme;
- sampling size and type;
- place and time of sampling;
- extraction and preparation of samples for testing;
- special precautions, especially for sterile and dangerous drugs or materials;

- list of sampling equipment used;
- requirements for cleaning and storage of sampling equipment, etc.;
- the type, characteristics, and labelling of containers for sample storage;
- environmental parameters for sampling and sample preparation for testing.

When preparing a sampling plan, it is necessary to take into account the specific goals of sampling, physical, chemical, biological and other properties of the object under study, its uniformity, stability, criticality, the number of samples taken; risks and consequences associated with erroneous decisions on the choice of the sampling plan.

The following products are subject to sampling:
- medicinal products (batches);
- intermediate products at critical stages of the production/manufacturing process;
- excipients;
- packaging and printing materials.

SAMPLING RULES

Samples are taken from the general population (batch/series) consisting of sample units.

When selecting samples that characterise the stages of the production (manufacturing) process, the general population is established by internal documents of the drug manufacturer.

The sampling process takes into account the factors that must be controlled in order to ensure reliability of the test results.

The sampling procedure must include a provision for preventing medicinal products and materials to be sampled, the samples themselves, as well as other drugs, materials, and the environment from contamination.

The material sampling procedure in the in-house process shall take into account the critical stages of the drug production (manufacturing) process and include preset control points for sampling (containers, sampling sites, etc.).

It is not allowed to take samples simultaneously from two or more drugs or materials, two or more series (batches) of finished products in order to avoid errors in sampling.

You can start selecting finished products or materials from the next batch only after completing the entire sampling procedure from the previous batch.

Before sampling, it is necessary to conduct an external inspection of each packaging unit of the entire series (batch) of finished products or materials. During the inspection it is necessary to pay attention to the compliance of packaging of finished products or materials and its labeling with requirements of a monograph or quality regulatory document, to determine the number of finished products and materials, integrity and availability of seals on the package, the correctness of supporting documentation and compliance with data series (batch) of products or materials intended for sampling.

Samples are taken only from undamaged, sealed and stoppered packaging units according to individual monograph or quality regulatory document. Finished products and materials in damaged containers or packages that do not meet the requirements of the monograph or quality regulation document must be rejected.

Note. Pursuant to the appropriate instructions in the manufacturer's documentation, it is allowed to take samples from each unit of finished products or materials from damaged containers to conduct full quality control of the analysed objects.

SAMPLING METHODS

Random sampling. Samples can be selected randomly from a specified quantity of sample units during sampling control, from each sample unit during continuous control, or by another technique in accordance with the developed statistically sound sampling plan.

To perform random sampling, each sample unit must be numbered sequentially, and then, using a table of random numbers (or computer-generated random numbers), determine from which randomly sampled units the required number of samples shall be taken.

Multi-stage sampling. Unless otherwise prescribed in the monograph, when taking samples of medicinal products for testing them for compliance with the requirements of a quality regulatory document, multi-stage sampling is carried out on the assumption that the series (batch) of the medicinal product represents a homogeneous product. Materials are sampled in the same way.

In multi-stage sampling, the sample is generated in stages and the finished products or materials in each stage are sampled randomly in proportional quantities from the packaging units selected in the previous stage. The number of stages is determined by the type of packaging (containers).

For example, if products in consumer (secondary) packaging are placed in a multipack, and then in a transport container, then three-stage sampling is possible.

Stage 1: sampling of transport container units (boxes, cases, bags, etc.).

Stage 2: sampling of multipack units (cartons, packs, rolls, etc.).

Stage 3: sampling of products in consumer (secondary) packaging (vials, tubes, blisters, etc.).

To calculate the number of selected packaging units (N) at each stage, use the formula for homogeneous products:

$$N = 0{,}4\sqrt{n}, \qquad (1)$$

where n is the total number of packaging units at a given stage in a single series (batch).

The fractional number obtained by counting using the formula (1) is rounded up to an integer, it must be not less than 3 and not greater than 30.

If there are not enough packaging units for the test, re-sample the packaging units as indicated above.

From packaging units selected in the final stage after visual control, take a sample to check the compliance of the drug with requirements of a monograph or quality regulatory document. The quantity of the sample shall be enough for a particular purpose (including testing for microbiological purity, test for sterility, a test of parenteral and ophthalmic solutions on mechanical inclusions, etc.).

Note. For solid dosage forms, the number of sample units for microbiological control is calculated by dividing the required amount of sample in grams (50 g) by the average weight of a tablet, dragee, capsule, or suppository.

If the identification of a homogeneous product is reliable, the following formula should be used to calculate the number of selected packaging units:

$$N = 1 + \sqrt{n}. \qquad (2)$$

The fractional number obtained as a result of counting by formula (2) is rounded up or down to an integer by simple rounding rules. If there are 4 or fewer packaging units, all of them shall be sampled.

Note. It is not recommended to use formula (2) for on-receipt (incoming) control of materials intended for the production of drugs.

If the product is heterogeneous and/or comes from an unknown source, the next formula can be used to calculate the number of packaging units selected:

$$N = 1{,}5\sqrt{n}. \qquad (3)$$

The fractional number obtained as a result of counting by formula (3) is rounded up to an integer.

Requirements for sampling from in-bulk drugs and materials.

A sample of in-bulk drug products or materials is a combination of spot samples taken in approximately equal quantities, mixed and, if necessary, reduced to the mass (volume) of the required sample for testing the drug product or materials for compliance with the requirements of a quality regulatory document for the implementation of a specific goal.

Note. If each spot sample is analysed separately, their masses (volumes) may not be the same, but not less than the quantity determined by the quality regulation document for a particular type of test.

For sampling, use samplers that correspond to the physical condition, type of product packaging, made of a material that does not contaminate the product, and does not react with it. The capacity of the sampler should be sufficient to take the entire spot sample and must be accessible for cleaning. The samplers used must be clean and dry, and if a sample is used to determine microbiological purity, they must be sterile.

Spot sampling is carried out with a suitable sampler from different levels: the upper, middle, and lower layers of each selected packaging unit. To take samples of liquids, they have to be thoroughly mixed; if mixing is difficult (large containers), point samples are taken without mixing from different layers.

In the case of sampling to check uniformity of products, spot samples of loose, viscous, heterogeneous, and other defined products are examined separately, and during visual inspection, the uniformity of the selected point samples is verified.

Note. Signs of heterogeneity may include differences in the shape, size, or colour of particles in the crystalline, granular, or powder mass of a solid substance, wet crusts on hygroscopic substances, detected solids in liquid substances, layer separation of liquid substances, etc.

If the point samples are homogeneous, they are combined by mixing thoroughly on a clean, dry surface or in a suitable container to obtain a bulk sample.

If necessary, reasonable manual or automated methods are used to divide (reduce) the bulk sample.

REQUIREMENTS FOR SAMPLING DRUGS IN CONSUMER PACKAGING

Medicinal products of the same batch from the same manufacturer received from the same supplier can be considered homogeneous.

The sample of drugs should consist of unopened packaging units.

The sample size of drugs is determined by the purpose of selection, the requirements of the test method, the type of dosage form, and other factors.

The sampling of drug products is carried out in accordance with the requirements of chapters for specific dosage forms, for test methods, or in accordance with the requirements of quality regulatory documents.

PACKAGING, LABELING AND STORAGE OF SAMPLES

The selected samples (final samples) of drugs and materials are placed in a prepared container and/or packed and, if necessary, sealed at the sampling site.

The packaging must ensure that the sample is suitable for subsequent testing and that it does not alter the quality parameters to be tested during transportation and storage.

Samples of in-bulk products or materials must be placed in sterile containers.

The selected samples must be duly identified using uniform labeling and issued with a selection certificate or other document that includes the date, time and place of sampling, the environmental conditions during sampling, the surname, first name, and patronymic of the person who conducted the sampling, and other necessary information.

Before and after testing, samples should be stored in a separate room as prescribed in the monograph or quality regulatory document for drugs or materials. The conditions in the room must ensure the safety of samples during the storage period.

The packaging units from which the samples were taken must be carefully opened and closed, they must be marked showing that samples were taken from this package (container), and the remaining amount of the analysed object must be specified.

If a package was punctured for sampling, then the puncture site must be sealed and labeled after sampling.

Requirements for sampling facilities, equipment, and personnel

All sampling operations should be performed properly in a separate room or designated area, using appropriate sampling equipment and tools. The test equipment and measuring instruments used in sampling must pass certification or verification in accordance with the established procedure.

Personnel carrying out the sampling should be suitably trained.

Documentation of the sampling method should be available at the sampling sites and accessible for personnel.

Before sampling, the personnel responsible for sampling should study the necessary documents related to safety and health, including the necessary precautions and safety requirements for personnel and the environment.

Samplers must strictly follow the instructions governing the state of health and personal hygiene requirements.

Samplers should wear appropriate protective clothing and special shoes for the task, using gloves, aprons, goggles, respirators, and other personal protective equipment if necessary.

When sampling, it is forbidden to eat, drink, smoke, or store food or smoking products in special clothing or in the sampling area.

When sampling, it is necessary to observe precautions and safety requirements, taking into account the toxicity, fire and explosion hazard, hygroscopicity, and other properties of the product, as well as measures aimed at protecting the samples from damage and contamination during handling, requirements for their packaging, transportation, warehousing, and storage, taking into account the requirements and methods of subsequent tests.

When sampling drugs and materials related to narcotic drugs, psychotropic substances and their precursors, the current legislative documents of the Eurasian Economic Union should be taken into account.

Persons responsible for sampling should have safe access to and exit from the sampling area and the sample storage area. The sample storage areas must have adequate lighting, ventilation, and an internal arrangement that meets the safety requirements related to the nature of the selected product samples.

Care should be taken to prevent the collapse of large numbers of packages stacked together.

201080001-2019

2.1.8.1. Ash Insoluble in Hydrochloric Acid

Ash insoluble in hydrochloric acid is the residue obtained after extracting the sulfated or total ash with hydrochloric acid, calculated with reference to 100 g of raw materials.

To the crucible containing the residue from the determination of sulfated or total ash, add 15 mL of *water R* and 10 mL of *hydrochloric acid R*, cover with a watch-glass, boil the mixture gently for 10 min, allow to cool, and filter through an ashless filter. Wash the residue with hot water until the filtrate is neutral, dry, and then ignite to dull redness, allow to cool in a desiccator, and weigh. Ignite until the difference between two consecutive weighings is not greater than 0.5 mg.

201080002-2019

2.1.8.2. Foreign Matter

Herbal drugs should be free from moulds. In herbal drugs, the presence of foreign matter is possible that are divided into acceptable and unacceptable impurities.

Acceptable impurities include:

- part of the raw material that has changed the colour inherent in this type of herbal drug/preparation (browned, blackened, faded, etc.);
- other parts of the plant that do not correspond to the established description of raw materials;
- organic impurity (parts of other non-poisonous plants);
- mineral impurity (earth, sand, stones).

Unacceptable impurities include:

- glass;
- rodent and bird droppings;
- parts of poisonous plants;
- pests that get into raw materials during harvesting, processing and storage.

DRIED PLANTS

Sampling and sample preparation. As prescribed in the general chapter. *Herbal drugs: sampling and sample preparation*.

Determination of acceptable impurities. Weigh 100 g to 500 g of drug or the minimum quantity prescribed in the monograph and spread it out in a thin layer. Examine for acceptable impurities by inspection with the unaided eye or by use of a lens (6x), separate, weigh it, and calculate the percentage present.

FRESHLY HARVESTED PLANTS

If the chapter *Herbal drugs: sampling and sample preparation* is not applicable, use one of the following methods: Method A, if the test can be performed for the entire batch; Method B, if the test cannot be performed for the entire batch.

METHOD A

Sampling and sample preparation. The test is performed for the entire batch.

Determination of acceptable impurities. The batch is distributed in a thin layer and the acceptable impurities are determined with the naked eye or using a magnifying glass (6x), separated, weighed and calculated as a percentage.

METHOD B

Sampling and sample preparation. If it is not possible to check the entire batch, perform the following.

Bulk sample. The bulk sample is prepared as specified in the general chapter. *Herbal drugs: sampling and sample preparation*.

Test sample. Use a bulk sample or, if it is greater than 1 kg, reduce it to a mass of 500-1000 g by a suitable method ensuring that the bulk sample is representative.

Determination of acceptable impurities. Use a sample or its minimum amount specified in the monograph, which is distributed in a thin layer and determine the acceptable impurities with the naked eye or with a magnifying glass (6x), separate, weigh and calculate their content as a percentage.

Determination of mineral impurity in powdered herbal drugs. Weigh the test sample to a precision of ±0.01 g, then place in a 1000 mL glass beaker, add 200 mL of *water R.* To eliminate lumps of adhered particles, stir the contents until the drug/preparation is completely wetted, evenly distribute in the volume of the liquid and allow it to stand for 3-5 min. After the mineral impurity settles, water with suspended particles is quickly drained from the sediment (without allowing the raw material particles to swell). The precipitate in the beaker is washed several times with *water R* until the suspended particles of the raw material are completely removed. At the end of washing, a mineral impurity precipitate with a minimum amount of water should remain in the beaker. The precipitate in the beaker is dried in a drying cabinet at 100-105 °C until the precipitate becomes friable. The dried precipitate (mineral impurity) is cooled and weighed with an accuracy to ±0.01 g. Calculate the mineral impurity content as a percentage.

As a rule, the content of organic impurities should be not greater than 1%, mineral impurities – not greater than 1%.

201080003-2019

2.1.8.3. Stomata and Stomatal Index

STOMATA

There are several types of stomata (see Figure 2.1.8.3.-1), distinguished by the form and arrangement of the surrounding cells.

1. The *anomocytic* (irregular-celled) type: the stoma is surrounded by a varying number of cells in no way differing from those of the epidermis generally.

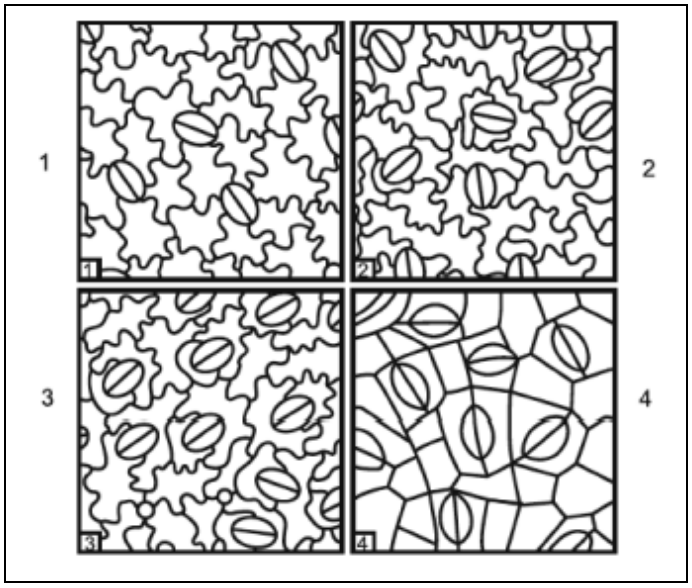


Figure 2.1.8.3.-1. – *Types of stomata.*

2. The *anisocytic* (unequal-celled) type: the stoma is usually surrounded by 3 subsidiary cells, of which one is markedly smaller than the others.

3. The *diacytic* (cross-celled) type: the stoma is accompanied by two subsidiary cells, whose common wall is at right angles to the guard cells.

4. The *paracytic* (parallel-celled) type: the stoma has on each side one or more subsidiary cells parallel to the long axis of the pore and guard cells.

STOMATAL INDEX

$$\text{Stomatal index} = \frac{100 \cdot S}{E \pm S},$$

where *S* is the number of stomata in a given area of leaf;

E is the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of leaf, make not fewer than 10 determinations and calculate the mean.

201080004-2019

2.1.8.4. Swelling Index

The swelling index is the volume in millilitres occupied by 1 gram of a herbal drug, including any adhering mucilage after it has swollen in an aqueous liquid for 4 h.

In a 25 mL ground-glass stoppered cylinder graduated over a height of 125 ± 5 mm in 0.5 mL divisions, place 1.0 g of the herbal drug, whole or of the degree of comminution prescribed in the monograph. Unless otherwise prescribed in the monograph, moisten the sample with 1.0 mL of *alcohol R*, add 25 mL of *water R* and close the cylinder, shake vigorously every 10 min for 1 h. Allow to stand for 3 h. At 90 min after the beginning of the test, release any large volumes of liquid retained in the layer of the drug and any particles of the raw material floating at the surface of the liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the raw material, including any adhering mucilage. Carry out three tests at the same time.

The swelling index is given by the mean of the three tests.

201080005-2019

2.1.8.5. Water in Essential Oils

Mix 10 drops of the essential oil with 1 mL of *carbon disulfide R.* The solution remains clear on standing.

201080006-2019

2.1.8.6. Foreign Esters in Essential Oils

Heat 1 mL of the essential oil for 2 min on a water bath with 3.0 mL of a freshly prepared 100 g/L solution of *potassium hydroxide R* in *alcohol R*. No crystals are formed within 30 min, even after cooling.

201080007-2019

2.1.8.7. Fatty Oils and Resinified Essential Oils in Essential Oils

The determination of fatty and resinified essential oils can be carried out using the following procedures:

a) Apply 1 drop of the essential oil to filter paper. The drop evaporates completely within 24 h without leaving any translucent or greasy spot.

b) Shake 1 mL of essential oil in a 20 mL test tube with 10 mL of *96% ethanol R*, the solution remains clear, and no fat drops are formed.

201080008-2019

2.1.8.8. Odor and Taste of Essential Oils

The following procedures can be used to determine the odour and taste of essential oils:

a) Mix three drops of the essential oil with 5 mL of *90% alcohol R* and stir in 10 g of powdered *sucrose R*. The odor and taste are similar to that of the plant or parts of the plant from which the essential oil has been obtained;

b) Apply two drops (about 0.1 mL) of essential oil to a strip of filter paper 12 cm long and 5 cm wide and compare its odor with the odour of a reference standard every 15 min. The odor of essential oil should not differ from the odour of the reference standard during 1 h.

201080009-2019

2.1.8.9. Residue on evaporation of Essential Oils

The residue on evaporation of an essential oil is the percentage by mass of the oil which remains after evaporation on a water bath under the conditions specified below.

The apparatus (see Figure 2.1.8.9.-1) consists of:

- a water bath with a cover having holes of 70 mm diameter;
- an evaporating dish of heat-resistant glass which is inert to its contents;
- a desiccator.

Procedure. Weigh the evaporating dish after having heated it on the water bath for 1 h and cooled it in the desiccator. Weigh into the evaporating dish 5.00 g of the essential oil, unless otherwise prescribed in the monograph (if the content of non-volatile residue in the oil is greater than 8%, a smaller weight may be indicated), heat the oil on the vigorously boiling water bath in a fume hood for the prescribed time. Allow to cool in the desiccator and weigh.

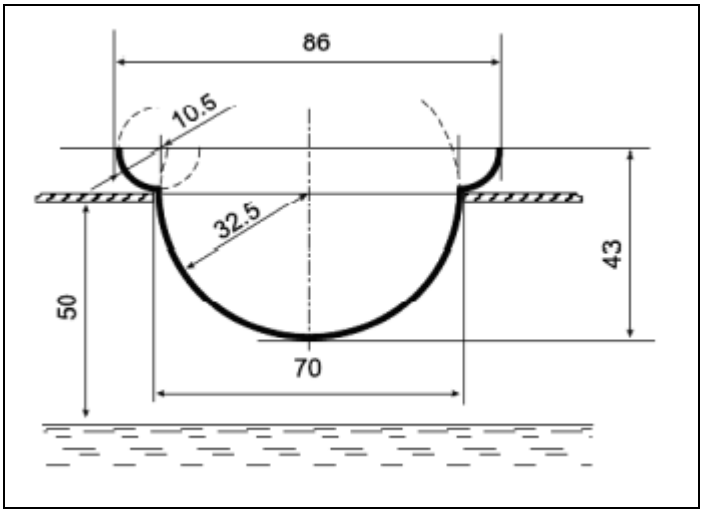


Figure 2.1.8.9.-1. – *Apparatus for the determination of essential oils (dimensions in millimetres)*

During the test, the level of water in the bath is maintained about 50 mm beneath the level of the cover.

201080010-2019

2.1.8.10. Solubility in Alcohol of Essential Oils

Place 1.0 mL of the essential oil in a 25 mL or 30 mL glass-stoppered cylinder. Place in a constant temperature device, maintained at a temperature of 20 ± 0.2 °C. Using a burette of at least 20 mL capacity, add the alcohol of the strength prescribed in the monograph by increments of 0.1 mL until the solution is complete and then continue adding by increments of 0.5 mL to a total of 20 mL, shaking frequently and vigorously. Record the volume of alcohol added when a clear solution has been obtained and, if the solution becomes cloudy or opalescent before 20 mL of alcohol has been added, record the volume added when the cloudiness or opalescence appears and, where applicable, the volume added when the cloudiness or opalescence disappears.

If a clear solution has not been obtained when 20 mL of alcohol of the prescribed strength has been added, repeat the test using the next highest concentration of alcohol.

An essential oil is said to be "soluble in *V* volumes and more of alcohol of given strength *t*" when the clear solution in *V* volumes remains clear when compared with the undiluted oil after further addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *V* volumes of alcohol of given strength *t*, becoming cloudy when diluted" when the clear solution in *V* volumes becomes cloudy in V_1 volumes (V_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *V* volumes of alcohol of given strength *t* with cloudiness between V_1 and V_2 volumes" when the clear solution in *V* volumes becomes cloudy in V_1 volumes (V_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of V_2 volumes of alcohol and then becomes clear (V_2 less than 20).

An essential oil is said to be "soluble with opalescence" when the alcoholic solution shows a bluish tinge, similar to that of a freshly prepared standard of opalescence. Preparation of the standard of opalescence: mix 0.5 mL of *silver nitrate solution R2* and 0.05 mL of *nitric acid R*, then add 50 mL of a 12 mg/L solution of *sodium chloride R*, mix and allow to stand protected from light for 5 min.

201080011-2019

2.1.8.11. Quantitation of 1,8-cineol in Essential Oils

Weigh 3.00 g of the oil, recently dried with *anhydrous sodium sulfate R*, into a dry test-tube and add 2.10 g of melted *cresol R*. Place the tube in the apparatus for the determination of freezing point (*2.1.2.17*) and allow to cool, stirring continuously.

When crystallisation takes place there is a small rise in temperature. Note the highest temperature reached (t_1).

Table 2.8.11.-1. – *Content of cineol corresponding to the highest observed crystallisation temperature*

t (°C)	Cineol % (m/m)	*t* (°C)	Cineol % (m/m)	*t* (°C)	Cineol % (m/m)	*t* (°C)	Cineol % (m/m)
24	45.5	31	56.0	40	67.0	48	82.0
25	47.0	33	57.0	41	68.5	49	84.0
26	48.5	34	58.5	42	70.0	50	86.0
27	49.5	35	60.0	43	72.5	51	88.5
28	50.5	36	61.0	44	74.0	52	91.0
29	52.0	37	62.5	45	76.0	53	93.5
30	53.5	38	63.5	46	78.0	54	96.0
31	54.5	39	65.0	47	80.0	55	99.0

Melt the mixture on a water-bath at a temperature that does not exceed t_1 by greater than 5 °C and place the tube in the apparatus, maintained at a temperature 5 °C below t_1. When crystallisation takes place, or when the temperature of the mixture has fallen 3 °C below t_1, stir continuously. Note the highest temperature at which the mixture crystallises t_2. Repeat the operation until the two highest values obtained for t_2 do not differ by greater than 0.2 °C. If supercooling occurs, induce crystallisation by adding a small crystal of the complex consisting of 3.00 g of *cineole R* and 2.10 g of melted *cresol R*. If t_2 is below 27.4 °C, repeat the determination after the addition of 5.10 g of the complex.

The content of cineole corresponding to the highest temperature observed (t_2) is given in Table 2.1.8.11.-1.

If 5.10 g of the complex has been added, calculate the cineole content percent (m/m) from the expression:

$$2 \cdot (A - 50),$$

where A is the value found in Table 2.1.8.11.-1.

The cineol retention corresponding to the highest observed temperature (t_2) is determined, if necessary, by interpolation.

2.1.8.12. Essential Oils in Herbal Drugs

The determination of essential oils in herbal drugs is carried out by steam distillation in a special apparatus in the conditions described below.

PROCEDURE 1

The distillate is collected in the graduated tube, using xylene to take up the essential oil; the aqueous phase is automatically returned to the distillation flask.

Apparatus. The apparatus comprises the following parts:

(a) a suitable round-bottomed flask with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;

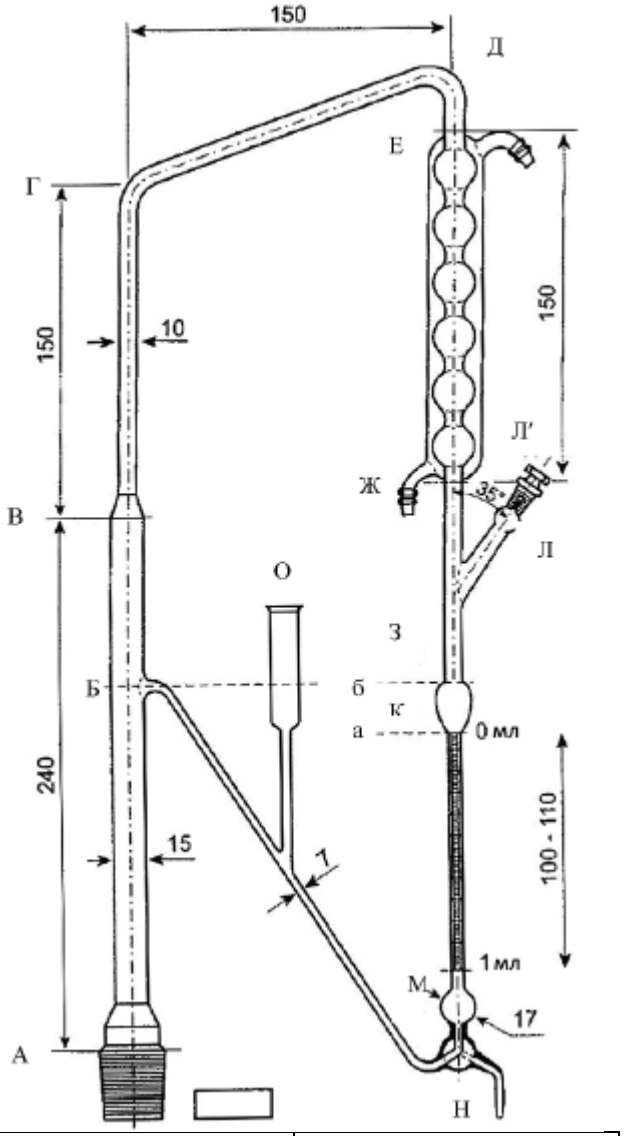
(b) a condenser assembly (see Figure 2.1.8.12.-1) that closely fits the flask; the different parts being fused into one piece; the glass used has a low coefficient of expansion:

- the stopper *K`* is vented and the tube *K* has an orifice of diameter about 1 mm that coincides with the vent; the wide end of the tube *K* is of ground-glass and has an internal diameter of 10 mm;
- a pear-shaped swelling *J*, of 3 mL capacity;
- a tube *JL* is graduated in 0.01 mL;
- the bulb-shaped swelling *L* has a capacity of about 2 mL;
- a three-way tap *M*;
- the junction *B* is at a level 20 mm higher than the uppermost graduation;

(c) a suitable heating device, allowing a fine control;

(d) a vertical support with a horizontal ring covered with insulating material.

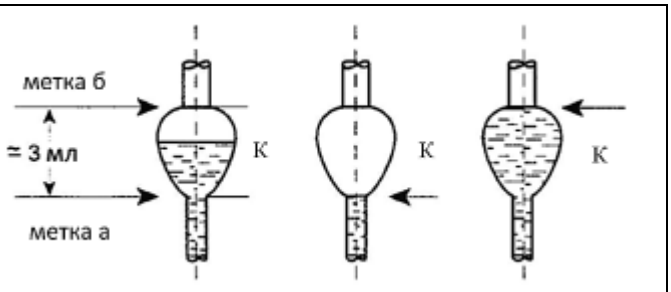
Procedure. Use a thoroughly cleaned apparatus. Carry out the quantitation according to the nature of the herbal drug.



	A B C D E F G H J K K' L M N
	1 mL
	0 mL

Figure 2.1.8.12.-1. – *Apparatus for determining essential oils in herbal drugs (dimensions in millimetres)*

The volume of distilled liquid specified in the monograph, together with several pieces of porous porcelain, is placed in a flask, which is then connected to the condensing system. Through the funnel-shaped expansion *N*, *water R* is poured until the level *B* is reached. Remove the stopper *K'* and add the amount of *xylene R* specified in the monograph using a pipette, lowering its tip to the bottom of the tube *K*. Close the tube *K* with a stopper *K'*, making sure that the holes are aligned. The liquid is heated in the flask to boiling and the distillation rate is adjusted to 2-3 mL/min unless otherwise specified in the monograph.



	mark b
	≈3 mL
	mark a
	K

Figure 2.1.8.12.-2.

To determine the rate of distillation, during distillation lower the level of the water by means of the three-way tap until the meniscus is at the level of the lower mark (a) (see Figure 2.1.8.12.-2). Close the tap and measure the time taken for the liquid to reach the upper mark (b). Open the tap and continue the distillation, modifying the heat to regulate the distillation rate. Distill for 30 min, then stop the heating and after at least 10 min read off the volume of xylene in the graduated tube.

Introduce into the flask the quantity of the raw material specified in the monograph and continue the distillation as described above for the time and at the rate prescribed. Stop the heating and after 10 min read the volume of liquid collected in the graduated tube and subtract the volume of xylene previously noted. The difference represents the quantity of essential oil in the mass of the raw material taken. Calculate the result as millilitres per kilogram of the raw material.

When the essential oil is to be used for other analytical purposes, the water-free mixture of xylene and essential oil may be recovered as follows: remove the stopper *K'* and introduce 0.1 mL of a 1 g/L solution of *sodium fluoresceinate R* and 0.5 mL of *water R*. Lower the mixture of xylene and essential oil into the bulb-shaped swelling *L* by means of the three-way tap, allow to stand for 5 min and lower the mixture slowly until it just reaches the level of the tap *M*. Open the tap anti-clockwise so that the water flows out of the connecting tube *BM*. Wash the tube with *acetone R* and with a little *toluene R* introduced through the filling funnel *N*. Turn the tap anti-clockwise in order to recover the mixture of xylene and essential oil in a suitable flask.

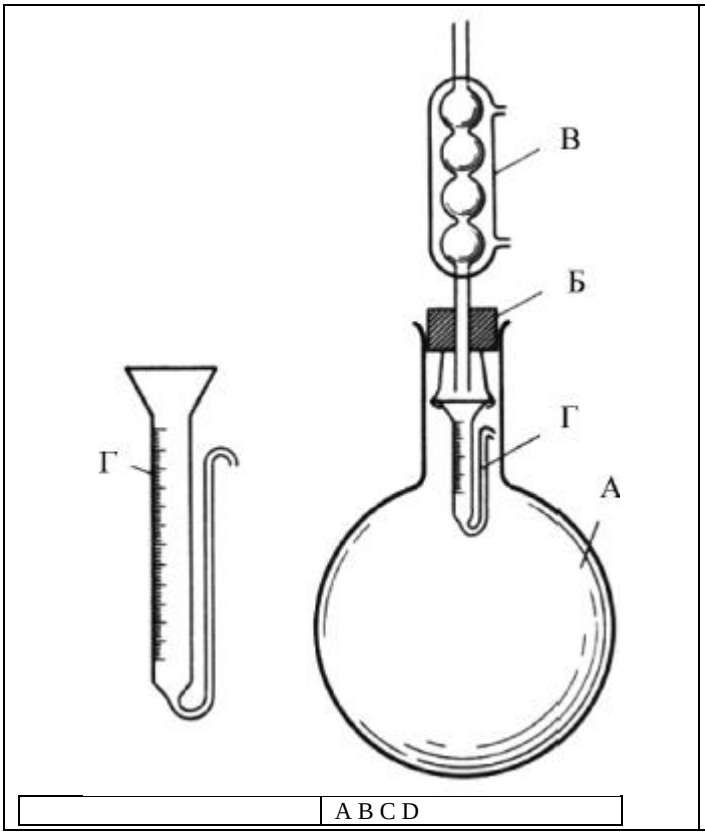


Figure 2.1.8.12.-3. – *Apparatus for determining the content of essential oil according to Procedure 1*

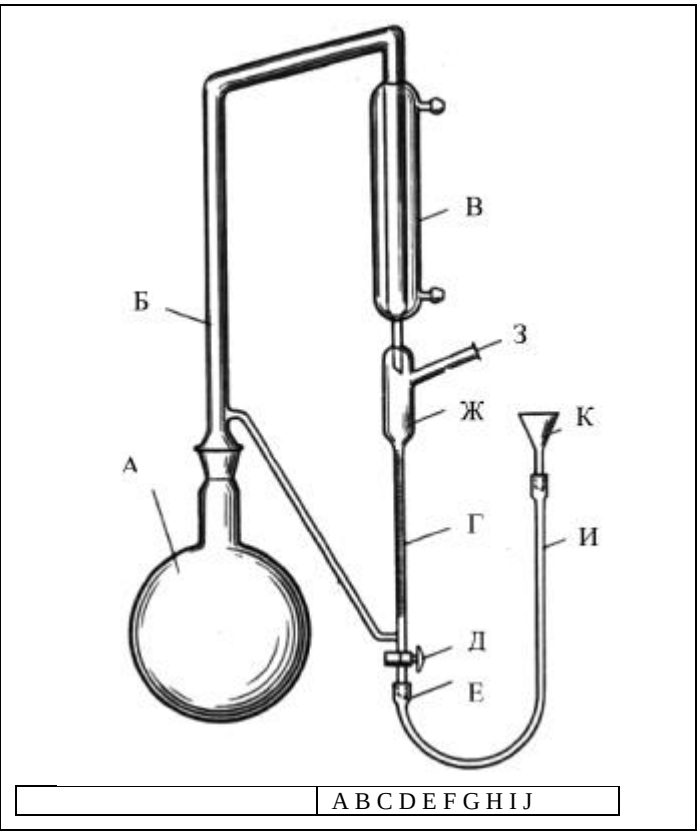


Figure 2.1.8.12.-4. – *Apparatus for determining the content of essential oil according to Procedure 3 and Procedure 4*

PROCEDURE 2

To determine the essential oil, use the apparatus shown in Figure 2.1.8.12.-3. Place the weighed amount of the powdered material in a 1000 mL wide-necked round-bottomed flask (*A*), add 300 mL of *water R*, and stopper (*B*) with a reflux condenser (*C*). In the bottom of the tube, fix metal hooks, and then suspend a graduated receiver (*D*) on them using a thin wire so that the end of the condenser is above the funnel-shaped extension of the receiver, without touching it. The receiver must fit freely in the neck of the flask, without touching the walls, and be at least 50 mm above the water level. The minimum value of the graduation of the receiver is 0.025 mL. The flask with contents is heated and boiled for the time specified in the monograph.

The volume of oil in the graduated part of the receiver is measured after the distillation and cooling of the apparatus to room temperature. After 6-8 determinations, the condenser and graduated receiver must be washed sequentially with *acetone R* and *water R*.

Calculate the content of essential oil as a percentage equivalent to absolutely dry raw materials using the formula:

$$\frac{V \cdot 100 \cdot 100}{m \cdot (100 - W)},$$

where *V* is the volume of essential oil, in millilitres
m is a weight of raw materials, in grams
W is a loss on drying, in percent.

PROCEDURE 3

To determine the essential oil, use the apparatus shown in Figure 2.1.8.12.-4. The apparatus consists of a 1000 mL round-bottom flask (*A*), a curved steam pipe (*B*), a condenser (*C*), a graduated tube (*D*) ending at the bottom with a drain tap (*E*) and a drain tube (*F*). The upper part of the receiver has an extension (*G*) with a side tube (*H*), which serves to introduce the essential oil solvent into the distillate and communicate the interior of the apparatus with the atmosphere. The flask and the steam pipe are connected through a joint. The minimum value of the graduated tube is 0.02 mL.

To fill the device with water, use a 450 mm long rubber tube (I) with an internal diameter of 4.5-5 mm and a funnel (*J)* with a diameter of 30-40 mm.

Before each determination, steam is passed through the device for 15-20 minutes. After 6-8 definitions, the device must be washed sequentially with *acetone R* and *water R.*

Place a weighed amount of crushed raw materials in a flask, add 300 mL of *water R.* Attach the flask to a steam pipe, fill the graduated tube and drain tube with water through a tap using a rubber tube ending in a funnel. The flask is heated and boiled at an intensity at which the distillate flow rate is 60-65 drops/min for the time specified in the monograph.

5 min after the distillation, open the tap, gradually lowering the distillate so that the essential oil occupies the graduated part of the receiver tube, and after 5 min, measure the volume of oil.

Calculate the content of essential oil as a percentage equivalent to absolutely dry raw materials using the formula:

$$\frac{V \cdot 100 \cdot 100}{m \cdot (100 - W)},$$

where V is the volume of essential oil in millilitres;

m is a weight of raw materials, in grams

W is a loss on drying, in percent.

PROCEDURE 4

To determine the essential oil, use the device shown in figure 2.1.8.12. -5. Place a weighed amount of crushed raw materials in a flask, add 300 mL of *water R.* Attach the flask to a steam pipe, fill the graduated tube and drain tube with water through a tap using a rubber tube ending in a funnel. Using a pipette, the exact volume of decalin (about 0.5 mL) is poured into the receiver through the side tube, lowering the liquid level to the graduated part of the tube. Then proceed as prescribed in Procedure 3.

Calculate the content of essential oil as a percentage equivalent to absolutely dry raw materials using the formula:

$$\frac{(V - V_1) \cdot 100 \cdot 100}{m \cdot (100 - W)},$$

where V is the volume of the oil solution in decalin in millilitres;

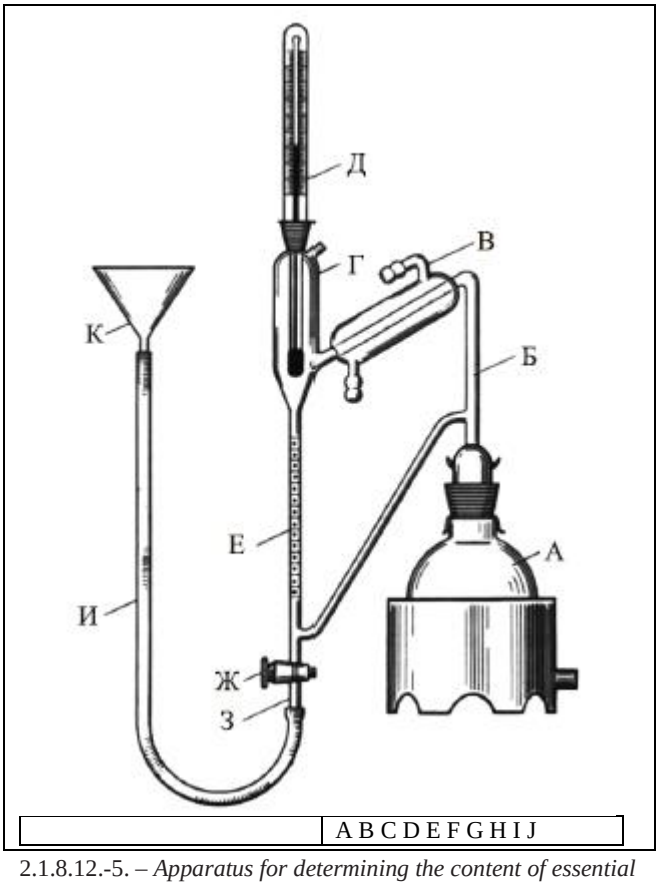
$V1$ is the volume of decalin in millilitres;

m is a weight of raw materials, in grams

W is a loss on drying, in percent.

PROCEDURE 5

To determine the essential oil, use the device shown in figure 2.1.8.12. -5. The apparatus consists of a 1000 mL round-bottom flask with a short neck (*A*), a steam pipe (*B*), a condenser (*C*), a catcher (*D*) with a 100 °C thermometer (*E*), the mercury ball of which is at the level of the condenser opening, a graduated tube (*F*) with a minimum value of 0.001 mL, a drain tap (*G*), and a drain tube (*H*). To fill the device with *water R*, use a 450 mm long rubber tube (I) with an internal diameter of 4.5 - 5 mm and a funnel with a diameter of 30 - 40 mm.



2.1.8.12.-5. – *Apparatus for determining the content of essential oil according to Procedure 5*

Before each determination, steam is passed through the device for 15-20 minutes. After 6-8 definitions, the device must be washed sequentially with *acetone R* and *water R*.

Place the weighed amount of the powdered material in the flask, add the required amount of *water R*. Connect the flask to a steam pipe, fill with *water R* the graduated tube and the drain tube ending in a funnel, until a layer of water 8-12 mm high is collected in the lower funnel-shaped part of the catcher. This water level must remain unchanged during distillation. The flask is heated and boiled for the time specified in the monograph. During distillation, the temperature in the catcher should not exceed 25 °C. 5 min after the end of distillation, open the tap, gradually lowering the distillate so that the essential oil fills the graduated part of the tube. After 5 min, measure the volume of essential oil.

Calculate the content of essential oil as a percentage equivalent to absolutely dry raw materials using the formula:

$$\frac{V \cdot 100 \cdot 100}{m \cdot (100 - W)},$$

where V is the volume of essential oil, in millilitres
m is a weight of raw materials, in grams
W is a loss on drying, in percent.

201080013-2019

2.1.8.13. Tannins in Herbal Drugs, Herbal Pharmaceutical Substances, and Herbal Drug Preparations

Carry out all the extraction and dilution operations protected from light.

METHOD A

In the case of herbal drugs or a dry extract, place the quantity of powdered materials or the extract specified in the monograph in a 250 mL round-bottomed flask, add 150 mL of *water R*, heat on a water bath for 30 min, then cool under running water, and transfer quantitatively to a 250 mL volumetric flask.

Rinse the round-bottomed flask with *water R* and collect the washings in the volumetric flask, then dilute to 250.0 mL with *water R*. Allow the solids to settle and filter the liquid through a filter paper 125 mm in diameter, discarding the first 50 mL of the filtrate.

In the case of a liquid extract or a tincture, dilute the amount of the liquid extract or tincture specified in the monograph to 250.0 mL with *water R*. Filter the mixture through a filter paper 125 mm in diameter, discarding the first 50 mL of the filtrate.

Test solution (a). Total polyphenols. Dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.1.2.24*) of this solution at 760 nm (A1), using *water R* as the compensation liquid.

Test solution (b). Polyphenols not adsorbed by hide powder. To 10.0 mL of the filtrate, add 0.10 g of *hide powder CRS* and shake vigorously for 60 min, filter, and dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.1.2.24*) at 760 nm (A_2), using *water R* as the compensation liquid.

Reference solution. Dissolve immediately before use 50.0 mg of *pyrogallol R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.1.2.24*) of this solution at 760 nm (A_3), using *water R* as the compensation liquid.

Calculate the percentage content of tannins expressed as pyrogallol from the equation:

$$\frac{62{,}5 \cdot (A_1 - A_2) \cdot m_2}{A_3 \cdot m_1},$$

where A_1 is the absorbance of the test solution (a);

A_2 is the absorbance of the test solution (b);

A_3 is the absorbance of the reference solution;

$m1$ is the weighted amount of the test sample in grams.

m_2 is the weighed amount of pyrogallol in grams.

METHOD B

Place 2.0 g of powdered herbal drugs in a 500 mL conical flask, filled with boiling *water R*, and boil under reflux on an electric stove with a closed spiral for 30 min, stirring periodically. Allow the resulting extraction to cool to room temperature and filter through a cotton swab into a 250 mL volumetric flask avoiding the ingress of plant product particles, dilute to 25.0 mL with *water R* and mix. Place 25. 0 mL of the filtrate in a 1000 mL conical flask, add 500.0 mL of *water R*, 25 mL of *indigosulfonic acid solution R*, and titrate with *0.02 M potassium permanganate*, stirring continuously, until golden yellow colour is obtained.

In parallel, a blank titration is carried out using 25.0 mL of *water R* instead of 25.0 mL of filtrate.

1 mL of *0.02 M potassium permanganate solution* is equivalent to 0.004157 g of tannins.

Calculate the content of tanning substances equivalent to tannin in absolutely dry raw material as a percentage using the formula:

$$\frac{(V_1 - V_2) \cdot 0{,}004157 \cdot 100000}{m \cdot (100 - W)},$$

where V_1 is the volume of *0.02 M of potassium permanganate* used for titration of the extraction, in millilitres;

V_2 is the volume of *0.02 M potassium permanganate* used for the blank titration, in millilitres;

m is weighed amount of herbal drug in grams;

W is the loss on drying for herbal drugs in grams.

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2.1.8.14. Bitterness Value

The requirements of this monograph are advisory.

The bitterness value is the reciprocal of the dilution of a substance, a liquid or an extract that still has a bitter taste. It is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200,000.

DETERMINATION OF THE CORRECTION FACTOR

A taste panel comprising at least six persons is recommended. The mouth must be rinsed with *water R* before tasting.

To correct for individual differences in tasting bitterness amongst the panel members it is necessary to determine an individual correction factor (for each person).

Stock solution. Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R.*

Reference solution. Prepare a series of dilutions by placing in a first tube 3.6 mL of the stock solution and increasing the volume by 0.2 mL in each subsequent tube to a total of 5.8 mL. Dilute the contents of each tube to 10.0 mL with *water R*.

Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 mL of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the individual correction factor *k* from the expression:

$$\frac{V}{5},$$

where: *V* is the volume of the stock solution in the dilution of the lowest concentration that is judged to be bitter, in millilitres.

Persons who are unable to taste any bitterness when using the reference solution prepared from 5.8 mL of stock solution have to be excluded from the panel.

SAMPLE PREPARATION

If necessary, reduce the sample to a powder. To 1.0 g of the test sample add 100 mL of boiling *water R*, heat on a water bath for 30 min, stirring continuously, allow to cool, and dilute to 100 mL with *water R*. Shake the mixture vigorously and filter, discarding the first 2 mL of the filtrate. The filtrate is labeled C-1 and has a dilution factor (DF) of 100.

DETERMINATION OF THE BITTERNESS VALUE

Test solutions:

10.0 mL of C-1 is diluted with water R to 100 mL: C-2 (DF = 1000)

10.0 mL of C-2 is diluted with water R to 100 mL: C-3 (DF = 10,000)

20.0 mL of C-3 is diluted with water R to 100 mL: C-3A (DF = 50,000)

10.0 mL of C-3 is diluted with water R to 100 mL: C-4 (DF = 100,000)

Starting with dilution C-4 each panel member determines the dilution which still has a bitter taste. This solution is designated D, and its DF is Y.

Starting with solution D prepare the following sequence of dilutions:

Solution D (mL)	1.2	1.5	2.0	3.0	6.0	8.0
Water R (mL)	8.8	8.5	8.0	7.0	4.0	2.0

Determine the number of millilitres of solution D which, when diluted to 10.0 mL with *water R*, still has a bitter taste (X).

Calculate the individual bitterness value from the expression:

$$\left(\frac{Y \cdot k}{X \cdot 0{,}1}\right).$$

Calculate the bitterness value of the test sample as the average value for all panel members.

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2.1.8.15. Dry Residue of Extracts

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, introduce rapidly 2.00 g or 2.0 mL of the extract to be examined, evaporate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h, allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage (*m/m*) or in grams per litre.

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2.1.8.16. Loss on Drying of Extracts

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 0.50 g of the extract to be examined, finely powdered; dry in an oven at 100-105 °C for 3 h, allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage (*m/m*).

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2.1.8.17. Microscopic and Microchemical Examination of Herbal Drugs

This general chapter sets out general requirements for the microscopic and microchemical examination of medicinal plant raw materials.

The technique for preparing microscopic preparations from medicinal plant raw materials depends on the morphological group of the object to be examined, as well as on the condition of the raw material — whole, crushed, cut, or powdered.

Chloral hydrate solution R is the most commonly prescribed reagent. However, certain features are not visible or not easily seen after mounting in this reagent. In this case, other reagents are used, for example, a 50% (*V/V*) solution of *glycerol R*, which makes it possible to visualise starch granules. It may also be necessary to prescribe specific reagents in a monograph, for example, *lactic reagent R* which is used to show the presence of various features (lignified elements, essential oils, resins, etc.), 10% (*V/V*) alcoholic solution of *phloroglucinol R* and *hydrochloric acid R*, which are used to identify the presence of lignin in cells or tissues, *ruthenium red solution R*, which is used to show the presence of mucilage in cells, *glycerol R* or *Lugol's solution R* used to show the presence of starch and inulin, solution of *Sudan III R* used to detect fatty and essential oils. In addition, examination under polarised light (between crossed nicol prisms) is used to identify starch granules (black cross phenomenon), calcium oxalate crystals (refringence), or lignified structures.

POWDERED HERBAL DRUGS

The preparation procedures given in this section are suitable for all morphological groups of powdered herbal drugs unless otherwise specified in the monograph.

Mounting in chloral hydrate solution

Place 2-3 drops of *chloral hydrate solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a coverslip. Heat the preparation very gently to boiling on a hot plate or a micro gas burner, maintaining gentle boiling for a short time and ensuring that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette, allow it to cool, and then examine under a microscope. Repeat the heating until the starch granules and the water-soluble contents of the cells are no longer visible. Examine under a microscope.

Chloral hydrate tends to crystallise as long as needles. To avoid this, proceed as follows: after heating, remove the coverslip; to the preparation add 1 drop of a 10% (*V/V*) mixture of *chloral hydrate solution R* in *glycerol R*; place a clean coverslip on the preparation and examine under a microscope.

Mounting in a 50% (*V/V*) solution of glycerol

Place 2 drops of a 50% (*V/V*) solution of *glycerol R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a coverslip, examine under a microscope.

Note: Clarify powdered herbal drugs having leathery leaves and stiff stems by boiling in a 5% (*m/V*) solution of *sodium hydroxide R*.

MICROCHEMICAL EXAMINATION

Mounting in a 10% (*V/V*) alcoholic solution of phloroglucinol and hydrochloric acid

Place a very small quantity of the powdered drug on a glass microscope slide. Add 1-2 drops of a 10% (*V/V*) alcoholic solution of *phloroglucinol R*.

Mix and allow the solvent to evaporate almost completely. Then add 1-2 drops of *hydrochloric acid R* and cover the preparation with a coverslip, and examine immediately under a microscope. The red colour indicates the presence of lignin.

Mounting in lactic reagent

On a glass microscope slide, apply 2-3 drops of *lactic reagent R.* Disperse a very small quantity of the powdered raw material in the liquid and cover the preparation with a coverslip. Heat the preparation very gently to boiling, maintaining gentle boiling for a short time and ensuring that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Lignified structures stain bright yellow; structures containing cellulose remain colourless. Starch granules stain more or less violet; certain secretions (e.g., essential oils, resins, oleoresins) stain orange and cork stains red.

Mounting in ruthenium red solution

Place 2 drops of *ruthenium red solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a coverslip. After about 1 minute, allow a drop of distilled *water R* to be taken up between the slide and the coverslip. Examine under a microscope. The mucilage stains violet red.

Preparation in Lugol solution

Place 2 drops of *Lugol solution R* on the slide. Distribute a small amount of powdered material in the liquid, close it with a cover glass, and examine under a microscope. Starch grains are coloured blue.

Preparation in black ink solution

Place 2 drops of *black ink solution R* on the slide. Distribute a small amount of powdered material in the liquid, close it with a cover glass, and immediately examine under a microscope (small magnification). The mucus is visible as colorless masses on a black background.

Preparation in Sudan III solution

Place 2-3 drops of *Sudan III solution R on the slide.* Distribute a small amount of powdered material in the liquid and close it with a cover glass, heat, and examine under a microscope. Drops of fat and essential oil are coloured orange-pink.

Preparation in a solution of β-naphthol (resorcinol or thymol)

Place about 0.1 g of powdered material on a slide, add 1-2 drops of *β-naphthol solution R (resorcinol R* or *thymol R*) and 1 drop of *sulfuric acid R*, and examine under a microscope. Inulin is coloured reddish-violet, or, when using resorcinol and thymol, orange-red. The presence of inulin is judged only in the absence of starch.

LEAVES, HERBS, FLOWERS

*Whole and cut materials***.** In the study of whole materials, pieces of a leaf plate with an edge and a vein are used; in herbs – a leaf, sometimes a piece of a stem, and a flower; in flowers – a calyx and a corolla are examined separately. In the study of cut materials, several different pieces are used, presumably related to the above-mentioned organs.

Clarification of the preparation can be carried out in two ways:

- place pieces of raw materials in a test tube, add a 5% (*m/V*) solution of *sodium hydroxide R* diluted with *water R* (1:1), and boil for 1-2 min. Then pour the contents into a silica crucible, drain the liquid, wash the pieces of the material thoroughly with *water R*, and allow to stand in *water R*. Remove the test material from the *water R* using a dissecting needle or spatula and place it on a slide in a drop of *chloral hydrate solution R* or *glycerol R*;

- boil pieces of the material in a solution of *chloral hydrate R* diluted with *water R* (1:1) for 5-10 min (until clarified), then place on a slide in a drop of *chloral hydrate solution R* or *glycerol R*, divide with a scalpel or a dissecting needle into two parts, carefully turn over one of them. Close the object with a cover glass, gently heat until air bubbles are removed, and after cooling, examine it from both sides under a microscope, first at low, then at high magnification. When preparing micro-preparations from thick leaves, crush them previously using a scalpel.

To study stems, boil their cuts in 5% (*m/V*) solution of *sodium hydroxide R*, thoroughly wash with *water R*, remove the epidermis using a scalpel or dissecting needles, and examine it from the surface; make a preparation from the remaining tissues, crushing the object with a scalpel on a slide in *chloral hydrate solution R* or *glycerol R.*

To prepare cross-sections of leaves and stems, after boiling in *chloral hydrate solution R* for 10 min, make sections by clamping pieces of the material in a cork or elder pith. Wash the ready sections with *water R* and prepare from them micro-preparations, placing in *chloral hydrate solution R.*

FRUITS AND SEEDS

Whole raw materials. Prepare preparations of the seed peel and pericarp from the surface or cross-sections.

Preparations of peel and pericarp from the surface. Boil 2-3 seeds or fruits in a test tube in a 5% solution of *sodium hydroxide R* for 2-3 min and thoroughly wash with *water R*. Place the object on a slide, separate the seed peel or pericarp tissue using dissecting needles, and examine in a solution of *chloral hydrate R* or *glycerol R.*

Sections. To prepare sections, previously soften dry fruits and seeds by placing them in a wet chamber for a day. The wet chamber is a desiccator with *water R*, to which a few drops of *chloroform R* are added. Depending on the hardness of the object, softening can be carried out with water vapour for 15-30 min or more.

Seal small fruits and seeds in a paraffin block measuring 0.5 cm × 0.5 cm × 1.5 cm. To do this, melt the paraffin with the tip of a heated dissecting needle and quickly immerse the object in the resulting hole (the object's surface must be dry). Make sections of the object together with paraffin and prepare micro-objects in a solution of *glycerol R* or *chloral hydrate R.*

To determine starch, fat and essential oils, or mucus, carry out additional microchemical studies of the powdered material as specified under *Microchemical examination.* If necessary, the powdered herbal drugs (fruits, seeds) are degreased and clarified.

BARK

Whole and cut materials. Boil pieces of bark measuring 2-3 cm × 0.5-1 cm in a test tube with *water R* for 5 min, then align them with a scalpel so that they have a strictly cross- or longitudinal section. Make sections and prepare micro-objects in a solution of *chloral hydrate R* or *glycerol R.*

To detect *lignified elements*, add a few drops of a solution of *phloroglucinol R* and 1 drop of a 25% solution of *sulfuric acid R* to the section on the slide. After 1 min, remove the liquid with a strip of filter paper, enclose the section in a *chloral hydrate solution R* or *glycerol R*, and close with a cover glass (examine without heating); lignified mechanical elements are coloured crimson-red. In addition, a solution of safranin may be used. Introduce the sections in a 10 g/L solution of safranin in *50% alcohol R* for 30 min (in a weighing bottle or on a watch glass), wash first with *50% alcohol R*, then with acidified *96% alcohol R;* add 2 drops of concentrated *hydrochloric acid R* per 100 mL of *96% alcohol R* and place on a slide in *glycerol R*; lignified shells are coloured red.

To detect *starch*, scrape off the dry bark and examine it in *Lugol solution R*; starch grains are coloured blue. Before use, dilute the solution with *water R* in the ratio (1:4). Protect the solution from light.

To establish the presence of tannins on the inner surface of the dry bark, apply 1 drop of 1% (*m/V*) solution of *ferric ammonium sulfate R* or 3% *(m/V*) solution of *ferric chloride R*; a black - blue or black-green colour develops.

Determine anthracene derivatives by applying 1-2 drops of a solution of *sodium hydroxide R* to the inner surface of the bark; a blood-red colour develops.

Carry out the microchemical analysis of powdered herbal drugs as prescribed in the *Microchemical examination* section.

ROOTS, RHIZOMES, TUBERS, BULBS, CORMS

Whole raw materials. Prepare cross and longitudinal sections. Place small pieces of underground organs in cold water and allow to stand for about a day, then place in a mixture of *96% alcohol R* and *glycerol R* (1:1) for 3 days. Align wet objects with a scalpel so that they have a strictly cross or longitudinal section. Make sections and prepare micro-objects in *chloral hydrate solution R* or *glycerol R* and examine diagnostic signs first at small, then at high magnification.

Cut or crushed materials. Boil pieces of underground organs for 3-5 min in a 5% (*m/V*) solution of *sodium hydroxide R*, thoroughly wash with *water R*, and prepare micro-objects, crushing the pieces in *chloral hydrate solution R* or *glycerol R*.

Determine the presence of lignified elements, starch, tannins, anthracene derivatives as prescribed in the *Bark* section.

Perform microchemical reactions with a scraping of dry underground organs or powdered materials to detect mucus, fat and essential oil, and inulin as prescribed in the *Microchemical examination* section.

FLUORESCENCE (LUMINESCENT) MICROSCOPY

The method of luminescent microscopy is used (where appropriate) to identify herbal drugs.

The advantage of this method is that it can be used to study dried herbal drugs, from which thick sections or powder preparations are prepared and primary (own) fluorescence is observed. Fluorescent microscopy is performed using a fluorescent microscope, equipped with special fluorescent lights.

Preparation of micro-objects

To prepare slides, use dried or powdered herbal drugs. Previously soaking of raw materials is excluded, as this leads to leaching of substances from the cells; only a short softening in a wet chamber is allowed.

Leaves. Usually, prepare preparations from leaf powder, which are viewed without inert liquid. The brightest luminescence is typical for lignified elements (vein, vessels, mechanical fibers), as well as cuticles and cutinised membranes of various epidermal formations (hairs, glands, etc.). The epidermal cells often contain flavonoids that cause brown, yellow, or greenish-yellow luminescence. Mesophyll cells, depending on their chemical composition, contain various inclusions — yellow, blue, greenish-yellow, brown. Chlorophyll and calcium oxalate crystals in dried herbal drugs do not luminesce. If it is necessary to prepare a section, the sheet is previously softened in a wet chamber and a thick section (2-3 mm) is made with a razor. The thinner sections are placed in an immersion liquid and closed with a cover glass.

Use *water R*, *glycerol R*, a 50 g/L solution of *polyvinyl alcohol R*, non-fluorescent *liquid paraffin R* as the immersion liquid. The immersion liquid should not dissolve the luminescent substances contained in the preparation.

Herbs. When analysing herbs, prepare micro-objects of leaves. If stem preparation is necessary, soften it in a wet chamber and make sections. Thick sections (2-3 mm) are fixed on a slide and examined without immersion liquid; thin sections are placed in a suitable liquid and closed with a cover glass.

Lignified elements of vascular bundles (vessels and mechanical fibers) and sclerenchyma cells found in the bark and core of the stem have the brightest luminescence. In the cells of the epidermis and cortex, flavonoids are often found; in some types of raw materials, the cells of the lining around the vascular bundles contain alkaloids, which, depending on the composition, have a diverse glow: blue, light blue, green, greenish-yellow, golden-yellow, orange-red.

Flowers. More often, preparations are prepared from the powder of flowers or individual parts of the flower (inflorescence), which are usually examined without immersion liquid. Flowers often contain flavonoids, carotenoids, and other substances that have fluorescence. Pollen grains are clearly visible, having a yellow, greenish-yellow, or bluish glow.

Fruits. Usually, prepare cross-sections of the fruit after preliminary softening in a wet chamber and view in the inert liquid or without it, depending on the thickness of the section. The fruits are characterised by the luminescence of the pericarp tissues (exocarp, mechanical mesocarp cells, vascular bundles). Secretory channels are clearly visible: their contents glow brightly; the cells of the lining layer usually have a yellowish-brown luminescence. The contents of the channels often show bright luminescent crystal inclusions, most often yellow or yellow-green.

Seeds. Usually, prepare cross-sections of the seed after preliminary softening in a wet chamber and view in the inert liquid or without it, depending on the thickness of the section. Pay attention to the nature of the luminescence of the seed peel, in which sclerenchyma layers are clearly distinguished. The cells of the epidermis that contain mucus usually have a blue or light blue glow. The endosperm and embryo tissues rich in fatty oil are characterised by blue luminescence.

Bark. Pre-soften the bark in a wet chamber, prepare thick cross-sections (up to 3-5 mm), fix them on a slide, and examine without immersion liquid; thin sections are enclosed in liquid.

For some types of raw materials, the luminescence of the cork layer of the cortex is characteristic: the shells of cork cells have an intense blue glow, their contents are dark red (anthocyanins). Mechanical elements (bast fibers and stony cells) have a bright and varied glow: blue, greenish-blue, yellowish-green. The luminescence of the cortical parenchyma depends on the chemical composition. Anthracene derivatives cause a bright orange or reddish-orange glow. Tannins have the ability to "extinguish" luminescence, so tissues containing tannins are dark brown, almost black.

A slide prepared from bark powder or scraping is examined without immersion liquid. Mechanical elements are most clearly visible there.

Roots, rhizomes, bulbs, tubers, corms. Prepare cross-sections, saws, powder, or scraping preparations. Prepare sections from the material previously softened in a wet chamber, make cuts of dry material (from thick roots and rhizomes) using a thin saw or cutter. Using a razor, remove a thin layer from the cut surface to remove the layer of cells covered with dust. Fix thick sections and cuts (up to 3-5 mm) on a slide and examine without immersion liquid. The cork layer of underground organs is usually dull, almost black. Wood (roots and rhizomes) and vascular bundles, as well as sclerenchyma elements, have bright luminescence. Their glow is very diverse: from brownish-green, yellow-green to light blue and intense blue, depending on the type of raw material. Even more diverse is the luminescence of parenchymal tissues and various secretory formations (conceptacles, channels, passages, milk vessels, various idioblasts), which is defined by their chemical composition. In secretory formations, there are crystalline inclusions of coumarins, alkaloids, and flavonoids with bright luminescence.

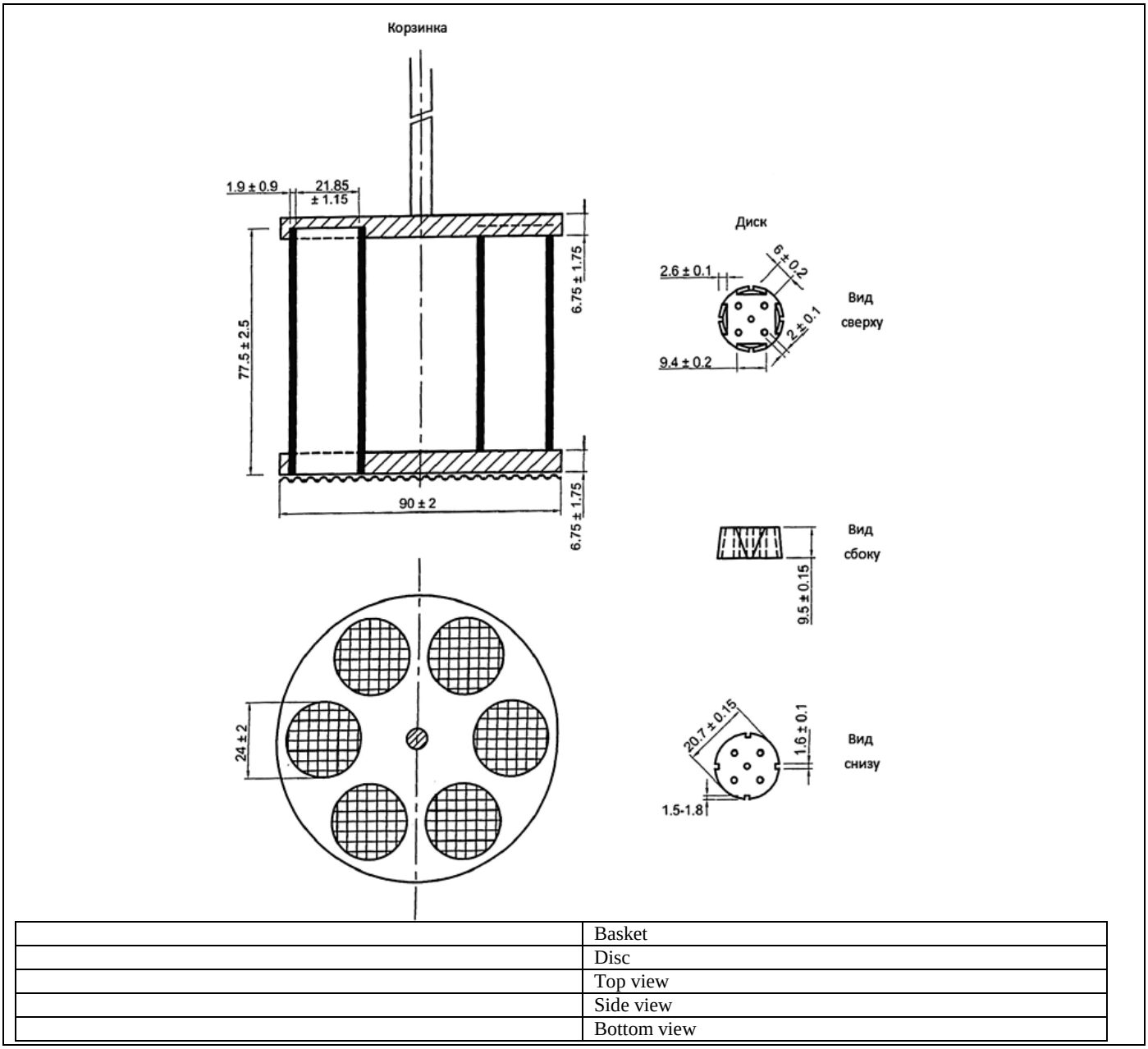
In powder preparations, individual vessels, groups of mechanical fibers, stony cells, individual secretory formations and their fragments, and brightly luminescent parenchyma cells containing certain substances are visible.

2.1.9.1. Disintegration of Tablets and Capsules

This test is provided to determine whether tablets or capsules, excluding vaginal tablets, rectal and vaginal capsules, disintegrate within the prescribed time when placed in a liquid medium under the experimental conditions presented below or in the monograph.

A sample is disintegrated when there is no residue of the unit, except fragments of an insoluble coating or capsule shell, remaining on the screen or adhering to the lower surface of the discs, if used, or a residue is a soft mass that collapses with a light touch of a glass rod. The presence of such a residue should be specified in the monograph.

Use apparatus A for tablets and capsules that are not greater than 18 mm long. For larger tablets or capsules use apparatus B.



	Basket
	Disc
	Top view
	Side view
	Bottom view

Figure 2.1.9.1.-1. – *Configuration and dimensions of the components of Apparatus A for determining the disintegration of tablets and capsules. Dimensions are shown in millimetres*

Test A for tablets and capsules not exceeding 18 mm

Apparatus. The Apparatus A (see Figure 2.1.9.1.-1) consists of a basket-rack assembly, a 1 L, low-form beaker for the immersion fluid, a thermostatic arrangement for heating the fluid in the range of (37 ± 2) °C, and an electromechanical device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute, through a distance of 53-57 mm in the vertical plane.

The main part of the apparatus is the basket-rack assembly with 6 cylindrical glass tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 21.85 ± 1.15 mm and a wall 1.9 ± 0.9 mm thick. The tubes are held in a vertical position by two overhead plastic plates, each 90 ± 2 mm in diameter and 6.75 ± 1.75 mm in thickness, with 6 holes, each 24 ± 2 mm in diameter. The holes are equidistant from the center of the plate and equally spaced from one another. Attached to the undersurface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 2.0 ± 0.2 mm mesh apertures and with a wire diameter of 0.615 ± 0.045 mm. The parts of the apparatus are assembled and rigidly held by metal vertical bolts passing through the two plates. Another metal rod is attached to the center of the upper plate which provided to suspend the basket-rack assembly from the raising and lowering device. The time required for the upward stroke is equal to the time required for the downward stroke; the change in stroke direction is a smooth transition.

The basket moves vertically along the axis. There should be no noticeable displacement of the axis in the horizontal plane.

The design of the apparatus provides for the use of discs. Each tube is provided with a cylindrical disc 9.5 ± 0.15 mm thick and 20.7 ± 0.15 mm in diameter, made of a transparent plastic material having a density of 1.18-1.20 g/cm^3. 5 parallel 2 ± 0.1 mm holes extend between the ends of the cylinder; one of the holes is centered on the cylindrical axis, the other holes are evenly around a circle with a radius of 6.0 ± 0.2 mm from the center of the disc. 4 identical trapezoidal-shaped symmetrical planes are cut into the wall of the cylinder, nearly perpendicular to the upper and lower surfaces of the cylinder. Parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes arranged in a circle. The length of the parallel side of the trapezoid on the lower surface of the disc is 1.5 to 1.8 mm, the plane is square. The parallel side of the trapezoid on the top of the cylinder has a length of 9.4 ± 0.2 mm and its center lies at a depth of 2.6 ± 0.1 mm from the cylinder's circumference. All surfaces of the disc are smooth.

The use of discs is specified in the general or individual monograph.

The basket is placed in a beaker, 149 ± 11 mm in height, and having an internal diameter of 106 ± 9 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid, and descends to not less than 25 mm from the bottom of the vessel on the downward stroke.

The design of the basket can be changed if the above requirements for glass tubes and wire mesh are met.

Procedure. 18 samples of tablets (or capsules) are selected for the test unless otherwise specified in the general or individual monograph. Place 1 sample in each of the 6 tubes and, if prescribed, add a disc. Immerse the basket into the vessel with the fluid specified in the general or individual monograph and operate the apparatus. At the end of the specified time, lift the basket from the fluid, and observe the conditions of tablets and capsules. All of the samples must be disintegrated completely. If 1 or 2 samples fail to disintegrate, repeat the test on 12 additional samples. Not less than 16 of the 18 samples tested must be disintegrated.

Test B for tablets and capsules larger than 18 mm

Apparatus. The disintegration Apparatus B (Figure 2.1.9.1.-2.) consists of a rigid basket-rack assembly, a 1 L glass vessel for a fluid, a thermostatic arrangement for heating the fluid in the range of (37 ± 2) °C, and an electromechanical device for raising and lowering the basket at a constant frequency rate between 29 and 32 cycles per minute, through a distance of 53-57 mm in the vertical plane.

The main part of the apparatus is a rigid basket-rack assembly supporting 3 cylindrical transparent tubes 77.5 ± 2.5 mm long, 33.0 mm ± 0.5 mm in internal diameter, and with a wall thickness of 2.5 ± 0.5 mm. The tubes are held vertically by two separate and superimposed rigid plastic plates 97 mm in diameter and 9 mm thick, with 3 holes. The holes are equidistant from the center of the plate and equally spaced from one another.

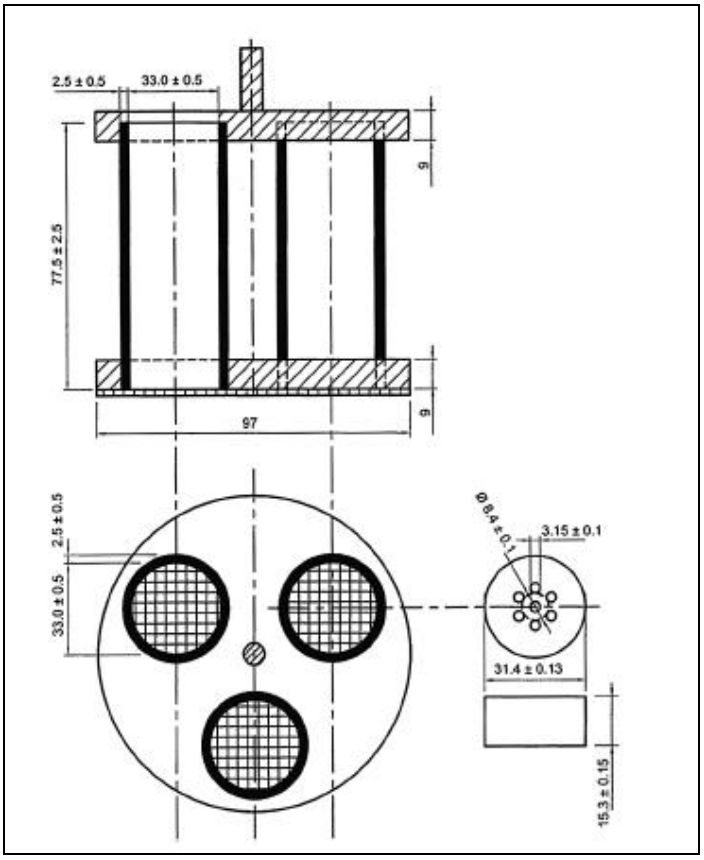


Figure 2.1.9.1.-2. – *Configuration and dimensions of the components of Apparatus B for determining the disintegration of tablets and capsules. Dimensions are shown in millimetres*

Attached to the underside of the lower plate is a piece of woven gauze made from stainless steel wire 0.63 ± 0.03 mm in diameter and having mesh apertures of 2.0 ± 0.2 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery. Another metal rod is attached to the center of the upper plate which provided to suspend the basket-rack assembly from the raising and lowering device. The time required for the upward stroke is equal to the time required for the downward stroke; the change in stroke direction is a smooth transition.

The basket moves vertically along the axis. There should be no noticeable displacement of the axis in the horizontal plane.

The design of the apparatus provides for the use of discs. Each tube is provided with a cylindrical disc 31.4 ± 0.13 mm in diameter and 15.3 ± 0.15 mm thick, made of transparent plastic with a density of 1.18-1.20 g/cm^3. Each disc is pierced by 7 holes, each 3.15 ± 0.1 mm in diameter, 1 in the center, and the other 6 spaced equally on a circle of radius 4.2 mm from the center of the disc. The discs correspond to the dimensions shown in Figure 2.1.9.1.-1.

The use of discs is specified in the general or individual monograph.

The use of automatic detection employing modified discs is permitted where the use of discs is specified or allowed. Such discs must meet the requirements of density and dimension given in this chapter.

Place the basket in the beaker. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid, and descends to not less than 25 mm from the bottom of the vessel on the downward stroke.

The design of the basket can be changed if the above requirements for glass tubes and wire mesh are met.

Procedure. Test 6 tablets (or capsules) either by using two basket-rack assemblies in parallel or by repeating the procedure. In each tube, place one sample and, if prescribed, add a disc. Immerse the basket into the vessel with the fluid specified in the general or individual monograph and operate the apparatus. At the end of the specified time, lift the basket from the fluid, and observe the conditions of tablets and capsules. All of the samples must be disintegrated completely.

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2.1.9.2. Disintegration of Suppositories and Vaginal Tablets, Rectal and Vaginal Capsules

The test determines whether the suppositories, vaginal tablets, rectal and vaginal capsules soften or disintegrate within the prescribed time when placed in a liquid medium in the experimental conditions described below, as well as in the monograph or corresponding general chapters.

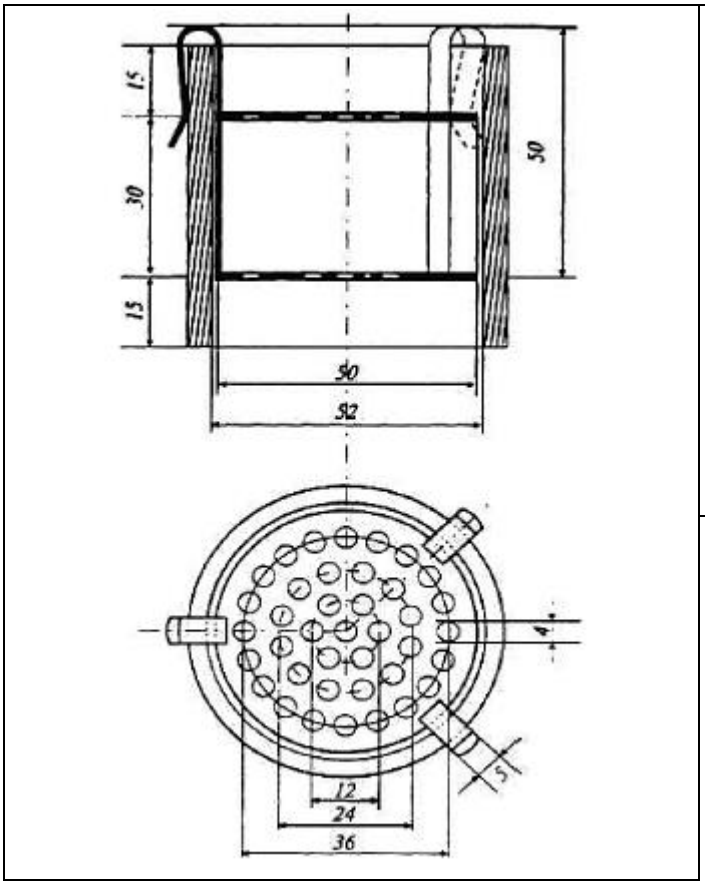

Figure 2.1.9.2.-1. – *Apparatus for disintegration of suppositories. Dimensions are shown in millimetres*

Apparatus. The apparatus (Figure 2.1.9.2.-1) consists of a sleeve of glass or suitable transparent plastic, of appropriate thickness, to the interior of which is attached by means of three hooks a metal device. The device consists of two perforated stainless metal discs that are about 30 mm apart. The diameter of the discs is similar to that of the interior of the sleeve, and each disc contains 39 holes 4 mm in diameter.

The test is carried out using three such apparatuses each containing a single sample. Each apparatus is placed in a beaker with a capacity of at least 4 L filled with a fluid medium.

Use water maintained at (36.5±0.5) °C as a fluid medium unless otherwise prescribed in the monograph.

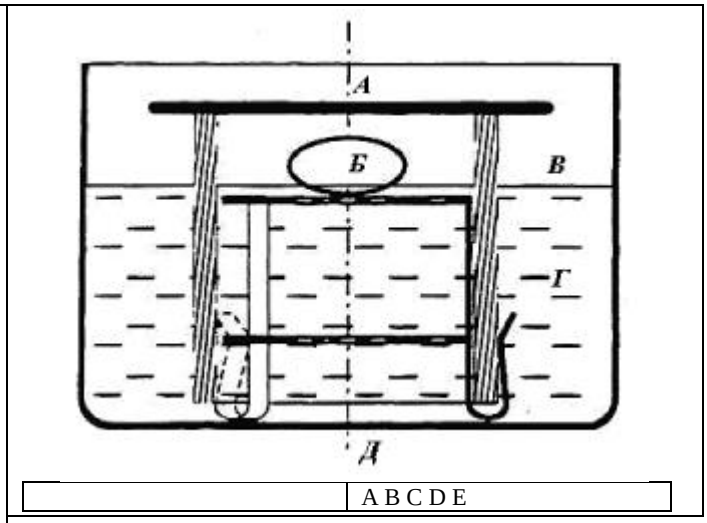

Figure 2.1.9.2.-2. – *Apparatus for disintegration of vaginal tablets, rectal and vaginal capsules. A – glass plate; B – vaginal tablet; C – water surface; D –water; E –dish, beaker*

The beaker is fitted with a slow stirrer and a device that will hold the cylinders vertically not less than 90 mm below the surface of the water and allow them to be inverted 180° without emerging from the water.

Three apparatuses may also be placed together in one vessel with a capacity of at least 12 L.

Test procedure for suppositories. Use three suppositories for the test. Place each sample on the lower disc of a device, install the device in the cylinder of the device, and fix it. Place the device in a vessel with a liquid medium and start the test. Invert the apparatuses every 10 min.

Examine the samples to be analysed after the period prescribed in the monograph.

The drug passes the test if all the samples have disintegrated.

Test procedure for vaginal tablets, rectal and vaginal capsules. Use the apparatus described above, arranged so as to rest on the hooks (see Figure 2.1.9.2.-2). Place it in a beaker of a suitable diameter containing a liquid medium with the level just below the upper perforated disc. Using a pipette, adjust the level with the prescribed fluid medium until a uniform film covers the perforations of the disc.

Use three vaginal tablets or three rectal or vaginal capsules for the test. Place each tablet or capsule on the upper plate of an apparatus and cover the latter with a glass plate to maintain appropriate conditions of humidity.

Examine the samples to be analysed after the period prescribed in the monograph.

The drug passes the test if all the samples have disintegrated.

Interpretation of results. Samples are considered to be disintegrated when:

a) the samples are completely dissolved;

b) the components of the suppository have separated: melted fatty substances collect on the surface of the liquid, insoluble powders fall to the bottom and soluble components dissolve, depending on the type of preparation, the components may be distributed in one or more of these ways;

c) there is softening of the sample that may be accompanied by an appreciable change of shape without complete separation of the components, the softening is such that the suppository or pessary no longer has a solid core offering resistance to the pressure of a glass rod;

d) no residue remains on the perforated disc or if a residue remains, it consists only of a soft or frothy mass having no solid core offering resistance to the pressure of a glass rod (vaginal tablets);

e) rupture of the gelatin shell of rectal or vaginal capsules occurs allowing the release of the contents.

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2.1.9.3. Dissolution Test for Solid Dosage Forms

The "Dissolution" test is intended to determine the amount of active substance that should be released into the dissolution medium from a solid dosage form over a certain period of time under the conditions specified below, as well as in the monograph and the corresponding chapters.

The "Dissolution" test is performed during quality control of solid dosage forms in order to confirm the constancy of their properties and the proper conditions of the manufacturing process.

Depending on the rate of release of active substances, all solid dosage forms (tablets, granules, the dissolution time of which exceeds 5 min, and capsules) are divided into groups:

Group 1: tablets; coated tablets; granules (the dissolution time of which exceeds 5 min); coated granules; capsules (conventional-release tablets, capsules, granules);

Group 2: delayed-release tablets, capsules, and granules;

Group 3: prolonged-release tablets, capsules, and granules.

In this section, the dosage unit is understood as one tablet, or one capsule, or the specified number of granules.

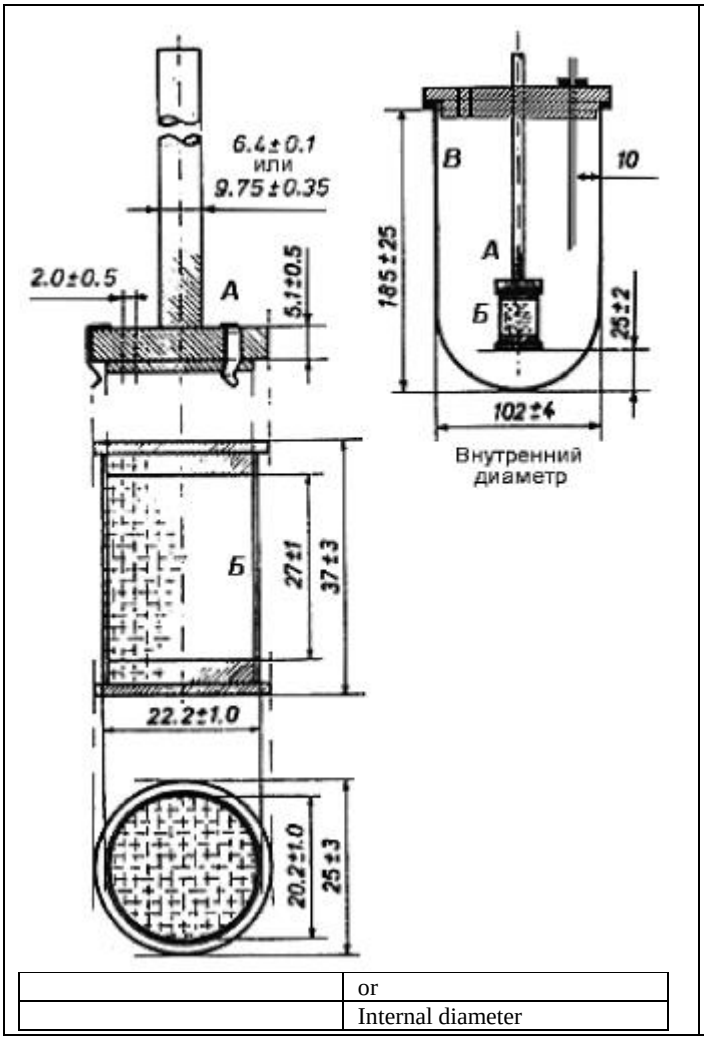
Apparatus 1 (Basket apparatus). The apparatus (see Figure 2.1.9.3.-1) consists of:

- a cylindrical dissolution vessel (*B*) with a hemispherical bottom made of borosilicate glass or other suitable transparent inert material. The nominal capacity of the dissolution vessel is 1000 mL; height from 160 to 210 mm; internal diameter between 98 and 106 mm;

- a motor with a speed-regulating device maintaining the basket rotation speed within ± 4% of that in the monograph;

- a stirring element which consists of a vertical shaft (*A*), to the bottom of which a cylindrical basket is attached (*B);* The axis of rotation of the shaft must not deviate from the vertical axis of the vessel by greater than 2 mm. The rotation of the shaft should be smooth, without significant wobbles.

The basket consists of two parts: the upper part, which has a hole with a diameter of 2.0 ± 0.5 mm, must be welded to the shaft and equipped with 3 elastic clips or another suitable appliance that allows removing the lower part of the basket for the introduction of the drug to be examined.



	or
	Internal diameter

Figure 2.1.9.3.-1. – *Apparatus 1, Basket stirring element (dimensions in millimetres)*

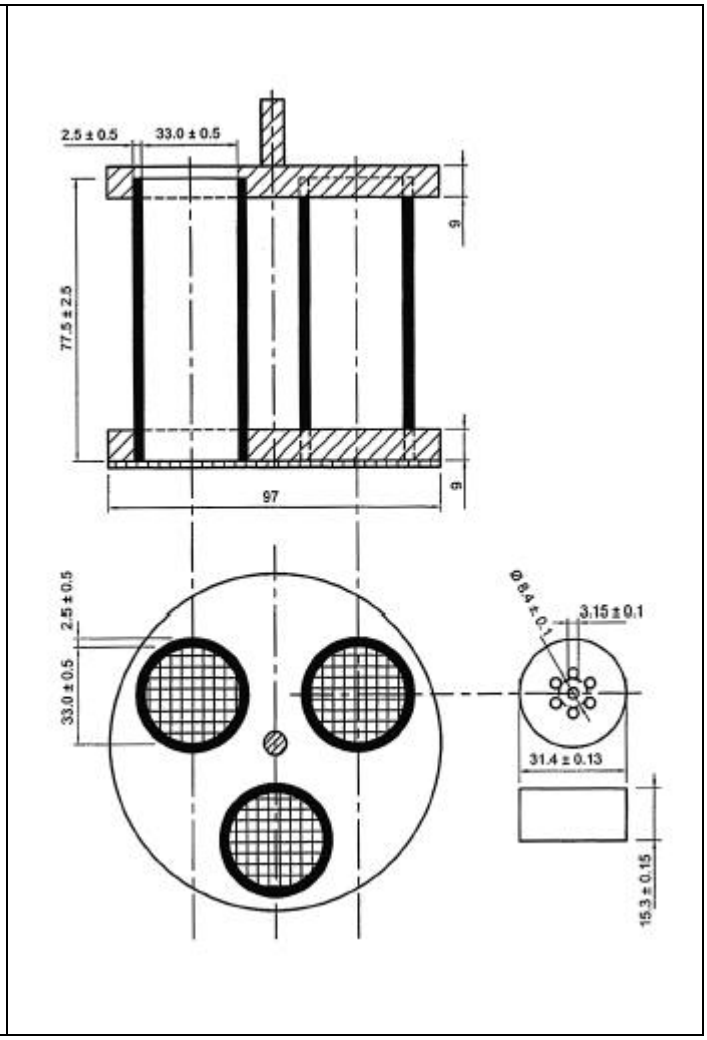


Figure 2.1.9.3.-2. – *Apparatus 2, Paddle stirring element (dimensions in millimetres)*

The removable part of the basket is made of straight-welded metal wire mesh, in which a wire with a diameter of 0.22-0.31 mm forms holes with a size of 0.36 - 0.44 mm. The mesh has the shape of a cylinder and is bounded by a metal frame at the top and bottom.

When using aggressive acidic solutions, a basket having a gold coating of about 2.5 μm thick may be used.

The distance between the bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm.

To prevent evaporation of the dissolution medium, the dissolution vessels should be closed with caps with a central hole for passing the basket axis, as well as with holes for the thermometer and sampling.

The water bath with a constant volume of the temperature-controlled liquid permits maintaining the temperature of the dissolution medium at (37 ± 0.5) °C during the test.

No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. Apparatus that permits observation of the preparation and stirring element during the test is preferable.

Apparatus 2 (Paddle apparatus). Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not greater than 2 mm from the vertical axis of the vessel, at any point, and rotates smoothly without significant wobble that could affect the results. The vertical centerline of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft.

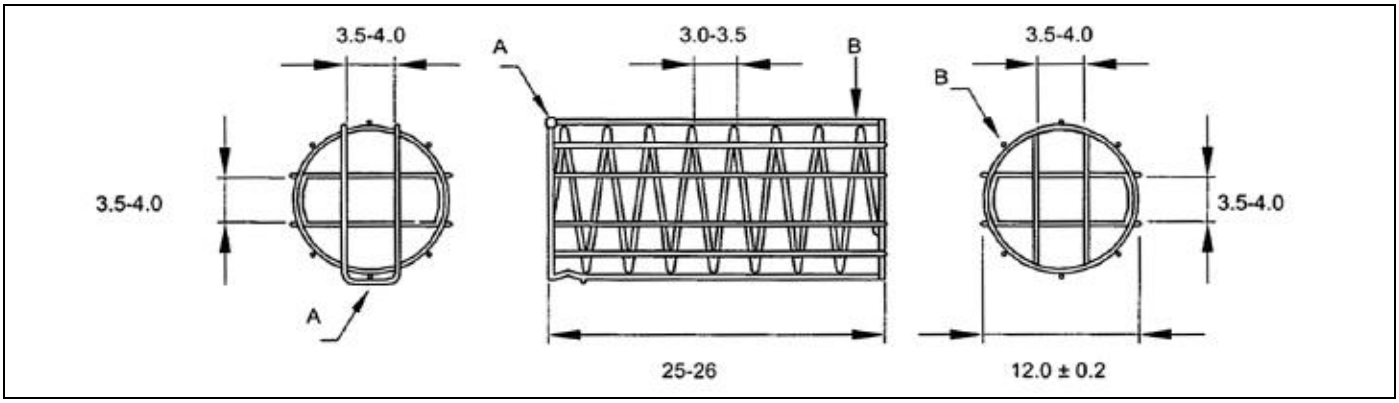


Figure 2.1.9.3.-3. – *Alternative sinker (dimensions in millimetres). A – acid-resistant wire clasp; B – acid-resistant wire support*

The paddle conforms to the specifications shown in Figure 2.1.9.3.-2. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test.

The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before the rotation of the blade is started. A small, loose piece of non-reactive material, such as not greater than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Figure 2.1.9.3.-3. Other validated sinker devices may be used.

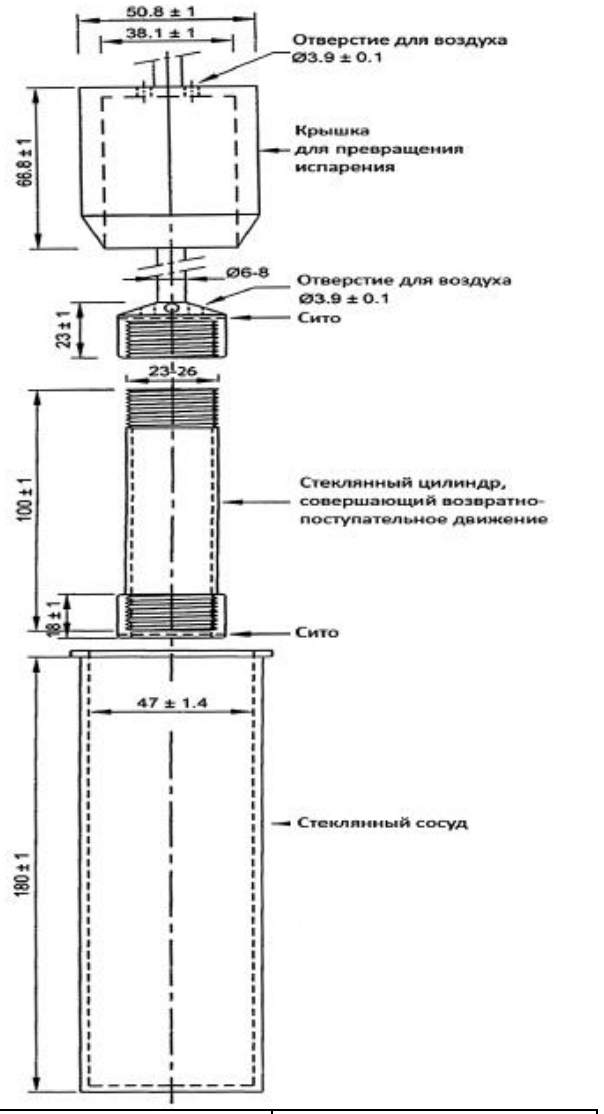
The difference in size between A and B should not exceed 0.5 mm when rotating around the central axis. Deviations may be ±1.0 mm unless otherwise indicated.

Apparatus 3 (Reciprocating cylinder). The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material) and screens that are made of suitable nonsorbing and nonreactive material, and that are designed to fit the tops and bottoms of the reciprocating cylinders; a motor and drive assembly to reciprocate the cylinders vertically inside the vessels, and if desired, index the reciprocating cylinders horizontally to a different row of vessels.

The vessels are partially immersed in a suitable water-bath of any convenient size that permits holding the temperature at 37 ± 0.5 °C during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation speed to be selected and maintained at the specified dip rate, within ± 5%. An apparatus that permits observation of the preparations and reciprocating cylinders is preferable. During the test, the vessels are closed with a cap to prevent evaporation. The components meet the dimensions shown in Figure 2.1.9.3.-4 unless otherwise specified in the monograph.

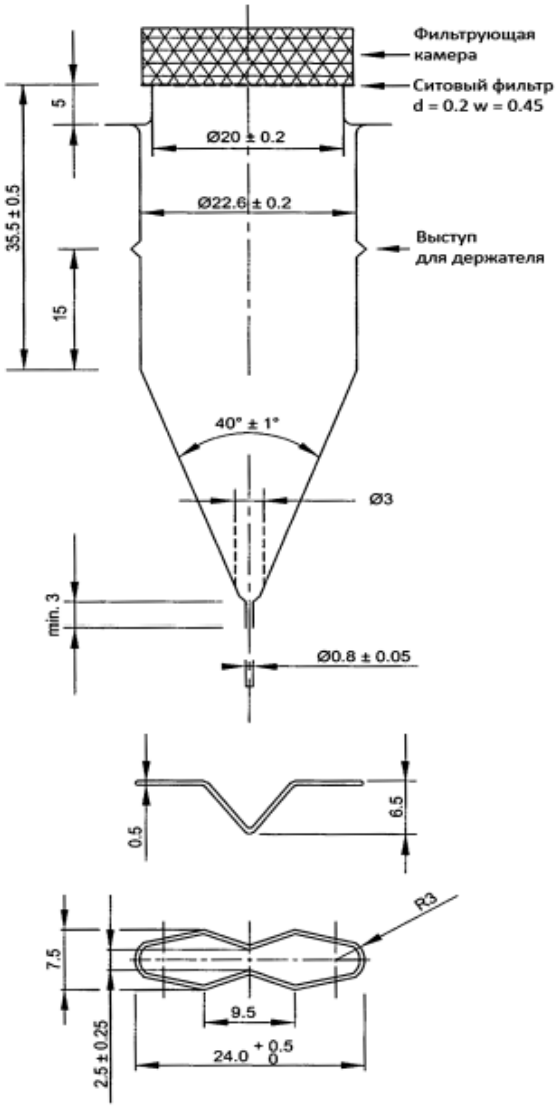
Apparatus 4 (Flow-through cell). The assembly consists of a reservoir and a pump for the dissolution medium; a flow-through cell; a water-bath that maintains the dissolution medium at 37 ± 0.5 °C. Use the cell size specified in the monograph.

The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range between 240 mL/h and 960 mL/h, with standard flow rates of 4 mL/min, 8 mL/min, and 16 mL/min. It must deliver constant flow with an accuracy of ± 5% of the nominal flow rate; the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses/min. A pump without pulsation may also be used. Dissolution test procedures using the flow-through cell must be characterised with respect to rate and any pulsation.



	Vent hole
	evaporation cap
	Vent hole
	Sieve
	Glass reciprocating cylinder

Figure 2.1.9.3.-4. – *Apparatus 3, glass vessel and reciprocating cylinder (dimensions in millimetres unless otherwise specified)*

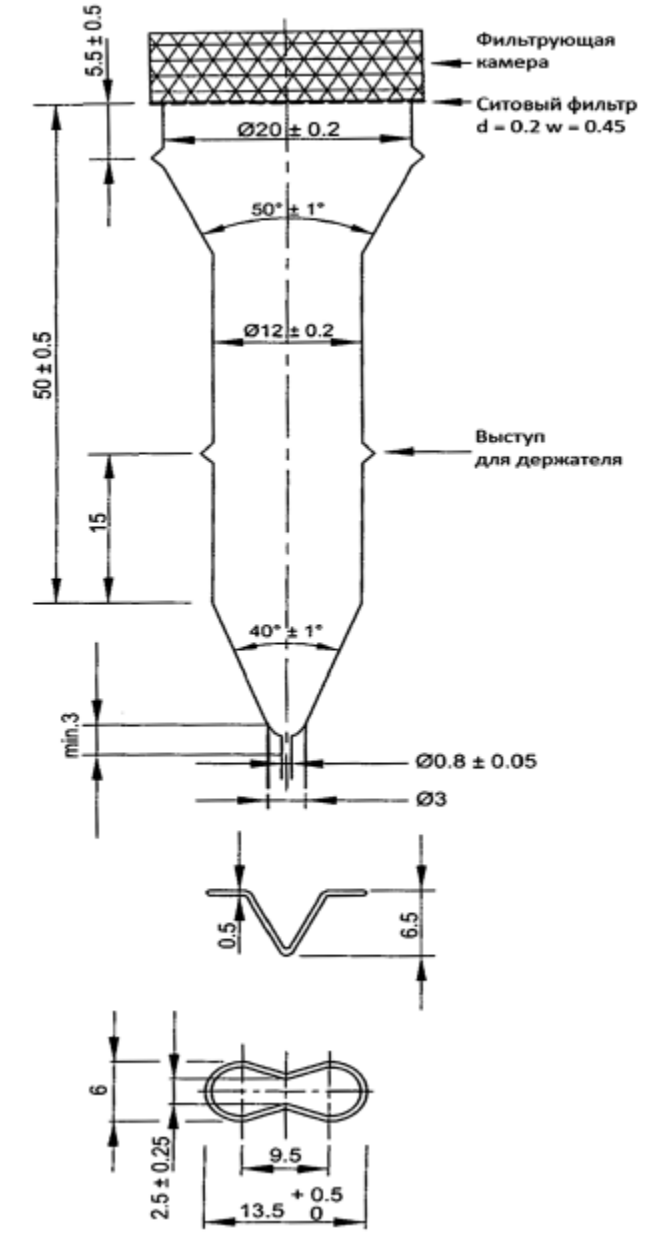


	Filter chamber
	Mesh screen
	Score for the holder

Figure 2.1.9.3.-5. – *Apparatus 4, large cell for tablets and capsules (top), tablet holder for the large cell (bottom) (dimensions in millimetres unless otherwise specified)*

The flow-through cell (see Figures 2.1.9.3.-5 and 2.1.9.3.-6) of transparent and inert material is mounted vertically, with a filter system that prevents the escape of undissolved particles from the top of the cell. Standard cell diameters are 12 mm and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1 mm diameter. One bead of about 5 mm in diameter positioned at the apex to protect the fluid entry tube. A tablet holder (see Figures 2.1.9.3.-5 and 2.1.9.3.-6) is available for positioning of special dosage forms. The cell is immersed in a water bath, and the temperature is maintained at 37 ± 0.5 °C.

The apparatus uses a clamp mechanism and two O-rings for the fixation of the cell assembly. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump must not be on a level higher than the reservoir flasks.



	Filter chamber
	Mesh screen
	Score for the holder

Figure 2.1.9.3.-6. – *Apparatus 4, small cell for tablets and capsules (top), tablet holder for the small cell (bottom) (dimensions in millimetres unless otherwise specified)*

The connecting tubes should be as short as possible and made of a suitable inert material, such as polytetrafluoroethylene, with an internal diameter of 1.6 mm and inert flange tips.

Test for suitability of the apparatus. The test should include compliance with the dimensions and requirements above.

In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotational speed (Apparatus 1 and 2), sinking rate (Apparatus 3), and flow rate of the medium (Apparatus 4). Determine the acceptable performance of the dissolution test assembly periodically.

Sampling

Sampling is carried out from the area of the dissolution vessel located at a distance S between the surface of the dissolution medium and the upper part of the removable element of the basket or agitator blade and at a distance of at least 1 cm from the walls of the dissolution vessel.

The sampling time must be specified in the monograph and must be observed with an accuracy of ± 2%.

For *Group 1* preparations, unless otherwise specified in the monograph, the sampling time is 45 min after the start of the test.

For *Group 2* drugs, 2 separate normalised time intervals should be specified — for the acid stage and the alkaline stage.

For *Group 3* drugs, at least 3 time intervals must be specified.

After each sampling, the volume of the dissolution medium must be replaced by the same solvent in a volume equal to the volume of the aliquot taken. If preliminary studies show that the replenishment of the solution medium is not mandatory, the decrease in the solution medium should be taken into account when calculating the amount of the drug released into the solution medium.

Filter the solution aliquot taken from the solution medium through an inert filter, which should not absorb the active substance from the solution and should not contain substances that can be extracted by the solution medium. The pore size of the filter should not exceed 0.45 μm, unless otherwise specified in the monograph.

Centrifugation of the aliquote is allowed only if it is not possible to filtrate the sample and validate the sample preparation procedure.

The analytical method for the quantitation of the active substance in solution should be described in the monograph and validated in accordance with the established requirements.

If the capsule shell affects the results of the analysis, then determine the correction factor, for which the "Dissolution" test is performed on capsules used in the production of this dosage form that do not contain the active substance. The correction factor is taken into account when calculating the content of the active substance released in the dissolution medium. The correction factor should not exceed 25% of the label content of the active substance.

Test procedure

Conventional-release tablets, capsules, granules

Place the specified volume of the dissolution medium in the vessel of the dissolution apparatus (Apparatuses 1 and 2). Bring the temperature of the dissolution medium to (37 ± 0.5) °C.

When using Apparatus 1 (basket apparatus) unless otherwise specified in the monograph, place one dosage unit in each of 6 dry baskets of the apparatus. Lower the baskets into the dissolution medium and turn on the motor that rotates the stirring device.

When using Apparatus 2 (paddle apparatus), unless otherwise specified in the monograph, place one dosage unit directly in each of 6 vessels with the dissolution medium before the stirring device begins to rotate. To prevent the tablets and capsules from floating to the surface of the dissolution medium, the complete set of the apparatus provides an appropriate device. Care must be taken to avoid air bubbles settling on the surface of the tablet or capsule.

When using Apparatus 3 (reciprocating cylinder), place the specified volume of the dissolution medium (± 1%) in each vessel of the apparatus, assemble the apparatus, equilibrate the dissolution medium to (37 ± 0.5) °C, and remove the thermometer. Place 1 dosage unit in each of the reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit. Then immediately operate the apparatus as specified. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9-10.1 cm. Within the time interval specified, or at each of the times stated, raise the cylinders and withdraw a portion of the medium from a zone midway between the surface of the dissolution medium and the bottom of each vessel.

When using Apparatus 4 (Flow-through cell), place 1 glass bead of (5.0 ± 0.5) mm in diameter and then glass beads of a suitable size, usually (1.0 ± 0.1) mm (supplied with the instrument), at the bottom of the conical part of the flow-through cell to prevent the passage of liquid into the tube. Place the dosage unit in the cell or directly in the layer of glass beads unless otherwise specified in the monograph. Close the apparatus with a filter system.

After sampling, filter the test samples immediately unless otherwise prescribed. Use an inert filter that does not adsorb the active substance and does not contain extractable substances that can affect the results of the analysis.

Analyse the sample using a suitable quantitation method. If necessary, repeat the test on an additional number of dosage units.

Delayed-release tablets, capsules or granules

For *Group 2* solid dosage forms, one of the 2 alternative procedures of conducting the "Dissolution" test using Apparatuses 1 or 2 can be used.

A reference to the procedure and apparatus used is given in the monograph.

Procedure 1. Carry out the test in two stages.

Acid stage 1 Place 750 mL of 0.1 M *hydrochloric acid* in each of the 6 dissolution vessels unless otherwise specified in the monograph. Bring the temperature of the dissolution medium to (37 ± 0.5) °C. Place 1 dosage unit, unless otherwise specified in the monograph, in each of the 6 dissolution vessels, operate the motor of the stirring device. After 2 h, unless otherwise specified in the monograph, select the aliquot and immediately continue the process of dissolution in an alkaline medium, as described below.

Analyse the selected aliquot part of the solution according to the procedure described in the monograph. The test results at stage 1 are considered satisfactory if the amount of active substance transferred to the dissolution medium meets the criteria of the "Interpretation of results" section (see Table 2.1.9.3.-2).

Table 2.1.9.3.-1. – *Interpretation of the results of the "Dissolution" test for Group 1 solid dosage forms*

Level	Number of test units	Acceptance criteria
S_1	6	For each test unit: not less than Q + 5% of the nominal active substance content must be released into the dissolution medium
S_2	6	The average amount of active substance released into the dissolution medium from 12 dosage units under the test (S_1 + S_2) should be not less than Q and there should not be a single unit where less than Q–15% of the nominal content of the active substance passed into the dissolution medium
S_3	12	The average amount of active substance released into the medium from 24 test dosage units ($S1$ + S_2 + S_3) should be not less than Q; only 2 units can be less than Q–15%, and no unit should be less than Q–25% of the nominal content of the active substance

Table 2.1.9.3.-2. – *Interpretation of the results of the "Dissolution" test for Group 2 solid dosage forms.*

Level	Number of test units	Acceptance criteria
		Acid stage 1
A_1	6	For each test unit: no greater than 10% of the nominal active substance content should be released in the dissolution medium
A_2	6	The average amount of active substance released in the medium of dissolution from 12 test units (A_1 + A_2) should not be greater than 10% of the declared content of the active substance and there should not be a single unit where the amount of active substance released exceeds 25% of the nominal content
A_3	12	The average amount of active substance released into the dissolution medium from 24 test units (A_1 + A_2 + A_3) should not be greater than 10% of the declared content of the active substance and there should not be a single unit where the amount of active substance released exceeds 25% of the nominal content
		Buffer stage 2
B_1	6	For each test unit: not less than Q + 5% of the nominal active substance content must be released into the dissolution medium
B_2	6	The average amount of active substance released in the dissolution medium from 12 test units (B_1 + B_2) should be at least Q and there should not be a single unit where less than Q– 15% of the nominal content of the active substance is released in the dissolution medium
B_3	12	The average amount of active substance released into the medium from 24 test units (B_1 + B_2 + B_3) must be not less than Q; only 2 units can be less than Q – 15%, and no unit should be less than Q – 25% of the nominal content of the active substance

Buffer stage 2. In each of the 6 dissolution vessels, add 250 mL of 0.2 M solution of *trisodium phosphate dodecahydrate R* ($Na_3PO_4 \cdot 12H_2O$), the temperature of which is (37 ± 0.5) °C (the stirring device of the apparatus works continuously), adjust the pH of the dissolution medium to 6.80 ± 0.05 with *2 M hydrochloric acid or 2 M sodium hydroxide solution.*

Continue the dissolution process for 45 min unless otherwise specified in the monograph, after sampling the solution, determine the content of the active substance in the solution according to the procedure described in the monograph. The test results at stage 2 are considered satisfactory if the amount of the active substance transferred to the dissolution medium meets the criteria of the "Interpretation of results" section (see Table 2.1.9.3.-2).

Note. The procedure for adding *0.2 M trisodium phosphate dodecahydrate* and adjusting the pH of the dissolution medium to the given value should be carried out for no greater than 5 min.

Procedure 2. Carry out the test in two stages.

Acid stage 1 Place 1000 mL of *0.1 M hydrochloric acid*, unless otherwise specified in the monograph, in each of the 6 vessels for dissolution. Adjust the temperature of the dissolution media to (37 ± 0,5) °C. Place a single dosage unit, unless otherwise specified in the monograph, in each of the 6 dissolution vessels, operate the motor of the stirring device. After 2 h, unless otherwise specified in the monograph, take an aliquot and immediately continue the process of dissolution under alkaline conditions, as described below.

Analyse the selected aliquot part of the solution according to the procedure described in the monograph.

The test results at stage 1 are considered satisfactory if the amount of active substance transferred to the dissolution medium meets the criteria of the "Interpretation of results" section (see Table 2.1.9.3.-2).

Buffer stage 2. Remove *0.1 M hydrochloric acid* from each dissolution vessel and introduce 1000 mL of phosphate buffer solution pH 6.8 (2) at 37 ± 0.5 °C.

It is permissible to transfer test solid dosage units from dissolution vessels containing *0.1 M hydrochloric acid* to dissolution vessels containing 1000 mL of phosphate buffer solution pH 6.8 (2) at 37 ± 0.5 °C.

Continue the dissolution process for 45 min unless otherwise prescribed in the monograph, then select an aliquot and immediately analyse according to the procedure described in the monograph. The test results at stage 2 are considered satisfactory if the amount of active substance released into the dissolution medium meets the criteria of the "Interpretation of results" section (see Table 2.1.9.3.-2).

Note. Preparation of phosphate buffer solution pH 6.8 (2). Mix *0.1 M hydrochloric acid* and *0.20 M solution of trisodium phosphate dodecahydrate R* ($Na_3PO_4 \cdot 12H_2O$) in a ratio of 3:1 (*V/V*) and, if necessary, adjust the pH of the resulting solution to 6.80 ± 0.05 with *2 M hydrochloric acid* or *2 M sodium hydroxide*.

When using Apparatus 3 (reciprocating cylinder), use Procedure 2.

For the acid stage, use one row of vessels, for the buffer stage, use the next row of vessels at the specified volume of the dissolution medium (usually 300 mL).

When using Apparatus 4 (flow-through cell), it is possible to use Procedures 1 and 2.

Prolonged-release tablets, capsules, granules

For *Group 3* solid dosage forms, the apparatus, test method, and analytical procedure for determining the active substance content in solution should be described in the monograph in accordance with the requirements specified above for conventional-release solid dosage forms.

Interpretation of results

Group 1. Conventional-release tablets, capsules, granules

Unless otherwise specified in the monograph, the amount of active substance released into the dissolution medium at 37 ± 0.5 °C for 45 min at a basket rotational speed of 100 rpm or a paddle blade rotational speed of 50 rpm must be at least 75% (*Q*) of the nominal content.

Quantity *Q* is the normalised amount of dissolved active substance, expressed as a percentage of the nominal content.

Perform the test on 6 dosage units. The test results are considered satisfactory if the amount of active substance released into the dissolution medium meets the criteria given in Table 2.1.9.3.-1, level *S*1.

If at least one result does not meet the criterion specified in Table 2.1.9.3.-1, level S_1, then the "Dissolution" test is repeated on another 6 solid dosage units. The results are interpreted according to Table 2.1.9.3.-1, level S_2.

If the results do not meet the established criteria during repeated testing, repeat the test on 12 additional units of the solid dosage form. The results are interpreted according to Table 2.1.9.3.-1, level S_3.

Unless otherwise prescribed in the monograph, reject the batch if the test results do not meet the established criteria at any stage of the examination.

Group 2. Delayed-release solid dosage forms.

The test is performed on 6 solid dosage units for each stage (acid and buffer).

The test results at each stage are considered satisfactory if the amount of active substance released into the dissolution medium meets the criteria given in Table 2.1.9.3.-2, level A1.

Unless otherwise specified in the monograph, the *Q* value is considered equal to 75%.

Table 2.1.9.3.-3. – *Interpretation of the results of the "Dissolution" test for group 3 solid dosage forms.*

Level	Number of test units	Acceptance criteria
L_1	6	There should be no test unit for which the amount of active substance released into the medium is outside the specified ranges and less than the value set for the final test time
L_2	6	The average amount of active substance released into the medium of dissolution from 12 test units ($L_1 + L_2$) must be within the established ranges and must be at least the value set for the final test time. No individual value shall be greater than 10% of the nominal content outside the specified ranges and greater than 10% of the nominal content below the value set for the final test time
L_3	12	The average amount of active substance released into the medium of dissolution from 24 test units ($L_1 + L_2 + L_3$) must be within the established ranges and must not be less than the value set for the final test time. For no greater than 2 out of 24 units, the amount of released substance may exceed the specified ranges by greater than 10% of the declared content and be greater than 10% of the nominal content below the value set for the final test time. For none of the units, the amount of released substance must be greater than 20% of the nominal content outside the specified ranges and greater than 20% of the nominal content below the value set for the final test time

If at least one result does not meet the standard specified in the monograph, the "Dissolution" test is repeated on another 6 solid dosage units. The results are interpreted according to Table 2.1.9.3.-2, level A_2.

If the results do not meet the established criteria during repeated testing, repeat the test on 12 additional units of the solid dosage form. The results are interpreted according to Table 2.1.9.3.-2, level A_3.

Unless otherwise prescribed in the monograph, reject the batch if the test results do not meet the established criteria at any stage of the examination.

Group 3. Prolonged-release solid dosage forms.

The test is performed on 6 solid dosage units. The test results are considered satisfactory if the amount of active substance released into the dissolution medium meets the criteria given in Table 2.1.9.3.-3, level L_1.

If at least one result does not meet the standard specified in the monograph, the "Dissolution" test is repeated on another 6 solid dosage units. The results are interpreted according to Table 2.1.9.3.-3, level L_2.

If the results do not meet the established criteria during repeated testing, repeat the test on 12 additional units of the solid dosage form. The results are interpreted according to Table 2.1.9.3.-3, level L_3.

If the test results do not meet the established criteria at any stage of the study, the batch is rejected.

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2.1.9.4. Dissolution Test for Transdermal Patches

This test is used to determine the dissolution rate of the active substance of transdermal patches under conditions specified below or in the individual monograph.

Dissolution of the active substance can occur as a result of its direct release from the transdermal patch into the dissolution medium, or as a result of its release into the dissolution medium through the polymer membrane (release rate).

The monograph indicates:

- type of apparatus;
- description of the transdermal patch holder;
- the contact area of the transdermal patch with the dissolution medium to determine the release rate of the active substance or the contact area of the transdermal patch with the polymer membrane with the dissolution medium to determine the delivery rate of the active substance;
- transdermal patch attachment method;
- for Apparatus 1 – the polymer membrane used (when determining the drug delivery rate from a transdermal patch);
- composition and volume of the dissolution medium;
- the rotational speed of the stirrer;
- sampling time;
- analytical method for quantitation of the active substance(s) released in the dissolution medium;
- acceptance criteria.

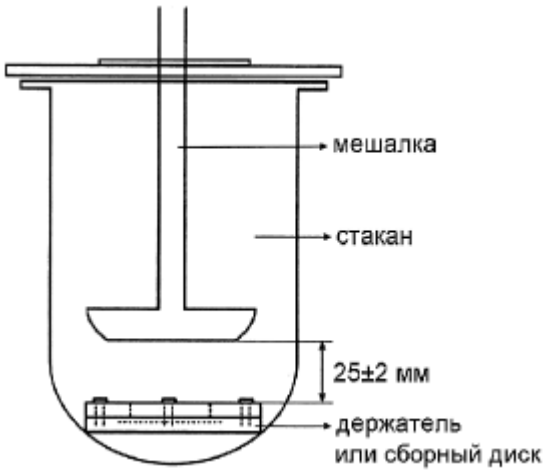
Apparatus

Use Apparatus 2 "Paddle apparatus" described in the chapter "Dissolution for solid dosage forms", which can be modified into three independent apparatuses by adding additional elements:

- Apparatus 1 – contains a transdermal patch holder;
- Apparatus 2 – equipped with a stainless steel disc for attaching a transdermal patch to its surface;
- Apparatus 3 – instead of a paddle blade, it contains a stainless steel cylinder.

Select the apparatus depending on the composition, size, and shape of the patch.

Apparatus 1. A transdermal patch holder (figure 2.1.9.4.-1) made of a chemically inert material is placed at the bottom of the dissolution vessel.



	stirrer
	beaker
	25±2 mm
	the holder or assembled disc

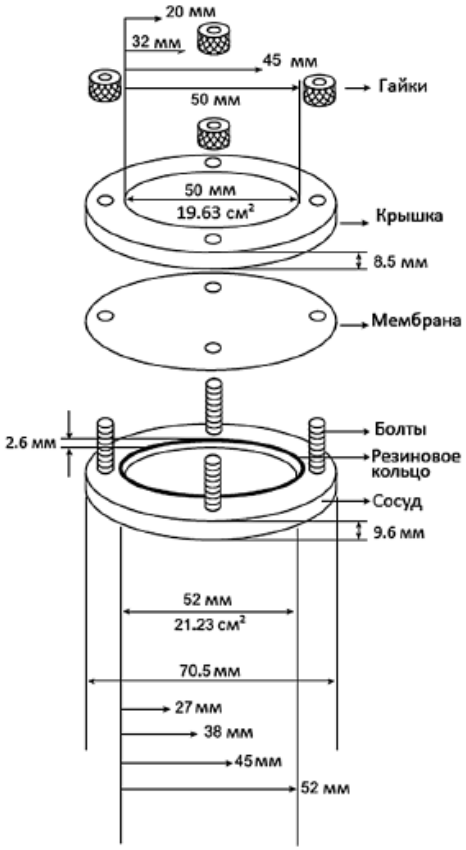
Figure 2.1.9.4.-1. – *Schematic diagram of Apparatus 1*

The holder (extraction cell) consists of a supporting part (base) intended for fixing the patch, and a cover part (lid) with a central hole of the required diameter, selected in accordance with the size of the transdermal patch. The design of the holder can also use a polymer membrane placed between the base and the lid. When determining the drug delivery rate to the dissolution medium from the transdermal patch through the polymer membrane, the holder design should not allow contact of the transdermal patch with the dissolution medium.

The polymer membrane is used when the direct contact of the surface of the transdermal patch that releases the active substance with the dissolution medium is unacceptable. The rate of drug diffusion in the membrane should be constant during the test and should not affect the kinetics of the process. The thickness of the membrane must ensure its mechanical strength and constant properties during the test.

Base. The central part of the base forms a cavity designed to fix the patch. The cavity has a depth of 2.6 mm and a diameter corresponding to the size of the test patch. The following diameters are allowed: 27 mm, 38 mm, 45 mm, 52 mm, corresponding to the volumes of 1.48 mL, 2.94 mL, 4.13 mL, 5.52 mL (Figure 2.1.9.4.-2).

Lid. The lid has a hole in the center with a diameter selected according to the size of the test patch. The patch can thus be positioned accurately in the center, and the surface of its release is limited.



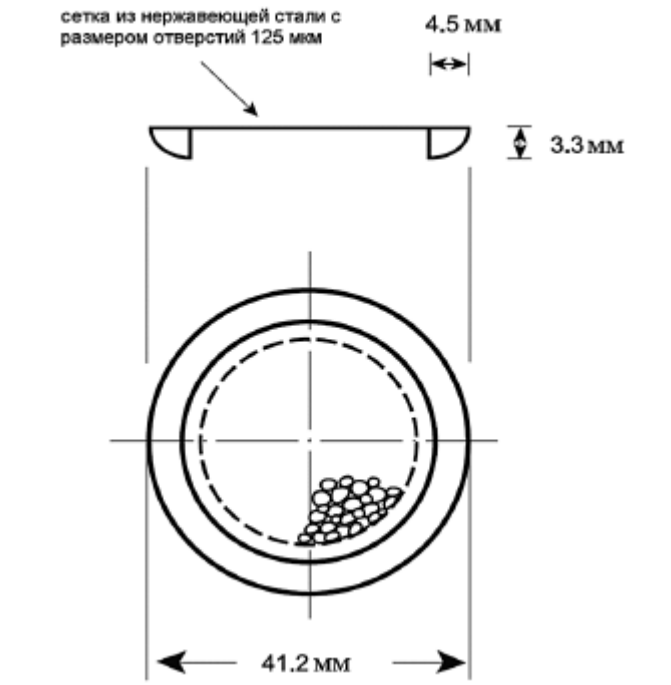
	mm
	Lid
	Membrane
	Bolts
	Rubber ring
	Vessel
	cm^2

Figure 2.1.9.4.-2. – *Extraction cell*

The following diameters are allowed: 20 mm, 32 mm, 40 mm, 50 mm, corresponding to the areas of 3.14 cm^2, 8.03 cm^2, 12.56 cm^2, 19.63 cm^2. The lid is held with nuts screwed onto bolts inserted into the base. The lid and base are sealed with a rubber ring, which is put on the vessel (Figure 2.1.9.4.-2).

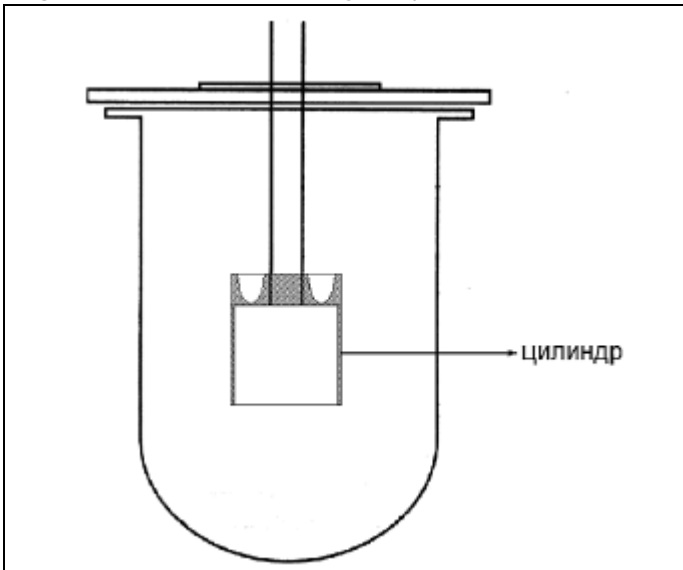
The holder with a attached transdermal patch (or a transdermal patch with a membrane) is placed on the bottom of the vessel with the releasing surface up, parallel to the lower edge of the stirring blade. The volume of the dissolution medium between the holder and the bottom of the vessel should be minimal, the distance between the surface of the holder and the lower edge of the stirring blade should be 25 ± 2 mm and not change during the test.

Apparatus 2. This apparatus uses a pre-assembled stainless steel disc in the form of a mesh with 125 μm openings (Figure 2.1.9.4.-3).



	stainless steel mesh with 125 μm openings
	mm

Figure 2.1.9.4.-3. – *Schematic diagram of the assembled disc*



cylinder	cylinder

Figure 2.1.9.4.-4. – *Schematic diagram of Apparatus 3*

Apparatus 3. The stirrer and shaft are replaced with a rotating stainless steel cylinder (Figures 2.1.9.4.-4, 2.1.9.4.-5). The distance between the inner surface of the vessel and the cylinder must be 25 ± 2 mm and not change during the test.

Dissolution medium

Water, buffer solutions with pH values in the range of 5.5 - 7.5 (permissible deviation of pH ±0.05), 0.9% sodium chloride solution, organic solvents (96% alcohol, isopropanol), and other media specified in the monograph can be used as a solution medium. Unless otherwise specified in the monograph, the volume of the solution medium is 500 mL, and the temperature of the solution medium in the vessel is (32.0 ± 0.5) °C.

Dissolved gases in the solution medium must be removed prior to testing by a validated solution degassing method.

To prevent evaporation of the dissolution medium, the dissolution vessels must be covered with appropriate lids.

The rotational speed of the stirrer

The rotational speed of the stirrer is 100 rpm unless otherwise specified in the monograph. The permissible deviation of the rotational speed is ± 4% of the value specified in the monograph.

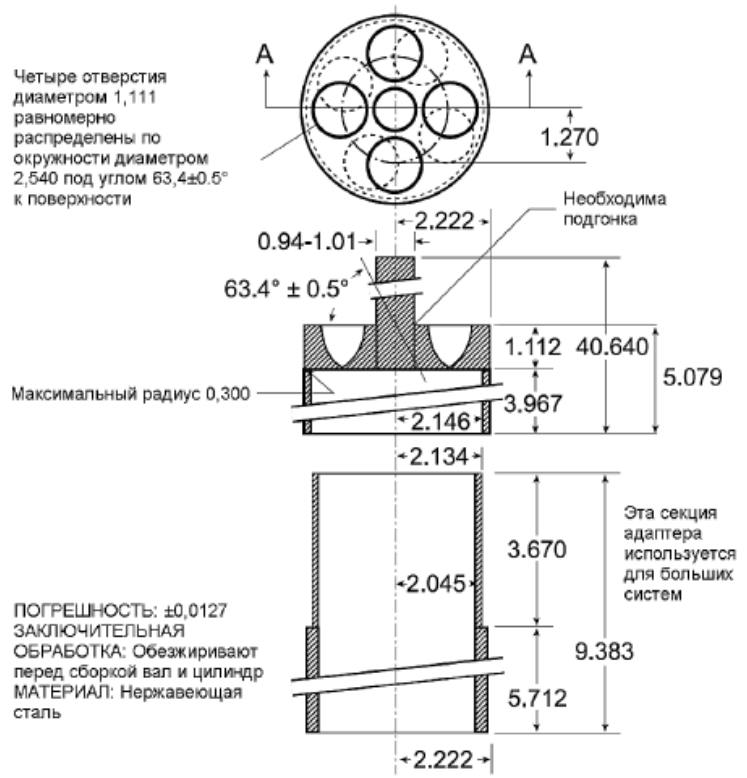
Test procedure

The test is performed on at least 6 transdermal patches (or 5 in the case of a dissolution test using a polymer membrane).

Place the volume of the dissolution medium specified in the monograph in a vessel and adjust the temperature to (32.0 ± 0.5) °C.

When using Apparatus 1, unless otherwise specified in the monograph, a transdermal patch is placed on the base of the cell, if necessary, the release surface outward, accurately in the center of the cell, if necessary, the transdermal patch is covered with a membrane, then the lid of the holder is placed on top. If necessary, use a hydrophobic substance (such as vaseline) to lubricate flat surfaces for a tighter connection and fixation of the patch. Place the cell on the bottom of the vessel with the release surface facing up.

When using Apparatus 2, the transdermal patch is placed on an assembled disc so that the release surface of the patch is as flat and even as possible. The transdermal patch can be attached to the disc using glue or double-sided adhesive tape. The patch is pressed with the release surface outward so that it does not extend beyond the disc.



	Four holes with a diameter of 1.111 are evenly distributed around a circle with a diameter of 2.540 at an angle of 63.4±0.5° to the surface
	Fitting is required
	Maximum radius 0.300
	ERROR: ±0.0127 FINAL PROCESSING: Degrease the shaft and cylinder before assembly MATERIAL: Stainless steel
	This adapter section is used for large systems

Figure 2.1.9.4.-5. – *Schematic diagram of the rotating cylinder. Dimensions are shown in centimeters*

A disc with the attached transdermal patch is placed on the bottom of the vessel with the release surface up.

When using Apparatus 3, remove the protective tape from the transdermal patch and place it with the sticky side on a clean surface of an inert porous membrane. The size of the membrane on all sides should be at least 1 cm larger than the patch. There are two ways to attach the transdermal patch to the cylinder:

- a transdermal patch with the attached membrane is placed with the membrane down on a clean surface and a suitable glue is applied to the free edges of the membrane, as well as, if necessary, to the outer cover layer of the patch;

- use double-sided adhesive tape, which is attached to the outer wall of the cylinder.

Carefully pressing, thoroughly attach the transdermal patch with the outer cover side to the cylinder so that the longitudinal axis of the transdermal patch is around the circumference of the cylinder.

For Apparatus 1, if a membrane is used, the effect of the membrane on the test results should be checked first. For Apparatuses 2 and 3, check the effect of glue or adhesive tape on the test results and exclude the possibility of adsorption of active substances on them.

Turn on the stirring device. From this point on, every hour or other time range specified in the monograph, select samples of the solution.

Note. If necessary, it is allowed to use a membrane made of various materials such as inert porous cellulose or silicones, which does not affect the kinetics of the release of the active substance(s) from the patch. In addition, the membrane must not contain materials that affect its functionality. The membrane can be subjected to special treatment before the test, for example, by holding it under test conditions for 24 h.

Table 2.1.9.4.-1. – *Interpretation of the results of the "Dissolution" test for transdermal patches*

Level	Number of experiments	Criteria
A	6(5)	None of the individual values of the release rate (or delivery rate) of the active substance from the transdermal patch does not lie outside the limits of the rate values specified in the monograph.
B	6(5)	The average value of individual values of the release rate (or delivery rate) of the active substance from the transdermal patch in 12 (10) vessels (A + B) is within the limits of the rate values specified in the monograph. None of the individual values of the release rate (or delivery rate) of the active substance from the transdermal patch deviates by greater than 10% from the average value of the established limit from the rate limits specified in the monograph.
C	12(10)	The average value of individual values of the release rate (or delivery rate) of the drug from the transdermal patch in 24 (20) vessels (A + B + C) lies within the limits of the rate values specified in the monograph. No greater than 2 out of 24 (20) results are outside the standard limits, and the deviation does not exceed 10% of the average value of the established limit, none of the results deviates from the average rate limit specified in the monograph by greater than 20%.

The membrane is applied to the release surface of the patch, while avoiding the formation of air bubbles.

Sampling

Sampling is carried out from the average height of the dissolution medium at a distance not closer than 10 mm from the inner wall of the vessel.

Each sample is analysed for the quantitative content of the active substance. The decrease in the volume of the solution medium is compensated either by returning the solution sample to the vessel, or by adding the solution medium, or is taken into account in calculations.

The sampling time must be specified in the monograph and must be observed with an accuracy of ± 2%.

Evaluation of results

The dosage form passes the test if the amount of active substance(s) released from the patch at certain time intervals of sampling, expressed as area unit per time unit, meets the established requirements.

The test results are considered satisfactory if the release rate meets the criteria in Table 2.1.9.4.-1, Level A.

If at least one result does not meet the standard specified in the monograph, the test is repeated on another 6 (5) samples, and the results are interpreted according to Table 2.1.9.4.-1, Level B.

If the results do not meet the established criteria during repeated testing, the test is repeated on 12 (10) additional samples, and the results are interpreted according to Table 2.1.9.4.-1, Level C. If the Level C requirement is not met, the analysed batch is rejected.

The amount of active substance released into the dissolution medium during the largest of the time periods specified in the monograph must be at least 75% of the label content of the active substance in the transdermal patch.

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2.1.9.5. Uniformity of Mass of Single-Dose Preparations

This test applies to drug dosage forms (tablets, capsules, suppositories, etc.) and single-dose preparations in individual containers (granules, powders, lyophilizates, etc.). This test is not used if a uniformity test of dosage forms of all active substances is performed as prescribed in chapter *2.1.9.14. Uniformity of dosage units.*

Unless otherwise specified in the monograph, this test is not performed for multivitamin drugs and for drugs with trace nutrients.

The test is performed on 20 dosage units of the drug or on the contents of 20 dosage units of the drug in single-dose individual containers selected randomly.

Procedure. Weigh 20 units taken at random or, for single-dose preparations presented in individual containers, the contents of 20 units, and determine the average mass: weigh each unit individually with an accuracy of 0.001 g, unless otherwise specified in the monograph, and calculate the average mass.

For drug products in the form of capsules and solid dosage forms in single-dose packages, determine the mass of the contents as described below.

Capsules. Weigh an intact capsule. Open the capsule and remove the contents as completely as possible. For soft-shell capsules, wash the shell with a suitable solvent and allow it to stand until the odour of the solvent is no longer perceptible. Weigh the shell. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 capsules.

Solid dosage forms (powders, granules, lyophilizates) in single-dose packages. If necessary, remove any paper labels from a container. Wash and dry the outside. Open the container and without delay weigh. Empty the container as completely as possible by gentle tapping, rinse it if necessary with water and then with *96% alcohol R* and dry at 100-105 °C for 1 h, or, if the nature of the container precludes heating at this temperature, dry at a lower temperature to constant mass. Allow to cool in a desiccator and weigh. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 containers.

Table 2.1.9.5.-1. – *Allowable deviations from the average mass of single-dose preparations*

Pharmaceutical form	Average mass, mg	Percentage deviation, %
Tablets (uncoated and film-coated)	80 or less	10
-	Greater than 80 and less than 250	7.5
	250 or greater	5
Tablets (pan coated)	All masses	15
Capsules and granules (uncoated), powders for oral administration and external use	Less than 300	10
	300 or greater	7.5
Solid dosage forms for preparation of dosage forms for parenteral administration	greater than 40	10
	40 or less*	—
Suppositories	All masses	5
Powders for eye-drops and powders for eye lotions (single-dose)	Less than 300	10
	300 or greater	7.5

Note. * When the average mass is equal to or below 40 mg, the preparation is submitted to the test for uniformity of content of single-dose preparations in accordance with general chapter *2.1.9.14. Uniformity of single-dose preparations* and is not submitted to the test for uniformity of mass in accordance with this general chapter.

Requirement. The drug preparation passes the test if not greater than 2 individual masses deviate from the average mass by a value exceeding the permissible percentage deviation indicated in the table. At the same time, no individual mass should deviate from the average mass by a value 2 times higher than that indicated in the table.

201090006-2019

2.1.9.6. Friability of Tablets

This test is used to determine the friability of compressed, uncoated tablets under defined conditions, i.e. the phenomenon whereby tablets are damaged when subjected to mechanical shock or attrition. Measurement of tablet friability supplements other physical strength measurements, such as tablet breaking force. The test procedures presented in this chapter are applicable to most compressed tablets.

The friability is expressed as the loss of mass and it is calculated as a percentage of the initial mass of the tested tablets.

Apparatus. Use a drum (Figure 2.1.9.6.-1) with an internal diameter of 287.0 ± 4.0 mm and a depth of 38.0 ± 2.0 mm, made of a clear synthetic polymer with polished internal surfaces and not subject to static build-up. One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius of 80.5 ± 5.0 mm that extends from the middle of the drum to the outer wall.

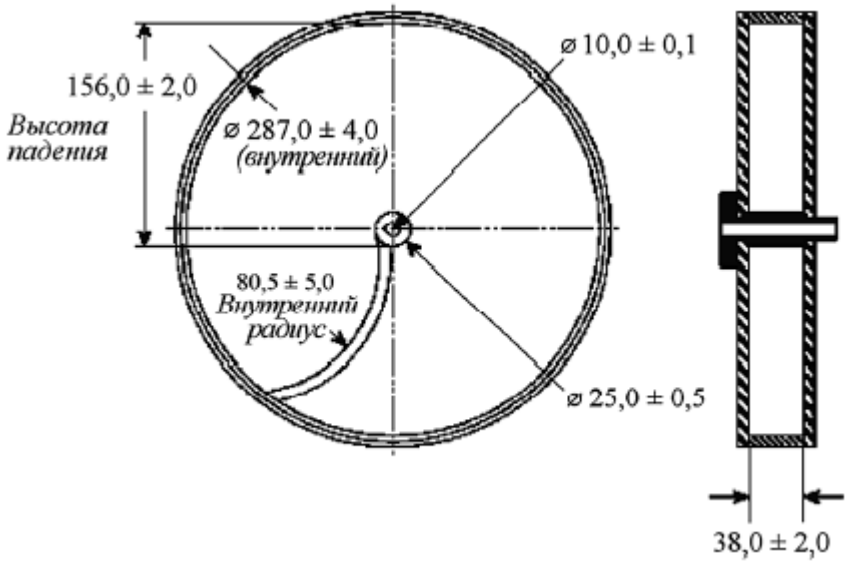
The outer diameter of the central shaft is 25.0 ± 0.5 mm. The drum is attached to the horizontal axis of a device that ensures the drum rotational speed of 25 ± 1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

Procedure. For tablets with a unit mass equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit mass of greater than 650 mg, take a sample of 10 whole tablets. The tablets are carefully dedusted prior to testing, weighed with an accuracy of 0.001 g and placed in the drum. Rotate the drum 100 times, and remove the tablets. Dedust the tablets as before and weigh with an accuracy of 0.001 g. The weight loss should not exceed 1.0%.

If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test.

If the results are difficult to interpret (there are only single minor cracks or chips, or if the weight loss is greater than the targeted value), the test is repeated twice. A maximum loss of mass obtained from a single additional test or from the mean of 3 tests should not exceed a standard value.

Effervescent tablets and chewable tablets may have different requirements as far as friability is concerned. In the case of hygroscopic tablets, a humidity-controlled environment is required for testing.



	Drop height
	(internal)
	Internal radius

Figure 2.1.9.6.-1. – *Tablet friability apparatus Dimensions are shown in millimetres*

A drum with dual scooping projections, or apparatus with greater than one drum, for the running of multiple samples at one time, are also permitted.

Note. If the shape or size of the tablets makes it difficult to move them inside the drum, adjust the apparatus so that the tablets may fall freely and do not bind together when lying next to each other. Adjusting the drum so that the axis forms a 10° angle with the base is usually satisfactory.

201090007-2019

2.1.9.7. Resistance to Crushing of Tablets

This test is intended to determine, under defined conditions, the resistance to crushing of tablets, measured by the force needed to disrupt them by crushing.

Determining and normalising the mechanical strength of tablets is necessary both in industrial production (for example, the process of coating tablets and packaging), and to ensure the consumer properties of the drug (preserving the integrity of the tablet when removed from the container).

Apparatus. The apparatus consists of two jaws facing each other, one of which moves towards the other. The flat surfaces of the jaws are perpendicular to the direction of movement. It is allowed to use an apparatus in which both jaws can move at a constant speed towards each other. The crushing surfaces of the jaws are flat and larger than the zone of contact with the tablet. The apparatus must stop crushing in case of any violation of the integrity of the tablets.

The apparatus is calibrated using a system with a precision of 1 N (newton).

Procedure. Unless otherwise specified in the monograph, place the tablet between the jaws with respect to the direction of application of the force. The tablet is compressed to destruction. Carry out the measurement on 10 tablets.

Table 2.1.9.7.-1 – *Minimum allowable strength depending on the tablet diameter.*

Diameter, mm	6	7	8	9	10	11	12	13
Strength, N	30	30	30	30	40	40	50	50

Note. This procedure does not apply when fully automated equipment is used.

Before each measurement, carefully remove all fragments of the previous tablet.

If tablets have break lines (engravures) or art prints, each tablet should be oriented identically in relation to the direction of the applied crushing force.

Oval or oblong tablets are placed between the jaws with a long edge perpendicular to the crushing surfaces of the apparatus (along the direction of the applied force) unless otherwise specified in the monograph.

Expression of results. Express the results as the mean, minimum and maximum values of the forces measured, all expressed in newtons (N); indicate the type of apparatus and, where applicable, the orientation of the tablets.

Round tablets should have a strength not lower than the values given in Table 2.1.9.7.-1 unless otherwise specified in the monograph.

201090008-2019

2.1.9.8. Ethanol content

The requirements of this monograph apply to medicinal products: substances (homeopathic matrix tinctures, liquid extracts, etc.) and liquids (tinctures, extracts, alcohol solutions, etc.) containing ethyl alcohol. Ethanol content in medicinal products, depending on their composition and physical and chemical properties, can be determined by one of the following methods: distillation followed by density determination using a pycnometer (method 1) or hydrometer (Procedure 2) or gas chromatography (Procedures 1, 2, 3). The quantitation method for ethanol should be specified in the monograph.

The ethanol content of a liquid expressed as the number of volumes of ethanol in 100 volumes of the liquid at a temperature of (20±0.1)°C is called the "percentage of ethanol by volume" (% *V/V*). The ethanol content of liquids expressed in grams per 100 g of the liquid at a temperature of (20±0.1)°C is called the "percentage of ethanol by mass" (% *m/m*).

Distillation method (Procedure 1)

This method consists of separating ethanol from the substances dissolved in it by distillation. If dissolved substances are present in the preparations, separate them by distillation. Where distillation would distil volatile substances other than ethanol and water, the appropriate pre-treatment should be carried out as stated in the monograph.

Diagrams of apparatuses (1 and 2) for the determination of ethanol content in liquids by distillation are shown in Figures 2.1.9.8.-1 and 2.1.9.8.-2.

Transfer an accurately measured amount of the preparation to a 200-250 mL round-bottom distillation flask (*1*). If the ethanol content in the preparation is up to 20%, take 75 mL of the preparation for determination; if the content is between 20 and 50%, take 50 mL; if the content is from 50% or higher, take 25 mL; dilute the liquid to 75 mL with water before distillation.

The flask is attached to a horizontally positioned straight condenser with a tube adapter (*4*) that directs the distillate to the receiver — a 50 mL volumetric flask (*5*), preferably placed in a vessel with cold water.

Heat the distillation flask on an electric heater (*6*). For even boiling, place capillaries, a few pieces of pumice or calcined porcelain in the flask with the drug preparation to be examined. The vapour temperature is measured with a thermometer (*2*) placed in the apparatus so that the mercury ball is located 0.5-1.0 cm below the outlet tube opening. If the temperature limits of the distillation are observed, an even boiling of the test solution is achieved.

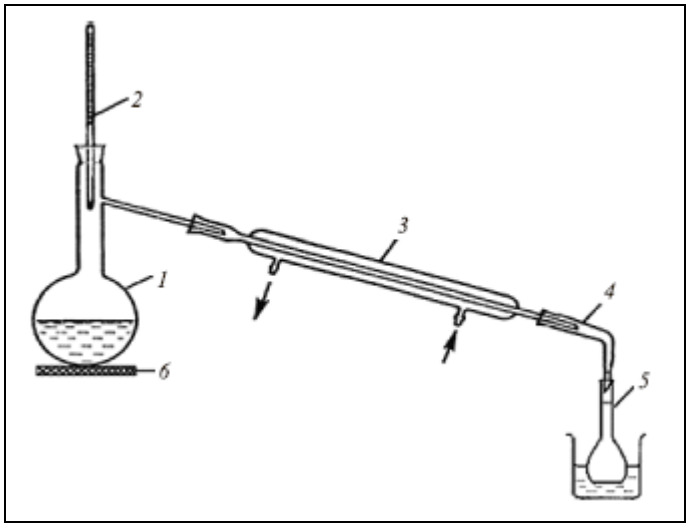


Figure 2.1.9.8.-1. – *Diagram of the apparatus (1) for the determination of ethanol content. 1 –round-bottom flask; 2 – thermometer; 3 – condenser; 4 – tube adapter; 5 – receiver; 6 – electric heater*

If the test solution foams a lot during distillation, add 2 to 3 mL of *phosphoric acid R* or *concentrated sulfuric acid R, calcium chloride R*, paraffin, and wax (2-3 g).

Collect about 48 mL of distillate, cool it to a temperature of 20 ^{O}C, dilute the solution to volume with water, and mix. The distillate may be clear or slightly cloudy.

Determine the density of the distillate with a pycnometer and find the ethanol content in volume percentages from alcoholometric tables.

Calculate the ethanol content in per cent V/V using the following expression:

$$X = \frac{50 \cdot a}{V},$$

where 50 is the distillate volume, mL;

a is the ethanol content in the distillate, % (*V/V*);

V is the volume of the test preparation taken for distillation, mL

The apparatus (Figure 2.1.9.8.-2) consists of a round-bottom flask (*A*) fitted with a distillation head (*B*) with a steam trap and attached to a vertical condenser (*C*).

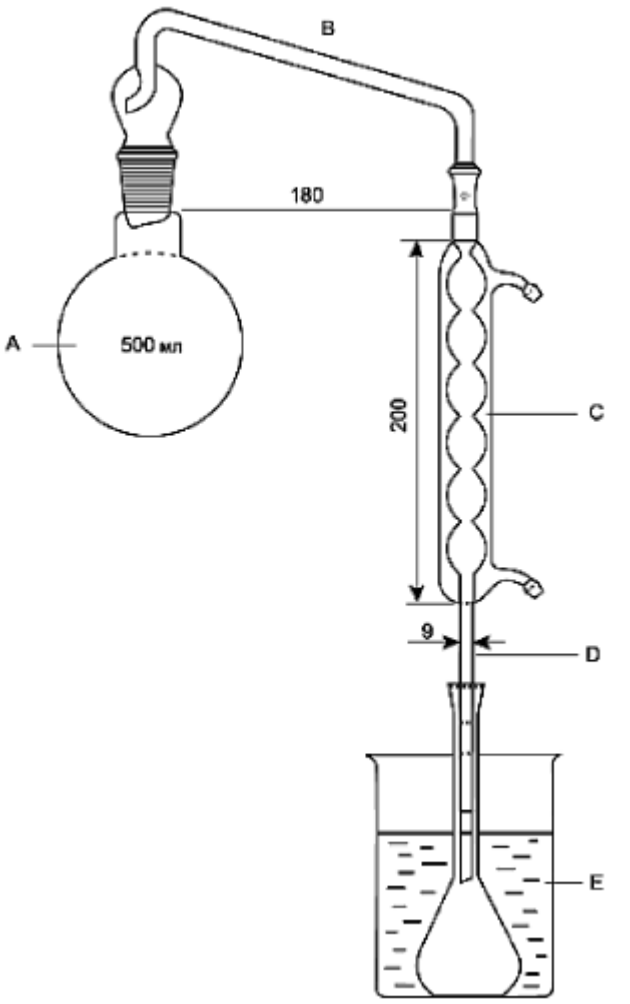


Figure 2.1.9.8.-2. – *Diagram of the apparatus (2) for the determination of ethanol content (dimensions in millimetres)*

The lower part of the condenser is connected to a tube adapter (*D*), through which the distillate enters the lower part of the 100-250 mL volumetric flask. During distillation, the measuring flask is immersed in a mixture of ice and water (*E*). A disc having a circular aperture 6 cm in diameter is placed under the flask (*A*) to prevent charring of any dissolved substances.

Procedure

Pycnometer method/oscillating transducer density meter method. Place 25.0 mL of the test sample, measured at a temperature of 20 ±0.1 °C, in the distillation flask, dilute to volume with 100-150 mL of *distilled water R* and add a few pieces of pumice. Attach the distillation head and condenser. Distill and collect NLT 90 mL of distillate in a 100 mL volumetric flask. Adjust the temperature to 20 ± 0.1 °C and dilute to 100 mL with *distilled water R* at 20±0.1 °C.

Determine the relative density of the distillate at a temperature of (20 ±0.1) °C using a pycnometer or an oscillating transducer density meter.

Using alcoholometric tables, find the ethanol content of the distillate and multiply by four to obtain the percentage of ethanol by volume (*V/V*) contained in the preparation. Round off the result to 1 decimal place.

Hydrometer method. Place 50.0 mL of the preparation to be examined, measured at 20 ± 0.1 °C in the distillation flask, add 200-300 mL of *distilled water R* and perform distillation as described above, collecting at least 180 mL of distillate in a 250 mL volumetric flask. Adjust the distillation temperature to 20 ± 0.1 °C and dilute to 250.0 mL with *distilled water R* at 20 ±0.1 °C.

Place the distillate in a cylinder with a diameter 6 mm wider than the bulb of the hydrometer. If the volume of the distillate is insufficient, double the quantity of the sample dilute the distillate to 500.0 mL with *distilled water R* at 20 ±0.1 °C.

Make an adjustment for dilution by multiplying the value of strength by five. Use alcoholometric tables to calculate the percentage of ethanol content (*V/V*) and round off the result to 1 decimal place.

If the drug preparation contains essential oils, chloroform, diethyl ether or camphor, add equal volumes of saturated *sodium chloride R* and *petroleum ether R* solution in the separating funnel. Shake the mixture for 3 minutes. After separation of the layers, drain the alcohol-water layer into another separating funnel and treat in the same way with half the quantity of *petroleum ether R*. Drain the alcohol-water layer into the distillation flask. Shake the combined essential extracts with half the volume of the saturated *sodium chloride R* solution, and then add to the liquid in the distillation flask.

If the drug contains less than 30% of alcohol, then carry out the salting not with a solution but with 10 g of *sodium chloride R*.

If the drug contains volatile acids, neutralise them with an alkali metal hydroxide solution, and if it contains volatile bases – with *phosphoric acid R* or *sulfuric acid R*.

Drug preparations containing free iodine are treated before distillation until discoloured with zinc dust or calculated quantity of *sodium thiosulfate R*. To bind volatile sulfur compounds, add a few drops of 10% (*m/v*) of *sodium hydroxide R* solution to the preparation.

Distillation method (Procedure 2)

Place 50.0 mL of the drug product sampled at 20 ±0.1 °C in the distillation flask, add 200-300 mL of *distilled water R* and distill as described above, collecting the distillate in a 250 mL volumetric flask until at least 180 mL is obtained. Adjust the distillate temperature to 20 ± 0.1 °C and dilute to volume with *distilled water R* at the same temperature. Transfer the distillate to a cylinder whose diameter must exceed the width of the hydrometer body by at least 6 mm. If the volume of the distillate is insufficient, double the amount of the sample and dilute the distillate to 500.0 mL with *distilled water R* at 20 ±0.1 °C. The values found in alcoholometric tables are multiplied by 5 to obtain the percentage of ethanol by volume (V/V) contained in the preparation. Round off the ethanol content calculated from alcoholometric tables to 1 decimal place.

If it is necessary to determine the ethanol content in ethyl alcohol of higher concentrations, use the alcoholometric tables given in the Annex, in accordance with general chapter 5.5. *The use of alcoholometric tables.*

Gas chromatography method
(Procedure 1)

Unless otherwise specified in the monograph, use Procedure 1 or Procedure 2 or headspace gas chromatography method (Procedure 3) for the test.

Test solution. Place in a 100 mL volumetric flask an accurately measured volume of the test preparation sufficient to obtain a solution containing 4 - 6% (*V/V*) ethanol, , add 5.0 mL of internal standard solution, mix, dilute to 100.0 mL volume with *water R*, and stir. Place 10.0 mL of the resulting solution into a 100 mL volumetric flask, dilute to 100.0 mL with *water R*, and mix.

Reference solution. Place 5.0 mL of not less than 96% (*V/V*) *ethanol R* and 5.0 mL of the internal standard solution in a 100 mL volumetric flask, dilute to 100.0 mL with *water R*, and mix. Dilute 10.0 mL of the resulting solution to 100.0 mL with *water R*, and mix.

Internal standard solution. Propanol R.

The chromatographic procedure may be carried out using a flame-ionisation detector under the following conditions:

- 150 x 0.4 cm column filled with divinylbenzene/ethylvinylbenzene sorbent (surface area 500-600 m^2/g) with a particle size of 100-120 μm;
- column temperature – 150 °C
- evaporator temperature – 170 °C
- detector temperature – 170 °C
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas;
- carrier gas velocity of 30 mL/min.

Chromatograph 1-2 μl of the test solution and the reference solution.

Calculate the retention of ethanol in the preparation as a percentage by volume (*V/V*) using the formula:

$$X = \frac{S \cdot S'_o \cdot 5.0 \cdot P}{S_o \cdot S'V_s},$$

where S is area of the peak due to ethanol in the chromatogram obtained with the test solution;

S' is area of the peak due to ethanol in the chromatogram obtained with reference solution;

S_o is area of the peak due to the internal standard in the chromatogram obtained with the test solution;

S'_o is area of the peak due to the internal standard in the chromatogram obtained with reference solution;

V_s is the volume of the preparation taken for analysis, mL;

P is the ethanol content in per cent (*V/V*).

Chromatographic system suitability test. A chromatographic system is considered suitable if in the chromatogram of the reference solution:

- the resolution between the peaks of ethanol and propanol is NLT 2.0;
- the symmetry coefficient calculated for the ethanol peak is NMT 1.5;
- the relative standard deviation calculated for the ratio of the ethanol peak area to the internal standard peak area is NMT 2%.

Gas chromatography method (Procedure 2)

Internal standard solution. Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*.

Test solution. Dilute the volume of the preparation corresponding to 1 g of ethanol to 50.0 mL with *water R*. To 1.0 mL of the resulting solution, add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

Reference solution (a). Dilute 1.0 mL of *anhydrous ethanol R* to 50.0 mL with *water R*.

Reference solution (b). Dilute 1.0 mL of *methanol R2* to 100,0 mL with *water R*. Dilute 1,0 mL of the resulting solution to 20,0 mL *with water R*.

Reference solution (c). To 1.0 mL of the internal standard solution, add 1.0 mL of the reference solution (a), 2.0 mL of the reference solution (b), and dilute to 20.0 mL with *water R*.

The chromatographic procedure may be carried out using a flame-ionisation detector under the following conditions:

quartz column size 30 m x 0.53 mm, covered with *poly[(cyanopropyl)(phenyl)][dimethyl]siloxane R* (film thickness 3 µm);

- *helium for chromatography R* as the carrier gas;
- carrier gas velocity is 3 mL/min;
- split ratio 1:50
- the temperature program mode:

	Time (min)	Temperature (°C)
Column	0-1.6	40
	1.6-9.9	40 > 65
	9.9-13.6	65 > 175
	13.6-2.0	175
Injection port		200
Detector		200

Chromatograph 1.0 µl each of the reference solution (b) and the test solution.

The elution order on the reference solution chromatogram: methanol, ethanol, 1-propanol.

The retention time of the ethanol peak is about 5.3 min. Relative peak retention times should be about 0.8 for methanol, about 1.6 for 1-propanol.

Chromatographic system suitability test. A chromatographic system is considered suitable if the chromatogram of the reference solution (b) has a resolution of at least 5.0 between the peaks of methanol and ethanol.

Calculate the ethanol content in per cent volume (*V/V*) using the following expression:

$$X = \frac{S_1 \cdot S'_2 \cdot 100}{S_2 \cdot S'_1 \cdot V_1},$$

where S_1 is area of the peak due to ethanol in the chromatogram obtained with the test solution;

S_2 is area of the peak due to ethanol in the chromatogram obtained with reference solution (c);

S'_1 is area of the peak due to the internal standard in the chromatogram obtained with the test solution;

S'_2 is area of the peak due to the internal standard in the chromatogram obtained with reference solution;

V_1 is the volume of the preparation in the test solution, mL

Headspace gas chromatography method (Procedure 3)

The internal standard solution. Dilute 1.0 mL of *propanol R* to 100.0 mL with *water R*. Dilute 1.0 mL of the resulting solution to 20.0 mL with *water R*.

Test solution. Dilute the volume of the preparation corresponding to 1 g of ethanol to 50.0 mL with *water R*. Dilute 1.0 mL of the resulting solution to 20.0 ml with *water R*. To 2.0 mL of this solution, add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

Reference solution (a). Dilute 5.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 25.0 mL of the resulting solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

Reference solution (b). Mix 0.5 mL of reference solution (a) with 1.0 mL of the internal standard solution and dilute the resulting solution to 20.0 mL with *water R*.

Reference solution (c). Mix 1.0 mL of reference solution (a) with 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

Reference solution (d). Mix 1.5 mL of reference solution (a) with 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

Reference solution (e). Dilute 1.0 mL of *methanol R2* to 100.0 mL with *water R*. Dilute 1.0 mL of the resulting solution to 20.0 mL with *water R*.

Reference solution (f). To 1.0 mL of the internal standard solution, add 2.0 mL of reference solution (a), 2.0 mL of reference solution (e), and dilute the solution to 20.0 mL with *water R*.

Test the sample using gas chromatograph equipped with a flame ionisation detector under the following conditions:

- quartz column size 30 m x 0.53 mm, covered with *poly[(cyanopropyl)(phenyl)][dimethyl]siloxane R* (film thickness 3 μm);
- helium for chromatography R *as the carrier gas*;
- carrier gas velocity is 3 mL/min;
- split ratio 1:50.

Static head-space conditions:

- equilibrium temperature: 85 °C;
- equilibration time: 20 min;
- the temperature program mode:

	Time (min)	Temperature (°C)
Column	0-1.6	40
	1.6-9.9	40 > 65
	9.9-13.6	65 > 175
	13.6-2.0	175
Injection port		200
Detector		200

Chromatograph 1.0 mL of the gas phase over the test solution, reference solutions (b), (c), (d), and (f) at least three times.

The elution order on the reference solution chromatogram: methanol, ethanol, 1-propanol. The retention time of the ethanol peak is about 5.3 min. Relative peak retention times should be about 0.8 for methanol, about 1.6 for 1-propanol.

Chromatographic system suitability test. A chromatographic system is considered suitable if the chromatogram of the reference solution (f) has a resolution of at least 5.0 between the peaks of methanol and ethanol.

Build a calibration curve by plotting on the abscissa axis the concentration of ethanol in reference solutions (b), (c), (d) and (f), and on the ordinate axis — the average values of the ratio of the area peaks of ethanol and internal standard on the corresponding chromatograms.

Calculate the ethanol content in the drug product in per cent volume (*V/V*).

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2.1.9.9. Test for extractable volume of parenteral preparations

The volume of the dosage form in the container should be sufficient to ensure the injection of the nominal volume indicated on the label. Oily and viscous preparations may be prewarmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20-25 °C before measuring the volume.

Compliance of parenteral preparations with the requirements of the test for extractable volume is achieved by filling containers with a small excess of the nominal volume (see Table 2.1.9.9.-1).

Suspensions and emulsions are shaken before withdrawal of the contents and before the determination of the density.

Single-dose containers

Select 1 container for the test if the nominal volume is 10 mL or greater, 3 containers if the nominal volume is greater than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding 3 times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm in length. Expel any air bubbles from the syringe and needle, then discharge the contents into a standard dry cylinder (graduated to contain) without emptying the needle. The capacity of the cylinder must be sufficient so that the volume to be measured occupies at least 40% of its graduated volume. Alternatively, the volume of the contents in millilitres may be calculated as the mass of the test drug (in grams) divided by the density.

For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume suitable for the measurement provided that a separate, dry syringe assembly is used for each container.

The contents of containers holding 10 mL or greater may be determined by opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in the case of containers examined individually.

Table 2.1.9.9.-1 – *Filling volume of injection solutions in single-dose containers*

Nominal volume, mL	Filling volume, mL	
	Non-viscous solutions	Viscous solutions
0.5	0.6	0.62
1.0	1.10	1.15
2.0	2.15	2.25
5.0	5.30	5.50
10.0	10.50	10.70
20.0	20.60	20.90
30.0	30.80	31.20
50.0	51.00	51.50
greater than 50	2% greater than the nominal value	3% greater than the nominal value

In the case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

Multidose containers

For injections in multidose containers labeled to yield a specific number of doses of a stated volume, select one container and proceed as directed for single-dose containers using the same number of separate syringe assemblies as the number of doses specified.

The measured volume is such that each syringe delivers not less than the stated dose.

Cartridges and Prefilled Syringes

Select 1 container for the test if the nominal volume is 10 mL or greater, 3 containers if the nominal volume is greater than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in millilitres calculated as the mass in grams divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

Infusion solutions

Select one container. Transfer the contents to a dry measuring cylinder, graduated for filling, of such capacity that the determined volume fills at least 40% of the nominal volume of the cylinder. Measure the transferred volume.

The resulting volume must be at least the specified nominal volume.

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2.1.9.10. Particulate Contamination : Sub-Visible Particles

The test for the presence of sub-visible particulate matter is intended for solid and liquid medicinal products for parenteral use.

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

For the determination of particulate contamination (less than 100 μm), use three methods.

Method 1 – Light obscuration particle count test.

Method 2 – electrical sensing zone analysis (Coulter principle).

Method 3 — Microscopic method

When examining parenteral preparations for sub-visible particles, Methods 1 and 2 are preferably applied. If necessary, method 3 should also be used to obtain a reasoned conclusion.

Method 1 is not applicable for the examination of preparations having reduced clarity (for example, emulsions, colloidal and liposomal preparations) or drug products that form air or gas bubbles when passing through the measuring cell. In this case, the test is carried out according to Method 3. In the study of cloudy drugs (for example, emulsions), Method 2 can be used after quantitative dilution of the drug with the appropriate solvent, which should be indicated in the monograph or a quality specification document.

When testing dark-coloured preparations, the use of Method 2 is a priority and is possible without adding a diluent.

If the viscosity of the preparation to be tested is sufficiently high, this is an obstacle to testing it by any of the methods. To reduce the viscosity, quantitative dilution is carried out with an appropriate particle-free solvent, which should be indicated in the monograph or a quality specification document.

The results obtained in examining a discrete unit or group of units for particulate contamination cannot be extrapolated with certainty to other units that remain untested. Therefore, sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterise the level of particulate contamination in a large group of units.

TEST CONDITIONS

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet. Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of surfactant. Immediately before use, wash again outside and inside with *particle-free water*.

Avoid getting air bubbles in the test product, especially in cases where samples of the preparation are placed in the container in which the test is conducted.

Testing of solutions for parenteral use in containers of 25 mL or greater is carried out on single containers. For containers containing less than 25 mL, the contents of 10 or more containers are combined in a clean vessel to produce a volume of at least 25 mL. The test solution can also be prepared by mixing the contents of an appropriate number of containers, followed by diluting up to 25 mL with *particle-free water* or aparticle-free solvent, if it is not possible to use p*article-free water,* which should be specified in the monograph.

Dissolve powders for the preparation of solutions for parenteral use with *particle-free water* or, if it is impossible to use *water*, in an appropriate particle-free solvent which should be specified in the monograph or a regulatory quality document.

The number of test samples should be sufficient to obtain a statistically significant result. Tests of samples with a volume of 25 mL or greater can be carried out on a batch of less than 10 pcs in accordance with the sampling rules.

1. Light obscuration particle count test

Apparatus. Use an apparatus based on the principle of light blockage which allows determination of the size of particles and the number of particles according to size. The apparatus is calibrated using dispersions of spherical particles (certified reference standard) of known sizes between 10 μm and 25 μm. These reference standard particles are dispersed in *particle-free water R*. Care must be taken to avoid aggregation of particles during dispersion.

Checking the suitability of test conditions. Pre-check the suitability of test conditions (environment, prepared glassware and water to be used). For this purpose, determine the particulate contamination of 5 samples of *particle-free water R*, each of 5 mL, according to the procedure described below. If the number of particles of 10 μm or greater size exceeds 25 for the combined 25 mL, the test conditions are not suitable.

The preparatory steps must be repeated until the environment, glassware and water are suitable for the test.

Procedure. Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container to be opened using a jet of *particle-free water* and remove the closure, avoiding any contamination of the contents. Prepare the test solution as described above, depending on the contents of the container. Eliminate air bubbles by allowing the prepared solution to stand for 2 min or sonicating.

In a bulk sample with a volume of at least 25 mL, the number of particles with a size equal to or greater than 10 and 25 μm is determined. Perform 4 measurements. In this case, the results of the determination for the first sample are not taken into account, and the average number of particles in the test sample is calculated.

Evaluation of results. Preparations with a nominal volume of 100 mL or less meet the requirements if the average number of particles in one container does not exceed 6000 for particles equal to or greater than 10 μm, and does not exceed 600 for particles equal to or greater than 25 μm.

Preparations with a nominal volume of 100 mL or greater meet the requirements if the average number of particles in 1 mL does not exceed 25 for particles equal to or greater than 10 μm, and does not exceed 3 for particles equal to or greater than 25 μm.

If the average number of particles exceeds the limits, test the preparation by the microscopic particle count test.

2. Method of electro-sensitive zones (Coulter's method)

Apparatus. The test is performed using a Coulter counter, which is based on recording electrical pulses that occur when a particle passes through an aperture (a calibrated hole with a diameter of 100 μm). The magnitude of the pulse is proportional to the size of the particle. The results obtained using the apparatus are not affected by the colour of the particles, the refractive index of the particle or liquid, or the shape of the particles.

The apparatus is calibrated using a dispersion of latex particles (reference standard) having a known size from 10 to 25 μm. The reference standard is dispersed in a *0.9% sodium chloride particle-free solution.*

Checking the suitability of test conditions. Pre-check the suitability of the test conditions (the environment, prepared glassware, and the *0.9% sodium chloride solution* used). To do this, determine the presence of mechanical inclusions in 3 samples of *0.9% sodium chloride solution*, each of 20 mL. If the number of particles with a size of 10 μm exceeds 25 in 60 mL for 3 combined samples, the conditions are not suitable for the test.

The preparatory steps for the test must be repeated until the environment, glassware, and *0.9% sodium chloride solution* become suitable for the test.

Procedure. Stir the contents of the sample, slowly turning it at least 20 times. Clean the outer surfaces of the package to be opened with a jet of *particle-free water*, open it, avoiding any contamination of the contents. Prepare the test solution in accordance with the instructions given in the "Test conditions" section.

A commercial or laboratory-prepared *0.9% sodium chloride solution* is generally used as a particle-free solvent.

Eliminate air bubbles by allowing the prepared solution to stand for 2 min or sonicating.

In a bulk sample with a volume of at least 20 mL, determine the number of particles with a size equal to or greater than 10 and 25 μm.

Take into account the results of at least three measurements, each of 1.0 mL, and calculate the average number of particles in the test sample.

Evaluation of results. Preparations with a nominal volume of 100 mL or less meet the requirements if the average number of particles in one container does not exceed 6000 for particles equal to or greater than 10 μm, and does not exceed 600 for particles equal to or greater than 25 μm.

Preparations with a nominal volume of 100 mL or greater meet the requirements if the average number of particles in 1 mL does not exceed 25 for particles equal to or greater than 10 μm, and does not exceed 3 for particles equal to or greater than 25 μm.

If the average number of particles exceeds the limits, test the preparation by the microscopic particle count test.

3. Microscopic method

Equipment*:* binocular microscope and filter unit.

The microscope is equipped with an eyepiece-micrometer and two illuminators. The microscope is set to 100x magnification.

The field of view of the eyepiece-micrometer is a circle divided by diameter, and consists of a large circle divided by crosshairs into quadrants, clear and black standard circles with a diameter of 10 and 25 μm at a 100-fold magnification, and a linear scale with a minimum value of 10 μm. The scale is calibrated using a certified stage micrometer. The relative error of the linear scale is allowed within ± 2%.

One of the illuminators is a bright episcopic illuminator built into the microscope, the other is an external, focussed illuminator that allows for reflected side lighting at an angle of 10-20°.

The filter unit is designed to hold mechanical inclusions and consists of a filter holder made of glass or other suitable material, a vacuum source and a membrane filter. The membrane filter must be of the appropriate size and colour, with or without markings, and with a pore size of 1.0 μm or less.

Checking the suitability of test conditions. Pre-check the suitability of the test conditions (environment, prepared glassware, filter equipment, and the water used). To do this, determine the presence of mechanical inclusions in 50 mL of *particle-free water*, according to the procedure described below. If the number of particles with a size of 10 μm or greater exceeds 20 or the number of particles with a size of 25 μm or greater exceeds 5 on the filter after passing 50 mL of *water*, the conditions are not suitable for the test.

The preparatory steps for the test must be repeated until the environment, glassware, filter equipment, and *water* become suitable for the test.

Procedure. Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container to be opened with a jet of *particle-free water* and remove the closure, avoiding any contamination of the contents. Prepare the test solution as described above, depending on the contents of the container.

The inner side of the filter holder with the attached membrane filter is wetted with several millilitres of *particle-free water*. Transfer the entire volume of the solution or the volume of one container to the filtration funnel and connect the vacuum. If necessary, add the solution in portions until the entire volume is filtered out. After the last addition of the solution, wash the inner walls of the filter holder with *particle-free water*. The vacuum is left on until the filter surface is free of liquid. Place the filter in a Petri dish and allow to air-dry with the cover slightly ajar. After drying, place the slide with the filter on the stage of the microscope and examine the entire filter surface in reflected light.

Determine the number of particles with a size of 10 µm or greater, and the number of particles with a size of 25 µm or greater. Examination of a part of the filter and following extrapolation of the result to the entire filter area is allowed. Calculate the average number of particles in the test preparation.

When counting particles by microscopic method, it is not necessary to determine the size or number of amorphous or other formations of indeterminate morphology such as spots or films. In this case, use Method 1 or 2.

Evaluation of results. Preparations with nominal volume 100 mL or less comply with the requirements if the average number of particles equal to or greater than 10 µm does not exceed 3000 per container, and the average number of particles equal to or greater than 25 µm does not exceed 300 per container.

Preparations with nominal volume 100 mL and greater comply with the requirements if the average number of particles equal to or greater than 10 µm in 1 mL does not exceed 12, and the average number of particles equal to or greater than 25 µm does not exceed 2.

The microscopic particle count test is a compliance test.

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2.1.9.11. Softening Time Determination of Lipophilic Suppositories

The test is intended to determine, under defined conditions, the time which elapses until a lipophilic suppository completely deform.

Apparatus 1 (see Figure 2.1.9.11.-1) consists of a glass tube (1) 15.5 mm in internal diameter with a flat bottom and a length of about 140 mm and a rod (2) 5.0 mm in diameter which becomes wider towards the lower end, reaching a diameter of 12 mm, with a free sliding support (3) having an opening 5.2 mm in diameter. A metal needle (4) 2 mm in length and 1 mm in diameter is fixed on the flat underside. The upper part of the rod carries a sliding mark ring (5).

The rod consists of 2 parts: a lower part made of plastic material and an upper part made of plastic material or metal with a weight disk. The weight of the entire rod is 30 ± 0.4 g.

Procedure. Set the zero position of the marking ring, for which insert the rod into the glass tube until the bottom is reached and fix this position with a supporting device. The marking ring is moved to the level of the upper edge of the supporting device of the rod (zero position).

Place the glass tube containing 10 mL of water in a water bath and equilibrate at (36.5 ± 0.5) °C, immerse it vertically to a depth of at least 7 cm below the surface but without touching the bottom of the water bath. Introduce a suppository, tip first, into the tube followed by the rod with the free gliding plastic cover into the glass tube until the metal needle touches the flat end of the suppository. From this moment, turn on a stopwatch. Note the time which elapses until the rod sinks down to the bottom of the glass tube and the mark ring reaches the zero position.

Apparatus 2 (see Figure 2.1.9.11.-2) consists of a water bath (A) into which a thermometer (B) and a glass tube (C) with a capillary transition, sealed at the shorter end, are inserted and fixed with a stopper, and an inset (D).

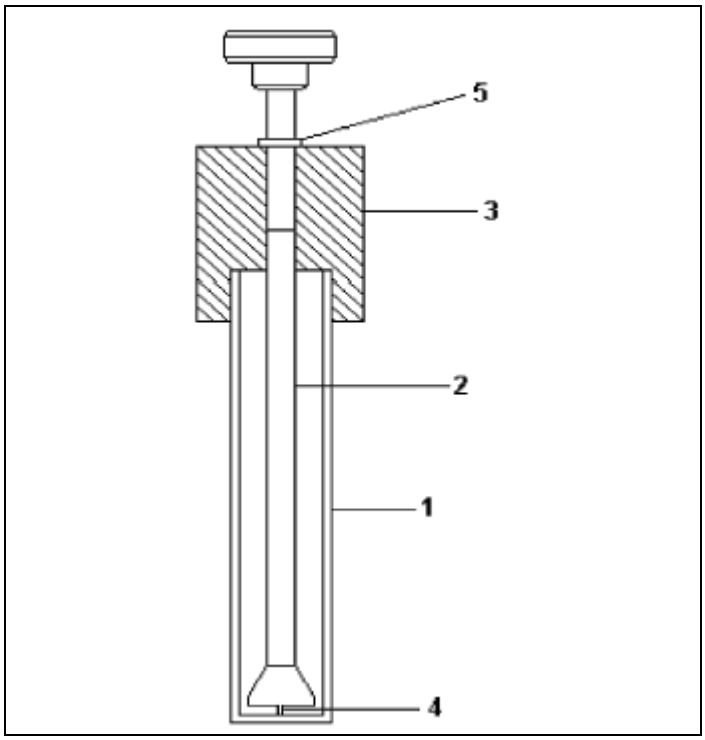

Figure 2.1.9.11.-1. *– Apparatus 1. 1 – glass tube; 2 – rod; 3 – supporting device of the rod; 4 – metal needle; 5 – marking ring in zero position*

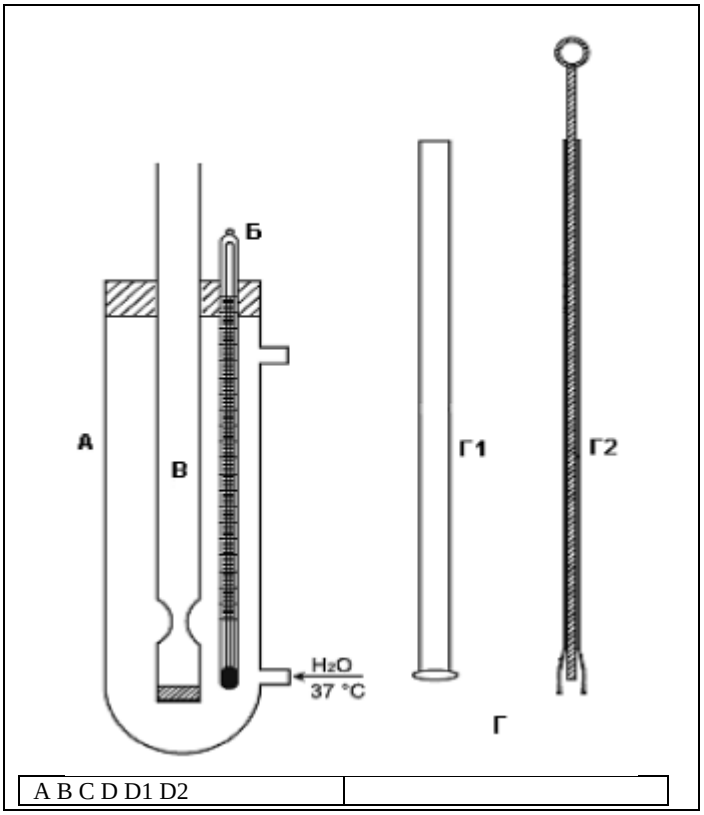

Figure 2.1.9.11.-2. – *Apparatus 2. A — water bath; B – thermometer; C – glass tube; D1 – glass rod; D2 – penetrating insert*

As an insert, you can use:

- a glass rod (D1) in the form of a tube, sealed at both ends, having a lead rim at the lower end. Rod weight 30 ± 0.4 g;

- penetrating insert (G2) consisting of a rod weighing 7.5 ±0.1 g in a shaft that has a downward extension for attaching the suppository; both parts are made of stainless steel.

Procedure. Set and maintain the temperature of the water bath in the range of (36.5 ± 0.5) °C. In tube (C), place 5 mL of water heated to 36.5 ± 0.5 °C, the suppository with the pointed end down, and introduce the insert (D1 or D2). Using a stopwatch, note the time required for the lower edge of the insert to reach the narrowed part of the glass tube.

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2.1.9.12. Uniformity of Mass of Delivered Doses from Multidose Containers

The following test is intended for the evaluation of oral dosage forms (granules, powders, and liquid dosage forms), which are supplied in multidose containers provided at manufacture with a measuring device.

Determine the mass of each of the 20 individual doses selected randomly from one or more multi-dose containers using a measuring apparatus, then calculate the average mass.

Acceptance criteria

A drug product is considered to have passed the test if no greater than two individual masses deviate from the average mass by greater than 10%. At the same time, no individual mass should deviate from the average mass by greater than 20%.

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2.1.9.13. Optical microscopy

Optical microscopy is a set of methods for observing and studying the particles of analysed drug samples that are invisible to the naked eye using an optical microscope.

The size of the particles that can be examined by this method is determined by the resolution of the microscope and is usually 1 μm or more. However, if necessary, microscopes with a total magnification of greater than 1500 can be used, which makes it possible to characterise objects from 0.5 μm in size with a resolution of individual object structures up to 0.1 μm.

Scope

In pharmacopoeial analysis, optical microscopy is used to determine the particle size when controlling the quality of semi-solid dosage forms, suspensions, emulsions, and aerosols; in dosage form technology, it is used to determine the degree of fineness of substances and excipients, as well as to study crystalline substances, since the shape, colour, and size of crystals are individual characteristics of the substance.

Apparatus

Typically, an optical microscope has a two-stage magnification system formed by a lens and an eyepiece.

All components of the microscope are mounted on a massive base. On the base, a tube holder is installed, in which a tube with a lens and an eyepiece is fixed. Under the lens is a stage, under which the lighting system (mirror, collector, condenser) is located. Both natural light and special light sources (built-in or external illuminators) can be used to illuminate the object of observation, for example, a 6V 30W halogen lamp.

The microscope can be equipped with additional instruments (phase-contrast devices, dark-field condensers, polarisers, analysers, etc.) and, depending on the selected observation method, can be light-field, dark-field, phase-contrast, polarisation, etc.

Place the test object on the stage of a microscope. Light from the light source, passing through the illuminating system, the test object and the lens, enters the eyepiece or the registration system installed in its place, a photo or video camera. Through the eyepiece, in the stream of passing light (e.g., centered by Keller), a visual study of the object is performed, and a digital photo or video camera connected to a computer allows registration of object images, after which they can be processed with special programs in semi- or fully automatic mode.

The magnification of the microscope (the product of magnifications of the lens, eyepiece, and additional accessories) should be sufficient to adequately describe and determine the size of the smallest particles of the sample.

For each zoom range, select the maximum numerical aperture of the lens. To control the contrast and detail of the image of coloured objects, it is recommended to use colour filters with a relatively narrow transmission spectrum. Colour filters can also be used for achromatic (colourless) objects.

Adjustment of all elements of the optical system, focusing and calibration are carried out in accordance with the microscope manufacturer's instructions.

Sample preparation

Test samples can be examined with or without immersion fluid. The nature of the immersion fluid used is largely determined by the physical properties of the test sample, which should not be dissolved in it. Unless otherwise specified, mineral oil is used as an immersion liquid in the examination of pharmaceutical substances and excipients.

The powder particles must be in the same plane and must be dispersed so that individual particles are visible (adhered particles are inadmissible).

Furthermore, when preparing a sample for microscopy (including when dispersing in an immersion liquid), the particles must be representative of their initial size and the distribution of sizes in the test sample.

Dosage forms are analysed without dilution or diluted as indicated in the monograph.

Procedure

When powders are examined, weigh 5-100 mg of powder and suspend it in 10 mL of immersion liquid, adding a wetting agent if necessary. Apply 1-2 drops of the resulting homogeneous suspension containing at least 10 μg of the substance on a counting cell and scan under a microscope.

The size limit and the permitted number of particles exceeding the limit are defined for each substance in the monograph, or it is determined by the purpose of the analysis.

The analysis of dosage forms is carried out as indicated in the corresponding monograph (under "Particle size").

Particle shape characterisation

The most frequently occurring forms of particles are presented in Figure 2.1.9.13.-1.

The particles may be of a different, uncertain shape.

Particle size characterisation

The way to determine the particle size depends on its shape. For spherical particles, size is defined by the diameter. The particle size shown in Figure 2.1.9.13.-1 is usually determined by the maximum length.

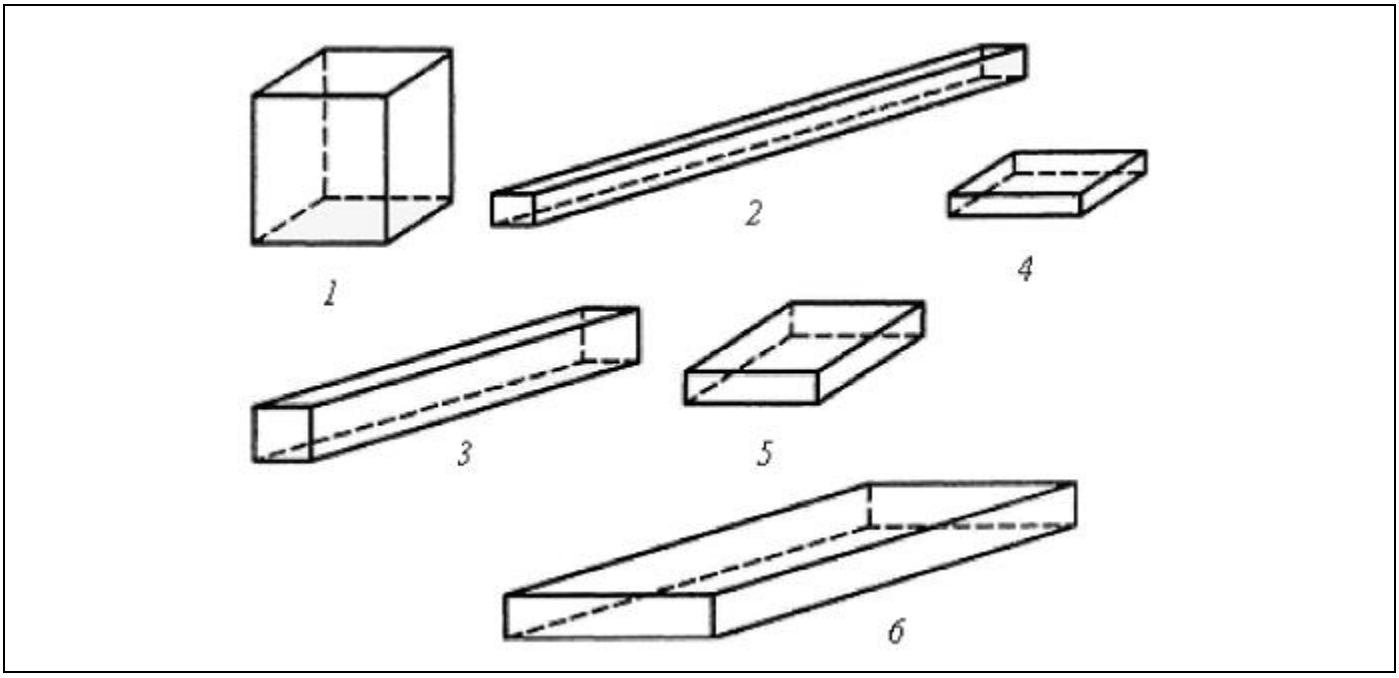

Figure 2.1.9.13.-1. – *Particle shapes. 1 – equilateral: particles with the same length, width, and thickness, including cubic and spherical particles; 2 – needle-shaped: thin, needle-like particles, or similar in length and thickness; 3 – columnar: long, thin particles with a width and thickness greater than the needle; 4 – scaly: thin, flat with the same width and length; 5 – lamellar: flat, equal in length and width, but with a greater thickness than the scaly; 6 – plank-like: large, thin, lamellar particles.*

Figure 2.1.9.13.-2. shows the dimensions usually used to characterise particles of irregular shape.

A single particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semi-solid droplet, a single crystal or polycrystalline, amorphous, or an agglomerate; particles may be associated.

By degree of ***association***, particles may be described by the following terms:

- *lamellars* – stacked plates;
- *aggregates* – mass of adhered particles;
- *agglomerate* – fused or cemented particles;
- *conglomerates* – mixture of two or more types of particles;
- *spherulites* — spherical cluster of thin needle-like crystals;
- *drusy* – particle covered with tiny particles

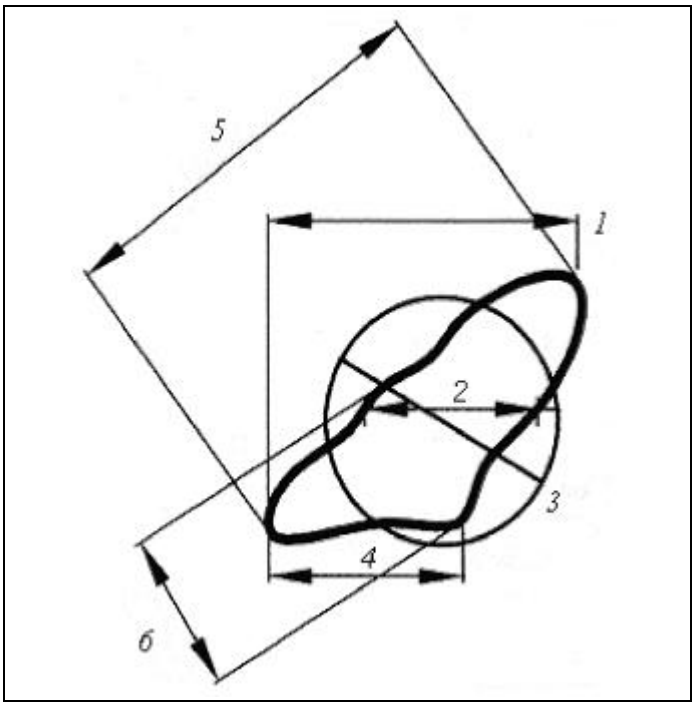

Figure 2.1.9.13.-2. – *Methods for determining the size of irregular particles. 1. Feret's diameter – the distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale; 2. Martin's diameter – the diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas; 3. Equivalent diameter – the diameter of a circle that has the same projected area as the particle; 4. Maximum horizontal size; 5. Length – the longest dimension from edge to edge of a particle oriented parallel to the ocular scale; 6. Width – the longest dimension of the particle measured at right angles to the length.*

The particle ***surface*** can be described as follows:

- *smooth* – free of irregularities, roughness, or projections;
- *rough* – uneven, not smooth;
- *cracked* – partial split, break, or fissure;
- *porous* – having openings or passageways;
- *pitted* – small indentations.

Particles can also be described:

- *edges* – angular, rounded, smooth, sharp, fractured;
- *optical* – properties-coloured, clear, translucent, opaque;
- *defects*: occlusions, inclusions.

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2.1.9.14. Uniformity of dosage units

Dosage units are dosage forms containing one or part of the dose of the active substance in each dosage unit. The purpose of the test for uniformity of dosage units is to control the uniformity of distribution of the active substance in individual units of the dosage form (tablets, capsules, suppositories, etc.). The results of this test allow quantifying the indicators that characterise the variation in the content of the active substance for individual units of the tested dosage form.

To ensure uniformity of dosage units (UDU), the content of the active substance in a dosage unit of each batch should be in a narrow range from the label amount.

A test for uniformity of the dosage units is applicable to dosage forms containing one or several active substances.

This test is usually not applied to vitamin preparations; drugs containing trace elements; drugs containing active components of plant or animal origin and other drugs if there is an appropriate justification, as well as suspensions, emulsions, gels in a single-dose containers intended for topical or external use.

The uniformity of dosage units can be demonstrated by either of two methods:

- quantitation of the active substance content separately in each dosage unit of the drug preparation selected for testing (content uniformity method, CU);
- accurate determination of the net weight of each dosage unit selected for testing (mass variation method, MV) (Table 2.1.9.14.-1).

The content uniformity method may be applied in all cases.

The test for mass variation is applicable for the following dosage forms:

(1) solutions enclosed in single-dose containers and in soft capsules;

(2) solids (including powders, granules and sterile solids) that are packaged in single-dose containers and contain no added active or inactive substances;

(3) solids (including sterile solids) that are packaged in single-dose containers, with or without added active or inactive substances, that have been prepared from true solutions and freeze-dried in the final containers and are labelled to indicate this method of preparation;

(4) hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of an active substance comprising 25% or more, by mass, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other active substances present in lesser proportions is demonstrated by meeting content uniformity (CU) requirements.

Determination of uniformity of dosage units

Select randomly 30 units from the tested batch of the drug and then 10 units from them for the first stage of the test. In each of the selected units, determine the content of the active substance by the CU or MV method. The remaining 20 units of the dosage form are retained for the second stage of the test.

Content uniformity

Carry out the determination as indicated for this dosage form. Where different procedures are used for the quantitation of the preparation and for the content uniformity test, it may be necessary to establish a correction factor to be applied to the results of the latter.

The active substance content in each of 10 units (n = 10) is determined individually using an appropriate analytical procedure given in the corresponding section of a monograph. When testing for liquid or soft dosage forms, the determination is performed with careful mixing of each container. Each of the results obtained is expressed as a percentage (x_i) of the label content of the active substance in one dose (i is the number of the dosage unit in the order of analysis).

Mass variation

Carry out a quantitation for the active substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result A, expressed as a percentage of label claim (see Calculation of Acceptance Value). Assume that the concentration (mass of an active substance per mass of dosage unit) is uniform for all dosage units. Select not fewer than 30 dosage units, and proceed as follows for each type of the dosage form designated.

Table 2.1.9.14.-1. – *Application of Content Uniformity (CU) and Mass Variation (MV) test for dosage forms*

Dosage form		Dose and ratio of active substance	
		>25 mg and >25%	<25 mg or <25%
Uncoated	tablets	MV	CU
	film-coated	MV	CU
	coated by pressing or build-up technique	cu	
Capsules	Solid:	MV	CU
	soft, containing a suspension, gel, or emulsion	CU	
	soft, containing a solution	MV	
Granules and powders in single-dose containers	single-component without excipients	MV	
	containing two or more active substances and/or excipients	cu	
Lyophilised preparations in single-dose containers		MV	
Solutions enclosed in single-dose containers		MV	
Suspensions, emulsions, gels in single-dose containers intended for parenteral use and oral administration		CU	
Suppositories		CU	
Transdermal systems		CU	
Others		CU	

Uncoated or film-coated tablets. Accurately weigh 10 tablets individually. Calculate the active substance content, expressed as a percentage of label claim, of each tablet from the mass of the individual tablets and the result of the quantitation.

Hard capsules. Accurately weigh 10 capsules individually. Remove the contents of each capsule by suitable means, then accurately weigh the emptied shell. Calculate for each capsule the net mass of its contents by subtracting the mass of the shell from the respective gross mass. Calculate the active substance content in each capsule from the mass of the contents from the individual capsules and the result of the quantitation.

Soft capsules. Accurately weigh 10 capsules individually. Cut open the capsules by means of a suitable clean, dry cutting instrument (scissors or a scalpel), and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature for 30 min, taking precautions to avoid uptake or loss of moisture. Then weigh the individual shells, and calculate the net contents. Calculate the active substance content in each capsule from the mass of the contents from the individual capsules and the result of the quantitation.

Other solid dosage forms. Proceed as directed for hard capsules, treating each unit as described therein.

Liquid or semi-solid dosage forms. Accurately weigh the amount of liquid or semi-solid that is removed from each of 10 individual containers. If necessary, calculate the equivalent volume, having previously determined the density. Calculate the active substance content in each container from the mass of the contents from the individual containers and the result of the quantitation.

Using the results obtained, calculate the content of the active substance in each dosage unit as a percentage (x_i) of the label claim:

$$x_i = w_i \cdot \frac{A}{\overline{W}},$$

where *i* is the number of the dosage unit in the order of weighing;

W_i is the net weight of the dosage unit to be examined;

W is the average net weight defined in the dosage units;

A is the content of the active substance in the dosage unit to be examined, obtained as specified under the "Quantitation" section and expressed as a percentage of the label claim.

Calculation of Acceptance Value

For the set of values *xi* obtained by any of the described methods, calculate the arithmetic mean (*X*) and standard deviation (*s*).

According to the found value *X*, select the standard value (*M*) and calculate the acceptable value (*AV*) for the first 10 dosage units (stage 1) and then, if necessary, for 30 dosage units (stage 2) using the expression:

$$|M - \overline{X}| + ks.$$

The symbols of values are given in Table 2.1.9.14.-2. Using the adopted value *M*, calculate an acceptable value as indicated in Table 2.1.9.14.-2.

Acceptance criteria

Unless otherwise specified in the monograph, the requirements for uniformity of dosage units are considered met if the acceptable value for the first 10 dosage units is less than or equal to L1. If the acceptance value is greater than L1, test the next 20 dosage units and calculate the acceptance value again.

The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to L1 and no individual content of an active substance in each dosage unit is less than (1 − L2 × 0.01) M or greater than (1 + L2 × 0.01) M in the calculation of acceptance value under content uniformity or under mass variation method. Unless otherwise specified in the monograph, L1 is 15.0 and L2 is 25.0.

Table 2.1.9.14.-2. – *Procedure for processing experimental data*

Symbol	Definition	Explanations (conditions)	Formula or value
X	The arithmetic mean of values (xi, x_2,..., x_n), expressed as % of the label claim		
x_1, x_2, ..., x_n	Individual contents of an active substance in a dosage unit of the medicinal product tested, expressed as % of the label claim		
n	Number of dosage units tested (sample size)		
k	Acceptability constant	if n = 10, then	2.4
		if n = 30, then	2.0
s	Standard deviation		$\sqrt{\frac{\sum_{i=1}^{n}(x_i-\bar{X})^2}{n-1}}$
RSD	Relative standard deviation		$\frac{100 \cdot s}{\bar{X}}$
M (case 1) applies if $T \leq 101.5$	Standard value	If 98.5% ≤ X ≤ 101.5%, then	X
		If $X \leq 98.5\%$, then	98.5%
		If$X > 101.5\%$, then	101.5%
M (case 2) applies if $T > 101.5$	Standard value	If 98.5% ≤ $X \leq T$, then	X
		If $X < 98.5\%$	98.5%
		If $X >$ T	T%
AV	Acceptable value, %		$\lvert M - X \rvert + k \cdot s$
$L1$	Maximum allowed AV value, %		15.0
$L2$	Maximum allowed range for deviation of each dosage unit tested from the calculated value of M	On the low side, 0.75 M while on the high side, 1.25 M; (based on $L2$ value of 25.0)	25.0 unless otherwise specified
T	Target content per dosage unit at time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, T = 100%. T > 100% when the excess is confirmed by stability data		

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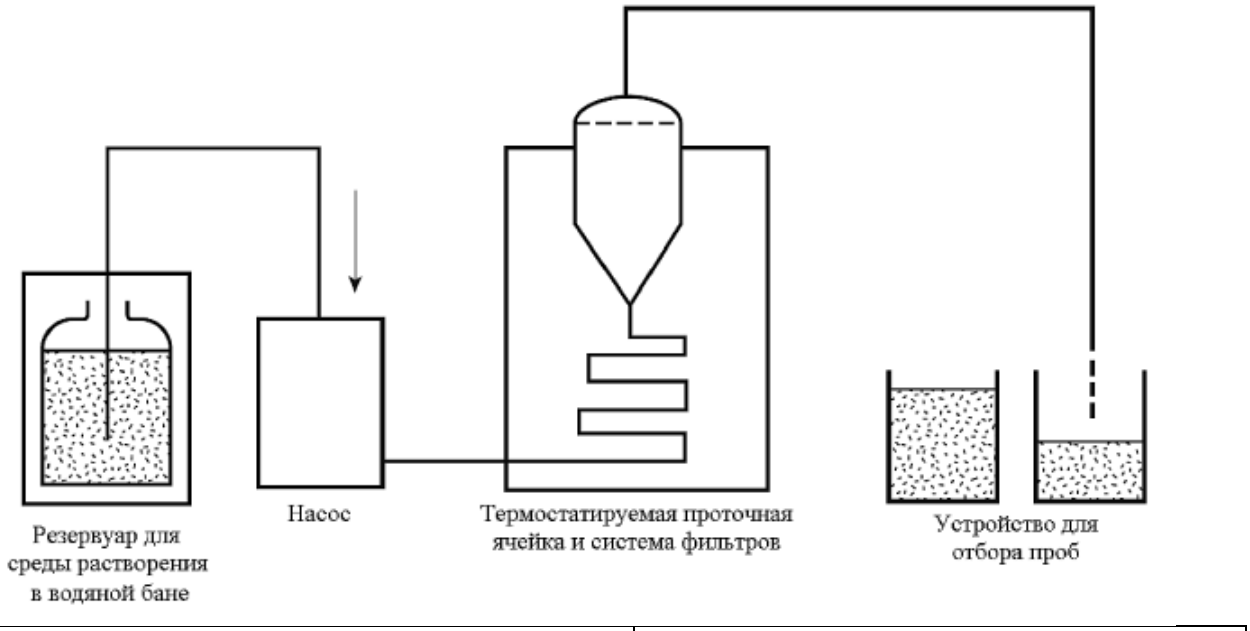
2.1.9.15. Dissolution Test for Lipophilic Solid Dosage Forms

This test is intended to determine the amount of active substance that should be released into the dissolution medium from lipophilic dosage forms (suppositories, soft capsules) during a defined period of time.

The conditions for this test are specified in the monograph and the corresponding general chapter, namely:

- dissolution medium – composition and volume;
- flow rate of the dissolution medium;
- temperature of the dissolution medium;
- sample volume;
- sampling time;
- analytical quantitation method for the active substance(s) released into the dissolution medium;



	Reservoir for the dissolution medium in a water bath
	Pump
	Thermostatically controlled flow-through cell and filter system
	Sampler

Figure 2.1.9.15.-1. – *Schematic diagram of the Flow-Through Cell*

- the quantity of the active substance dissolved in a specified time and expressed as a percentage of the stated content.

Apparatus

Use the flow-through cell (Figure 2.1.9.15.-1), which consists of:

- a reservoir for the dissolution medium placed in a water bath;
- a water bath that maintains the temperature of the dissolution medium at (37.0 ± 0.5) °C;
- a pump that forces the dissolution medium through the flow-through cell;
- a thermostatically controlled flow-through cell with a filter system;
- a sampler.

The flow-through cell consists of 3 transparent parts that fit into each other (Figure 2.1.9.15.-2):

I. The lower part is made up of 2 adjacent chambers (A and B) connected to an overflow device (1).

The dissolution medium passes through Chamber A with an upward flow, then goes to chamber B where the flow is downwards directed and passes through a small-size outlet (2) which leads upwards to a filter assembly. A conical sieve with a tip (3) can be placed in front of the outlet to collect large particles.

II. The middle part of the cell has a cavity designed to collect lipophilic excipients (4) which float on the surface of the dissolution medium. A metal grid (5) serves as a course filter.

III. The filtration assembly includes a filter (6) made of paper, glass fiber, or cellulose.

Dissolution medium

If the dissolution medium is buffered, adjust its pH to the prescribed value (within ± 0.05 units). Remove any dissolved gases from the dissolution medium before the test.

Test procedure

Place 1 unit of the preparation to be examined in Chamber A. Close the cell with the prepared filter assembly. Heat the dissolution medium to an appropriate temperature. Using a suitable pump, introduce the warmed dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed flow rate deviation of ± 5%. When the dissolution medium reaches the overflow, air starts to escape through the capillary (7) connected to the filter assembly, and chamber B fills with the dissolution medium. The active substance spreads through the dissolution medium according to its physicochemical properties.

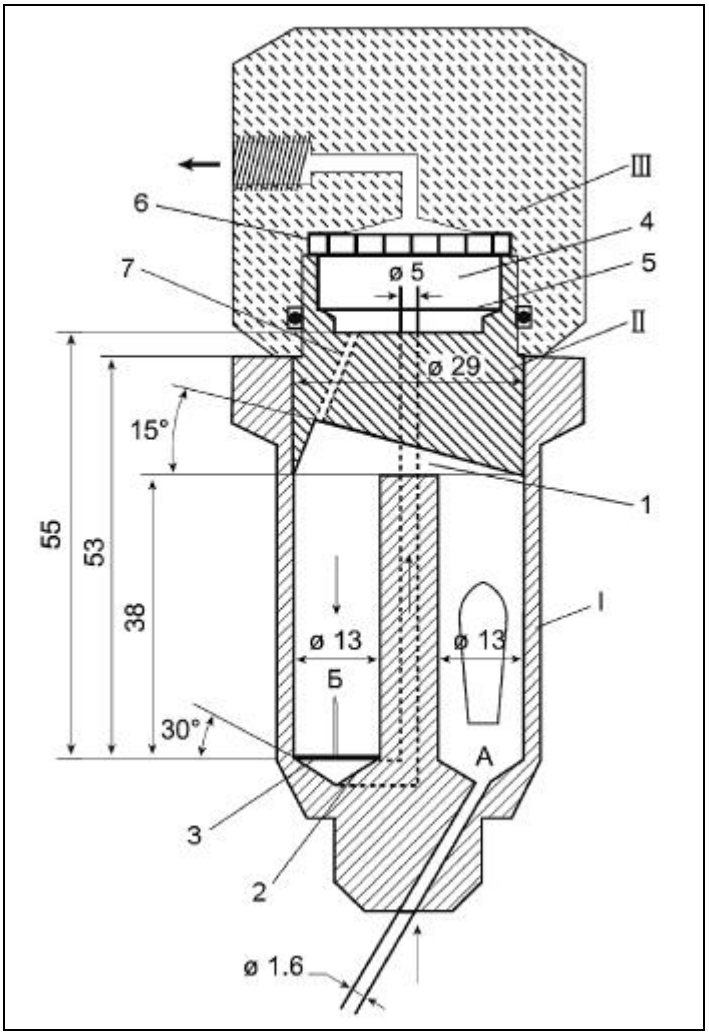


Figure 2.1.9.15.-2 – *Flow-through cell. Dimensions are shown in millimetres. A – upward flow chamber; B – downward flow chamber; 1 – overflow orifice; 2 – outlet; 3 – conical sieve with a tip; 4 – cavity for collecting lipophilic excipients; 5 – metal grid; 6 – filter; 7 –capillary*

Sampling

Samples are always collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the active substance from the solution and does not contain substances extractable by the dissolution medium that would interfere with the analytical method prescribed in the monograph.

Interpretation of results

The quantity of the active substance dissolved in a specified time is expressed as a percentage of the stated content. Unless otherwise specified in the monograph, at least 75% (*Q*) of the active substance should be released into the dissolution medium after 45 min.

Perform the test on 6 dosage units. The test results are considered satisfactory if the amount of active substance released into the dissolution medium meets the criteria given in Table 2.1.9.15.-1, stage *S*1.

If at least one result fails requirements specified in the monograph, perform the "Dissolution" test again on 6 dosage units. The results are assessed according to the Table, stage S_2.

Table 2.1.9.15.-1. – *Assessment of the results of the "Dissolution" test for lipophilic solid dosage forms*

Stage	Number of test samples	One dosage unit
S_1	6	For each test unit: not less than Q + 5% of the nominal active substance content must be released into the dissolution medium
S_2	6	The average amount of the active substance released into the dissolution medium from 12 test units of the dosage form (S_1 + S_2) should be at least Q, and there should not be a single unit where less than Q — 15% of the nominal content of the active substance passed into the dissolution medium
S_3	12	The average amount of active substance released into the medium from 24 test dosage units (S_1 + S_2 + S_3) should be not less than Q; only 2 units can be less than Q – 15% and no unit should be less than Q – 25% of the nominal content of the active substance

If the re-test results do not meet the established criteria, perform the test again on 12 additional dosage units. The results are evaluated according to the Table, stage S_3.

Unless otherwise prescribed in the monograph, reject the batch if the test results do not meet the established criteria at any stage of the examination.

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2.1.9.16. Test for Extractable Volume of Oral Liquid Dosage Forms

This chapter applies to oral liquid dosage forms. These tests are applicable to dosage forms regardless of whether they are delivered as liquid dosage forms or obtained by dissolving solid substances in a certain volume of the specified solvent. Tests are not carried out for dosage forms in single-dose containers, provided the test for dosage uniformity is included in the monograph or regulatory documentation.

DOSAGE FORMS FOR CONTAINER FILL VOLUME < 250 mL

Select 30 containers, unless otherwise specified in the monograph, and perform the test as described below for a specific dosage form.

Solutions, suspensions, emulsions and other oral liquid dosage forms. Shake the contents of each of the 10 containers and conduct testing as prescribed in the procedure.

Powders for the preparation of oral solutions and suspensions. To the contents of each of the 10 packages, add the measured volume of solvent specified on the label in accordance with the instructions for use. Shake the contents of each container and perform the test according to the procedure.

Procedure. Unless otherwise specified in the monograph, the test is performed according to the following procedure. Carefully, avoiding the formation of air bubbles, gently pour the contents of each container into separate dry graduated and calibrated cylinders of a rated capacity not exceeding 2.5 times the volume to be measured. Unless otherwise specified in the monograph or regulatory documentation, allow each container to drain for a period not exceeding 30 min, for multiple-unit containers and 5 s for single-unit containers. When the air bubbles disappear, measure the volume of liquid in each cylinder.

In the case of low-volume drug preparations, the extractable volume can be determined as follows:

- discharge the container contents into a suitable dry and pre-weighed weigning bottle, allowing drainage for not greater than 5 s;
- determine the fill weight;
- compute the volume after determining the density.

Acceptance criteria

For multiple-unit containers. The average fill volume obtained from the 10 containers is not less than 100%, and the volume of no container is less than 95% of the volume declared in the labelling.

If the average volume of the contents of 10 containers is less than 100% of that declared in the labelling, but the volume of no container is less than 95% of the labelled amount, or the average volume of the contents of 10 packages is NLT 100% and the volume of not greater than one container is less than 95%, but is not less than 90% of the labelled volume, perform the test on 20 additional containers.

The average volume of liquid obtained from 30 containers is NLT 100% of the volume declared in the labelling, and the volume obtained from NMT 1 of the 30 containers is less than 95%, but not less than 90% of the labelled volume.

For single-unit containers. If the requirements of chapter 2.1.9.14 "Uniformity of dosage units" are not applicable, the average volume of the contents of the 10 containers is NLT 100%, and the volume of each of the 10 containers lies within the range of 95 to 110% of the volume declared in the labelling.

If the average volume of the contents of 10 containers is less than 100% of the labelled volume, but the volume on no container is outside the range of 95 to 110%, or if the average volume is not less than 100% and the volume of not greater than one container is outside the range of 95 to 110%, but within the range of 90 to 115%, perform the test on 20 additional containers.

The average volume of liquid obtained from the 30 containers is not less than 100% of the volume declared in the labelling; and the volume obtained from not greater than 1 of the 30 containers is outside the range of 95 to 110%, but within the range of 90 to 115% of the labelled volume.

DOSAGE FORMS FOR CONTAINER FILL VOLUME > 250 mL

Determination is carried out using a single container in accordance with the above procedure.

Acceptance criteria

The volume of liquid obtained from one container is not less than 100% of the labelled volume.

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2.1.9.17. Weight (volume) of container contents

This test applies to non-dosage forms: ointments, liquid dosage forms for external and topical use, powders, aerosols, sprays and other dosage forms, except for oral liquid dosage forms and parenteral dosage forms.

Procedure for dosage forms other than aerosols and sprays

For containers labelled by weight. Unless otherwise specified in the monograph, select a sample of 10 filled containers and remove any labelling. Thoroughly cleanse and dry the outside of each container and weigh individually. Quantitatively remove the contents from each container and wash all parts of the container with the solvent specified in the monograph. Dry and again weigh each empty container. The difference between the two weights is used to calculate the container contents.

For containers labelled by volume. Unless otherwise specified in the monograph, pour the contents of each of the 10 containers into 10 suitable dry calibrated cylinders, and allow the liquid to drain completely. Determine the volume of the contents of each container.

Acceptance criteria

The average net content (weight, volume) of the 10 containers is not less than the labelled amount, and the net content (weight, volume) of any single container is NLT 90% of the labelled amount where the labelled amount is 60 g or 60 mL or less, or NLT 95% of the labelled amount where the labelled amount is greater than 60 g or 60 mL.

If this requirement is not met for 2 or more containers, the test is considered failed. If the requirement is not met for only one container, but the weight (volume) of the contents of this container is not less than 85% of the labelled amount, determine the content (weight, volume) of 20 additional containers.

The average content (weight, volume) of the 30 containers is not less than the labelled amount, and the net content (weight, volume) of not greater than 1 of the 30 containers is less than 90% (but NLT 85%) of the labelled amount where the labelled amount is 60 g or 60 mL or less, or less than 95% (but NLT 90%) of the labelled amount where the labelled amount is greater than 60 g or 60 mL.

Procedure for aerosols and sprays. Unless otherwise specified in the monograph, select a sample of 10 filled containers and remove any labelling. Thoroughly cleanse and dry the outside of each container and weigh individually. Remove the contents from each container by employing any safe technique (e.g., cool to reduce internal pressure, remove the valve, and pour). Remove any residual contents and rinse with a suitable solvent specified in the monograph. Heat the reservoir, valve and all associated parts at 100 °C for 5 min. Cool and again weigh each of the containers together with their corresponding parts. The difference between the original weight and the weight of the empty aerosol container is the net fill weight.

Acceptance criteria.

The net weight of the contents of each of the 10 containers must be not less than the labelled amount.

2.2. REAGENTS

2.2. REAGENTS, STANDARD SOLUTIONS, BUFFER SOLUTIONS

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2.2.1.1. REAGENTS

Where the name of a substance or a solution is followed by the letter R (the whole in italics), this indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicinal products. Also, characteristics, properties, permissible impurities of reagents, the titles and numbers of general and individual monographs are italicised.

The description may also include a CAS number (Chemical Abstract Service Registry Number) recognisable by its typical format, for example, [9002-93-1].

Some of the reagents included in the list are toxic and are to be handled in conformity with good quality control laboratory practice.

The storage periods for reagents are calculated by the manufacturers or users in accordance with the valid quality system.

In the description of dyes (indicators), after the "colour index" inscription and before the number, the designation for the colour is given according to the Colour Index (C.I.) system (in parentheses).

Reagents in aqueous solutions are prepared using *water R*. Where a reagent solution is described using an expression such as “hydrochloric acid (10 g/L HCl)”, the solution is prepared by appropriate dilution with *water R* of a more concentrated reagent solution specified in this chapter. Reagent solutions used in the limit tests for barium, calcium, and sulfates are prepared using *distilled water R.* Where the name of the solvent is not stated, an aqueous solution is intended.

The reagents and reagent solutions are to be stored in well-closed containers. The labeling should meet the relevant national legislation and international agreements.

Agarose for chromatography. [9012-36-6].

Swollen beads 60 to 140 μm in diameter presented as a 4% suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular masses of 3×10^3 to 5×10^6.

Agarose for chromatography, cross-linked.[61970-08-9].

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

Swollen beads 60 to 140 μm in diameter presented as a 4% suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular masses of 3×10^3 to 5×10^6.

Agarose for chromatography, cross-linked R1. [65099-79-8].

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

Swollen beads 60 to 140 μm in diameter presented as a 4% suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 7×10^4 to 40×10^6 and of polysaccharides with relative molecular masses of 1×10^5 to 2×10^7.

Agarose for electrophoresis. [9012-36-6].

A neutral, linear polysaccharide, the main component of which is derived from agar.

White or almost white powder, practically insoluble in cold water, very slightly soluble in hot water.

Agarose-DEAE for ion-exchange chromatography. [57407-08-6].

Cross-linked agarose substituted with diethylaminoethyl groups, presented as beads.

Agarose/cross-linked polyacrylamide.

Agarose trapped within a cross-linked polyacrylamide network; it is used for the separation of globular proteins with relative molecular masses of 2 × 104 to 35 × 104.

Agnuside. $C_{22}H_{26}O_{11}$. (*Mr* 466.4). [11027-63-7]. (1*RS*,4a*SR*,5*RS*,7a*R5*)-5-Hydroxy7-[[(4-hydroxybenzoyl)oxy]methyl]-1,4a,5,7atetrahydrocyclopenta[c]pyran-1-yl β-D-glucopyranoside.

White or almost white crystals.

Adenine. $C_5H_5N_5$. (M_r 135,1). [73-24-5].

Adenine contains NLT 98.5% and NMT 101.0% of 7H-purine-6-amine calculated on a dry substance basis.

White or almost white powder.

Very slightly soluble in water and 96% ethanol, soluble in dilute mineral acids and in dilute solutions of alkaline hydroxides.

Adenosine. $C_{10}H_{13}N_5O_4$. (M_r 267.24). [58-61-7]. 6-Amino-9-β-D-ribofuranosyl-9*H*-purine.

White or almost white, crystalline powder, slightly soluble in water, practically insoluble in acetone and in 96% ethanol, dissolves in dilute solutions of acids.

The melting point is about 234 °C.

Adipic acid. $C_6H_{10}O4$. (M_r 146.14). [124-04-9].

Crystals in the form of prisms, freely soluble in methanol, soluble in acetone, practically insoluble in petroleum ether.

The melting point is about 152 °C.

Adrenaline. $C_9H_{13}NO_3$. (*Mr* 183.20). [51-43-4]. (1*R*)-1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanol. 4-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol.

White or almost white powder, gradually becoming brown on exposure to light and air, very slightly soluble in water and in 96% ethanol, insoluble in acetone, dissolves in dilute solutions of mineral acids and alkali hydroxides.

The melting point is about 215 °C.

Azomethine H. $C_{17}H_{12}NNaO_8S_2$. (M_r 445.4). [5941-07-1]. Sodium hydrogeno-4-hydroxy-5-(2-hydroxybenzylideneamino)-2,7-naphthalenedisulfonate.

Azomethine H solution.

Dissolve 0.45 g of *azomethine H R* and 1 g of *ascorbic acid R* with gentle heating in *water R* and dilute to 100 mL with the same solvent.

Nitrogen. N_2. (M_r 28.01). [7727-37-9]. Nitrogen, washed and dried.

Nitrogen, **oxygen-free**.

Nitrogen R which has been freed from oxygen by passing it through *alkaline pyrogallol solution R*.

Nitrogen R1. N_2. (M_r 28.01). [7727-37-9].

Contains not less than 99.999% (*V/V*) of N_2.

Carbon monoxide. Less than 5 ppm.

Oxygen. Less than 5 ppm.

Nitrogen for chromatography. N_2. (M_r 28.01). [7727-37-9].

Contains not less than 99.95% (*V/V*) of N_2.

Nitrogen dioxide. NO_2. (M_r 46.01). [10102-44-0]. Nitrogen(IV) oxide.

Contains not less than 98.0% (*V/V*) of NO_2.

Nitrogen monoxide. NO. (M_r 30.01). Nitrogen(II) oxide.

Contains not less than 98.0% (*V/V*) of NO.

Nitric acid. HNO_3. (M_r 63.01). [7697-37-2].

Contains no less than 63.0% (*m/m*) and no greater than 70.0% (*m/m*) of HNO_3.

Clear, colourless, or almost colorless liquid, miscible with water.

d_{20}^{20} is 1.384 to 1.416.

A 10 g/L solution is strongly acid and gives the reaction of nitrates (*2.1.3.1*).

Clarity (*2.1.2.1*). Nitric acid is clear.

Colour Index (*2.1.2.2*, *Method II).* Not more intensely coloured than the reference solution Y_6.

Chlorides (*2.1.4.4*). Maximum 0.5 ppm.

To 5 g add 10 mL of *water R* and 0.3 mL of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared in the same manner using 13 mL of *water R*, 0.5 mL of *nitric acid R*, 0.5 mL of *chloride standard solution (5 ppm Cl^-) R*, and 0.3 mL of *silver nitrate solution R2*.

Sulfates (*2.1.4.13*). Maximum 2 ppm.

evaporate 10 g to dryness with 0.2 g of *sodium carbonate R*. Dissolve the residue in 15 mL of *distilled water R*. Prepare the standard using a mixture of 2 mL of *sulfate standard solution (10 ppm SO_4^{2-}) R* and 13 mL of *distilled water R*.

Arsenic (*2.1.4.2, Method A*). Maximum 0.02 ppm.

Gently heat 50 g with 0.5 mL of *sulfuric acid R* until white fumes begin to evolve; to the residue, add 1 mL of a 100 g/L solution of *hydroxylamine hydrochloride R* and dilute to 2 mL with *water R*. Prepare the standard using 1.0 mL of *arsenic standard solution (1 ppm As^{3+}) R.*

Iron (*2.1.4.9*). Maximum 1 ppm.

Dissolve the residue from the determination of sulfated ash in 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Dilute 5 mL of this solution to 10 mL with *water R.*

Heavy metals (*2.1.4.8, Method A*). Maximum 2 ppm.

Dilute 10 mL of the solution obtained in the test for iron to 20 mL with *water R*. 12 mL of the solution complies with the test on heavy metals. Prepare the reference solution using a *lead standard solution (2 ppm Pb^{2+}) R.*

Sulfated ash. Maximum 10^{-3}%.

Carefully evaporate 100 g of *nitric acid* to dryness; moisten the residue with a few drops of *sulfuric acid R* and heat to dull red.

Quantitation. To 1.50 g add about 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 63.0 mg of HNO_3.

Store in a place protected from light.

Nitric acid, fuming. [52583-42-3].

Clear, slightly yellowish liquid, fuming on contact with air

d_{20}^{20} is about 1.5.

Nitric acid, dilute.

Contains about 125 g/L of HNO_3 (M_r 63.01).

Dilute 20 g of *nitric acid R* to 100 mL with *water R*.

Nitric acid, diluted R1.

Dilute 40 g of *nitric acid R* to 100 mL with *water R*.

Nitric acid, dilute R2.

Dilute 30 g of *nitric acid R* to 100 mL with *water R*.

Nitric acid, **lead-free**.

Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

Lead. Maximum 0.1 ppm.

Determination is performed by atomic absorption spectrometry (*2.1.2.22, Method II).*

Test solution. To 100 g of *nitric acid R* add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness; dissolve the residue in *water R* heating slightly, and dilute to 50.0 mL with the same solvent.

The absorption intensity is measured at a wavelength of 283.3 nm or 217.0 nm, using a lead hollow-cathode lamp and an air-acetylene flame as the radiation source.

Nitric acid, **lead-free R1**.

Nitric acid R containing not greater than 1 µg/kg of lead.

Nitric acid, **lead-free, dilute.**

Dilute 5 g of *lead-free nitric acid R1* to 100 mL with *deionised distilled water R*.

Nitric acid, **cadmium- and lead-free.**

Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

Test solution. To 100 g of *nitric acid R*, add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness; dissolve the residue in *water R* heating slightly, and dilute to 50.0 mL with the same solvent.

Cadmium. Maximum 0.1 ppm.

The cadmium content is determined by atomic absorption spectrometry (*2.1.2.22, Method II).* The absorption intensity is measured at a wavelength of 228.8 nm using a cadmium hollow-cathode lamp and an air-acetylene or air-propane flame as the radiation source.

Lead. Maximum 0.1 ppm.

The lead content is determined by atomic absorption spectrometry (*2.1.2.22, method II).*

The absorption intensity is measured at a wavelength of 283.3 nm or 217.0 nm using a lamp with a lead hollow-cathode lamp and an air-acetylene flame.

Nitric acid, **heavy metal-free.**

Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

As NMT 0.005 ppm.
Cd NMT 0.005 ppm.
Cu NMT 0.001 ppm.
Fe NMT 0.02 ppm.
Hg NMT 0.002 ppm.
Ni NMT 0.005 ppm.
Pb NMT 0.001 ppm.
Zn NMT 0.01 ppm.

Nitric acid, **dilute, heavy metal-free.**

Complies with the requirements prescribed for *dilute nitric acid R* and with the following additional tests. Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

As NMT 0.005 ppm.
Cd NMT 0.005 ppm.
Cu NMT 0.001 ppm.
Fe NMT 0.02 ppm.
Hg NMT 0.002 ppm.
Ni NMT 0.005 ppm.
Pb NMT 0.001 ppm.
Zn NMT 0.01 ppm.

Acrylamide. C_3H_5NO. (*Mr* 71,08). [79-06-1]. Prop-2-enamide.

Colourless or white flakes or white or almost white crystalline powder. Very soluble in water and methanol, freely soluble in anhydrous ethanol.

The melting point is about 84 °C.

30% acrylamide/bisacrylamide (29:1) solution.

Dissolve 290 g of *acrylamide R* and 10 g of *methylene bisacrylamide R* in 1 L of *water R* and filter.

30% acrylamide/bisacrylamide (36.5:1) solution.

Dissolve 292 g of *acrylamide R* and 8 g of *methylene bisacrylamide R* in 1L of *water R* and filter.

Acrylic acid. $C_3H_4O_2$. (M_r 72,06). [79-10-7]. Prop-2-enoic acid. Phenylformic acid.

Contains not less than 99% of $C_3H_4O_2$.

Stabilised with 0.02% solution of hydroquinone monomethyl ether.

Corrosive liquid. Miscible with water and 96% ethanol. Readily polymerises in the presence of oxygen.

d_{20}^{20} is about 1.05.
d_{20}^{20} is about 1.421.

The boiling point is about 141 °C.

The melting point is from 12 °C to 15 °C.

Alanine. $C_3H_7NO_2$. (M_r 89,1). [56-41-7].

Alanine contains not less than 98.5% and not greater than 101.0% (2*S*)-2-aminopropanoic acid calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Soluble in water, very slightly soluble in 96% ethanol.

Store in a place protected from light.

β-Alanine. [107-95-9].

See *3-Aminopropionic acid R*.

Aleuritic acid. $C_{16}H_{32}O_5$. (M_r 304.43). [533-87-9].

(9*RS*,10*SR*)-9,10,16-Trihydroxyhexadecanoic acid.

White or almost white powder, greasy to the touch. Soluble in methanol.

The melting point is about 101 °C.

Alizarin S. $C_{14}H_7NaO_7S{\cdot}H_2O$. ($M_r$ 360.27). [130-22-3].

Schultz No. 1145.

Colour Index (C.I.) No. 58005.

Sodium 1,2-dihydroxyanthraquinone-3-sulfonate monohydrate. Sodium 3,4-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate monohydrate.

Orange-yellow powder. Freely soluble in water and 96% ethanol.

Alizarin S solution.

A 1 g/L solution.

Test for sensitivity. The reagent shows a colour change from yellow to orange-red when it is tested according to the standardisation of *0.05 M barium perchlorate.*

Colour change. From yellow to violet in the pH range of 3.7-5.2.

Albumin, bovine. [9048-46-8]. Bovine serum albumin. Contains about 96% of protein.

White to light yellowish-brown powder.

Water (*2.1.5.12*). Maximum 3.0%.

Determined on 0,800 g of bovine albumin.

Albumin, human.

Human serum albumin contains not less than 96% of albumin.

Albumin solution, human. [9048-46-8].

Human albumin solution is a sterile liquid preparation from the plasma protein fraction containing human albumin.

Clear, slightly viscous liquid of yellow, amber, or green colour, or almost colourless.

Albumin solution, human R1.

Dilute *human albumin solution R* with a 9 g/L *sodium chloride R* solution to a 1 g/L protein concentration. Adjust the pH of the solution with *glacial acetic acid R* to 3.5-4.5.

Aldehyde dehydrogenase.

Enzyme obtained from baker's yeast which oxidises acetaldehyde to acetic acid in the presence of nicotinamide-adenine dinucleotide, potassium salts, and thiols, at pH 8.0.

Aldehyde dehydrogenase solution.

Dissolve in *water R* a quantity of *aldehyde dehydrogenase R* equivalent to 70 units and dilute to 10 mL with the same solvent. The solution is stable for 8 h at 4 °C.

Aluminum. Al. (A_r 26.98). [7429-90-5].

White or almost white, malleable, flexible, bluish metal, available as bars, sheets, powder, strips or wire. In moist air an oxide film forms which protects the metal from corrosion.

Analytical grade.

Aluminum-potassium sulfate. $AlK(SO_4)_2 \cdot 12H_2O$. (M_r 474.4). [7784-24-9]. Alum.

Contains NLT 99.0% and NMT 100.5% of $AlK(SO_4)_2 \cdot 12H_2O$.

Granular powder or colourless, clear, crystalline mass.

Soluble in water, very soluble in boiling water, soluble in glycerol, practically insoluble in 96% ethanol.

Aluminum nitrate. $Al(NO_3)_3 \cdot 9H_2O$. (M_r375.13). [7784-27-2]. Aluminum nitrate nonahydrate.

Crystals, deliquescent. Very soluble in water and 96% ethanol, very slightly soluble in acetone.

Store in an airtight container.

Aluminum oxide, anhydrous. Al_2O_3. (M_r 101.96). [1344-28-1]. Aluminum oxide.

Aluminum oxide consisting of γ-Al_2O_3, dehydrated and activated by heat treatment. The particle size is from 75 μm to 150 μm.

Aluminum oxide, basic.

A basic grade of *aluminum oxide anhydrous R* suitable for column chromatography.

pH (*2.1.2.3*). From 9 to 10.

Measure the pH of the suspension obtained by shaking 1 g with 10 mL of c*arbon dioxide-freewaterR*for 5 min.

Aluminum oxide, neutral. Aluminum oxide, hydrated.

Contains not less than 47.0% and not greater than 60.0% of Al2O3 (*Mr* 102.0).

Amorphous white or almost white powder.

Practically insoluble in water, soluble in dilute mineral acids and solutions of alkaline hydroxides.

Aluminum chloride. $AlCl_3 \cdot 6H_2O$. (M_r 241.43). [7784-13-6]. Aluminum chloride hexahydrate.

Contains not less than 98.0% of $AlCl_3 \cdot 6H_2O$.

White to slightly yellowish crystalline powder, hygroscopic. Freely soluble in water and 96% ethanol.

Store in an airtight container.

Aluminum chloride solution.

Dissolve 65.0 g of *aluminum chloride R* in *water R* and dilute to 100 mL with the same solvent. Add 0.5 g of *activated carbon R*, stir for 10 minutes, and filter. Add a sufficient amount of a 10 g/L solution of *sodium hydroxide R*(about 60 mL) to the filtrate stirring continuously until a pH value of about 1.5 is obtained.

Aluminum chloride reagent.

Dissolve 2.0 g of*aluminum chloride R* in 100 mL of a 5% (*V/V)* solution of *glacial acetic acid R* in *methanol R.*

Amido black 10B. $C_{22}H_{14}N_6Na_2O_9S_2$. ($M_r$ 617). [1064-48-8].

Schultz No. 299.

Colour Index (C.I.) No. 20470.

Disodium 5-amino-4-hydroxy-6-[(4-nitrophenyl)azo]-3-(phenylazo)naphthalene-2,7-disulfonate.

Dark brown to black powder. Sparingly soluble in water, soluble in 96% ethanol.

Amido black 10B solution.

A 5 g/L solution of *amido black 10B R* in a mixture of *acetic acid R/methanol R* (10:90).

α-Amylase. 1,4-α-D-Glucan-glucanohydrolase.

White to light brown powder.

α-Amylase solution.

A solution of *α-amylase R* with an activity of 800 FAU (Franco-American units)/g.

Aminoazobenzene. $C_{12}H_{11}N_3$. (M_r 197.24). [60-09-3]. 4-(Phenylazo)aniline.

Colour Index (C.I.) No. 11000.

Brownish-yellow needles with a bluish tinge. Slightly soluble in water, freely soluble in 96% ethanol.

The melting point is about 128 °C.

2-Aminobenzoic acid. $C_7H_7NO_2$. (M_r 137.14). [118-92-3]. Anthranilic acid.

A white or pale-yellow, crystalline powder. Sparingly soluble in cold water, freely soluble in hot water, in 96% ethanol, and in glycerol.

Solutions in 96% ethanol or in ether and, particularly, in glycerol show a violet fluorescence.

The melting point is about 145 °C.

3-Aminobenzoic acid. $C_7H_7NO_2$. (M_r 137.14). [99-05-8].

White or almost white crystals. An aqueous solution turns brown on standing in air.

The melting point is about 174 °C.

Store in an airtight container, protected from light.

4-Aminobenzoic acid. $C_7H_7NO_2$. (M_r 137.14). [150-13-0].

White or almost white crystalline powder. Slightly soluble in water, freely soluble in 96% ethanol, practically insoluble in light petroleum.

The melting point is about 187 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Procaine hydrochloride*; the chromatogram shows only one principal spot.

Store in a place protected from light.

4-Aminobenzoic acid solution.

Dissolve 1 g of *4-aminobenzoic acid R* in a mixture of 18 mL of *anhydrous acetic acid R*, 20 mL of *water R*, and 1 mL of *phosphoric acid R*. Immediately before use, mix the resulting solution with *acetone R* (2:3).

***N*-(4-Aminobenzoyl)-L-glutamic acid.** $C_{12}H_{14}N_2O_5$. (M_r 266.25). [4271-30-1]. (2 *S*)-2-[(4-Aminobenzoyl)amino]pentanedioic acid.

White or almost white crystalline powder.

The melting point is about 175 °C with decomposition.

Aminobutanol. C_4H_nNO. (M_r 89.14). [5856-63-3]. 2-Aminobutanol.

Oily liquid. Miscible with water, soluble in 96% ethanol.

d_{20}^{20} is about 0.94.

n_D^{20} is about 1.453.

The boiling point is about 180 °C.

6-Aminohexanoic acid. $C_6H_{13}NO_2$. (M_r 131.17). [60-32-2].

Colourless crystals. Freely soluble in water, sparingly soluble in methanol, practically insoluble in anhydrous ethanol.

The melting point is about 205 °C.

Aminohydroxynaphthalenesulfonic acid. $C_{10}H_9NO_4S$. (M_r 239.3). [116-63-2]. 4-Amino-3-hydroxynaphthalene-1-sulfonic acid.

White or grey needle-like crystals that turn pink under exposure to light, especially when wet. Practically insoluble in water and 96% ethanol, soluble in solutions of alkali metal hydroxides and hot sodium metabisulfite solutions.

Store in a place protected from light.

Aminohydroxynaphthalenesulpfonic acid solution.

Mix 5.0 g of *anhydrous sodium sulfite R*, 94.3 g of *sodium hydrosulfite R* and 0.7 g of *aminohydroxynaphthalenesulfonic acid R*. Dissolve 1.5 g of the resulting mixture in *water R* and dilute with to 10.0 mL the same solvent.

Prepare the solution daily.

Aminohippuric acid. $C_9H_{10}N_2O_3$. (M_r 194.2). [61-78-9]. (4-Aminobenzamido)acetic acid.

White or almost white powder. Sparingly soluble in water, soluble in 96% ethanol.

The melting point is about 200 °C.

Aminohippuric acid reagent.

Dissolve 3 g of *phthalic acid R* and 0.3 g of *aminohippuric acid R* in *96% ethanol R* and dilute to 100 mL with the same solvent.

Aminomethylalizarindiacetic acid. $C_{19}H_{15}NO_8 \cdot 2H_2O$. ($M_r$ 421.4). [3952-78-1]. 2,2'[(3,4-dihydroxyanthraquinon-3-yl)methylene-nitrilo]diacetic acid dihydrate.

Fine, pale brownish-yellow or orange-brown powder. Practically insoluble in water, soluble in solutions of alkali metal hydroxides.

The melting point is about 185 °C.

Loss on drying (*2.1.2.31*). NMT 10.0%.

Determined from 1,000 g.

Aminomethylalizarindiacetic acid solution.

Dissolve 0.192 g *aminomethylalizarindiacetic acid R* in 6 mL of freshly prepared *1 M sodium hydroxide*, add 750 mL of *water R*, 25 mL *succinate buffer solution pH 4.6 R* and, dropwise, *0.5 M hydrochloric acid* until the solution changes colour from violet-red to yellow (pH 4.5 to 5), then add 100 mL of *acetone R* and dilute to 1000 mL with *water R*.

Aminomethylalizarindiacetic acid reagent.

Solution A. Dissolve 0.36 g of *cerous nitrate R* in *water R* and dilute to 50 mL with the same solvent.

Solution B. Suspend 0.7 g of *aminomethylalizarindiacetic acid R* in 50 mL of *water R*, add about 0.25 mL of *concentrated ammonia solution R* until dissolved, then add 0.25 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

Solution C. Dissolve 6 g of *sodium acetate R* in 50 mL of *water R*, add 11.5 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

To 33 mL of *acetone R*, add 6.8 mL of solution C, 1.0 mL of solution B, 1.0 mL of solution A and dilute the resulting solution to 50 mL with *water R*.

Test for sensitivity. To 1.0 mL of the *standard fluoride solution (10 ppm F) R*, add 19.0 mL of *water R* and 5.0 mL of the aminomethylalizarindiacetic acid reagent. After 20 min, the solution turns blue.

Storage: use within 5 days.

4-Aminomethylbenzoic acid. $C_8H_9NO_2$. (M_r 151.16). [56-91-7].

Aminonitrobenzophenone. $C_{13}H_{10}N_2O_3$. (M_r 242.23). [1775-95-7]. 2-Amino-5-nitrobenzophenone.

Yellow crystalline powder. Practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methanol.

The melting point is about 160 °C.

$E_{1cm}^{1\%}$ from 690 to 720. The determination is carried out at a wavelength of 233 nm, using a 0.01 g/L solution in *methanol R*.

Aminopyrazolone. $C_{11}H_{13}N_3O$. (M_r 203.2). [83-07-8]. 4-Amino-2,3-dimethyl-1-phenylpyrazolin-5-one.

Light-yellow needle-like crystals or powder. Sparingly soluble in water, freely soluble in 96% ethanol.

The melting point is about 108 °C.

Aminopyrazolone solution.

1 g/L solution *in buffer solution pH 9.0 R*.

Aminopolyether. $C_{18}H_{36}N_2O_6$. (M_r 376.49). [23978-09-8]. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8,8,8]hexacosan.

The melting point is from 70 °C to 73 °C.

3-Aminopropanol. C_3H_9NO. (M_r 75,11). [156-87-6]. 3-Aminopropan-1-ol. Propanolamine.

Transparent colorless viscous liquid.

d_{20}^{20} is about 0.99.

n_D^{20} is about 1.461.

The melting point is about 11 °C.

3-Aminopropionic acid. $C_3H_7NO_2$. (M_r 89.09). [107-95-9]. Aminopropanoic acid. β-Alanine.

Contains not less than 99% of $C_3H_7NO_2$.

White or almost white crystalline powder. Freely soluble in water, slightly soluble in 96% ethanol, practically insoluble in acetone.

The melting point is about 200 °C with decomposition.

2-Aminophenol. C_6H_7NO. (M_r 109.13). [95-55-6].

Pale yellowish-brown crystals which rapidly turn brown.

Sparingly soluble in water, soluble in 96% ethanol.

The melting point is about 172 °C.

Store in an airtight container, protected from light.

3-Aminophenol. C_6H_7NO. (M_r 109.13). [591-27-5].

Pale yellowish-brown crystals. Sparingly soluble in water.

The melting point is about 122 °C.

4-Aminophenol. C_6H_7NO. (M_r 109.13). [123-30-8].

Contains minimum 95% of C_6H_7NO.

White or slightly coloured, crystalline powder, becoming coloured on exposure to air and light. Sparingly soluble in water, soluble in anhydrous ethanol.

The melting point is about 186 °C with decomposition.

Store in a place protected from light.

4-Aminofolic acid. $C_{19}H_{20}N_8O_5$. (*Mr* 440.4). [54-62-6]. (2*S*)-2-[[4-[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid. *N*-[4-[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]-L-glutamic acid. Aminopterine.

Yellowish powder.

The melting point is about 230 °C.

Aminochlorbenzophenone. $C_{13}H_{10}ClNO$. (M_r231.68). [719-59-5]. 2-Amino-5-chlorbenzophenone.

Yellow crystalline powder. Practically insoluble in water, freely soluble in acetone, soluble in 96% ethanol.

The melting point is about 97 °C. Contains minimum 95.0% of $C_{13}H_{10}ClNO$. Store in a place protected from light.

Concentrated ammonia solution.

NH_3. (*Mr* 17.03).

Concentrated ammonia solution contains not less than 25.0% *(m/m)* and not greater than 30.0% *(m/wm of ammonia*.

Clear, colourless, very alkaline fluid.

Miscible with water and 96% ethanol.

Ammonia solution.

Contains not less than 170 g/L and not greater than 180 g/L of NH_3 (*M*r 17.03).

Dilute 67 g of *concentrated ammonia solution R* to 100 mL with *water R*.

d_{20}^{20} is from 0.931 to 0.934.

Ammonia solution R used in the iron limit test must meet the following additional requirements: evaporate 5 mL of *ammonia solution R* in a water bath to dryness. To the dry residue, add 10 mL of *water R*, 2 mL of a 200 g/L solution of *citric acid R*, 0.1 mL of *thioglycolic acid R* and *ammonia solution R* to an alkaline reaction, dilute the resulting solution to 20 mL with *water R*. The solution should not be discoloured to pink.

Store at a temperature below 20 °C, protecting from atmospheric carbon dioxide.

Ammonia solution, diluted R1.

Contains minimum 100 g/L and maximum 104 g/L of NH_3 (M_r 17.03).

Dilute 41 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia solution, diluted R2.

Contains minimum 33 g/L and maximum 35 g/L of NH_3 (M_r 17.03).

Dilute 14 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia solution, diluted R3.

Contains minimum 1.6 g/L and maximum 1.8 g/L of NH_3 (M_r 17.03).

Dilute 0.7 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia solution, diluted R4.

Contains minimum 8.4 g/L and maximum 8.6 g/L of NH_3 (M_r 17.03).

Dilute 3.5 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia, **lead-free**.

Complies with the requirements prescribed for *dilute ammonia R1* with the following additional tests.

To 20 mL of lead-free ammonia solution, add 1 mL of *lead-free potassium cyanide solution R*, dilute to 50 mL with *water R*, and add 0.10 mL of *sodium sulfide solution R*. The solution is not more intensely coloured than a reference solution prepared without sodium sulfide.

Concentrated ammonia solution R1.

Contains minimum 30.0% (*m/m*) of NH_3 (M_r 17.03).

A clear, colorless liquid.

d_{20}^{20} is less than 0.892.

Quantitation. Introduce 50.0 mL of *1 M hydrochloric acid* in a glass-stoppered flask, accurately weigh, add 2 mL of *concentrated ammonia R1*, and weigh again. Titrate with *1 M sodium hydroxide* using 0.5 mL of *mixed methyl red solution R as indicator*.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of NH_3.

Store at a temperature not exceeding 20 °C, protecting from atmospheric carbon dioxide.

Ammonium acetate. $C_2H_7NO_2$. (M_r 77.08). [631-61-8]. Ammonium acetate.

Colourless crystals, very deliquescent in the air. Very soluble in water and 96% ethanol.

Store in an airtight container.

Ammonium acetate solution.

Dissolve 150 g of *ammonium acetate R* in *water R*, add 3 mL of *glacial acetic acid R* and dilute to 1000 mL with *water R*.

Storage: use within 7 days.

Ammonium vanadate. NH_4VO_3. (M_r 116.98). [7803-55-6]. Ammonium trioxovanadate (V).

White to slightly yellowish crystalline powder. Slightly soluble in water, soluble in *dilute ammonia R1*.

Ammonium vanadate solution.

Dissolve 1.2 g of *ammonium vanadate R* in 95 mL of *water R* and dilute to 100 mL with *sulfuric acid R*.

Ammonium bicarbonate. NH_4HCO_3. (M_r 79.06). [1066-33-7]. Ammonium bicarbonate.

Contains minimum 99% of NH_4HCO_3.

Ammonium hydrogen phosphate. $(NH_4)_2HPO_4$. (*Mr* 132.06). [7783-28-0]. Diammonium hydrogen phosphate.

White or almost white crystals or granules. Hygroscopic, very soluble in water, practically insoluble in 96% ethanol.

pH (*2.1.2.3*). About 8. Measure the pH of a 200 g/L solution.

Store in an airtight container.

Ammonium dihydrogen phosphate. $(NH_4)H_2PO_4$. (M_r 115.03). [7722-76-1]. Ammonium dihydrogen phosphate.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water.

pH (2.1.2.3). About 4.2. Measure the pH of the solution 23 g/L.

(1R)-(-)-Ammonium 10-camphorosulfonate. $C_{10}H_{19}NO_4S$. (M_r 249.3).

Contains minimum 97.0% of $C_{10}H_{19}NO_4S$.

$[\alpha]_D^{20}$ — 18 ± 2. Determined using a 50 g/L solution.

Ammonium carbonate. [506-87-6]. Ammonium carbonate.

A mixture of ammonium bicarbonate (NH_4HCO_3, M_r 79.1) and ammonium carbamate (NH_2COONH_4, M_r 78.1) in various quantitative ratios.

White or almost white translucent mass. Slowly soluble in about four volumes of water. Decomposes in boiling water. Ammonium carbonate in the free state releases not less than 30% (*m/m*) NH_3 (M_r 17.03).

Quantitation. Dissolve 2.00 g of ammonium carbonate in 25 mL of *water R*, slowly add 50.0 mL of *1 M hydrochloric acid*, and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl orange solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of NH_3.

Store at a temperature below 20 °C.

Ammonium carbonate solution.

158 g/L solution.

Ammonium carbonate solution R1.

Dissolve 20 g of *ammonium carbonate R* in 20 mL of *dilute ammonia solution R1* and dilute to 100 mL with *water R*.

Ammonium molybdate. $(NH_4)_6Mo_7O_{24}·4H_2O$. (M_r 1235.9). [12054-85-2]. Hexaammonium heptamolybdate tetrahydrate.

Colurless crystals or yellowish to greenish crystals. Soluble in water, practically insoluble in 96% ethanol.

Ammonium molybdate solution.

1000 m/L solution.

Ammonium molybdate solution R2.

Dissolve 5.0 g of *ammonium molybdate R* by heating in 30 mL of *water R*, then cool and adjust the pH to 7.0 with *dilute ammonia R2* and dilute the resulting solution to 50 mL with *water R*.

Ammonium molybdate solution R3.

Solution A. Dissolve 5 g of *ammonium molybdate R* in 20 mL of *water R with heating.*

Solution B. Mix 150 mL of *96% ethanol R* with 150 mL of *water R*. When cooling, add 100 mL of *sulfuric acid R*.

Immediately before use, add 80 volumes of solution B to 20 volumes of solution A.

Ammonium molybdate solution R4.

Dissolve 1.0 g of *ammonium molybdate R* in *water R*, dilute to 40 mL with the same solvent; add 3 mL of *hydrochloric acid R*, 5 mL of *perchloric acid R* and dilute to 100 mL with *acetone R*.

Store in a place protected from light.

Use within 1 month.

Ammonium molybdate solution R5.

Dissolve 1.0 g of *ammonium molybdate R* in a solution of 40.0 mL of a 15% (*V/V*) solution of *sulfuric acid R*.

The solution is prepared daily.

Ammonium molybdate solution R6.

To about 40 mL of *water R*, carefully add 10 mL of *sulfuric acid R*, mix, allow to cool and dilute to 100 mL with *water R*. Add 2.5 g of *ammonium molybdate R* and 1 g of *cerium sulfate R*, and shake for 15 min until dissolved.

Ammonium molybdate reagent.

Successively mix 1 volume of a solution of 25 g/L of *ammonium molybdate R*, a solution of 100 g/L of *ascorbic acid R*, and a solution of 294.5 g/L of *sulfuric acid R* (H_2SO_4), then add 2 volumes of *water R*.

Storage: use within 1 day.

Ammonium molybdate reagent R1.

Mix 10 mL of a 60 g/L of *disodium arsenate R* solution, 50 mL of *ammonium molybdate R solution*, 90 mL of *dilute sulfuric acid R*. Dilute to 200 mL in *water R*.

Store in dark glass containers at 37 °C for 24 hours.

Ammonium molybdate reagent R2.

Dissolve 50 t of *ammonium molybdate R* in 600 mL of *water R*. To 250 mL of cold *water R*, add 150 mL of *sulfuric acid R* and cool. Mix both solutions.

Storage: use within 1 day.

Ammonium nitrate. NH_4NO_3. (M_r 80.04). [6484-52-2]. Ammonium nitrate.

White or almost white crystalline powder or colorless crystals.

Hygroscopic, very soluble in water, freely soluble in methanol, soluble in 96% ethanol.

Store in an airtight container.

Ammonium nitrate R1.

Complies with the requirements prescribed for *ammonium nitrate R* with the following additional tests.

Acidity (*2.1.2.4*). The solution should have a slightly acidic reaction.

Chlorides (*2.1.4.4*). NMT 100 ppm.

Determined on 0.50 g.

Sulfates (*2.1.4.13*). NMT 150 ppm.

Determined on 1.0 g.

Sulfated ash (*2.1.4.14*). NMT 0.05%.

Determined on 1.0 g.

Ammonium oxalate. $C2H_8N_2O_4 \cdot H_2O$. (M_r 142.11). [6009-70-7]. Oxalate ammonium monohydrate.

Colourless crystals. Soluble in water.

Ammonium oxalate solution.

A 40 g/L solution.

Ammonium persulfate. $(NH_4)_2S_2O_8$. (*Mr* 228.2). [7727-54-0]. Diammonium peroxodisulfate.

Crystalline powder or white or almost white granules. Freely soluble in water.

Ammonium pyrrolidine dithiocarbamate. $C_5H_{12}N_2S_2$. (M_r 164.29). [5108-96-3]. Ammonium 1-pyrrolidinyl-dithioformiate.

White to light yellow crystalline powder. Sparingly soluble in water, very slightly soluble in 96% ethanol.

Store in a container with a small amount of ammonium carbonate in a linen bag.

Ammonium reineckate.

$NH_4[Cr(NCS)_4(NH_3)_2] \cdot H_2O$. ($M_r$ 354.44). [13573-16-5]. Ammonium diamine-tetrakis(isothiocyanato)chromate(III) monohydrate.

Red powder or crystals. Sparingly soluble in cold water, soluble in hot water and 96% ethanol.

Ammonium reineckate solution.

A 10 g/l solution.. Prepare immediately before use.

Ammonium sulfamate. NH_2SO_3NH4. (M_r 114.12). [7773-06-0]. Ammonium sulfamate.

White or almost white crystalline powder or colorless crystals.

Hygroscopic, very soluble in water, slightly soluble in 96% ethanol.

The melting point is about 130 °C.

Store in an airtight container.

Ammonium sulfate. $(NH_4)_2SO_4$. (M_r 132.14). [7783-20-2]. Diammonium sulfate.

Colourless crystals or white or almost white granules. Very soluble in water, practically insoluble in acetone and 96% ethanol.

pH (*2.1.2.3*). From 4.5 to 6.0.

Measure the pH of a 50 g/L solution in *carbon dioxide-free water R*.

Sulfated ash (*2.1.4.14*). NMT 0.1%.

Ammonium sulfide solution.

To 120 mL of *dilute ammonia solution R1*, saturated with *hydrogen sulfide R*, add 80 mL of *dilute ammonia solution R1*. Prepare immediately before use.

Ammonium thiocyanate. NH_4SCN. (M_r 76,12). [1762-95-4]. Ammonium thiocyanate.

Colourless crystals, deliquescent. Very soluble in water, soluble in 96% ethanol.

Store in an airtight container.

Ammonium thiocyanate solution.

A 76 g/L solution.

Ammonium formiate. CH_5NO_2. (M_r 63.06). [540-69-2]. Ammonium formiate.

Deliquescent crystals or granules. Very soluble in water, soluble in 96% ethanol.

The melting point is from 119 °C to 121 °C.

Store in an airtight container.

Ammonium chloride. NH_4CI. (M_r 53.49). [12125-02-9].

Ammonium chloride contains not less than 99.0% and not greater than 100.5% of NH_4CI calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water.

Ammonium chloride solution.

A 107 g/L solution.

Ammonium and cerium nitrate. $(NH_4)_2Ce(NO_3)_6$. (M_r 548.2). [16774-21-3]. Diammonium-cerium(IV) hexanitrate.

Orange-yellow crystalline powder or orange clear crystals. Soluble in water.

Ammonium and cerium sulfate. $(NH_4)_3Ce(SO_4)_4 \cdot 2H_2O$. ($M_r$ 633). [10378-47-9]. Tetraammonium-cerium(IV) tetrasulfate dihydrate.

Orange-yellow crystal powder or crystals. Slowly soluble in water.

Ammonium citrate. $C_6H_{14}N_2O_7$. (M_r 226.18). [3012-65-5]. Diammonium hydrocitrate.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, slightly soluble in 96% ethanol.

pH (*2.1.2.3*). About 4.3.

Measure the pH of the 22.6 g/L solution.

Amoxicillin trihydrate. $C_{16}H_{19}N_3O_5S \cdot 3H_2O$. ($M_r$ 419.4).

Amoxicillin trihydrate contains not less than 95.0% and not greater than 102.0% (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl] amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid trihydrate calculated on the dry substance basis.

A semi-synthetic product obtained from a fermentation product.

White or almost white crystalline powder.

Slightly soluble in water, very slightly soluble in 96% ethanol, practically insoluble in fatty oils. Soluble in dilute acids and dilute solutions of alkali metal hydroxides.

Anethole. $C_{10}H_{12}O$. (M_r 148.20). [4180-23-8]. 1-Methoxy-4-(propen-1-yl)-benzene.

White or almost white crystalline mass at a temperature between 20 °C and 21 °C, liquid at a temperature above 23 °C. Practically insoluble in water, freely soluble in anhydrous ethanol, soluble in ethyl acetate and petroleum ether.

n_D^{25} about 1.56.

The boiling point is about 230 °C.

Anethole used in gas chromatography must be able to withstand the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Anise oil*, using anethole as the test solution.

The *trans*-anethole content calculated by the internal normalisation method should be minimum 99.0% (retention time is about 41 min).

***p*-Anisidine**. C_7H_9NO. (M_r 123.15). [104-94-9]. 4-Methoxyaniline.

Contains minimum 97.0% of C_7H_9NO.

White or almost white crystals. Sparingly soluble in water, soluble in anhydrous ethanol.

Causes skin irritation; sensitiser.

Store in a dark place at a temperature from 0 °C to 4 °C.

During storage, *p*-anisidine darkens due to oxidation. Oxidised *p*-anisidine can be reduced and discoloured as follows: Dissolve 20 g of *p-anisidine R* in 500 mL of *water R* at 75 °C, add 1 g of *sodium sulfite R* and 10 g of *activated carbon R*, stir for 5 min, and filter. Cool the resulting filtrate and allow to stand at about 0 °C for at least 4 h, then filter. Wash the resulting crystals with a small amount of *water R*, cool to 0 °C, and dry in vacuo over *phosphorus pentoxide R*.

Aniline. C_6H_7N. (M_r 93.13). [62-53-3]. Benzenamine.

A colorless or slightly yellowish liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 1.02.

The boiling point is from 183 °C to 186 °C. Store in a place protected from light.

Aniline hydrochloride. C_6H_8C1N. (M_r 129.59).

[142-04-1]. Benzenamine hydrochloride. Contains not less than 97.0% of C6H8C1N.

Crystals. Darkens when exposed to air and light.

The melting point is about 198 °C. Store in a place protected from light.

Anion-exchange resin.

Resin in a chlorinated form containing quaternary ammonium groups $[CH_2N^+(CH_3)_3]$ attached to a polymer lattice consisting of polystyrene cross-linked with 2% of divinylbenzene. It is available as spherical beads, and the particle size is specified in the monograph.

Wash the resin on a glass filter (40) (*2.1.2*) with *1 M sodium hydroxide* until a negative reaction to the chlorides in the washing solution, then wash with *water R* until a neutral reaction is obtained in the washing water. Suspend in freshly prepared *ammonium-free water R* and protect from atmospheric carbon dioxide.

Anion-exchange resin R1.

Resin containing quaternary ammonium groups $[CH_2N^+(CH_3)_3]$ attached to a lattice consisting of methacrylate.

Anion-exchange resin, strongly basic.

Gel-type resin in hydroxide form containing quaternary ammonium groups $[CH_2N^+(CH_3)_3$, type 1] attached to a polymer lattice consisting of polystyrene cross-linked with 8% of divinylbenzene.

Brown transparent beads.

Particle size: 0.2 mm to 1.0 mm.

The moisture content is about 50%.

Total exchange capacity. NLT

1.2 meq/mL.

Chromatographic grade highly basic anion exchange resin.

Resin with quaternary ammonium groups attached to a lattice of latex cross-linked with divinylbenzene.

Anisaldehyde. $C_8H_8O_2$. (M_r 136,15). [123-11-5]. 4-Methoxybenzaldehyde.

Oily liquid. Very slightly soluble in water, miscible with 96% ethanol.

The boiling point is about 248 °C.

Anisaldehyde used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) under the conditions specified in the monograph *Anise oil*, using anisaldehyde as the test solution.

The content of anisaldehyde calculated by the internal normalisation method should be minimum 99.0%.

Anisaldehyde solution.

Successively mix 0.5 mL of *anisaldehyde R*, 10 mL of *glacial acetic acid R*, 85 mL of *methanol R*, and 5 mL of *sulfuric acid R*.

Anisaldehyde solution R1.

To 10 mL of *anisaldehyde R*, add 90 mL of *96% ethanol R*, mix and add 10 mL of *sulfuric acid R*, and mix again.

Antithrombin III. [90170-80-2].

Antithrombin III is isolated from human plasma chromatographically using a heparin-agarose column. The specific activity should be not less than 6 IU/mg.

Antithrombin III solution R1.

Treat *antithrombin III R* in accordance with the manufacturer's instructions and dilute to the activity of 1 IU/mL with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Antithrombin III solution R2.

Treat *antithrombin III R* in accordance with the manufacturer's instructions and dilute to the activity of 0.5 IU/mL with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Antithrombin III solution R3.

Treat *antithrombin IIIR* in accordance with the manufacturer's instructions and dilute to the activity of 0.3 IU/mL with *phosphate buffer solution pH 6.5 R*.

Antithrombin III solution R4.

Treat *antithrombin III R* in accordance with the manufacturer's instructions and dilute to an activity of 0.1 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R*.

Antithrombin III solution R5.

Treat *antithrombin III R* in accordance with the manufacturer's instructions and dilute to an activity of 0.125 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

Antithrombin III solution R6.

Treat *antithrombin III R* in accordance with the manufacturer's instructions and dilute to an activity of 1.0 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

Anthracene. $C_{14}H_{10}$. (M_r 178.22). [120-12-7].

White or almost white crystalline powder. Practically insoluble in water, slightly soluble in chloroform.

The melting point is about 218 °C.

Anthrone. $C_{14}H_{10}O$. (M_r 194.23). [90-44-8]. 9-(10*H*) - Anthracenone.

Light yellow crystalline powder.

The melting point is about 155 °C.

Apigenin. $C_{15}H_{10}O_5$. (M_r 270.24). [520-36-5]. 4′,5,7-Trihydroxyflavone

Light yellowish powder. Practically insoluble in water, sparingly soluble in 96% ethanol.

The melting point is about 310 °C with decomposition.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Roman chamomile flower*, using 10 µl of a 0.25 g/L solution in *methanol R*.

In the upper third of the chromatogram, the main zone with yellowish green fluorescence is detected.

Apigenin 7-glucoside. $C_{21}H_{20}O_{10}$. (M_r 432.6). [578-74-5]. Apigenin. 7-(β-D-glucopyranosyl-oxy)-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

Light yellowish powder. Practically insoluble in water, sparingly soluble in 96% ethanol.

The melting point is from 198 °C to 201 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Roman chamomile flower*, using 10 µl of a 0.25 g/L solution in *methanol R*. In the middle third of the chromatogram, the main area with yellowish fluorescence is detected.

Apigenin 7-glucoside used in liquid chromatography complies with the following test.

Quantitation. Determination is carried out by liquid chromatography (*2.1.2.28*) as prescribed in the monograph *Chamomile flower*.

Test solution. Dissolve 10.0 mg of apeginin 7-glucoside in *methanol R* and dilute to 100.0 mL with the same solvent.

The content of apigenin 7-glucoside calculated by the internal normalisation method should be minimum 95.0%.

Aprotinin. $C_{284}H_{432}N_{84}O_{79}S_7$. ($M_r$ 6511). [9087-70-1].

Aprotinin is a polypeptide consisting of a chain of 58 amino acids. It inhibits the stoichiometric activity of several proteolytic enzymes, such as chymotrypsin, kallikrein, plasmin, and trypsin. Contains not less than 3.0 PEU of aprotinin activity per milligram, calculated on a dry substance basis.

Almost white powder, hygroscopic.

Soluble in water and isotonic solutions, practically insoluble in organic solvents.

Arabinose. $C_5H_{10}O_5$. (M_r 150.13). [87-72-9]. (3*R*,4*S*,5*S*)-тетрагидро-2*H*-пиран-2,3,4,5-тетрол. L-Arabinopyranose. E-(+)-Arabinose.

White or almost white crystalline powder. Freely soluble in water.

$[\alpha]_D^{20}$ is between +103 and +105. Perform determination using a 50 g/l solution in *water R* containing about 0.05% of NH_3.

Arbutin. $C_{12}H_{16}O_7$. (M_r 272.25). [497-76-7]. Arbutoside. 4-Hydroxyphenyl-β-D-glucopyranoside.

White or almost white small shiny needle-like crystals. Freely soluble in water, very soluble in hot water, soluble in 96% ethanol.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Bearberry leaf*. The chromatogram must show only one principal spot.

Arginine. $C_6H_{14}N_4O_2$. (M_r 174.2). [74-79-3].

Contains not less than 98.5% and not greater than 101.0% of (2*S*)-2-amino-5-guanidinopentanoic acid calculated on a dry substance basis.

It is a fermentation product or protein hydrolysate.

White or almost white crystalline powder or colorless crystals, hygroscopic.

Freely soluble in water, very slightly soluble in 96% ethanol.

Argon. Ar. (*Ar* 39.95). [7440-37-1].

Contains minimum 99.995% (*V/V*) of Ar.

Carbon monoxide (*chapter "Carbon monoxide", Method I).*NMT 0.6 ppm *(V/V)*.

No greater than 0.5 mL of *0.002 M sodium thiosulfate* solution should be used for titration after passing 10 L of *argon R* at a flow rate of 4 L/h.

Argon R1. Ar. (A_r 39.95). [7440-37-1].

Contains minimum 99.99990% (*V/V*) of Ar.

Chromatographic grade argon. Ar. (A_r 39.95). [7440-37-1].

Contains minimum 99.95% (*V/V*) of Ar.

Ascorbic acid. $C_6H_8O_6$. (M_r 176.1). [50-81-7].

Contains minimum 99.0% and maximum 100.5% of (5*R*)-5-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one.

White or almost white crystalline powder or colorless crystals that change colour under the influence of air and moisture.

Freely soluble in water, sparingly soluble in 96% ethanol.

The melting point is about 190 °C with decomposition.

Ascorbic acid solution.

Dissolve 50 mg of *ascorbic acid R* in 0.5 mL of *water R* and dilute to 50 mL with *dimethylformamide R*.

Aspartic acid. $C_4H_7NO_4$. (M_r 133.1). [56-84-8].

Contains minimum 98.5% and maximum 101.5% of (2*S*)-2-aminobutanedioic acid calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Slightly soluble in water, practically insoluble in alcohol. Soluble in dilute mineral acids and dilute solutions of alkali metal hydroxides.

L-Aspartyl-L-phenylalanine. $C_{13}H_{16}N_2O_5$. (M_r 280.28). [13433-09-5]. (*S*)-3-Amino-*N*-[(*S*)-1-carboxy-2-phenylethyl]-succinic acid.

White or almost white powder.

The melting point is about 210 °C with decomposition.

Acetal. $C_6H_{14}O_2$. (M_r 118.17). [105-57-7].

Acetaldehyde diethylacetal. 1,1-Diethoxyethane.

A clear, colorless, volatile liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.824.

n_D^{20} is about 1.382.

The boiling point is about 103 °C.

Acetaldehyde. C_2H_4O. (M_r 44.05). [75-07-0]. Ethanal.

A clear, colorless, flammable liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.788.

n_D^{20} is about 1.332.

The boiling point is about 21 °C.

Acetylacetamide. $C_4H_7KO_2$. (M_r 101.1). [5977-14-0]. 3-Oxobutanamide.

The melting point is from 53 °C to 56 °C.

Acetylacetone. $C_5H_8O_2$. (M_r 100.12). [123-54-6]. 2,4-Pentanedione.

Colourless or slightly yellowish, highly flammable liquid. Freely soluble in water, miscible with acetone, 96% ethanol and glacial acetic acid.

n_D^{20} is 1.452 to 1.453.

The boiling point is from 138 °C to 140 °C.

Acetylacetone reagent R1.

To 100 mL of a solution of *ammonium acetate R*, add 0.2 mL of *acetylacetone R*.

Acetylacetone reagent R2.

Dissolve 0.2 mL of *acetylacetone R*, 3 mL of *glacial acetic acid R* and 25 g of *ammonium acetate R* in *water R* and dilute to 100 mL with the same solvent.

***N*-Acetyl-ε-caprolactam.** $C_8H_{13}MO_2$. (M_r 155.19). [1888-91-1]. *N*-Acetylhexane-6-lactam.

A colorless liquid. Miscible with anhydrous ethanol.

d_{20}^{20} is about 1.100.

n_D^{20} is about 1.489.

The boiling point is about 135 °C.

***N*-Acetylneuraminic acid.** $C_{11}H_{19}O_9$. M_r 309.27). [131-48-6]. *O*-Sialic acid.

White or almost white needle-like crystals. Soluble in water and methanol, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

$[\alpha]_D^{20}$ is about 36. Determination is carried out using a 10 g/L solution.

The melting point is about 186 °C with decomposition.

Acetyltyrosine ethyl ether.

$C_{13}H_{17}NO_4 \cdot H_2O$. ($M_r$ 269.29). [36546-50-6]. *N*-Acetyl-L-tyrosine ethyl ester monohydrate. Ethyl-(*S*)-2-acetamido-3-(4-hydroxyphenyl)propanoate monohydrate.

White or almost white crystalline powder; suitable for the quantitation of chymotrypsin.

$[\alpha]_D^{20}$ is between +21 and +25. Determination is carried out using a 10 g/L solution in *96% ethanol R*.

$E_{1cm}^{1\%}$ from 60 to 68. Determination is carried out at 278 nm in *96% ethanol R*.

Acetyltyrosine ethyl ester a 0.2 M solution.

Dissolve 0.54 g of *acetylthyrosine ethyl ether R* in *96% ethanol R* and dilute to 10.0 mL with the same solvent.

***N*-Acetyltryptophan.** $C_{13}H_{14}M_2O_3$. (M_r 246.26). [1218-34-4]. 2-Acetylamino-3-(indole-3-yl)propanoic acid.

White or almost white powder or colorless crystals. Slightly soluble in water, soluble in dilute solutions of alkali metal hydroxides.

The melting point is about 205 °C.

Quantitation. Determination is performed by liquid chromatography (*2.1.2.28*) as prescribed in the monograph *Tryptophan*.

Test solution. Dissolve 10.0 mg in a mixture of solvents *acetonitrile R/water R* (10:90) and dilute to 100.0 mL with the same mixture.

N-acetyltryptophan content calculated by the internal normalisation method should be minimum 99.0%.

Acetyl chloride. C_2H_3ClO. (M_r 78.50). [75-36-5].

A clear, colorless, flammable liquid. Decomposes in water and 96% ethanol, miscible with ethylene chloride.

d_{20}^{20} is about 1.10.

Distillation range (*2.1.2.11*). From 49 °C to 53 °C; not less than 95% must be distilled.

Acetylcholine chloride. $C_7H_{16}C1NO_2$. (M_r 181.66). [60-31-1].

Crystalline powder. Very soluble in cold water and 96% ethanol, decomposes in hot water and alkali solutions.

Store at -20 °C.

Acetyleugenol. $C_{12}H_{14}O_3$. (M_r 206.24). [93-28-7]. 2-Methoxy-4-(2-propenyl)phenylacetate.

Yellow oily liquid. Freely soluble in 96% ethanol, practically insoluble in water.

n_D^{20} is about 1.521.

The boiling point is from 281 °C to 282 °C.

Acetyleugenol used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Clove oil*, using acetyl eugenol as the test solution.

Acetyleugenol content, calculated by internal normalisation, should be not less than 98,0%.

Acetone. C_3H_6O. (M_r 58.08). [67-64-1].

Acetone is a propanone. A clear, colorless, volatile liquid. Miscible with water, 96% alcohol. Fumes are flammable.

Acetonitrile. C2H3N. (M_r 41, 05). [75-05-8]. Methyl cyanide. Ethanenitrile.

A clear, colorless liquid. Miscible with water, acetone and methanol.

d_{20}^{20} is about 0.78.

n_D^{20} is about 1.344.

A 100 g/L acetonitrile solution is neutral to litmus paper.

Distillation range (*2.1.2.11*). From 80 °C to 82 °C; not less than 95% must be distilled.

Acetonitrile used for spectrophotometry complies with the following additional test.

Absorbance (*2.1.2.24*). Maximum at 0.01.

The determination is carried out in the range of wavelengths from 255 nm to 420 nm, using *water R* as compensation liquid.

Chromatographic grade acetonitrile.

See *Acetonitrile R.*

Acetonitrile used for spectrophotometry complies with the following additional tests.

Absorbance (*2.1.2.24*). Maximum at 0.01.

Determination is carried out at 240 nm, using *water R* as compensation liquid.

Content (*2.1.2.27*). NLT 99.8%.

Acetonitrile R1.

Complies with the requirements prescribed for *acetonitrile R* with the following additional requirements.

Content. NLT 99.9% of C_2H_3N.

Absorbance (*2.1.2.24*). NMT 0.01.

Measure at 200 nm, using *water R* as compensation liquid.

Barbaloin. $C_{21}H_{22}O_9 \cdot H_2O$. ($M_r$ 436.4). [1415-73-2]. Aloin. 1,8-Di-hydroxy-3-hydroxy-methyl-10-β-D-glucopyranosyl-10*H*-anthracene-9-one.

Yellow to dark yellow crystalline powder or needle-like crystals, that darken under exposure to light and air. Sparingly soluble in water and 96% ethanol, soluble in acetone, solutions of ammonia and alkali metal hydroxides.

$E_{1cm}^{1\%}$ values:

1 cm

- about 192 at a wavelength of 269 nm;
- about 226 at a wavelength of 296.5 nm;
- about 259 at a wavelength of 354 nm.

Determination is carried out using *methanol R* as a solvent equivalent to an anhydrous substance.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Frangula bark*; the chromatogram shows only one principal spot.

Barbital. $C_8H_{12}N_2O_3$. (M_r 184.2). [57-44-3].

Contains minimum 99.0% and maximum 101.0% of 5,5-diethylpyrimidine-2,4,6(1H,3H,5H)-trione calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Slightly soluble in water, soluble in boiling water and alcohol, forms water-soluble compounds with alkaline hydroxides, carbonates, and ammonia.

Barbital sodium. $C_8H_{11}N_2NaO_3$. (M_r 206.2). [144-02-5]. Sodium derivative of 5,5-diethyl-1*H*,3*H*,5*H*-pyrimidine-2,4,6-trione.

Contains not less than 98,0% of $C_8H_{11}N_2NaO_3$.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, slightly soluble in 96% ethanol.

Barbituric acid. $C_4H_4N_2O_3$. (M_r 128.09). [67-52-7]. 1*H*,3*H*,5*H*-Pyrimidine-2,4,6-trione.

White or almost white powder. Slightly soluble in water, freely soluble in boiling water and dilute acids.

The melting point is about 253 °C.

Barium acetate. $C_4H_6BaO_4$. (M_r 255.42). [543-80-6]. Barium acetate.

White or almost white powder. Soluble in water.

d20 is equal to 2.47.

Barium hydroxide. $Ba(OH)_2$-$8H_2O$. (M_r 315.47). [12230-71-6]. Barium hydroxide octahydrate.

Colourless crystals. Soluble in water. Poisonous.

Barium hydroxide solution.

A 47.3 g/L solution.

Barium carbonate. $BaCO_3$. (M_r 197.34). [513-77-9]. Barium carbonate.

White or almost white powder or loose mass. Practically insoluble in water. Poisonous.

Barium nitrate. $Ba(NO_3)_2$. (M_r 261.3). [10022-31-8]. Barium nitrate.

Crystals or crystalline powder, freely soluble in water, very slightly soluble in 96% ethanol and acetone.

The melting point is about 590 °C.

Barium sulfate. $BaSO_4$. (M_r 233.4). [7727-43-7].

White or almost white fine powder, does not contain solid particles.

Practically insoluble in water and organic solvents. Very little soluble in acids and solutions of alkali metal hydroxides.

Barium chloride. $BaCl_2 \cdot 2H_2O$. (M_r 244.3). [10326-27-9]. Barium chloride dihydrate.

Colourless crystals. Freely soluble in water, slightly soluble in 96% ethanol. Poisonous.

Barium chloride solution R1.

A 61 g/L solution.

Barium chloride solution R2.

A 36.5 g/L solution.

Benzaldehyde. C_2N_6O. (M_r 106.1). [100-52-7].

Colourless or slightly yellowish liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 1.05.

n_D^{20} is about 1.545.

Distillation range (*2.1.2.11*). From 177 °C to 180 °C; not less than 95% must be distilled.

Store in a place protected from light.

Benzil. C14H10O2. (*Mr* 210.2). [134-81-6]. Diphenylethanedione.

Yellow crystalline powder. Practically insoluble in water, soluble in 96% ethanol, ethyl acetate, and toluene.

The melting point is 95 °C.

Benzil benzoate. $C_{14}H_{12}O$. (M_r 212.2). [120-51-4].

Contains minimum 99.0% and maximum 100.5% of phenylmethyl benzoate.

Colourless or almost colorless crystals or colorless or almost colorless oily liquid.

Chromatography. Determination is carried out by thin-layer chromatography *(2.1.2.26)* as prescribed in the monograph *Peru balsam*, using 20 µl of 0.3% (*V/V*) solution in *ethyl acetate R*. After spraying and heating, the chromatogram demonstrates a baseband with an R_F of about 0.8.

Benzil alcohol. C_7H_8O. (M_r 108,1). [100-51-6]. Phenylmethanol.

Contains minimum 98.0% and maximum 100.5% of C_7H_8O.

A clear, colourless, oily liquid.

Soluble in water, miscible with 96% ethanol, with fatty and essential oils.

Relative density from 1.043 to 1.049.

Benzylpenicillin sodium salt. $C_{16}H_{17}N_2NaO_4S$. (M_r 356.4). [69-57-8].

Contains not less than 95.0% and not greater than 102.0% sodium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-tia-1-azabicyclo[3.2.0]heptane-2-carboxylate calculated on a dry substance basis.

The substance is produced by the growth of certain strains of *Penicilliumnotatum* or related organisms.

White or almost white crystalline powder. Hygroscopic.

Very soluble in water, slightly soluble in 96% ethanol, practically insoluble in methylene chloride.

2-Benzylpyridine. $C_{12}H_{11}N$. (M_r 169.2). [101-82-6].

Contains NLT 98.0% of $C_{12}H_{11}N$.

Yellow liquid.

The melting point is from 13 °C to 16 °C.

4-Benzylpyridine. $C_{12}H_{11}N$. (M_r 169.2). [2116-65-6].

Contains NLT 98.0% of $C_{12}H_{11}N$.

Yellow liquid.

The melting point is from 72 °C to 78 °C.

Benzylcinnamate $C_{16}H_{14}O_2$. (M_r 238.3). [103-41-3]. Benzyl-3-phenyl-prop-2-enate.

Colourless or yellowish crystals. Practically insoluble in water, soluble in 96% ethanol.

The melting point is about 39 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Peru balsam*, using 20 µl of a 3 g/L solution in *ethyl acetate R*. After spraying and heating, the chromatogram should show a baseband with an R_F of about 0.6.

Benzoylarginine ethyl ester hydrochloride. $C_{15}H_{23}ClN_4O_3$. (M_r 342.8). [2645-08-1]. *N*-Benzoyl-L-arginine ethyl ester hydrochloride. Ethyl-(*S*)-2-benzamido-5-guanidinovalerate hydrochloride.

White or almost white crystalline powder. Very soluble in water and anhydrous ethanol.

$[\alpha]_D^{20}$ is between -15 and -18. Determination is carried out using a 10 g/L solution.

The melting point is about 129 °C.

$E_{1cm}^{1\%}$ is from 310 to 340. Determination is carried out at 227 nm, using a 0.01 g/L solution.

***N*-Benzoyl-L-prolyl-E-phenylalanyl-L-arginine 4-nitroanilide acetate.** $C_{35}H4_2N_8O_8$. (M_r 703).

Benzoyl chloride. C_7H_5ClO. (M_r 140.6). [98-88-4].

Colourless tear fluid. Decomposes in water and 96% ethanol.

d_{20}^{20} is about 1.21.

The boiling point is about 197 °C.

Benzene. C_6H_6. (M_r 78.1). [71-43-2].

A clear, colorless, flammable liquid. Practically insoluble in water, miscible with 96% ethanol.

The boiling point is about 80 °C.

Benzoin. $C_{14}H_{12}O_2$. (M_r 212.3). [579-44-2]. 2-Hydroxy-1,2-diphenyl-ethanol.

Slightly yellowish crystals. Very slightly soluble in water, freely soluble in acetone, soluble in hot 96% ethanol.

The melting point is about 137 °C.

Benzoic acid. $C_7H_6O_2$. (M_r 122.1). [65-85-0].

Contains not less than 99.0% and not greater than 100.5% of benzene carboxylic acid.

White or almost white crystalline powder or colorless crystals.

Slightly soluble in water, soluble in boiling water, freely soluble in 96% ethanol and fatty oils.

Benzophenone. $C_{13}H_{10}O$. (M_r 182.2). [119-61-9]. Diphenylmethanone.

Prismatic crystals. Practically insoluble in water, freely soluble in 96% ethanol.

The melting point is about 48 °C.

1,4-Benzoquinone. C_6H_4O. (M_r 108.1). [106-51-4]. Cyclohexa-2,5-diene-1,4-dione.

Contains NLT 98.0% of $C_6H_4O_2$.

Benzethonium chloride. $C_{27}H_{42}ClNO_2,H_2O$. (M_r 466.1). [121-54-0]. Benzyl-dimethyl[2-[2-[4-(1,1,3,3-tetramethyl-butyl)phenoxy]ethoxy]ethyl]ammonium chloride monohydrate.

Fine white or almost white powder or colorless crystals. Soluble in water and 96% ethanol.

The melting point is about 163 °C.

Store in a place protected from light.

Bergapten. $C_{12}H_8O_4$. (M_r 216.2). [484-20-8]. 5-Methoxypsoralen.

Colourless crystals. Practically insoluble in water, sparingly soluble in 96% ethanol, slightly soluble in glacial acetic acid.

The melting point is about 188 °C.

Betulin. $C_{30}H_{50}O_2$. (M_r 442.7). [473-98-3]. Lup-20(39)-ene-3β,28-diol.

White or almost white crystalline powder.

The melting point is from 248 °C to 251 °C.

Bisbenzimide. $C_{25}H_{27}Cl_3N_6O,5H_2O$. ($M_r$ 624). [23491-44-3]. 4-[5-[5-(4-Methylpiperazine-1-yl)benzimidazol-2-yl] benzimidazol-2-yl]phenol trihydrochloride pentahydrate.

Bisbenzimide stock solution.

Dissolve 5 mg of *bisbenzimide R* in *water R* and dilute to 100 mL with the same solvent.

Store in a dark place.

Bisbenzimide working solution.

Immediately before use, dilute 100 µL of the *basic bisbenzimide solution R* to 100 mL volume with *phosphate buffered saline solution pH 7.4 R*.

Biuret. $C_2H_5N_3O_2$. (M_r 103.1). [108-19-0].

White or almost white crystals, hygroscopic. Soluble in water, sparingly soluble in 96% ethanol.

The melting point is from 188 °C to 190 °C with decomposition.

Store in an airtight container.

Biuret reagent.

Dissolve 1.5 g of *copper(II) sulfate pentahydrate R* and 6.0 g of *potassium-sodium tartrate R* in 500 mL of *water R*, add 300 mL of a 100 g/L solution of carbonate-free *sodium hydroxide R*, dilute to 1000 mL with the same solvent, and mix.

Biphenyl. $C_{12}H_{10}$. (M_r 154.2). C12H10. (154.2 Mg). [92-52-4].

The melting point is from 68 °C to 70 °C.

Blocking solution.

10% (*V/V*) solution of *acetic acid R*.

Boron trifluoride. BF_3. (M_r 67.8). [7637-07-2]. Boron trifluoride.

Colorless gas.

Boron trifluoride-methanol solution.

A 140 g/L solution of *boron trifluoride R* in *methanol R*.

Boron trichloride. BCl_3. (M_r 117.2). [10294-34-5]. Boron trichloride.

Colorless gas. Reacts violently with water. Use as solutions in suitable solvents (2-chloroethanol, methylene chloride, hexane, heptane, methanol).

$n^{\wedge}$ is about 1.420.

The boiling point is about 12.6 °C.

Caution. Toxic, corrosive.

Boron trichloride-methanol solution.

A 120 g/L solution of BCl_3 in *methanol R*.

Store in a dark place at a temperature of -20 °C, mainly in ampoules.

Boric acid. H_3BO_3. *(Mr* 61.8). [10043-35-3].

Contains NLT 99,0% and NMT 100.5% of H_3BO_3.

White or almost white crystalline powder, shiny colorless plates, greasy to the touch, or white or almost white crystals.

Soluble in water, 96% ethanol, freely soluble in boiling water, and 85% glycerol.

Borneol. $C_{10}H_{18}O$. (M_r 154.3). [507-70-0]. *endo*-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol.

Colourless crystals. Easily sublimated, practically insoluble in water, freely soluble in 96% ethanol and petroleum ether.

The melting point is about 208 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel G R* as the coating substance. On the start line of the chromatographic plate, apply 10 µL of a 1 g/L solution in *toluene R* . Develop in *chloroform R*. When the solvent front passes 10 cm from the start line, the plate is removed from the chamber, air-dried, and sprayed with *anisaldehyde solution R*, using 10 mL per plate with an area of 200 mm^2, dried at 100-105 °C for 10 min. The chromatogram must show only one principal spot.

Bornyl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). [5655-61-8]. *endo*-1,7,7-Trimethylbicyclo[2.2.1] hept-2-yl acetate.

Colourless crystals or colorless liquid. Very slightly soluble in water, soluble in 96% ethanol.

The melting point is about 28 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel G R* as the coating substance. On the start line of the chromatographic plate, apply 10 µL of a 2 g/L solution in *toluene R.* Develop in *chloroform R.* When the solvent front passes 10 cm, the plate is removed from the chamber, air-dried, and sprayed with *anisaldehyde solution R*, using 10 mL per plate with an area of 200 mm^2, dried at 100-105 °C for 10 min. The chromatogram must show only one principal spot.

Brilliant blue. [6104-59-2].

See *Acid blue 83 R.*

Bromine. Br_2. (*Mr* 159.8). [7726-95-6].

Brownish-red fuming liquid. Slightly soluble in water, soluble in 96% ethanol.

d_{20}^{20} about 3.1.

Bromine solution.

Dissolve 30 g of b*romine R* and 30 g of *potassium bromide R* in *water R* and dilute to 100 mL with the same solvent.

Bromine water.

Shake 3 mL of *bromine R* with 100 mL of *water R* until saturated.

Store over excess *bromine R*, protected from light.

Bromine water R1.

Shake 0.5 mL of *bromine R* with 100 mL of *water R*.

Store in a place protected from light.

Storage: use within 7 days.

5-Bromo-2'-deoxyuridine. $C_9H_{11}BrN_2O_5$. (M_r 307.1). [59-14-3]. 5-Bromo-1-(2-deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione.

The melting point is about 194 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Iodoxyuridine (0669);* apply 5 µl of 0.25 g/L solution; the chromatogram shows only one principal spot.

Bromelains. [37189-34-7].

Concentrate of proteolytic enzymes derived from *Ananas comosus* Merr.

Dull yellow powder.

Activity: 1 g of bromelains should release about 1.2 g of amine nitrogen from a solution of *gelatin R* for 20 min at a temperature of 45 °C and a pH of 4.5.

Bromelains solution.

10 g/L solution of *bromelain R* in a mixture of *phosphate buffer solution pH 5.5 R* / 9 g/L solution of *sodium chloride R* (1:9).

Hydrobromic acid 30%. [10035-10-6].

30% hydrobromic acid in *glacial acetic acid R.*

Degas with caution the contents before opening.

Hydrobromic acid, diluted.

Place 5.0 mL of 30% *hydrobromic acid R* in amber vials, seal under *argon R* with polyethylene stoppers, and store protected from light.

Immediately before use, add 5.0 mL of *glacial acetic acid R* and mix.

Store in a dark place.

Hydrobromic acid 47%.

47% (*m/m*) hydrobromic acid in the *water R.*

Dilute hydrobromic acid R1.

Contains 7,9 g/l of HBr.

Dissolve 16.81 g of 47% *hydrobromic acid R* in *water R* and dilute to 1000 mL with the same solvent.

Bromocresol green. $C_{21}H_{14}Br_4O_5S$. (M_r 698). [76-60-8]. 3',3'',5',5''-Tetrabromo-*m*-crezolsulfonphthalein. 4,4'-(3*H*-2,1-Benzoxatiol-3-ylidene)bis(2,6-dibromo-3-methylphenol)-*S,S*-dioxide.

White to brownish powder. Slightly soluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Bromocresol green solution.

Dissolve 50 mg of *bromocresol green R* in 0.72 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R,* dilute to 100 mL with *water R.*

Test for sensitivity. To 100 mL of *carbon dioxide-free water R,* add 0.2 mL of a solution of bromocresol green; a blue colour appears, which should turn green when no greater than 0.2 mL of *0.02 M hydrochloric acid* is added.

Colour change. Yellow to blue in the pH range of 3.6-5.2.

Bromocresol green and methyl red solution.

Dissolve 0.15 g of *bromocresol green R* and 0.1 g of *methyl red R* in 180 mL of *anhydrous ethanol R* and dilute to 200 mL with *water R.*

Bromocresol purple. $C_{21}H_{16}Br_2O_5S$. (M_r 540.2). [115-40-2]. 3',3''-Dibromo-*o*-cresol-sulfonphthalein. 4,4'-(3*H*-2,1-Benz-oxatiol-3-ylidene)bis(2-bromo-6-methylphenol)-*S,S*-dioxide.

Pinkish powder. Practically insoluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Bromocresol purple solution.

Dissolve 50 mg of *bromocresol purple R* in 0.92 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R,* dilute to 100 mL with *water R.*

Test for sensitivity. To 100 mL of *carbon dioxide-free water R,* add 0.2 mL of a solution of *bromocresol purple* and 0.05 mL of *0.02 M sodium hydroxide*; a bluish-violet colour appears, which should turn yellow when no greater than 0.2 mL of *0.02 M hydrochloric acid* is added.

Colour change. Yellow to bluish-violet in the pH range of 5.2-6.8.

Bromothymol blue. $C_{27}H_{28}Br_2O_5S$. (M_r 624). [76-59-5]. 3',3''-Dibromothymolsulfone-phthalein. 4,4'-(3*H*-2,1-Benzoxatiol-3-ylidene) bis (2-bromo-6-isopropyl-3-methylphenol)-*S,S*-dioxide.

Reddish pink to brownish powder. Practically insoluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Bromothymol blue solution R1.

Dissolve 50 mg of *bromothymol blue R* in a mixture of 4 mL of *0.02 M sodium hydroxide* and 20 mL of *96% ethanol R,* dilute to 100 mL with *water R.*

Test for sensitivity. To 100 mL of *carbon dioxide-free water R,* add 0.3 mL of *bromothymol blue solution R1; a yellow colour appears, which should turn blue when no greater than 0.1 mL of* 0.02 M sodium hydroxide is added.

Colour change. Yellow to blue in the pH range of 5.8-7.4.

Bromothymol blue solution R2.

A 10 g/L solution in *dimethylformamide R.*

Bromothymol blue solution R3.

To 0.1 g of *bromothymol blue R,* add 3.2 mL of *0.05 M sodium hydroxide,* and 5 mL of *ethanol* (*90%, V/V) R,* heat until dissolved, cool the resulting solution, and dilute to 250 mL with *ethanol (90%, V/V) R.*

Bromothymol blue solution R4.

Dissolve 100 mg of *bromothymol blue R* in a mixture of equal volumes of *96% ethanol* and *water R* and dilute to 100 mL with the same mixture of solvents. Filter, if necessary.

BRP indicator solution.

Dissolve 0.1 g of *bromothymol blue R*, 20 mg of *methyl red R*, and 0.2 g of *phenolphthalein R* in *96% ethanol R*, dilute to 100 mL with the same solvent, and filter.

Bromphenol blue. $C_{19}H_{10}Br_4O_5S$. (M_r 670). [115-39-9]. 3',3'',5',5''-Tetrabromphenol-sulfone-phthalein. 4,4'-(3*H*-2,1-Benzoxatiol-3-ylidene)bis(2,6-dibromphenol)-*S*,*S*-dioxide.

Light orange-yellow powder. Very slightly soluble in water, slightly soluble in 96% ethanol, freely soluble in solutions of alkali metal hydroxides.

Bromphenol blue solution.

Dissolve 0.1 g of *bromphenol blue R* in a mixture of 1.5 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R*, dilute to 100 mL with *water R*.

Test for sensitivity. To 20 mL of *carbon dioxide-free water R*, add 0.05 mL of a solution of bromphenol blue and 0.05 mL of *0.1 M hydrochloric acid*; a yellow colour appears, which should turn bluish-violet when no greater than 0.1 mL of *0.1 M sodium hydroxide* is added.

Colour change. From yellow to bluish-violet in the pH range 2.8-4.4.

Bromophenol blue solution R1.

Dissolve 50 mg of *bromphenol blue R* under careful heating in 3.73 mL of *0.02 M sodium hydroxide* to 100 mL with *water R*.

Bromophenol blue solution R2.

Dissolve 0.2 g of *bromphenol blue R* under careful heating in 3 mL of *0.1 M sodium hydroxide* and 10 mL of *96% ethanol R*; cool the resulting solution and dilute to 100 mL with *96% ethanol R*.

Brucine. $C_{23}H_{26}N_2O_4$. (M_r 394.5). [357-57-3]. 2,3-Dimethoxystrichnidine-10-one. 2,3-Dimethoxy-strychnine.

Colourless crystals. Slightly soluble in water, freely soluble in 96% ethanol.

The melting point is about 178 °C.

Butanol. $C_4N_{10}10O$. (M_g 74.12). [71-36-3]. Butane-1-ol.

A clear, colorless liquid. Miscible with 96% ethanol.

d_{20}^{20} is about 0.81.

The boiling point is from 116 °C to 119 °C.

2-Butanol R1. $C_4H_{10}O$. (M_r 74.12). [78-92-2].

Butan-2-ol. *sec*-Butyl alcohol.

Contains NLT 99.0% of $C_4H_{10}O$.

A clear, colorless liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.81.

Distillation range (*2.1.2.11*). From 99 °C to 100 °C; not less than 95% must be distilled.

Quantitation. Determination is performed by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Isopropyl alcohol*.

Butylamine. C4H11N. (M_r 73.14). [109-73-9]. Butan-1-amine.

Distill and use during 1 month.

A colorless liquid. Miscible with water and 96% ethanol.

n_D^{20} is about 1.401.

The boiling point is about 78 °C.

tert-Butylamine. [75-64-9].

See *1,1-Dimethylethylamine R*.

Butyl acetate. $C_6H_{12}O_2$. (M_r 116.16). [123-86-4].

A clear, colorless, flammable liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.88.

n_D^{20} is about 1.395.

Distillation range (*2.1.2.11*). 123 °C to 126 °C; not less than 95% must be distilled.

Butyl acetate R1.

Butyl acetate content determined by gas chromatography should be minimum 99.5%.

A clear, colorless, flammable liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.883.

n_D^{20} is about 1.395.

Butanol. NMT 0.2%. Carry out determination by gas chromatography.

n-Butyl formate. NMT 0.1%. Carry out determination by gas chromatography.

n-Butyl propionate. NMT 0.1%. Carry out determination by gas chromatography.

Water. NMT 0.1%.

Butylboronic acid. $C_4H_{11}BO_2$. (M_r 101.94). [4426-47-5].

Contains NLT 98% of $C_4H_{11}BO_2$.

The melting point is from 90 °C to 92 °C.

tert-Butylhydroperoxide. $C_4H_{10}O_2$. (M_r 90.12). [75-91-2]. 1,1-Dimethylethylhydroperoxide.

Flammable liquid. Soluble in organic solvents.

d_{20}^{20} is about 0.898.

n_D^{20} is about 1.401.

The melting point is 35 °C.

Butylhydroxytoluene. $C_{15}H_{24}O$. (M_r 220.4). [128-37-0].

Butylhydroxytoluene is a 2,6-bis(1,1-dimethylethyl)-4-methylphenol.

White or yellowish white crystalline powder.

Practically insoluble in water, very soluble in acetone, freely soluble in alcohol and vegetable oils.

Butylhydroxytoluene. [128-37-0].

See *Butylhydroxytoluene R.*

tert-Butylmethyl ether. [1634-04-4].

See *1,1-dimethylethylamine ether R.*

Butyl parahydroxybenzoate. $C_{11}H_{14}O_3$. (M_r 194.2). [94-26-8].

Contains not less than 98.0% and not greater than 102.0% of butyl-4-hydroxybenzoate.

White or almost white crystalline powder or colorless crystals.

Very slightly soluble in water, freely soluble in 96% ethanol and methanol.

Butyrolactone. $C_4H_6O_2$. (M_r 86.09). [96-48-0].

Dihydro-2(3*H*)-furanon. γ-Butyrolactone.

Oily liquid. Miscible with water, soluble in methanol.

n_D^{25} is about 1.435.

The boiling point is about 204 °C.

Valerenic acid. $C_{15}H_{22}O_2$. (M_r 234.33). [3569-10-6]. (2*E*)-3-[(4*S*,7*R*,7a*R*)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl]-2-methylprop-2-enoic acid.

The boiling point is from 134 °C to 138 °C.

Valeric acid. $C_5H_{10}O_2$. (M_r 102.13). [109-52-4]. Pentanoic acid.

A colorless liquid. Soluble in water, freely soluble in 96% ethanol.

d_{20}^{20} is about 0.94.

n_D^{20} is about 1.409.

The boiling point is about 186 °C.

Vanillin. $C_8H_8O_3$. (*Mr* 152.1). [121-33-5].

Contains not less than 99.0% and not greater than 101.0% of 4-hydroxy-3-methoxybenzaldehyde calculated on a dry substance basis.

White or slightly yellowish crystalline powder or needle-like crystals.

Slightly soluble in water, freely soluble in 96% ethanol and methanol. Soluble in dilute solutions of alkali metal hydroxides.

Vanillin reagent.

To 100 mL of a 10 g/L solution of *vanillin R* in *96% ethanol R*, carefully add 2 mL of *sulfuric acid R* dropwise.

Use within 2 days.

Vanillin solution, phosphoric.

Dissolve 1.0 g of *vanillin R* in 25 mL of *96% ethanol R*, add 25 mL of *water R* and 35 mL of *phosphoric acid R*.

Tartaric acid. $C_4H_6O_6$. (M_r 150.1). [87-69-4].

Contains not less than 99.5% and not greater than 101,0% (2*R*,3*R*)-2,3-dihydroxybutanedioic acid calculated on a dry substance basis.

The substance is a product of natural origin obtained by extraction in a wine-making process.

White or almost white crystalline powder or colorless crystals.

Very soluble in water, freely soluble in 96% ethanol.

Vinyl acetate. $C_4H_6O_2$. (*Mr* 86.09). [108-05-4]. Ethenyl acetate.

d_{20}^{20} is about 0.930.

The boiling point is about 72 °C.

2-Vinylpyridine. C_7H_7X. (*M*r 105.14).

[100-69-6].

Yellow liquid. Water-miscible.

d_{20}^{20} is about 0.97.

n_D^{20} is about 1.549.

1-Vinylpyrrolidin-2-one. C_6H_9NO. (M_r 111.14). [88-12-0]. 1-Ethenylpyrrolidine-2-one.

Contains minimum 99.0% of C_6H_9NO.

A clear, colorless liquid.

Water (*2.1.5.12*). NMT 0.1%. Determination is carried out from 2.5 g, using a mixture of 50 mL of *anhydrous methanol R* and 10 mL of *butyrolactone R* as a solvent.

Quantitation. The determination is carried out by gas chromatography (*2.1.2.27*).

The chromatographic procedure may be carried out using a flame-ionisation detector under the following conditions:

- quartz capillary column with a size of 30 m x 0.5 mm, covered with a layer of *macrogol 20 000 R*.
- *helium for chromatography R* as the carrier gas;
- temperature:

	Time (min)	Temperature (°C)
Column	0-1	80
	1 - 12	80 → 190
	12-27	190
Sample injection device		190

Chromatograph 0.3 µl of the test substance, adjusting the flow rate of the carrier gas so that the retention time of the peak corresponding to 1-vinylpyrrolidine-2-one is about 17 min.

Chromatographic grade octadecylsilyl vinyl polymer. Vinyl alcohol-octadecylsilane copolymer in the form of spherical particles (5 µm). Contains 17% of carbon.

Vinyl chloride. C_2H_3Cl. (M_r 62.50). [75-01-4].

Colorless gas. Slightly soluble in organic solvents.

Bismuth subnitrate.

$4BiNO_3(OH)_2$-$BiO(OH)$. (M_r 1462). [1304-85-4].

White or almost white powder. Practically insoluble in water.

Bismuth subnitrate R1.

Contains not less than 71.5% and not greater than 74.0% of bismuth (Bi) and not less than 14.5% but not greater than 16.5% of nitrate equivalent to nitrogen pentoxide (N_2O_5).

Bismuth subnitrate solution.

Dissolve 5 g of *basic bismuth nitrate R1* in a mixture of 8.4 mL of *nitrc acid R* and 50 mL of *water R*, dilute to 250 mL with *water R*, and filter if necessary.

Acidity. To 10 mL, add 0.05 mL of *methyl orange solution R*; the colour of the solution should change with the addition of 5.0 mL to 6.25 mL of *1 M sodium hydroxide*.

Water. H_2O. (M_r 18.02). [7732-18-5].

Purified water intended for the preparation of drug products, other than sterile and non-pyrogenic, unless otherwise specified in the monograph.

Highly purified water. H_2O. (*Mr* 18.02).

The water of high biological quality intended for the preparation of drug products, except for the cases of the use of *Water for injections.*

Obtained from drinking water by double reverse osmosis in combination with other suitable methods, such as ultrafiltration and deionisation. The water treatment system must be properly maintained.

To ensure the proper quality of highly purified water, validated procedures are used and regular monitoring of electrical conductivity and microbiological purity is carried out during the production process.

Store and use in conditions that prevent the growth of microorganisms and any other contamination.

Water R1.

Prepared from *distilled water R* by multiple distillation. Remove carbon dioxide by boiling for at least 15 min before use in a boiling flask of fused silica or borosilicate glass and cool. Any other suitable method may be used. The boiling flask has been already used for the test or has been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h prior to first use. When tested immediately before use, *water R1* is neutral to *methyl red solution R*, i.e. it shall produce an orange-red (not a violet-red or yellow) colour corresponding to pH 5.5 ± 0.1 when 0.05 mL of *methyl red solution R1* is added to 50 mL of the water to be examined.

Conductivity determined by an in-line conductivity meter at 25 °C, maximum 1 µS·cm^{-1} (see *Water*).

Distilled water.

Water R obtained by distillation.

Distilled deionised water.

Deionised *water R* obtained by distillation with a resistance of at least 0.18 MOhm· m.

Water for injections. H_2O. (M_r 18.02).

Water intended as a solvent for the preparation of parenteral drug products (bulk water for injections or for dissolving or diluting substances and parenteral drug products before use (sterile water for injections)).

Water for chromatography.

Deionised water R with a resistivity of not less than 0.18 MOhm-m, obtained by distillation, ion exchange, reverse osmosis or other suitable method, using water that meets the requirements for drinking water established by the competent authority. The water quality should be such that when used in chromatography, there are no significant interfering peaks or loss of response. For isocratic elution with UV detection at low wavelengths (i.e. less than 230 nm) with evaporative detectors (for example, light scattering detector, particle detector, charged aerosol detector) or mass spectrometric detectors or gradient elution, it may be necessary to use water with a total organic carbon content of not greater than 5 µg/kg.

Water, ammonium-free.

To 100 mL of *water R*, add 0.1 mL of *sulfuric acid R*, distill using an apparatus described for the determination of *Distillation range* (*2.1.2.11*); reject the first 10 mL and collect the following 50 mL.

Water, nitrate-free.

To 100 mL of *water R*, add a few milligrams of *potassium permanganate R* and *barium hydroxide R;* distill using an apparatus described for the determination of *Distillation range* (*2.1.2.11*); reject the first 10 mL and collect the following 50 mL.

Water, carbon dioxide-free.

Boil *water R* for several minutes, protecting it from the atmosphere during cooling and storage or deionise *water R* with a resistivity of not less than 0.18 MOhm·m.

Water, particle-free.

Filter *water R* through a 0.22 µm membrane filter.

Hydrogen for chromatography. H_2. (M_r 2,016). [1333-74-0].

Contains minimum 99.95% (*v/v*) of H_2.

Hydrogen peroxide solution, strong. [7722-84-1].

Hydrogen peroxide solution (30%) contains not less than 29.0% (*w/w*) and not greater than 31.0% (*w/w*) H_2O_2 (M_r 34.01).

One volume of the substance corresponds to approximately 110 volumes of oxygen. A suitable stabiliser can be added.

Colourless clear liquid.

Diluted hydrogen peroxide solution. [7722-84-1].

Hydrogen peroxide solution (3%) contains not less than 2.5% (*w/w*) and not greater than 3.5% (*w/w*) H_2O_2 (M_r 34.01).

One volume of this solution corresponds to approximately 10 volumes of oxygen. A suitable stabiliser can be added.

Colourless clear liquid.

Reducing mixture.

Grind the substances added in the following order to obtain a homogeneous mixture: 20 mg of *potassium bromide R*, 0.5 g of *hydrazine sulfate R* and 5 g of *sodium chloride R*.

Galactose. $C_6H_{12}O_6$. (M_r 180.16). [59-23-4]. D-(+)-Galactose.

White or almost white crystalline powder. Freely soluble in water.

$[\alpha]_D^{20}$ is between +79 and +81. Determination is carried out using a 100 g/L solution in *water R* containing about 0.05% of NH_3.

1,6-Galactosylgalactose. $C_6H_{22}O_{11}$. (M_r 342.30). [5077-31-6]. 6-*O*-β-D-Galactopyranosyl-D-galactopyranose.

White or almost white powder.

Galacturonic acid. $C_6H_{10}O_7$. (M_r 194.14). [685-73-4]. D-(+)-galacturonic acid. (2*S*,3*R*,4*S*,5*R*)-2,3,4,5-Tetrahydroxy-6-oxo-hexanoic acid.

$[\alpha]_D^{20}$ is about + 53. Determination is carried out using a 100 g/L solution.

Gallic acid. $C_7H_6O_5 \cdot H_2O$. (M_r 188.13). [5995-86-8]. 3,4,5-Trihydroxybenzoic acid monohydrate.

Colourless or slightly yellowish crystalline powder or long needle-like crystals. Soluble in water, freely soluble in hot water, 96% ethanol, and glycerin. Gallic acid loses its crystallisation water at 120 °C.

The melting point is about 260 °C with decomposition.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Bearberry leaf;* the chromatogram shows only one principal spot.

Harpagoside. $C_{24}H_{30}O_{11}$. (M_r 494.5).

White or almost white crystalline powder, very hygroscopic. Soluble in water and 96% ethanol.

The melting point is from 117 °C to 121 °C.

Store in an airtight container.

Guaiazulene. $C_{15}H_{18}$. (M_r 198.30). [489-84-9]. 1,4-Dimethyl-7-isopropylazulene.

Dark blue crystals or blue liquid. Very slightly soluble in water, miscible with fatty and essential oils and liquid paraffin, sparingly soluble in 96% ethanol, soluble in a 500 g/L sulfuric acid solution and 80% (*m/m*) phosphoric acid to form a colorless solution.

The melting point is about 30 °C.

Store in a dark place protected from light and air.

Guaiacum resin.

Resin obtained from the heartwood of *Guaiacum officinale L.* and *Guaiacum sanctum L.* Reddish brown or greenish brownish, hard, smooth fragments, shiny on the cut.

Hexadimethrine bromide. $(C_{13}H_{30}Br_2N)_n$. [28728-55-4]. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide. Poly(1,1,5,5-tetramethyl-1,5-azoniaundecamethylene dibromide).

White or almost white amorphous powder, hygroscopic. Soluble in water.

Store in an airtight container.

Hexacosane. $C_{26}H_{54}$. (M_r 366,70). [630-01-3].

Colourless or white or almost white flakes.

The melting point is about 57 °C.

2,2',2",6,6',6"-Hexa(1,1-dimethylethyl)- 4,4',4"-[(2,4,6-trimethyl-1,3,5-benzoltriyl) trimethylen]triphenol. $C_{54}H_{78}O_3$. (M_r 775). 2,2',2",6,6',6"-Hexa-*tert*-butyl-4,4',4"-[(2,4,6-tri-methyl-1,3,5-benzoltriyl)trimethylen]triphenol.

Crystalline powder. Practically insoluble in water, soluble in acetone, slightly soluble in 96% ethanol.

The melting point is about 144 °C.

Hexamethyldisilazane. $C_6H_{19}NSi_2$. (M_r 161,39). [999-97-3].

A clear, colorless liquid.

d_{20}^{20} is about 0.78.

n_D^{20} is about 1.408.

The boiling point is about 125 °C.

Store in an airtight container.

Hexamethylenetetramine. $C_6H_{12}N_4$. (M_r 140,19). [100-97-0]. Hexamine. 1,3,5,7-Tetraazatricyclo(3.3.1.1^{3,7})decane

Colorless crystalline powder. Very soluble in water.

Hexane. C_6H_{14}. (M_r 86,18). [110-54-3].

Colourless flammable liquid. Practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20} is from 0.659 to 0.663.

n_D^{20} from 1.375 to 1.376.

Distillation range (*2.1.2.11*). Between 67 °C and 69 °C; minimum 95% must be distilled.

Hexane used in spectrophotometry complies with the following test.

Absorbance (*2.1.2.24*). Maximum at 0.01. Carry out the determination between 260 nm and 420 nm, using *water R* as compensation liquid.

Hexylamine. $C_6H_{15}N$. (M_r 101,19). [111-26-2]. Hexane-1-amine.

A colorless liquid. Slightly soluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.766.

n_D^{20} is about 1.418.

The boiling point is from 127 °C to 131 °C.

Helium for chromatography. He. (A_r 4.003). [7440-59-7].

Contains not less than 99.995% (*V/V*) of He.

Haemoglobin. [9008-02-0].

Nitrogen. Between 15% and 16%.

Iron. Between 0.2% and 0.3%.

Loss on drying (*2.1.2.31*). NMT 2%.

Sulfated ash (*2.1.4.14*). NMT 1.5%.

Haemoglobin solution.

Place 2 g of *hemoglobin R* in a 250 mL beaker, add 75 mL of *dilute hydrochloric acid R2*. Stir until solution is complete. Adjust the pH to 1.6 ± 0.1 using *1 M hydrochloric acid.* Transfer the resulting solution to a 100 mL flask with the aid of *dilute hydrochloric acid R2* and add 25 mg of *thiomersal R*.

Prepare daily, store at 5 ± 3 °C; readjust to pH 1.6 before use.

Store at 2 °C to 8 °C.

Heparin. [9041-08-1]. Heparin sodium.

Substance containing the sodium salt of sulfated glycosaminoglycan from mammalian tissues. The substance is obtained both from the lung tissue of cattle and from the intestinal mucosa of pigs, cattle or sheep. When the substance is completely hydrolysed, D-glucosamine, d-glucuronic acid, L-iduronic acid, acetic acid, and sulfuric acid are formed. The substance has the characteristic property of slowing blood clotting. The activity should be at least 180 IU/mg calculated on a dry substance basis.

White or almost white hygroscopic powder.

Freely soluble in water.

Heparinase I. [9025-39-2]. Heparinase (EC 4.2.2.7).

An enzyme from *Flavobacteriumheparinum* that cleaves polysaccharides containing (1→4)-bound residues of D-glucuronate or L-iduronate and (1→4)-bound residues of 2-sulfamino-2-deoxy-6-sulfo-D-glucose to form oligosaccharides with 4-deoxy-α-D-gluco-4-enuronosyl groups at non-reducing ends.

Heparinase II. [149371-12-0].

An enzyme from *Flavobacteriumheparinum* that causes depolymerisation of sulfated polysaccharide chains containing (1→4)-bonds between hexosamine residues and uronic acid (iduronic and glucuronic acid residues). Oligosaccharides (mainly disaccharides) containing unsaturated uronic acids are formed as a result of the reaction.

Heparinase III. [37290-86-1]. Heparin-sulfate lyase.

An enzyme from *Flavobacteriumheparinum* that causes depolymerisation of selectively sulfated polysaccharide chains containing (1→4)-bonds between hexosamine and uronic acid residues to form oligosaccharides (mainly disaccharides) containing unsaturated uronic acids.

Heptane. C_7H_{16}. (M_r 100.2). [142-82-5].

Colourless flammable liquid. Practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20} is from 0.683 to 0.686.

n_D^{20} from 1.387 to 1.388.

Distillation Range

(*2.1.2.11*). Between 97 °C and 98 °C; minimum 95% must be distilled.

Geranyl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). [105-87-3]. (*E*)-3,7-Dimethylocta-2,6-dien-1-yl acetate.

Colourless or slightly yellow liquid, with a faint smell of rose and lavender.

Geranyl acetate used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Bitter-orange-flower oil*, using geranyl acetate as the test solution.

The content of geranyl acetate calculated by the internal normalisation procedure should be minimum 98.0%.

Hyaluronidase diluent.

Dissolve 0.140 g of *hydrolysed gelatin R* at 37 °C in 200 mL of a mixture of equal volumes of *phosphate buffer solution pH 6.4 R* and *water R.*

Use within 2 h.

Hydrazine sulfate. $H_6N_2O_4S$. (M_r 130.12). [10034-93-2]. Hydrazine sulfate.

Colourless crystals. Sparingly soluble in cold water, soluble in hot water (50 °C) and freely soluble in boiling water, practically insoluble in 96% ethanol.

Arsenic (*2.1.4.2, Method A*). Maximum 1 ppm. Determination is carried out from 10 g.

Sulfated ash (*2.1.4.14*). NMT 0.1%.

Hydrocortisone acetate. $C_{23}H_{32}O_6$. (M_r 404.5). [50-03-3].

Hydrocortisone acetate contains not less than 97.0% and not greater than 102.0% of 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate calculated on a dry substance basis.

White or almost white crystalline powder.

Practically insoluble in water, slightly soluble in anhydrous ethanol and methylene chloride.

Hydroxylamine hydrochloride. NH_4C_{10}. (*Mr* 69.5). [5470-11-1].

White or almost white crystalline powder. Very soluble in water, soluble in 96% ethanol.

Hydroxylamine hydrochloride solution R2.

Dissolve 2.5 g of *hydroxylamine hydrochloride R* in 4.5 mL of hot *water R*, add 40 mL of *96% ethanol R*, 0.4 mL of a solution of *bromphenol blue R2* and a sufficient volume of *0.5 M alcoholic potassium hydroxide solution* to obtain a greenish-yellow staining, dilute to 50.0 mL with *96% ethanol R*.

Hydroxylamine solution, alcoholic.

Dissolve 3.5 g of *hydroxylamine hydrochloride R* in 95 mL of *ethanol (60%, V/V) R*, add 0.5 mL of a 2 g/L solution of *methyl orange R* in *ethanol (60%, V/V) R* and a sufficient amount of *0.5 M alcoholic (60%, V/V) potassium hydroxide solution* to obtain a clear yellow staining of the solution, dilute to 100 mL with *ethanol (60%, V/V) R*.

Hydroxylamine solution, alkaline.

Immediately before use, mix equal volumes of a 139 g/L solution of *hydroxylamine hydrochloride R* and a 150 g/L solution of *sodium hydroxide R*.

Hydroxylamine, alkaline R1.

Solution A. Dissolve 12.5 g of *hydroxylamine hydrochloride R* in *methanol R* and dilute with the same solvent to 100 mL.

Solution B. Dissolve 12.5 g of *sodium hydroxide R* in *methanol R* and dilute to 100 mL with the same solvent.

Mix equal volumes of solutions A and B immediately before use.

4-Hydroxybenzoic acid. $C_7H_6O_3$. (M_r 138.12). [99-96-7].

Crystals. Very slightly soluble in water, very soluble in 96% ethanol, soluble in acetone.

The melting point is from 214 °C to 215 °C.

4-Hydroxyisophthalic acid. $C_8H_6O_5$. (M_r 182.13). [636-46-4]. 4-Hydroxybenzene-

1,3-dicarboxylic acid.

Needles or platelets. Very slightly soluble in water, freely soluble in 96% ethanol.

The melting point is about 314 °C with decomposition.

Hydroxymethylfurfural. $C_6H_6O_3$. (M_r 126.11). [67-47-0]. 5-Hydroxymethylfurfural..

Acicular crystals. Freely soluble in water, in acetone, and 96% ethanol.

The melting point is about 32 °C.

Hydroxynaphthol blue, sodium salt. $C_{20}H_{11}N_2Na_3O_{11}S_3$. ($M_r$ 620.5). [63451-35-4]. Trisodium 2,2'-dihydroxy-1,1'-azonaphthalene-3',4,6 '-trisulfonate.

12-Hydroxystearic acid. $C_{18}H_{36}O_3$. (M_r 300.48). [106-14-9]. 12-Hydroxyoctadecanoic acid.

White or almost white powder.

The melting point is from 71 °C to 74 °C.

5-Hydroxyuracil. $C_4H_4N_2O_3$. (M_r 128.09). [496-76-4]. Isobarbituric acid. Pyrimidine-2,4,5-triol.

White or almost white crystalline powder.

The melting point is about 310 °C with decomposition.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Fluorouracil*; the chromatogram shows a principal spot with R_F of about 0.3.

Store in an airtight container.

Hydroxyquinoline. C_9H_7NO. (M_r 145.16). [148-24-3]. 8-Hydroxyquinoline. Quinoline-8-ol.

White or slightly yellowish crystalline powder. Slightly soluble in water, freely soluble in acetone, in 96% ethanol and dilute mineral acids.

The melting point is about 75 °C.

Sulfated ash (*2.1.4.14*). NMT 0.05%.

Hydroquinone. $C_6H_6O_2$. (M_r 110.11). [123-31-9]. Benzene-1,4-diol.

Colorrless or white, needle-like, small crystals that darken under exposure to air and light. Soluble in water and in 96% alcohol.

The melting point is about 173 °C.

Store in a dark place protected from light and air.

Hydroquinone solution.

Dissolve 0.5 g of *hydroquinone R* in *water R*, add 20 µL of *sulfuric acid R*, and dilute to 50 mL with *water R*.

Hyoscyamine sulfate. $C_{34}H_{48}N_2O_{10}S{\cdot}2H_2O$. ($M_r$ 713). [620-61-1]. Bis-[(1R,3r,5S)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2S)-3-hydroxy-2-phenylpropanoate]sulfate dihydrate.

Contains not less than 98.0% and not greater than 101.0% of $C_{34}H_{48}N_2O_{10}S$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless needles.

Very soluble in water, sparingly soluble or soluble in 96% ethanol.

The melting point is about 203 °C with decomposition.

Hyoscine hydrobromide. $C_{17}H_{22}BrNO_4{\cdot}3H_2O$. ($M_r$ 438.3). [6533-68-2]. (1R,2R,4S,5S,7s)-9-Methyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]non-7-yl(2S)-3-hydroxy-2-phenylpropanoate hydrobromide trihydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_{17}H_{22}BrNO_4$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, soluble in 96% ethanol.

Hyperoside. $C_{21}H_{20}O_{12}$. (M_r 464.4). 2-(3,4-Dihydroxyphenyl)-3-β-D-galactopyranosyloxy-5,7-dihydroxychromene-4-one.

Needle-like light yellow crystals. Soluble in methanol.

The melting point is about 240 °C with decomposition.

Absorbance (*2.1.2.24*). The solution in *methanol R* has two absorption maxima at wavelengths of 259 nm and 364 nm.

Hypoxanthine. $C_5H_4N_4O$. (M_r 136.11). [68-94-0]. 1-*H*-Purine-6-one.

White or almost white crystalline powder. Very slightly soluble in water, sparingly soluble in boiling water, soluble in dilute acids and dilute solutions of alkali metal hydroxides, decomposes without melting at a temperature of about 150 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Mercaptopurine*; the chromatogram shows only one principal spot.

Hypophosphite reagent.

Dissolve with the aid of a gentle heat, 10 g of *sodium hypophosphite R* in 20 mL of *water R* and dilute to 100 mL with *hydrochloric acid R*; allow it to stand and decant or filter through glass wool.

Histamine dihydrochloride. $C_5H_9N_3{\cdot}HCl$ (M_r 184.1). [56-92-8]. 2-(1H-Imidazole-4-yl)ethane-1-amine dihydrochloride.

Contains not less than 98.5% and not greater than 101.0% of $C_5H_{11}Cl_2N_3$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals. Hygroscopic. Very soluble in water, soluble in 96% alcohol.

Histamine solution.

A 9 g/L solution of *sodium chloride R* containing 0.1 µg/mL of histamine phosphate or histamine dihydrochloride equivalent to histamine base.

Histidine hydrochloride monohydrate. $C_6H_1{\cdot}ClN_3O_2{\cdot}H_2O$. ($M_r$ 209.6). [123333-71-1]. (*R5'*)-2-Amino-3-(imidazole-4-yl)propanoic acid hydrochloride monohydrate.

colorless crystals or crystalline powder. Soluble in water.

The melting point is about 250 °C with decomposition.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Histamine dihydrochloride;* the chromatogram shows only one principal spot.

Gitoxin. $C_{41}H_{64}O_{14}$. (M_r 781). [4562-36-1]. Glycoside of *Digitalis purpurea L.* 3β-(*O*-2,6-Dideoxy-β-D-*ribo*-hexopyranosyl-(14)-*O*-2,6-dideoxy-β-D-*ribo* hexopyranosyl-(1 4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyloxy)-14,16 β-DI1'idroxy-5β,14β-card-20(22)enolide.

White or almost white crystalline powder. Practically insoluble in water and most organic solvents, soluble in pyridine.

$[\alpha]_D^{20}$: +20 and +24. Determination is carried out using a 5 g/L solution in a mixture of equal volumes of *chloroform R* and *methanol R.*

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Digitalis lanata leaves*; the chromatogram shows only one principal spot.

Glycollic acid. $C_2H_4O_3$. (M_r 76.05). [79-14-1]. 2-Hydroxyacetic acid.

Crystals. Soluble in water, acetone, 96% ethanol, and methanol.

The melting point is about 80 °C.

Glyoxalhydroxyanil. $C_{14}H_{12}M_2O_2$. (M_r 240.26). [1149-16-2]. Glyoxal-bis(2-hydroxyanil).

White or almost white crystals. Soluble in hot 96% ethanol.

The melting point is about 200 °C.

Glyoxal solution. [107-22-2].

Contains about 40% (*m/m*) of glyoxal.

Quantitation. Place 1.000 g of glyoxal solution in a ground-glass-stoppered flask, add 20 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* and 50 mL of *water R.* Allow to stand for 30 min and titrate with *1 M sodium hydroxide* until the colour of the solution changes from red to green, using 1 mL of a mixed solution of *methyl red R* as indicator. Carry out a blank titration.

1 mL of *1 M sodium hydroxide solution* is equivalent to 29.02 mg of glyoxal ($C_2H_2O_2$).

Glycerol. $C_3H_8O_3$. (M_r 92,1). [56-81-5].

Contains not less than 98.0% (*m/m*) and not greater than 101.0% (*m/m*) of propane-1,2,-triol calculated with reference to anhydrous substance.

Syrupy, oily to the touch, colorless or almost colorless clear liquid. Very hygroscopic.

Miscible with water and 96% ethanol, slightly soluble in acetone, practically insoluble in fatty and essential oils.

Glycerol (85%).

An aqueous solution of propane-1,2,3-triol containing not less than 83,5% (*m/m*) and not greater than 88,5% (*m/m*) of propane-1,2,3-triol ($C_3H_8O_3$, M_r 92,1).

Syrupy, oily to the touch, colorless or almost colorless, clear liquid. Very hygroscopic.

Miscible with water and 96% ethanol, slightly soluble in acetone, practically insoluble in fatty and essential oils.

Glycine. $C_2H_5O_2$. (M_r 75.1). [56-40-6].

Contains not less than 98.5% and not greater than 101.0% of 2-aminoacetic acid calculated on a dry substance basis.

White or almost white crystalline powder.

Freely soluble in water, very slightly soluble in 96% ethanol.

Glycyrrhetic acid. $C_{30}H_{46}O_4$.

(M_r 470.7). [471-53-4]. Glycyrrhetinic acid. 12,13-Didehydro-3βhydroxy-11-oxo-olean-30-oic acid.

A mixture of α and β-glycyrrhetic acids, in which the β-isomer predominates.

White to yellowish brownish powder. Practically insoluble in water, soluble in anhydrous ethanol and glacial acetic acid.

$[\alpha]_D^{20}$: +145 and +155. Determination is carried out using a 10.0 g/L solution in *anhydrous ethanol R.*

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel* GF_{254} *R* as the coating substance, the suspension of which is prepared using a 0.25% (*V/V*) solution of *phosphoric acid R.* On the chromatographic plate, apply 5 µL of a 5 g/L solution of the glycyrrhetic acid in a mixture of equal volumes of *chloroform R* and *methanol R.*

Chromatograph in a mixture of solvents *methanol R*/*chloroform R* (5:95). When the solvent front passes 10 cm, the chromatogram is viewed in UV light at a wavelength of 254 nm. The chromatogram shows a dark spot (R_F about 0.3) corresponding to the β-glycyrrhetic acid, and a smaller spot (R_F about 0.5) corresponding to the α-glycyrrhetic acid. The plate is sprayed with *anisaldehyde solution R* and heated at a temperature of 100 °C to 105 °C for 10 min. Both spots should be coloured bluish-violet; between them a smaller bluish-violet spot may be present.

Glutamic acid. $C_5H_9NO_4$. (M_r 147.1). [56-86-0].

Contains not less than 98,5% and not greater than 100.5% of (2*S*)-2-aminopentanedioic acid calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Freely soluble in boiling water, sparingly soluble in cold water, practically insoluble in acetic acid, acetone, and alcohol.

Glutaraldehyde. $C_5H_8O_2$. (M_r 100.1). [111-30-8].

Oily liquid. Soluble in water.

n_D^{25} is about 1.434.

The boiling point is about 188 °C.

Glutaric acid. $C_5H_8O_4$. (M_r 132.12). [110-94-1]. Pentanedioic acid.

White or almost white crystalline powder.

Glucose. $C_6H_{12}O_6$. (M_r 180.2). [50-99-7].

Contains not less than 97.5% and not greater than 102.0% of D-glucopyranose calculated with reference to the anhydrous substance. Obtained from starch.

White or almost white crystalline powder.

Freely soluble in water, very slightly soluble in 96% ethanol.

Glucosamine hydrochloride. $C_6H_{14}ClNO_5$. (M_r 215.6). [66-84-2]. D-Glucosamine hydrochloride.

Crystals. Soluble in water.

$[\alpha]_D^{20}$: +100, decreasing to +47.5 after 30 min. Determination is carried out using a 100 g/L solution in *water R*.

D-Glucuronic acid. $C_6H_{10}O_7$. (M_r 194.14). [6556-12-3].

Contains not less than 96.0% $C_6H_{10}O_7$ calculated on the dried basis of the substance dried *in vacuo* (*2.1.2.31*). Soluble in water and 96% ethanol.

Shows mutarotation: $[\alpha]_d^{24}$ + 11.7 → + 36.3.

Quantitation. Dissolve 0.150 g in *anhydrous methanol R* while stirring under nitrogen and titrate with *0.1 M tetrabutylammonium hydroxide* potentiometrically (*2.1.2.19*), protecting the solution from atmospheric carbon dioxide during solubilisation and titration.

1 mL of *0.1 M tetrabutylammonium hydroxide solution* is equivalent to 19.41 mg of $C_6H_{10}O_7$.

Holmium(III) oxide. Ho_2O_3. (M_r 377.86). [12055-62-8]. Diholmium trioxide.

Yellowish powder. Practically insoluble in water.

Holmium perchlorate solution.

A 40 g/L solution of *holmium oxide R* in a solution of *perchloric acid R* containing 141 g/L of $HClO_4$.

Guanidine hydrochloride. CH_5N_3HCl. (M_r 95.53). [50-01-1]. Guanidine hydrochloride.

Crystalline powder. Freely soluble in water and 96% ethanol.

Guanine. C_5H_5N5O. (M_r 151.13). [73-40-5]. 2-Amino-1,7-dihydro-6*H*-purine-6-one.

Amorphous white or almost white powder. Practically insoluble in water, slightly soluble in 96% ethanol, soluble in ammonia solutions and dilute solutions of alkali metal hydroxides.

Gum arabic.

Air-solidifying, sticky mass released from natural or specially made incisions in the trunk and branches of acacia *Acaciasenegal* L. Willd. (syn. *Senegalia Senegal* (L.) Britton), other species of *Acacia* of African origin and *Acacia seyal Delile*.

Gum arabic is almost completely but very slowly, within 2 hours, soluble in double the volume of water to its weight. Very small pieces of plant particles remain on the surface. The resulting liquid is colorless or yellowish, dense, viscous, sticky, translucent, and has a slightly acidic reaction on blue litmus paper.

Practically insoluble in 96% ethanol.

Gum Arabic solution.

Dissolve 100 g of *gum arabic R* in 1000 mL of *water R* while stirring with a mechanical stirrer for 2 h. Centrifuge at an acceleration of about 2000 *g* for 30 min until a clear solution is obtained.

Store in 250 mL plastic containers at a temperature from 0 °C to -20 °C.

Dantron. $C_{14}H_8O_4$. (M_r 240.21). [117-10-2]. 1,8-Dihydroxyanthraquine.1,8-Dihydroxyan - tracene-9,10-dione.

Orange crystalline powder. Practically insoluble in water, slightly soluble in ethanol (96%), soluble in solutions of alkaline hydroxides.

The melting point is about 195 °C.

Deuterium oxide. ^2H_2O. (M_r 20.03). [7789-20-0]. Deuterated water. The degree of deuteration is not less than 99.7%.

d_{20}^{20} is about 1.11

n_D^{20} is about 1.328.

The boiling point is about 101 °C.

Deuterated acetic acid. $C_{22}H_4O_2$. (M_r 64.08). [1186-52-3]. Tetradeuteroacetic acid. Acetic-d_3 acid-*d*. The degree of deuteration is not less than 99.7%.

d_{20}^{20} is about 1.12

n_D^{20} is about 1.368.

The boiling point is about 115 °C. The melting point is about 16 °C.

Deuterated acetone. $C_3{}^2H_6O$. (M_r 64.12). [666-52-4]. Acetone-d_6. ($^2H_6$)-Acetone.

Degree of deuteration is not less than 99.5%. A clear, colorless liquid. Miscible with water, dimethylformamide, ethanol, and methanol.

d20 is about 0.87.

*n*D about 1.357.

The boiling point is about 55 °C.

Water and deuterium oxide. NMT 0.1%.

Deuterated dimethyl sulfoxide. $C_2{}^2H_6OS$. (M_r 84.17). [2206-27-1]. ($^2H_6$)-Dimethyl sulfoxide. Dimethyl sulfoxide-d_6

The degree of deuteration is not less than 99.8%.

A viscous, almost colourless, highly hygroscopic liquid. Soluble in water, acetone, and anhydrous ethanol.

d_{20}^{20} is about 1.18.

The melting point is about 20 °C.

Water and deuterium oxide. NMT 0.1%.

Store in an airtight container.

Deuterated methanol. C^2H_4O. (M_r 36.07). [811-98-3]. ($^2H_4$)-Methanol. Methanol-*d*.

The degree of deuteration is not less than 99.8%. A clear, colorless liquid. Miscible with water, 96% ethanol, and methylene chloride.

d_{20}^{20} is about 0.888.

n_D^{20} is about 1.326.

The boiling point is about 65.4 °C.

Deuterated chloroform. C^2HCl_3. (M_r 120.38). [865-49-6]. ($^2H_4$)-Chloroform. Chloroform-*d*.

The degree of deuteration is not less than 99.7%. A clear, colorless liquid. Practically insoluble in water, miscible with acetone, 96% ethanol. Can be stabilised with silver foil.

d_{20}^{20} is about 1.51.

n_D^{20} is about 1.445.

The boiling point is about 60 °C.

Water and deuterium oxide. NMT 0.05%.

Decane. $C_{10}H_{22}$. (M_r 142.28). [124-18-5].

A colorless liquid. Practically insoluble in water.

n_D^{20} is about 1.411.

The boiling point is about 174 °C.

Decanol. $C_{10}H_{22}O$. (M_r 158.28). [112-30-1]. Decan-1-ol.

A viscous liquid that solidifies at a temperature of 6 °C. Practically insoluble in water, soluble in 96% ethanol.

n_D^{20} is about 1.436.

The boiling point is about 230 °C.

Blue Dextran 2000. [9049-32-5].

Prepared from dextran, which has an average molecular mass of 2 x 10^6, by introducing a polycyclic chromophore that turns the substance blue. The degree of substitution is 0.017. Freeze-dried. Quickly and completely dissolves in *water R* and aqueous salt solutions.

A 1 g/L solution in *phosphate buffer solution pH 7 R* shows an absorption maximum (*2.1.2.24*) at 280 nm.

Dextran for chromatography, cross-linked R2.

Bead-form dextran suitable for the separation of peptides and proteins with molecular masses of 15×10^2 to 30×10^3. When dry, the beads have a diameter of 20-80 µm.

Dextran for chromatography, cross-linked R3.

Bead-form dextran suitable for the separation of peptides and proteins with molecular masses of 4×10^3 to 15×10^4. When dry, the beads have a diameter of 40-120 µm.

Dextrose. [50-99-7]. See *Glucose R*.

2'-Deoxyuridine. $C_9H_{12}N_2O_5$. (M_r 228.2). [951-78-0]. 1-(2-Deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione.

The melting point is about 165 °C.

Chromatography. Determination is carried out by TLC (*2.1.2.26*) as prescribed in the monograph *Iodoxyuridine*; 5 µl of 0.25 g/L solution is applied; the chromatogram shows only one principal spot.

Diazobenzenesulfonic acid solution R1.

Dissolve 0.9 g of *sulfanilic acid R* in a mixture of 30 mL of *dilute hydrochloric acid R* and 70 mL of *water R*. To 3 mL of the resulting solution, add 3 mL of a 50 g/L solution of *sodium nitrite R*, cool in an ice bath for 5 min, then add 12 mL of sodium nitrite solution, cool again, and dilute to 100 mL with *water R*. Place the reagent in an ice bath. Prepare extemporaneously but allow to stand for 15 min before use.

3,3′-Diaminobenzidine tetrahydrochloride.

$C_{12}H_{18}Cl_4N_4 \cdot 2H_2O$. ($M_r$ 396.14). [7411-49-6]. 3,3′,4,4′-Biphenyl-tetramine.

Almost white or slightly pink powder. Soluble in water.

The melting point is about 280 °C with decomposition.

Diammonium hydrogen phosphate.. $(NH_4)_2HPO_4$. (M_r 132.06). [7783-28-0]. Diammonium hydrogen phosphate.

White crystals or granules. Hygroscopic, very soluble in water, practically insoluble in 96% ethanol.

pH (*2.1.2.3*). About 8. Measure the pH of a 200 g/L solution.

Store in an airtight container.

Diatomaceous earth. [91053-39-3].

White or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms.

Practically insoluble in water and in 96% ethanol. Identified by microscopic examination with a magnification of × 500.

Diatomaceous earth for gas chromatography.

White or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms. Practically insoluble in water and in 96% ethanol. Identified by microscopic examination with a magnification of × 500; purified by treating with *hydrochloric acid R* and washing with *water R*. *Particle size.* Maximum 5% is retained on a sieve No. 180. Maximum 10% passes a sieve No. 125.

Diatomaceous earth for gas chromatography R1.

White or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms. Practically insoluble in water and in 96% ethanol. Identified by microscopic examination with a magnification of × 500; purified by treating with *hydrochloric acid R* and washing with *water R*.

Particle size. Maximum 5% is retained on a sieve No. 250. Maximum 10% passes a sieve No. 180.

Diatomaceous earth for gas chromatography R2.

White or almost white, fine granular powder with a specific surface area of about 0.5 m^2/g, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms. Practically insoluble in water and in 96% ethanol. Identified by microscopic examination with a magnification of × 500; purified by treating with *hydrochloric acid R* and washing with *water R*.

Particle size. Maximum 5% is retained on a sieve No. 180. Maximum 10% passes a sieve No. 125.

Diatomaceous earth for gas chromatography, silanised.

Diatomaceous earth for gas chromatography R, silanised with dimethyldichlorosilane or other suitable silanising agents.

Diatomaceous earth for gas chromatography R1.

Prepared from crushed pink firebricks and silanised with dimethyldichlorosilane or other suitable silanising agents. Purified by treating with *hydrochloric acid R* and washing with *water R*.

Dibenzyl. $C_{14}H_{14}$. (M_r 182.26). [103-29-7]. 1,2-Diphenylethane.

White crystalline powder. Practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in 96% ethanol.

The melting point is from 50 °C to 53 °C.

Dibutyl ether. $C_8H_{18}O$. (M_r 130.23). [142-96-1].

Colourless flammable liquid. Practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20} is about 0.77.

n_D^{20} of about 1,399.

Do not distill if the dibutyl ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter, fill completely with the tested ether, stopper, and mix. Allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Dibutyl phthalate. $C_{16}H_{22}O_4$. (M_r 278.34). [84-74-2]. Dibutyl(benzene-1,2-dicarboxylate).

Clear, colorless or slightly coloured oily liquid. Very slightly soluble in water, miscible with acetone and in 96% alcohol.

d_{20}^{20}: from 1.043 to 1.048.

n_D^{20}: from 1.490 to 1.495.

10,11-Dihydrocarbamazepine. $C_{15}H_{14}N_2O$. (M_r 238.28). [3564-73-6]. 10,11-Dihydro-5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide.

The melting point is from 205 °C to 210 °C.

Dihydroxynaphthalene. [132-86-5].

See *1,3-Dihydroxynaphthalene R*.

1,3-Dihydroxynaphthalene. $C_{10}H_8O_2$. (M_r 160.17). [132-86-5]. Naphthalene-1,3-diol.

Usually brownish-violet crystalline powder. Freely soluble in water and 96% ethanol.

The melting point is about 125 °C.

2,7-Dihydroxynaphthalene. $C_{10}H_8O_2$. (M_r 160.2). [582-17-2]. Naphthalene-2,7-diol.

Acicular crystals. Soluble in water and 96% ethanol.

The melting point is about 190 °C.

2,7-Dihydroxynaphthalene solution.

Dissolve 10 mg of *2,7-dihydroxynaphthalene R* in 100 mL of *sulfuric acid R* and allow to stand until the solution is decolourised. Use within 2 days.

Digitoxin. $C_{41}H_{64}O_{13}$. (M_r 765). [71-63-6]. (3β,5β)-3-{[2,6-Dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl]oxy}-14-hydroxycard-20(22)-enolide.

Contains not less than 95.0% and not greater than 103.0% of $C_{41}H_{64}O_{13}$ calculated on a dry substance basis.

White or almost white powder.

Practically insoluble in water, freely soluble in a mixture of equal volumes of methanol and methylene chloride, slightly soluble in alcohol and methanol.

Digitonin. $C_{56}H_{92}O_{29}$. (M_r 1229). [11024-24-1]. 3β-[*O*-β-O-Glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyloxy]-(25R)-5α-spirostan-2α,15β-diol.

Crystals. Practically insoluble in water, sparingly soluble in anhydrous ethanol, slightly soluble in 96% ethanol.

Didodecyl-3,3'-thiodipropanate. $C_{30}H_{58}O_4S$. (M_r 514.8). [123-28-4].

White or almost white crystalline powder. Practically insoluble in water, freely soluble in acetone and petroleum ether, slightly soluble in 96% ethanol.

The melting point is about 39 °C.

Di-isobutyl ketone. $C_9H_{18}O$. (M_r 142.24). [108-83-8].

A clear, colorless liquid. Slightly soluble in water, miscible with most organic solvents.

n_D^{20} is about 1.414.

The boiling point is about 168 °C.

Di-isopropyl ether. $C_6H_{14}O$. (M_r 102.17). [108-20-3].

A clear, colorless liquid. Very slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is from 0.723 to 0.728.

The boiling point is from 67 °C to 69 °C.

Do not distill if the diisopropyl ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter, fill completely with the tested ether, stopper, and mix. Allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label. Store in a place protected from light.

Dipotassium hydrogen phosphate. K_2HPO_4. (M_r 174.18). [7758-11-4]. Dipotassium hydrogen phosphate.

White or almost white crystalline powder, hygroscopic. Very soluble in water, slightly soluble in 96% ethanol.

Store in an airtight container.

Dipotassium hydrogen phosphate trihydrate $K_2HPO_4 \cdot 3H_2O$. (M_r 228.22). [16788-57-1]. Dipotassium hydrogen phosphate trihydrate.

colourless, white or off-white, hygroscopic powder or crystals. Freely soluble in water.

Dicarboxylic acid hydrochloride. $C_{20}H_{26}C1_2N_2O_6$. (M_r 461.3). [56455-90-4]. 4,4'-[(4,4'-Diaminodi-phenyl-3,3'-diyl)dioxy]dibutanoic acid dihydrochloride.

Dimeticone. [9006-65-9]. α-Trimethylsilyl-ω-methylpoli[oxy(dimethylsilanediyl)].

Poly(dimethylsiloxane) is prepared by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. There are brands of dimethicone that differ in the number after the name indicating the nominal kinematic viscosity.

The degree of polymerisation (n = 20-400) of dimeticones should meet the nominal kinematic viscosity from 20 $mm^2 \cdot s^{-1}$ 1300 $mm^2 \cdot s^{-1}$.

Dimeticones with a nominal viscosity of 50 $mm^2 \cdot s^{-1}$ or lower are intended for external use only.

A clear, colorless liquid of various viscosity.

Practically insoluble in water, very slightly soluble or practically insoluble in anhydrous ethanol, miscible with ethyl acetate, methyl ethyl ketone, and toluene.

Dimethylaminobenzaldehyde. $C_9H_{11}XO$. (M_r 149.2). [100-10-7]. 4-Dimethylaminobenzaldehyde.

White or yellowish white crystals. Soluble in 96% ethanol and dilute acids.

The melting point is about 74 °C.

Dimethylaminobenzaldehyde solution R1.

Dissolve 0.2 g of *dimethylaminobenzaldehyde R* in 20 mL of *96% ethanol R*, add 0.5 mL of *hydrochloric acid R*, shake the resulting solution with *activated carbon R*, and filter. The colour of the solution should not be more intense than the colour of *iodine solution R3*. Prepare immediately before use.

Dimethylaminobenzaldehyde solution R2.

Dissolve 0.2 g of *dimethylaminobenzaldehyde R* without heating in a mixture of 4.5 mL of *water R* and 5.5 mL of *hydrochloric acid R*. Prepare immediately before use.

Dimethylaminobenzaldehyde solution R6.

Dissolve 0.125 g of *dimethylaminobenzaldehyde R* in a cooled mixture of 35 mL of *water R* and 65 mL of *sulfuric acid R*, add 0.1 mL of a 50 g/L solution of *ferric chloride R*. Before use, allow to stand for 24 h protected from light.

Store at room temperature for 7 days; in a refrigerator — for several months.

Dimethylaminobenzaldehyde solution R7.

Dissolve 1.0 g of *dimethylaminobenzaldehyde R* in 50 mL of *hydrochloric acid R* and add 50 mL of *96% ethanol R*. Store protected from light.

Use within 1 month.

Dimethylaminobenzaldehyde solution R8.

Dissolve 0.25 g of *dimethylaminobenzaldehyde R* in a mixture of 5 g of *phosphoric acid R*, 45 g of *water R*, and 50 g of *anhydrous acetic acid R*. Prepare immediately before use.

4-Dimethylaminocinnamaldehyde. $C_{11}H_{13}NO$. (M_r 175.23). [6203-18-5]. 3-(4-dimethylaminophenyl)prop-2-enal.

Orange to orange-brown crystals or powder. Sensitive to light.

The melting point is about 138 °C.

4-Dimethylaminocinnamaldehyde solution.

Dissolve 2 g of 4-dimethylaminocinnamaldehyde R *in a mixture of 100 mL of* hydrochloric acid R1 *and 100 mL of* anhydrous ethanol R.

Dilute the solution to four times its volume with *anhydrous ethanol R* immediately before use.

Dimethylaminonaphthalenesulfonyl chloride. $C_{12}H_{12}C1NO_2S$. (M_r 269.75). [605-65-2]. 5-Dimethylamino-1-naphthalenesulfonyl chloride.

Yellow crystalline powder. Slightly soluble in water, soluble in methanol.

The melting point is about 70 °C.

Dimethylaniline. [121-69-7].

See *N,N-Dimethylaniline R.*

N,N-Dimethylaniline. $C_8H_{11}N$. (M_r 121.18). [121-69-7].

A clear oily liquid. Freshly distilled almost colorless, darkens to a reddish brown colour when stored. Practically insoluble in water, freely soluble in 96% ethanol.

d_{20}^{20} is about 1.558.

Distillation range (*2.1.2.11*). Between 192 °C and 194 °C; not less than 95% must be distilled.

2,6-Dimethylaniline. $C_8H_{11}N$. (M_r 121.18). [87-62-7]. 2,6-Xylidine.

A colorless liquid. Sparingly soluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.98.

2,3-Dimethylaniline. $C_8H_{11}N$. (M_r 121.18). [87-59-2]. 2,3-Xylidine.

A yellowish liquid. Sparingly soluble in water, soluble in 96% ethanol.

d_{20}^{20}: from 0.993 to 0.995.

n_D^{20} is about 1.569.

The boiling point is about 224 °C.

Dimethylacetamide. C_4H_9NO. (M_r 87,12). [127-19-5]. *N,N*-Dimethylacetamide.

Contains not less than 99.5% of C_4H_9NO.

A colorless liquid. Miscible with water and most organic solvents.

d_{20}^{20} is about 0.94.

n_D^{20} is about 1.437.

The boiling point is about 165 °C.

Dimethylglyoxime. $C_4H_8N_2O_2$. (M_r 116,12). [95-45-4]. 2,3-Butanedione dioxime

White or almost white crystalline powder or colorless crystals. Practically insoluble in cold water, very slightly soluble in boiling water, soluble in 96% ethanol.

The melting point is about 240 °C with decomposition.

Sulfated ash (*2.1.4.14*). NMT 0.05%.

Dimethyldecylamine. $C_{17}H_{27}N$ (M_r 185.4). [1120-24-7]. *N,N*-Dimethyldecylamine.

Contains not less than 98.0% (*m/m*) of C17H27N.

The boiling point is about 234 °C.

Dimethyl carbonate. $C_3H_6O_3$. (M_r90.1). [616-38-6].

Dimethyl ether of carbonic acid.

Liquid. Insoluble in water, miscible with 96% ethanol.

d_4^{17} about 1.065

n_D^{20} is about 1.368.

The boiling point is about 90 °C.

Dimethyl yellow. $C_{14}H_{15}N_3$. (M_r 225.29). [60-11-7].

Schultz No. 28.

Colour index (C. I.) No. 11020.

4-(Dimethylamino)azobenzene. Methyl yellow.

Small yellow crystals or yellow or orange flakes. Practically insoluble in water, very slightly soluble in 96% ethanol.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel G R* as the coating substance. On the chromatographic plate, apply 10 µL of a 0.1 g/L solution in *methylene chloride R* and develop over a path of the solvent front NLT 10 cm with the same solvent; the chromatogram shows only one principal spot.

Store in an airtight container.

Dimethyl yellow and oracet blue solution.

Dissolve 10 mg of dimethyl yellow R *and 10 mg of* oracet blue B R *in 300 mL of* methylene chloride R.

N,N-Dimethyloctylamine. $C_{10}H_{23}N$. (M_r 157.30). [7378-99-6]. Octyl dimethylamine.

A colorless liquid.

d_{20}^{20} is about 0.765.

n_D^{20} is about 1.424.

The boiling point is about 195 °C.

1,3-Dimethyl-2-imidazolidinone. $C_5H_{10}N_2O$. (*Mr* 114.15). [80-73-9].

N,N-Dimethylethyleneurea.1,3-dimethyl-2-imidazolidone.

n_D^{20} is about 1.4720.

The boiling point is about 224 °C.

Dimethyl carbonate. $C_3H_6O_3$. (M_r 90.08). [616-38-6]. Dimethyl ether of carbonic acid.

Liquid. Insoluble in water, miscible with 96% ethanol.

d_4^{17} about 1.065

n_D^{20} is about 1.368.

The boiling point is about 90 °C.

Dimethylpiperazine. $C_6H_{14}N_2$. (M_r 114.19). [106-58-1]. 1,4-Dimethylpiperazine.

A colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.85.

n_D^{20} is about 1.446.

The boiling point is about 131 °C.

Dimethylstearylamide. $C_{20}H_{41}NO$. (M_r 311.55).

N,N-Dimethyl stearylamide.

White or almost white solid mass. Soluble in most organic solvents, including acetone.

The melting point is about 51 °C.

Dimethyl sulfoxide. C_2H_6OS. (M_r 78.1). [67-68-5]. Cylfinylbismethane.

A colorless liquid or colorless crystals, hygroscopic.

Miscible with water and 96% ethanol.

Dimethyl sulfoxide used in spectrophotometry should meet the following additional test.

The *absorbance* (*2.1.2.24*) is determined using *water R* as the compensation liquid:

maximum 1.00 at a wavelength of 262 nm

maximum 0.46 at a wavelength of 270 nm

maximum 0.16 at a wavelength of 290 nm

maximum 0.01 at a wavelength of 340 nm or greater.

Dimethyl sulfone. $C_2H_6O_2S$. (M_r 94.13). [67-71-0].

White or almost white crystalline powder. Freely soluble in water, soluble in acetone and 96% ethanol.

The melting point is from 108 °C to 110 °C.

Dimethyltetradecylamine. $C_{16}H_{35}N$. (*Mr* 241.46). *N,N*-Dimethyltetradecylamine.

Contains not less than 98.0% (*m/m*) and not greater than 101.0% (*m/m*) C16H35N.

A clear or almost clear colorless or yellowish liquid. Practically insoluble in water, miscible with acetone, 96% ethanol, and methanol.

d_{20}^{20} is about 0.80.

The boiling point is about 260 °C.

Water (*2.1.5.12*). NMT 0.3% (*m/m*).

Quantitation. Dissolve 0.200 g in 10 mL of *96% ethanol R* and titrate with *0.1 M hydrochloric acid* until the red colour develops, using 0.1 mL of a solution of *methyl red R* as indicator.

1 mL of *0.1 M hydrochloric acid* is equivalent to 24.15 mg of $C_{16}H_{35}N$.

2,6-Dimethylphenol. $C_8H_{10}O$. (*Mr* 122.16). [576-26-1].

Colourless needle-like crystals. Slightly soluble in water, freely soluble in 96% ethanol.

The boiling point is about 203 °C.

The melting point is from 46 °C to 48 °C.

3,4-Dimethylphenol. $C_8H_{10}O$. (M_r 122.16). [95-65-8].

White or almost white crystals. Slightly soluble in water, freely soluble in 96% ethanol.

The boiling point is about 226 °C.

The melting point is from 25 °C to 27 °C.

Dimethylformamide. C_3H_7NO. (M_r 73.09). [68-12-2].

A clear, colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20}: from 0.949 to 0.952.

The boiling point is about 153 °C.

Water (*2.1.5.12*). NMT 0.1%.

Dimethylformamide diethylacetal. $C_7H_{17}NO_2$. (*Mr* 147.22). [1188-33-6]. *N*,*N*-dimethylformamide diethylacetal.

n_D^{20} is about 1.40.

The boiling point is from 128 °C to 130 °C.

1,1-Dimethylethylamine. $C_4H_{11}N$. (M_r 73.14). [75-64-9]. 2-Amino-2-methylpropane. *tert*-Butylamine.

Liquid. Miscible with 96% ethanol.

d_{20}^{20} is about 0.694.

n_D^{20} is about 1.378.

The boiling point is about 46 °C.

1,1-Dimethyletyl methyl ether. $C_5H_{12}O$. (M_r 88,15). [1634-04-4]. 2-Methoxy-2-methylpropane. tert-Butyl methyl ether.

A colorless clear flammable liquid.

n_D^{20} is about 1.376.

Determine the *minimum transmission* (*2.1.2.24*) using *water R* as compensation liquid.:

minimum 0.30 at a wavelength of 240 nm

minimum 0.10 at a wavelength of 255 nm

minimum 0.01 at a wavelength of 280 nm.

Dimethoxypropane. $C_5H_{12}O_2$ (M_r 104,15). [77-76-9]. 2,2-Dimethoxypropane.

A colorless liquid. Decomposes under exposure to moist air or water.

d_{20}^{20} is about 0.847.

n_D^{20} is about 1.378.

The boiling point is about 83 °C.

Dimidium bromide. $C_{20}H_{18}BrN_3$. (M_r 380.28). [518-67-2]. 3,8-Diamino-5-methyl-6-phenylphenanthridinium bromide.

Dark red crystals. Slightly soluble in water at 20 °C, sparingly soluble in water at 60 °C and 96% ethanol.

Dimidium bromide-sulfane blue mixed solution.

Separately dissolve 0.5 g of *dimidium bromide R* and 0.25 g of *sulfane blue R* in 30 mL of a hot mixture of solvents *ethanol R*/*water R* (1:9), and mix. Mix both solutions and dilute to 250 mL with the same mixture of solvents. Mix 20 mL of the resulting solution with 20 mL of 14.0% (*V/V*) solution of *sulfuric acid R*, previously diluted with about 250 mL of *water R*, dilute to 500 mL with *water R*.

Store in a place protected from light.

Disodium arsenate. $Na_2HAsO_4{\cdot}7H_2O$. (M_r 312.01). [10048-95-0]. Hydroarsenate (V) disodium heptahydrate.

Crystals efflorescent in the air. Freely soluble in water, soluble in glycerol, slightly soluble in 96% alcohol. An aqueous solution that has an alkaline litmus reaction.

*d*20 about 1.87.

The melting point is about 57 °C (when heated rapidly).

Disodium hydrogen phosphate. $Na_2HPO_4{\cdot}12H_2O$. (M_r 358.1). [10039-32-4]. Disodium phosphate dodecahydrate.

Contains not less than 98.5% and no greater than 102.5% of $Na_2HPO_4{\cdot}12H_2O$.

colourless, clear, efflorescent crystals.

Very soluble in water, practically insoluble in 96% ethanol.

Disodium hydrogen phosphate solution.

90 g/L solution.

Disodium hydrogen phosphate, anhydrous. Na_2HPO_4. (*Mr* 141.96). [7558-79-4]. Disodium hydrogen phosphate.

Disodium hydrogen phosphate dihydrate. $Na_2HPO_4{\cdot}2H_2O$. (*Mr* 178.0). [10028-24-7]. Phosphate disodium dihydrate.

Contains NLT 98.0% and NMT 101.0% calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Soluble in water, practically insoluble in 96% ethanol.

Disodium hydrogen phosphate dodecahydrate. $Na_2HPO_4{\cdot}12H_2O$. (*Mr* 358.1). [10039-32-4]. Disodium phosphate dodecahydrate.

Contains not less than 98.5% and no greater than 102.5% of $Na_2HPO_4{\cdot}12H_2O$.

colourless, clear, efflorescent crystals.

Very soluble in water, practically insoluble in 96% ethanol.

Disodium hydrogen citrate. $C_6H_6Na_2O_7{\cdot}1{,}5H_2O$. ($M_r$ 263.11). [144-33-2]. Sodium acid citrate. Disodium hydrogen 2-hydroxypropane-1,2,3-tricarboxylate sesquihydrate.

White or almost white powder. Soluble in less than 2 parts of water, practically insoluble in 96% ethanol.

Disodium tetraborate. $Na_2B_4O_7 \cdot 10H_2O$. (M_r 381.4). [130396-4]. Disodium tetraborate decahydrate. Borax.

Contains not less than 99.0% and not greater than 103.0% of $Na_2B_4O_7 \cdot 10H_2O$.

White or almost white crystalline powder, or colorless crystals, or faded crystalline mass.

Soluble in water, very soluble in boiling water, freely soluble in glycerol.

Borax solution.

Dissolve 9.55 g of *disodium tetraborate R* in *sulfuric acid R* under heating in a water bath and dilute to 1 L with the same acid.

Dinitrobenzoyl chloride. $C_7H_3C1N_2O_5$. (M_r 230.56). [99-33-2]. 3,5-Dinitrobenzoyl chloride.

Translucent yellow or greenish-yellow powder or yellowish crystals, soluble in acetone and toluene.

The melting point is about 68 °C.

Suitability test. To 1 mL of *anhydrous ethanol R*, add 0.1 g of *dinitrobenzoyl chloride R*, 0.05 mL of *dilute sulfuric acid R*, and boil under reflux for 30 min. Then evaporate in a water bath, add 5 mL of *heptane R* to the resulting residue, heat to boiling, and filter the hot solution. Cool the resulting solution to room temperature, wash the resulting crystals with small portions of *heptane R* and dry in a drying cabinet.

The melting point of the crystals (*2.1.2.14.*) should be from 94 °C to 95 °C.

Dinitrobenzoic acid. $C_7H_4N_2O_6$. (M_r 212.12). [99-34-3]. 3,5-Dinitrobenzoic acid.

Almost colorless crystals. Slightly soluble in water, very soluble in 96% alcohol.

The melting point is about 206 °C.

Dinitrobenzoic acid solution.

A 20 g/L solution in *96% ethanol R.*

Dinitrobenzene. $C_6H_4N_2O_4$. (M_r 168.11). [99-65-0]. 1,3-Dinitrobenzene.

Yellowish crystalline powder or crystals. Practically insoluble in water, slightly soluble in 96% alcohol.

The melting point is about 90 °C.

Dinitrobenzene solution.

A 10 g/l solution of *96% ethanol R.*

Dinitrophenylhydrazine. $C_6H_6N_4O_4$. (M_r 198.14). [119-26-6]. 2,4-Dinitrophenylhydrazine.

Reddish orange crystals. Very slightly soluble in water, slightly soluble in 96% *ethanol*.

The melting point is about 203 °C (instantaneous melting method).

Dinitrophenylhydrazine acetic-hydrochloric solution.

Dissolve 0.2 g of *dinitrophenylhydrazine R* in 20 mL of *methanol R*, add 80 mL of a mixture of equal volumes of *acetic acid R* and *hydrochloric acid R1*, and mix.

Prepare immediately before use.

Dinitrophenylhydrazine hydrochloric solution.

Dissolve 0.50 g of *dinitrophenylhydrazine R* under heating in *dilute hydrochloric acid R*, dilute to 100 mL with the same solvent, allow to cool and filter.

Prepare immediately before use.

Dinonyl phthalate. $C_{26}H_{42}O_4$. (M_r 418.6). [28553-12-0].

A colorless or light yellow viscous liquid.

d_{20}^{20} is from 0.97 to 0.98.

n_D^{20} from 1.482 to 1.489.

Acidity. Shake 5.0 g with 25 mL of *water R* for 1 min. After separating the layers, take the aqueous layer, add 0.1 mL of *phenolphthalein solution R*; the colour of the solution should change with the addition of no greater than 0.3 mL of *0.1 M sodium hydroxide* (0.05% equivalent to phthalic acid).

Water (*2.1.5.12*). NMT 0.1%.

Dioxan. $C_4H_8O_2$. (M_r 88.11). [123-91-1]. 1,4-Dioxan.

A clear, colorless liquid. Miscible with water and most organic solvents.

d_{20}^{20} is about 1.03.

Freezing point (*2.1.2.17*). Between 9 °C and 11 °C.

Water (*2.1.5.12*). NMT 0.5%.

Do not distill if the dioxan does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter, fill completely with dioxan, and mix. Allow to stand protected from light for 30 min. No colour is produced. *Dioxan used for liquid scintillation is of a suitable analytical grade.*

Dioxan stock solution.

Dissolve 1.00 g of *dioxan R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the resulting solution to 50.0 mL with *water R* (1.0 mg/mL).

Dioxan solution.

Dilute 50.0 mL of the initial solution of *dioxan R* to 100.0 mL with *water R* (0.5 mg/mL of dioxan).

Dioxan solution R1.

Dilute 10.0 mL of a solution of *dioxan R* to 50.0 mL with *water R* (0.1 mg/mL of dioxan).

Di(octadecyl)-3,3'-thiodipropionate. $C_{42}H_{82}O_4S$. (M_r 683.2). [693-36-7].

White or almost white crystalline powder. Practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, 96% ethanol, and petroleum ether.

The melting point is from 58 °C to 67 °C.

2,2'-Di(octadecyloxy)-5,5'-spirobi(1,3,2-dioxaphosphorinane). $C_{41}H_{82}O_6P_2$. (M_r 733.0).

White or almost white solid waxy substance. Practically insoluble in water, soluble in hydrocarbons.

The melting point is from 40 °C to 70 °C.

Dioctadecyl disulfide. . $C_{36}H_{74}S_2$. (M_r 571,1). [2500-88-1].

White or almost white powder. Practically insoluble in water.

The melting point is from 53 °C to 58 °C.

Dithizone. $C_{13}H_{12}N_4S$. (M_r 256.33). [60-10-6]. 1,5-Diphenylthiocarbazone.

Bluish black or brownish black or black powder. Practically insoluble in water, soluble in 96% ethanol.

Store in a place protected from light.

Dithizone solution.

A 0.5 g/L solution in *chloroform R*.

Prepare immediately before use.

Dithizone solution R2.

Dissolve 40.0 mg of *dithizone R* in *chloroform R* and dilute to 1000 mL with the same solvent. Dilute 30.0 mL of the resulting solution to 100.0 mL with *chloroform R*.

Standardisation. Dissolve a quantity of *mercuric(II) chloride R* equivalent to 0.1354 g of $HgCl_2$ in a mixture of equal volumes of *dilute sulfuric acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 2.0 mL of the resulting solution to 100.0 mL with a mixture of equal volumes of *dilute sulfuric acid R* and *water R* (solution contains 20 ppm of Hg^{2+}). Place 1.0 mL of the resulting solution R in a separating funnel, add 50 mL of *dilute sulphuric acid R*, 140 mL of *water R*, and 10 mL of a 200 g/L solution of *hydroxylamine hydrochloride R*. Titrate the prepared solution with dithizone; after each addition of titrant, shake the mixture twenty times, by the end of the titration, allow the mixture to separate, then discard the chloroform layer and continue the titration until the bluish-green colour is obtained. The amount of mercury (E) in milligrams equivalent to the content of dithizone per millilitre of the solution is calculated by the formula: $E = 20/V$, where V is the volume of dithizone solution used for titration, in millilitres.

Dithizone R1. [60-10-6].

See *Dithizone R*.

5,5'-Dithiobis(2-nitrobenzoic acid). $C_{14}H_8N_2O_8S_2$. (M_r 396.35). [69-78-3]. 3-carboxy-4-nitrophenyl disulfide. Elman's reagent.

Yellow powder. Sparingly soluble in 96% ethanol.

The melting point is about 242 °C.

Dithiol. $C_7H_8S_2$. (M_r 156.26). [496-74-2]. Toluene-3,4-dithiol. 4-Methylbenzene-1,2-dithiol.

White or almost white crystals, hygroscopic. Soluble in methanol and solutions of alkali metal hydroxides.

The melting point is about 30 °C.

Store in an airtight container.

Dithiol reagent.

To 1 g of *dithiol R*, add 2 mL of *thiolicolic acid R* and dilute to 250 mL with a 20 g/L solution of *sodium hydroxide R*. Prepare immediately before use.

Dithiotreitol. $C_4H_{10}O_2S_2$. (M_r154,24). [27565-41-9]. *Treo*-1,4-Dimercaptobutane-2,3-di-ol.

Needle-like, slightly hygroscopic crystals. Freely soluble in water, acetone, and anhydrous ethanol. Store in an airtight container.

Diphenylamine. C12H11N. (*Mr* 169.22). [122-39-4].

White or almost white crystals. Slightly soluble in water, soluble in 96% ethanol.

The melting point is about 55 °C.

Store in a place protected from light.

Diphenylamine solution.

A 1 g/L solution of *sulfuric acid R*. Store protected from light.

Diphenylamine solution R1.

A 10 g/L solution in *sulfuric acid R*. The solution should be colourless.

Diphenylamine solution R2.

Dissolve 1 g of *diphenylamine R* in 100 mL of *glacial acetic acid R* and add 2.75 mL of *sulfuric acid R* . Use the solution immediately.

Diphenylanthracene. $C_{26}H_{18}$. (M_r 330,42). [1499-10-1]. 9,10-Diphenylanthracene.

Yellowish to yellow crystalline powder. Practically insoluble in water.

The melting point is about 248 °C.

Diphenylbenzidine. $C_{24}H_{20}N_2$. (M_r 336.43). [531-91-9]. *N*,*N*-Diphenylbenzidine. *N*,*N*-Diphenylbiphenyl-4,4'-diamine.

White or greyish white crystalline powder. Practically insoluble in water, slightly soluble in acetone and 96% ethanol.

The melting point is about 248 °C.

Nitrates. Dissolve 8 mg in a cooled mixture of 5 mL of *water R* and 45 mL of *nitrogen-free sulfuric acid R*; the resulting solution should be colorless or slightly bluish.

Sulfated ash (*2.1.4.14*). NMT 0.1%.

Store in a place protected from light.

Diphenylboric acid aminoethyl ether. $C_{14}H_{16}BNO$. (*Mr* 225.09). [524-95-8].

White or slightly yellowish crystalline powder. Practically insoluble in water, soluble in 96% ethanol.

The melting point is about 193 °C.

Diphenylcarbazide. . $C_{13}H_{14}N_4O$. (M_rC13H14N4O. (Mr 242.3). [140-22-7]. 1,5-Diphenylcarbondihydrazide.

White or almost white crystalline powder that gradually turns pink in the air. Very slightly soluble in water, soluble in acetone, 96% ethanol, and glacial acetic acid.

The melting point is about 170 °C.

Sulfated ash (*2.1.4.14*). NMT 0.1%.

Store in a place protected from light.

Diphenylcarbazide solution.

Dissolve 0.2 g of *diphenylcarbazide R* in 10 mL of *glacial acetic acid R* and dilute to 100 mL with *ethanol R*. Prepare immediately before use.

Diphenylcarbazone. $C_{13}H_{12}N_4O$. (M_r 240.26). [538-62-5]. 1,5-Diphenylcarbazone.

Orange-yellow crystalline powder.

Practically insoluble in water, very soluble in 96% ethanol.

The melting point is about 157 °C with decomposition.

Diphenylcarbazone mercuric reagent.

Solution A. Dissolve 0.1 g of *diphenylcarbazone R* in *ethanol R* and dilute to 50 mL with the same solvent.

Solution B. Dissolve 1 g of *mercuric(II) chloride R* in *ethanol R* and dilute to 50 mL with the same solvent. Mix equal volumes of solutions A and B.

Diphenyloxazole. $C_{15}H_{11}NO$. (M_r 221.25). [92-71-7]. 2,5-Diphenyloxazole.

White or almost white powder. Practically insoluble in water, soluble in methanol, sparingly soluble in dioxane and glacial acetic acid.

The melting point is about 70 °C.

$E_{1cm}^{1\%}$ is about 1260. Determination is carried out at 305 nm, using *methanol R* as a solvent.

Diphenyloxazole used for liquid scintillation must be of an appropriate grade.

Diphenylphenylene oxide polymer.

A polymer of 2,6-diphenyl-p-phenylene oxide.

White or almost white porous spherical granules; the size of the granules is indicated in the tests in which they are used.

Dichlorobenzene. $C_6H_4Cl_2$. (M_r 147.00). [95-50-1]. 1,2-Dichlorobenzene.

A colorless oily liquid.

Practically insoluble in water, soluble in anhydrous ethanol.

d_{20}^{20} is about 1.31.

The boiling point is about 180 °C.

Dichlorvos. $C_4H_7ClO_4P$. (M_r 220.98). [62-73-7]. 2,2-Dichlorovinyldimethylphosphate.

Colourless to brownish yellow liquid.

Soluble in water, miscible with most organic solvents.

n_D^{20} is about 1.452.

Dichloroacetic acid. $C_2H_2Cl_2O_2$. (M_r 128.94). [79-43-6].

A colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 1.566.

n_D^{20} is about 1.466.

The boiling point is about 193 °C.

Dichloroacetic acid solution.

Dilute 67 mL of *dichloroacetic acid R* to 300 mL with *water R* and neutralise with *ammonia solution R* on *blue litmus paper R*. Cool, add 33 mL of *dichloroacetic acid R*, and dilute to 600 mL with *water R*.

Dichlorophenolindophenol, sodium salt. $Cl_2H_6Cl_2NNaO_2{\cdot}2H_2O$. ($M_r$ 326.11). [620-45-1]. Sodium 2,6-dichloro-*N*-(4-hydroxyphenyl)-1,4-benzoquinonemonoimine dihydrate.

Dark green powder.

Freely soluble in water and ethanol. The aqueous solution has a dark blue colour, which turns pink when the solution is acidified.

Dichlorphenolindophenol volumetric solution.

Dissolve 50.0 mg of *dichlorophenolindophenol sodium salt R* in 100.0 mL of *water R* and filter.

Standardisation. Dissolve 20.0 mg of *ascorbic acid R* in 10 mL of a freshly prepared 200 g/L solution of *metaphosphoric acid R* and dilute to 250.0 mL with *water R*.

Titrate 5.0 mL of the resultant solution rapidly with the dichloro-phenolindophenol standard solution, added from a microburette graduated in 0.01 mL, until the pink colour persists for 10 s, the titration occupying not greater than 2 min. Dilute the dichlorophenolindophenol solution with *water R* to make 1 mL of the solution equivalent to 0.1 mg of ascorbic acid (C_6H_8O6).

Use within 3 days.

Standardise immediately before use.

Dichlorofluorescein. $C_{20}H_{10}Cl_2O_5$. (M_r 401.2). [76-54-0]. 2,7-Dichlorofluorescein. 2-(2,7-Dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid.

Yellowish brown to yellow-orange powder.

Slightly soluble in water, freely soluble in 96% ethanol and dilute solutions of alkali metal hydroxides to form a solution with yellowish green fluorescence.

Dichloroquinonechlorimide. $C_6H_2Cl_3NO$. (M_r210.44). [101-38-22]. 2,6-Dichloro-*N*-chloro-1,4-benzoquinone monoimine.

Pale yellow to greenish yellow crystalline powder.

Practically insoluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

The melting point is about 66 °C.

Dicyclohexylamine. $C_{12}H_{23}N$. (M_r 181.32). [101-83-7]. *N*,*N*-Dicyclohexylamine.

A colorless liquid.

Sparingly soluble in water, miscible with common organic solvents.

n_D^{20} is about 1.484.

The boiling point is about 256 °C.

Freezing point (*2.1.2.17)*. Between 0 °C and 1 °C.

Dicyclohexylurea. $C_{13}H_{24}N_2O$. (M_r 224.34). [2387-23-7]. 1,3-Dicyclohexylurea.

White or almost white crystalline powder.

The melting point is about 232 °C.

Diethanolamine. $C_4H_{11}NO_2$. (M_r 105,14). [111-42-2]. 2,2'-Iminobisethanol.

Viscous, clear, slightly yellow liquid or deliquescent crystals melting at about 28 °C.

Very soluble in water, acetone, and methanol.

d_{20}^{20} is about 1.09.

pH (*2.1.2.3*). 10.0 to 11.5.

Measure the pH for a 50 g/L solution.

Diethanolamine used in the test for alkaline phosphatase complies with the following additional test.

Ethanolamine. NMT 1.0%.

Determination is carried out by gas chromatography (*2.1.2.27*), using *propanolamine R* as the internal standard.

Internal standard solution. Dissolve 1.00 g of *3-aminopropanolamine R* in *acetone R* and dilute to 10,0 mL with the same solvent.

Test solution (a). Dissolve 5.00 g of diethylamine in *acetone R* and dilute to 10.0 mL with the same solvent.

Test solution (b). Dissolve 5.00 g of diethylamine in *acetone R*, add 1.0 mL of the internal standard solution, and dilute to 10.0 mL with the same solvent.

Reference solutions. Dissolve 0.50 g of *ethanolamine R* in *acetone R* and dilute to 10.0 mL with the same solvent. To 0.5 mL, 1.0 mL and 2.0 mL of the resulting solution, add 1.0 mL of the internal standard solution and dilute each solution to 10.0 mL with *acetone R*.

Chromatography is performed on a gas chromatograph with a flame ionisation detector. Chromatograph 1.0 µl of each test solution and 1.0 µl of each reference solution under the following conditions:

- a 1m x 4 mm column filled with *polymer of diphenylphenylene oxide R* with a particle size from 180 µm to 250 µm;
- *nitrogen for chromatography R as* carrier gas;
- the carrier gas velocity of 40 mL/min.;

	Time (min)	Temperature (°C)
Column	0 → 3	125
	3 → 17.6	125 → 300
Sample injection device		250
Detector		280

Diethylamine. $C_4H_{11}N$. (M_r 73.14). [109-89-7].

A clear, colorless, flammable liquid. Strongly alkaline, miscible with water and 96% ethanol.

d_{20}^{20} is about 0.71.

The boiling point is about 55 °C.

Diethylaminoethyldextran.

Anion-exchange resin presented as the hydrochloride. Powder forming gels *with* water.

N,N-Diethylaniline. $Cl_0H_{15}N$. (M_r 149.23). [91-66-7].

d_{20}^{20} is about 0.938.

The boiling point is about 217 °C.

The melting point is about -38 °C.

Di(2-ethylhexyl)phthalate. $C_{24}H_{38}O_4$. (M_r 390.56).

Di(2-ethylhexyl)benzene-1,2-dicarboxylate.

A clear oily liquid. Practically insoluble in water, soluble in organic solvents.

d_{20}^{20} is about 0.98.

n_D^{20} is about 1.486.

Viscosity (*2.1.2.9*). About 80 MPa·s.

Diethylene glycol. $C_4H_{10}O_3$. (M_r 106.12). [111-46-6]. 2,2'-diethylene glycol.

Contains not less than 99.5% (*m/m*) of C4H10O3.

Clear colorless hygroscopic liquid. Miscible with water, acetone and 96% ethanol.

d_{20}^{20} is about 1.118.

n_D^{20} is about 1.447.

The boiling point is from 244 °C to 246 °C. Store in an airtight container.

Diethylphenylenediamine sulfate.

$C_{10}H_{18}N_2O_4S$. (M_r 262.3). [6283-63-2]. N,N'-Diethyl-p-phenylenediamine sulfate. *N,N*-Diethyl-benzene-1,4-diamine sulfate.

White or slightly yellowish powder. Soluble in water.

The melting point is about 185 °C with decomposition.

Store in a place protected from light.

Diethylphenylenediamine sulfate solution.

To 250 mL of *water R*, add 2 mL of *sulfuric acid R* and 25 mL of *0.02 M disodium edetate*. Dissolve 1.1 g of *diethylphenylenediamine sulfate R*in the resulting solution and dilute to 1000 mL with *water R*. Use only a colorless solution.

Store in a place protected from light.

Use within 1 month.

N,N-Diethylethane-1,2-diamine. [100-36-7].

See *N,N-diethylethylenediamine R*.

N,N-Diethylethylenediamine. $C_6H_{16}N_2$. (M_r 116.20). [100-36-7].

Contains not less than 98.0% of $C_6H_{16}N_2$.

Colourless or slightly yellowish, slightly oily liquid with a strong odor of ammonia. Irritating to skin, eyes, and mucous membranes.

d_{20}^{20} is about 0.827.

The boiling point is from 145 °C to 147 °C.

Water (*2.1.5.12*). NMT 1.0%. Determination is carried out from 0.500 g.

Dietoxitetrahydrofuran. $C_8H_{16}O_3$. (M_r 160.2). [3320-90-9]. 2,5-Diethoxytetrahydrofuran. A mixture of *cis*- and *trans*-isomers.

Clear, colorless or slightly yellowish liquid. Practically insoluble in water, soluble in 96% ethanol, and most other organic solvents.

d_{20}^{20} is about 0.98.

n_D^{20} is about 1.418.

Docusate sodium. $C_{20}H_{37}NaO_7S$. (M_r 444.6). [577-11-7]. Sodium 1,4-bis [(2-ethylhexyl)oxy]-1,4-dioxobutane-2-sulfonate.

Contains not less than 98.0% and not greater than 101.0% of $C_{20}H_{37}NaO_7S$ calculated with reference to the anhydrous substance.

White or almost white waxy mass or flakes. Hygroscopic.

Sparingly soluble in water, freely soluble in 96% ethanol and methylene chloride.

Dotriacontane. $C_{32}H_{66}$. (M_r 450.9). [544-85-4]. *n*-Dotriacontane.

White or almost white plates. Practically insoluble in water, sparingly soluble in hexane.

The melting point is about 69 °C.

Impurities. No greater than 0.1% of impurities with a retention time t_R, characteristic of α-tocopherol acetate; carry out the determination by gas chromatography as specified in the individual monograph *α-Tocopherol Acetate*.

Gelatin. [9000-70-8].

Purified protein obtained by partial alkaline or acid hydrolysis and/or enzymatic hydrolysis and/or thermal hydrolysis of animal collagen.

Hydrolysis leads to the formation of gelling and non-gelling types of gelatin. Different types of gelatin form aqueous solutions with different degrees of clarity and colour. For special-use gelatin, a suitable clarity and colour specification is allowed.

Gelling gelatin. Pale yellow or slightly yellowish brown solid substance, usually found in the form of translucent leaves, shreds, granules, or powder. Practically insoluble in common organic solvents, swells in cold water, forms a colloidal solution when heated, and takes the form of a more or less dense gel when cooled.

Non-gelling gelatin. Pale yellow or white granules or powder. Soluble in cold or warm water, practically insoluble in common organic solvents.

Gelatin hydrolysed.

Dissolve 50 g of *gelatin R* in 1000 mL of *water R*. Treat with saturated steam in an autoclave at 121 °C for 90 min, and lyophilise.

Iron. Fe. (*A*r 55.85). [7439-89-6].

Grey powder or wire. Soluble in dilute mineral acids.

Ferric ammonium sulfate. $FeNH_4(SO_4)_2 \cdot 12H_2O$. ($M_r$ 482.2). [7783-83-7]. Ferric ammonium disulfate dodecahydrate.

Pale violet crystals, efflorescencent in the air. Very soluble in water, practically insoluble in 96% ethanol.

Ferric ammonium sulfate solution R2.

A 100 g/l solution.

Filter before use, if necessary.

Ferric ammonium sulfate solution R5.

Shake 30.0 g of *ferric ammonium sulfate R* with 40 mL of *nitric acid R* and dilute to 100 mL with *water R*. If the solution is cloudy, it is centrifuged or filtered.

Store in a place protected from light.

Ferric ammonium sulfate solution R6.

Dissolve 20 g of *ferric ammonium sulfate R* is in 75 mL of *water R*, add 10 mL of 2.8% (*V/V*) solution of *sulfuric acid R*, and dilute to 100 mL with *water R*.

Ferric nitrate. $Fe(NO_3)_3 \cdot 9H_2O$. (M_r 404.0). [7782-61-8]. Ferric nitrate nonahydrate.

Contains not less than 99.0% (*m/m*) of $Fe(NO_3)_3 \cdot 9H_2O$.

Light violet crystals or crystalline mass. Very soluble in water.

Free acid: not greater than 0.3% (in the form of HNO_3).

Iron salicylate solution.

Dissolve 0.1 g of *ferrous ammonium sulfate R* in a mixture of 2 mL of *dilute sulfuric acid R* and 48 mL of *water R*, dilute to 100 mL with *water R*. To the resultant solution, add 50 mL of an 11.5 g/L *sodium salicylate R* solution, 10 mL of *dilute acetic acid R*, 80 mL of a 136 g/L *sodium acetate R* solution, and dilute to 500 mL with *water R*. Prepare immediately before use.

Store in an airtight container, protected from light.

Ferric sulfate. $Fe_2(SO_4)_3 \cdot xH_2O$. [15244-10-7]. Iron(III) sulfate hydrated.

Yellowish-white highly hygroscopic powder, decomposes in air. Slightly soluble in water and 96% ethanol.

Store in an airtight container, protected from light.

Ferric chloride. $FeCl_3 \cdot 6H_2O$. (M_r 270.30). [10025-77-1]. Iron trichloride hexahydrate.

Yellow-orange or brownish crystalline mass, deliquescent. Very soluble in water, soluble in 96% ethanol. On exposure to light, ferric chloride and its solutions are partly reduced.

Store in an airtight container.

Ferric chloride solution R1.

A 105 g/L solution.

Ferric chloride solution R2.

A 13 g/L solution.

Ferric chloride solution R3.

Dissolve 2.0 g of *ferric chloride R* in *ethanol R* and dilute to 100.0 mL with the same solvent.

Ferric chloride and sulfamic acid reagent.

The solution contains 10 g/L of *ferric chloride R* and 16 g/L of *sulfamic acid R*.

Ferrous sulfate. $FeSO_4 \cdot 7H_2O$. (M_r 278.0). [7782-63-0]. Ferrous sulfate heptahydrate.

Contains not less than 98.0% and not greater than 105.0% of $FeSO_4 \cdot 7H_2O$.

Light green crystalline powder or bluish green crystals. Efflorescent in air.

Freely soluble in water, very soluble in boiling water, practically insoluble in 96% ethanol.

Oxidised in humid air, turning brown.

Ferrous sulfate solution R2.

Dissolve 0.45 g of *ferrous sulfate R* in 50 mL of *0.1 M hydrochloric acid* and dilute to 100 mL with *carbon dioxide-free water R*.

Prepare immediately before use.

Gastric juice, artificial.

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*, add 80 mL of *1 M hydrochloric acid*, and dilute to 1 L with *water R*.

Substitute for platelets.

To 0.5-1 g of *phospholipids R*, add 20 mL of *acetone R*, and shake for 2 h, then centrifuge for 2 min, and drain the supernatant. Dry the residue in vacuo (water pump), add 20 mL of *chloroform R*, shake for 2 h, and filter under vacuum; suspend the resulting residue in 5-10 mL of a 9 g/L solution of *sodium chloride R*.

For use in the quantitation of factor IX, prepare a dilute suspension in a 9 g/L solution of *sodium chloride R* in such a way that the difference in coagulation time between successive dilutions of suspensions BRP is about 10 s.

Store dilute suspensions at a temperature of -30 °C.

Use within 6 weeks.

Isatin. $C_8H_5NO_2$. [M_r 147.13). [91-56-5]. Indoline-2,3-dione.

Yellowish red small crystals.

Slightly soluble in water, soluble in hot water, 96% ethanol, soluble in solutions of alkali metal hydroxides to form a violet discolouration, turning yellow when allowed to stand.

The melting point is about 200 °C, with partial sublimation.

Sulfated ash (*2.1.4.14*). NMT 0.2%.

Isatin reagent.

Dissolve 6 mg of *ferric sulfate R* in 8 mL of *water R*, add 50 mL of *sulfuric acid R* with stirring; add 6 mg of *isatin R* to the resulting solution, and mix until dissolved.

The solution should be light yellow, but not orange or red.

Isoamyl alcohol. $C_5H_{12}O$. (M_r 88.15). [123-51-3]. 3-Methylbutane-1-ol.

A colorless liquid. Slightly soluble in water, miscible with 96% ethanol.

The boiling point is about 130 °C.

Isoandrosterone. $C_{19}H_{30}O_2$. (M_r 290.44). [481-29-8]. Epiandrosterone. 3β-Hydroxy-5*α*-androstan-17-one.

White or almost white powder. Practically insoluble in water, soluble in organic solvents.

$[\alpha]_{20}^{20}$ + 88. Determination is carried out using a 20 g/L solution in *methanol R*.

The melting point is from 172 °C to 174 °C.

ΔA: $14{,}24 \cdot 10^3$. Determination is carried out at a wavelength of 304 nm, using a 1.25 g/L solution.

Isomenthol. C10H20O. (M_r 156.27). [23283-97-8].

(+)-Isomenthol: (*1S,2R,5R*)-2-isopropyl-5-methylcyclohexanol.

(±)- *Isomentol*: a mixture of equal parts (*1S,2R,5R*) and (*1R,2S,5S*)-2-isopropyl-5-methyl-cyclohexanol. Colourless crystals. *Practically insoluble in water, very soluble in 96% ethanol.*

$[\alpha]_D^{20}$ (+)-*isomenthol* is about +24. Determination is carried out using a 100 g/L solution in 96% *ethanol R*.

The boiling point of (+)-*isomenthol* is about 218 °C.

The boiling point of (±)-*isomenthol* is about 218 °C.

The melting point of (+)-*isomenthol* is about 80 °C.

The melting point of (±)-*isomenthol* is about 53 °C.

(+)-Isomenthone. $C_{10}H_{18}O$. (M_r 154.25). (1*R*)-*cis*-*n*-Mentan-3-one. (1*R*)-*cis*-2-Isopropyl-5-methylcyclohexanone.

Contains variable amounts of menthone. A colorless liquid. Very slightly soluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.904.

n_D^{20} is about 1.453.

$[\alpha]_D^{20}$ is about +93.2.

The isomenthone used in gas chromatography complies with the following additional test.

Quantitation. Determination is performed by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil*, using (+)- isomenthone as the test solution.

The contents of isomenthone calculated by the internal normalisation procedure should be minimum 80.0%.

Isopropylamine. C_3H_9N. (M_r 59.11). [75-31-0]. Propan-2-amine.

Colourless, highly volatile, flammable liquid.

n_D^{20} is about 1.374.

The boiling point is from 32 °C to 34 °C.

Isopropyl myristate. $C_{17}H_{34}O_2$. (M_r 270.5). [110-27-0]. 1-Methyl ethyl tetradecanoate.

Contains not less than 90.0% of $C_{17}H_{34}O_2$.

A clear, colourless, oily liquid.

Immiscible with water, miscible with 96% ethanol, methylene chloride, fatty oils, and liquid paraffin.

The relative density is about 0.853.

4-Isopropylphenol. $C_9H_{12}O$. (M_r 136.19). [99-89-8].

Contains not less than 98% of $C_9H_{12}O$.

The boiling point is about 212 °C.

The melting point is 59 °C to 61 °C.

Imidazole. $C_3H_4N_2$. (M_r 68.08). [288-32-4].

White or almost white crystalline powder; soluble in water and 96% ethanol.

The melting point is about 90 °C.

Iminodibenzyl. $C_{14}H_{13}N$ (M_r 195.26).

[494-19-9]. 10,11-Dihydrodibenz[*b,f*]azepine.

Pale yellow crystalline powder. Practically insoluble in water, freely soluble in acetone.

The melting point is about 106 °C.

Indigo carmine**.** $C_{16}H_8N_2Na_2O_8S_2$. (M_r 466.4). [860-22-0].

Schultz No. 1309.

Colour index (C. I.) No. 73015.

Disodium 3,3'-dioxo-2,2'-bisindolidene-5,5 '-disulfonate. Usually contains sodium chloride.

Blue to violet-blue powder or blue granules with a coppery lustre. Sparingly soluble in water, practically insoluble in 96% ethanol. Precipitated from an aqueous solution with sodium chloride.

Indigo carmine solution.

Dissolve 0.2 g of *indigocarmine R* in a mixture of 10 mL of *hydrochloric acid R* and 990 mL of a 200 g/L solution of *nitrogen-free sulfuric acid R.*

The solution complies with the following test.

Add 10 mL of the resulting solution to a solution of 1.0 mg of *potassium nitrate R* in 10 mL of *water R,* immediately add 20 mL of *nitrogen-free sulfuric acid R,* and heat to boiling. The blue staining of the solution is discharged within 1 min.

Indigo carmine solution R1.

Dissolve 4 g of *indigo carmine R* in *water R,* adding water in separate portions to 900 mL, then add 2 mL of *sulfuric acid R,* and dilute to 1000 mL with *water R.*

Standardisation. Place 10.0 mL of *standard nitrate solution (100 ppm NO_3) R* in a 100 mL wide-neck conical flask, add 10 mL of *water R,* 0.05 mL of *indigo carmine solution R1,* and add immediately (at once but cautiously) 30 mL of *sulfuric acid R*. Titrate the resulting solution immediately with prepared *indigo carmine solution R1* until the stable blue colour is obtained.

The volume in millilitres (V) used for titration corresponds to 1 mg of NO_3.

Indigo sulfonic acid solution.

Dissolve 1 g of *indigo carmine R* in 25 mL of *sulfuric acid R,* then add another 25 mL of *sulfuric acid R* and dilute to 1000 mL with *water R,* carefully pouring the solution into water.

Indometacin. $C_{19}H_{16}ClNO_4$. (M_r 357.8). [53-86-1]. 2-[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-yl]acetic acid.

Contains not less than 98.0% and not greater than 102.0% of $C_{19}H_{16}ClNO_4$calculated on a dry substance basis.

White or yellow crystalline powder.

Practically insoluble in water, sparingly soluble in 96% ethanol.

Indophenol blue. $C_{18}H_{16}N_2O$. (M_r 276.33). [132-31-0]. *N*-[4-(Dimethylamino) phenyl)]1,4-naphthoquinone monoimine.

Schultz No. 939.

Colour index (C.I.) No. 49700.

violet-black powder. Practically insoluble in water.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel G R* as the coating substance. Apply 10 µL of 0.1 g/L solution in *methylene chloride R* to the chromatographic plate and develop with the same solvent. The solvent front must develop at least 10 cm. The chromatogram must show only one principal spot. A spot at the start is allowed.

Ion-exchange resin, highly acidic.

Resin in protonated form with sulfonic acid groups attached to a lattice consisting of polystyrene cross-linked with 8% of divinylbenzene. Produced in the form of spherical granules; unless otherwise specified, the particle size is between 0.3 mm and 1.2 mm.

Reservoir. Between 4.5 mmol/g and 5 mmol/g with a water content of 50% to 60%.

Column preparation. Unless otherwise specified, use a tube with a disk of porous glass fused inside, 400 mm long, 20 mm in internal diameter, and a filling height of about 200 mm. Pre-mix the resin with *water R*, inject the resulting suspension into the tube, preventing the formation of air bubbles between the particles. During operation, the liquid must not fall below the surface of the resin.

If the resin is in a protonated form, wash with *water R* until no greater than 0.05 mL of *0.1 M sodium hydroxide* is used to neutralise 50 mL.

Use 0.1 mL of *methyl orange solution R* as indicator. If the resin is in sodium form or needs to be regenerated, pass about 100 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R* slowly through the column and then wash with *water R* as described above.

Iodine. I_2. (M_r 253.8). [7553-56-2].

Contains no less than 99.5% and no greater than 100.5% of I2.

Greyish violet crystal plates or small crystals with a metallic lustre.

Very slightly soluble in water, soluble in 96% ethanol, slightly soluble in glycerol, very soluble in concentrated solutions of iodides.

Slowly fluorescing at room temperature.

Iodine solution R1.

To 10.0 mL of 0.05 M iodine solution, add 0.6 g of *potassium iodide R* and dilute to 100.0 mL with *water R*.

Prepare immediately before use.

Iodine solution R2.

To 10.0 mL of 0.05 M iodine solution, add 0.6 g of *potassium iodide R* and dilute to 1000 mL with *water R*.

Prepare immediately before use.

Iodine solution R3.

Dilute 2.0 mL of *iodine solution R1* to 100.0 mL with *water R*.

Prepare immediately before use.

Iodine solution R4.

Dissolve 14 g of *iodine R* in 100 mL of a 400 g/L solution of *potassium iodide R*, add 1 mL of *dilute hydrochloric acid R*, and dilute with *water R* to

1000 mL.

Store in a place protected from light.

Iodine solution, alcoholic.

A 10 g/L solution of in *96% ethanol R*. Store protected from light.

Iodine solution, chloroformic.

A 5 g/L solution in *chloroform R*.

Store in a place protected from light.

Starch iodide paper.

Immerse filter paper strips to 100 mL of *potassium iodide and starch solution R*. Excess liquid is removed. Dry in a place protected from light.

Test for sensitivity. Mix 0.05 mL of *0.1 M sodium nitrate* with 4 mL of *hydrochloric acid R* and dilute to 100 mL with *water R*. Apply one drop of the solution to the Iodosulfurous paper; blue staining should appear.

Iodine bromide. IBr. (M_r 206.8). [7789-33-5]. Iodine bromide.

Bluish-black to brownish-black crystals. Freely soluble in water, 96% ethanol, and glacial acetic acid.

The boiling point is about 116 °C.

The melting point is about 40 °C.

Store in a place protected from light.

Iodine bromide solution.

Dissolve 20 g of *iodine bromide R* in *glacial acetic acid R* and dilute to 1000 mL with the same solvent.

Store in a place protected from light.

Iodine pentoxide, recrystallised. I_2O_5. (*Mr* 333.81). [12029-98-0]. Iodine(V) oxide.

Contains not less than 99.5% of I_2O_5.

White or almost white crystalline powder or white to greyish white granules. Hygroscopic, very soluble in water to form HIO_3.

Stability on heating. Dissolve 2 g of iodine pentoxide, previously heated for 1 h at 200 °C, in 50 mL of *water R*; a colorless solution is obtained.

Quantitation. Dissolve 0.100 g of recrystallised pentoxide iodine in 50 mL of *water R*, add 3 g of *potassium iodide R* and 10 mL of *dilute hydrochloric acid R*. Titrate the liberated iodine with a *0.1 M sodium thiosulfate solution*, using 1 mL of a *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 2.782 mg of I_2O_5.

Store in an airtight container, protected from light.

2-Iodobenzoic acid. $C_7H_5O_2$.

(*Mr* 248.02). [88-67-5].

White to light yellow crystalline powder. Slightly soluble in water, soluble in 96% ethanol.

The melting point is about 160 °C.

Chromatography. Carry out the determination by thin-layer chromatography (2.1.2.26), using *cellulose F_{254} for chromatography R* as the coating substance. On the start line of the chromatographic plate, apply 20 µL of a 2-iodobenzoic acid solution prepared by dissolving 40 mg in 4 mL of a *0.1 M sodium hydroxide* and diluting with *water R* to 10 mL. Chromatograph using as a mobile phase the top layer obtained by shaking a mixture of *water R*/*glacial acetic acid R*/*toluene R* (20:40:40). When the solvent front passes 12 cm, the plate is viewed in UV light at 254 nm. The chromatogram must show only one principal spot.

2-Iodohippuric acid. $C_9H_8INO_3 \cdot 2H_2O$. (M_r 341.10). [147-58-0]. 2-(2-Iodobenzamido)acetic acid dihydrate.

White or almost white crystalline powder. Sparingly soluble in water.

The melting point is about 170 °C.

Water (*2.1.5.12*). 9% to 13%. Determined from 1,000 g.

Chromatography. Carry out the determination by thin-layer chromatography (*2.1.2.26*), using *cellulose F_{254} for chromatography R* as the coating substance. On the start line of the chromatographic plate, apply 20 µL of a 2-iodohippuric acid solution prepared by dissolving 40 mg in 4 mL of a *0.1 M sodium hydroxide* and diluting with *water R* to 10 mL. Chromatograph using as a mobile phase the top layer obtained by shaking a mixture of *water R*/*glacial acetic acid R*/*toluene R* (20:40:40). When the solvent front passes 12 cm, the plate is viewed in UV light at 254 nm. The chromatogram must show only one principal spot.

Hydroiodic acid. HI. (M_r 127.91). [10034-85-2].

Distill hydroiodic acid over red phosphorus, passing *carbon dioxide R* or *nitrogen R* during distillation. Use a colorless or almost colorless mixture (from 55% to 58% of HI) boiling at a constant temperature and distilled at 126-127 °C. Place the acid in small amber bottles, previously purged with *carbon dioxide R* or *nitrogen R*, fitted with glass stoppers, and seal them with paraffin.

Store in a place protected from light.

Periodic acid. H_5IO_6. (M_r 227.94). [10450-60-9].

Crystals. Freely soluble in water, soluble in 96% ethanol.

The melting point is about 122 °C.

Iodoplatinate reagent.

To 3 mL of a 100 g/L solution of *chloroplatinic acid R*, add 97 mL of *water R* and 100 mL of a 60 g/L solution of *potassium iodide R*.

Store in a place protected from light.

Iodosulfurous reagent.

The apparatus for preparation of the reagent, consisting of a 3-4 L round-bottom flask with three inlet holes for a stirrer, a thermometer and a tube filled with a desiccant, must be closed and dry during preparation. Introduce 700 mL of *anhydrous pyridine R* and 700 mL of *ethylene glycol monomethyl ether R* in the flask, add 220 g of *finely powdered iodine R* with continuous stirring, previously dried over *phosphorus pentoxide R*. Continue to mix until the iodine is completely dissolved (about 30 min), then cool the flask to -10 °C, and quickly add with constant stirring 190 g of *sulfur dioxide R*. The temperature of the reaction mixture should not exceed 30 °C. Cool.

Standardisation. Place about 20 mL of *anhydrous methanol R* in a titration vessel and titrate with a prepared iodosulfurous reagent (*2.1.5.12*). Add a sufficient, accurately weighed quantity of *water R* and repeat the determination of water. Calculate the quantity of water in milligrams equivalent to 1 mL of Iodosulfurous reagent.

1 mL of iodine-sulphuric reagent is equivalent to at least 3.5 mg of water.

Precautions should be taken to prevent the solutions from being exposed to atmospheric moisture. Standardise immediately before use.

Store in a dry container.

Iodoacetic acid. $C_2H_3IO_2$. (M_r 185.95). [64-69-7].

colorless or white or almost white crystals. Soluble in water and 96% ethanol.

The melting point is from 82 °C to 83 °C.

5-Iodouracil. $C_4H_3IN_2O_2$. (M_r 237.98). [696-07-1]. 5-Iodine-1H,3H-pyrimidine-2,4-dione.

The melting point is about 276 °C with decomposition.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) in accordance with the instructions of the monograph *Iodoxyuridine*. Apply 5 µl of a 0.25 g/L solution to the chromatographic plate; the resulting chromatogram shows only one principal spot.

Iodoethane. C_2H_5I. (M_r 155.97). [75-03-6].

Content: not less than 99%.

colorless to slightly yellowish liquid that darkens on exposure to air and light. Miscible with 96% ethanol and most organic solvents.

d_{20}^{20} is about 1.95.

n_D^{20} is about 1.513.

The boiling point is about 72 °C.

Store in an airtight container.

Cadmium. Cd. (A_r 112.41). [7440-43-9].

Shiny silver-white metal. Practically insoluble in water, freely soluble in nitric acid and hot hydrochloric acid.

Casein. [9000-71-9].

A mixture of related phosphoproteins derived from milk.

White or almost white amorphous powder or granules. Very slightly soluble in water and non-polar organic solvents, soluble in concentrated hydrochloric acid, with a pale violet discolouration. Forms salts with acids and bases. Its isoelectric point is at about pH 4.7. Alkaline solutions are laevorotatory.

Potassium bicarbonate. [298-14-6].

See *Potassium hydrogen carbonate R.*

Potassium bicarbonate solution, saturated methanolic.

See *Potassium bicarbonate, saturated solution, methanolic R.*

Potassium bromate. $KBrO_3$. (M_r 167.00). [7758-01-2]. Potassium bromate.

White or almost white crystals or granular powder. Soluble in water, slightly soluble in 96% ethanol.

Potassium bromide. KBr. (M_r119.0). [7758-02-3].

Contains not less than 98.50% and not greater than 101.0% of KBr calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water and glycerin, slightly soluble in 96% ethanol.

Potassium bromide used in infrared absorption spectrophotometry (2.1.2.23) complies with the following additional test.

The IR spectrum of a 2 mm thick disc of potassium bromide pre-dried at 250 °C for 1 h should have an almost even baseline in the wavelength range from 4000 cm^{-1} to 620 cm^{-1}. Must not have maxima with an absorption greater than 0.02 above the baseline, except for maxima for water at wavelengths of 3440 cm^{-1} and 1630 cm^{-1}.

Potassium bicarbonate. $KHCO_3$. (M_r 100.11). [298-14-6]. Potassium bicarbonate.

Clear, colorless crystals. Freely soluble in water, practically insoluble in 96% ethanol.

Potassium bicarbonate, saturated solution, methanolic.

Dissolve 0.1 g of *potassium bicarbonate R* in 0.4 mL of *water R* when heated in a water bath, add 25 mL of *methanol R*, and swirl with continuous heating until dissolved.

Prepare immediately before use.

Potassium hydroxide.KOH. (M_r 56.11). [1310-58-3]. Potassium hydroxide.

Contains not less than 85.0% and not greater than 100.5% of the alkali mixture calculated with reference to KOH.

White or almost white solid crystalline mass in the form of sticks, plates, or shapeless pieces. Deliquescent. Hygroscopic. Absorbs carbon dioxide.

Very soluble in water, freely soluble in 96% ethanol.

Potassium hydroxide, alcoholic, 2 M.

Dissolve 12 g of *potassium hydroxide R* in 10 mL of *water R* and dilute to 100 mL with *96% ethanol R*.

Potassium hydroxide in alcohol (10% *V/V*), 0.5 M.

Dissolve 28 g of *potassium hydroxide R* in 100 mL of *96% alcohol R* and dilute to 1000 mL with *water R*.

Potassium hydroxide, alcoholic.

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100 mL with *aldehyde-free alcohol (96%) R*. Decant the clear solution. The solution should be almost colourless.

Potassium hydroxide, alcoholic R1.

Dissolve 6.6 g of *potassium hydroxide R* in 50 mL of *water R* and dilute to 1000 mL with *ethanol R*.

Potassium hydrogen sulfate. $KHSO_4$. (M_r 136.17). [7646-93-7]. Potassium hydrogen sulfate.

Clear, colourless, hygroscopic crystals. Freely soluble in water to form a highly acidic solution.

Store in an airtight container.

Potassium hydrotartrate. $C_4H_5KO_6$. (M_r 188.18). [868-14-4]. Potassium hydro(2*R*, 3*R*)-2,3-dihydroxybutane-1,4-dioate.

White or almost white crystalline powder or colourless, slightly opaque crystals. Slightly soluble in water, soluble in boiling water, very slightly soluble in 96% ethanol.

Potassium hydrogen phthalate. $C_8H_5KO_4$. (M_r 204.22). [877-24-7]. Potassium hydrobenzo-1,2-dicarboxylate.

White or almost white crystals. Soluble in water, slightly soluble in 96% ethanol.

0.2 M potassium hydrophthalate solution.

A solution of *potassium hydrophthalate R* contains 40.84 g of potassium hydrophthalate equivalent to $C_8H_5KO_4$ per 1000 mL.

colourless, white or off-white, hygroscopic powder or crystals. Freely soluble in water.

Potassium dihydrogen phosphate. KH_2PO_4. (M_r 136.1). [7778-77-0]. Potassium dihydrogen phosphate.

Contains not less than 98.0% and not greater than 100.5% of KH_2PO_4 calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, practically insoluble in 96% ethanol.

0.2 M potassium dihydrogen phosphate solution.

A solution of *potassium dihydrogen phosphate R* contains 27.22 g equivalent to KH_2PO_4 per 1000 mL.

Potassium dichromate. $K_2Cr_2O_7$. (M_r 294.2). [7778-50-9]. Dipotassium dichromate.

Potassium dichromate used for calibration of spectrophotometers (*2.1.2.24*) must contain at least 99.9% of $K_2Cr_2O_7$ calculated on the dried basis of the substance dried at 130 °C.

Orange-red crystals. Soluble in water, practically insoluble in 96% ethanol.

Quantitation. Dissolve 1.000 g of potassium dichromate in *water R* and dilute to 250.0 mL with the same solvent. Place 50.0 mL of the resulting solution in a 500 mL flask, add a freshly prepared solution consisting of 4 g of *potassium iodide R*, 2 g of *sodium hydrogen carbonate R*, and 6 mL of *hydrochloric acid R* in 100 mL of *water R*. Stopper the flask, allow to stand in a place protected from light for 5 min, and titrate with *0.1 M sodium thiosulfate*, using 1 mL of *iodine-free starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 4.903 mg of $K_2Cr_2O_7$.

Potassium dichromate solution.

A 106 g/L solution.

Potassium dichromate solution R1.

A 5 g/L solution.

Potassium iodate. KIO_3. (M_r 214.0). [7758-05-6].

White or almost white crystalline powder. Soluble in water.

Potassium iodide. KI. (M_r 166.0). [7681-11-0]. Potassium iodide.

Contains not less than 99.0% and not greater than 100.5% of KI calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Very soluble in water, freely soluble in glycerol, soluble in 96% ethanol.

Potassium iodide solution. A 166 g/L solution.

Potassium iodide solution, iodinated.

Dissolve 2 g of *iodine R* and 4 g of *potassium iodide R* in 10 mL of *water R*. When solution is complete, dilute to 100 mL with *water R*.

Potassium iodide solution, saturated.

A saturated solution of *potassium iodide R* in *carbon dioxide-free water R* must contain undissolved crystals. Mix 0.5 mL of the saturated solution of *potassium iodide* with 30 mL of a mixture of *chloroform R*/*acetic acid R* (2:3), add 0.1 mL of *starch solution R*; if a blue colour develops, it should disappear with the addition of 0.05 mL of *0.1 M sodium thiosulfate*.

Store in a place protected from light.

Potassium iodobismuthate solution.

To 0.85 g of bismuth subnitrate R, *add 40 mL of* water R, *10 mL of glacial acetic acid R, and 20 mL of a 400 g/L solution of* potassium iodide R.

Potassium iodobismuthate solution R1.

Dissolve 100 g of *tartaric acid R* in 400 mL of *water R*, add 8.5 g of *bismuth subnitrate R*, shake for 1 h, add 200 mL of a 400 g/L solution of *potassium iodide R* and shake vigorously. Allow to stand for 24 h and filter.

Store in a place protected from light.

Potassium iodobismuthate solution R2.

Stock solution. Suspend 1.7 g of *bismuth subnitrate R* and 20 g of *tartaric acid R* in 40 mL of *water R*. Add 40 mL of a 400 g/L solution of *potassium iodide R* to the suspension, stir for 1 h, and filter.

The solution may be kept for several days in amber glass vials.

Solution for spraying. Mix immediately before use 5 mL of the stock solution with 15 mL of *water R*.

Potassium iodobismuthate solution, diluted.

Dissolve 100 g of tartaric acid R *in 500 mL of* water R *and add 50 mL of* potassium iodovismutate solution R1.

Store in a place protected from light.

Potassium carbonate. K_2CO_3. (M_r 138.21). [584-08-7]. Dipotassium carbonate.

White or almost white granular powder; hygroscopic. Very soluble in water, practically insoluble in anhydrous ethanol.

Store in an airtight container.

Potassium sodium tartrate. $C_4H_4KNaO_6 \cdot 4H_2O$. (M_r282.22). [6381-59-5].

Colourless prismatic crystals. Very soluble in water.

Potassium nitrate. KNO_3. (M_r 101.1). [7757-79-1].

Colourless crystals. Very soluble in water.

Potassium periodate. KIO_4. (M_r 230.0). [7790-21-8].

White or almost white crystalline powder or colorless crystals. Soluble in water.

Potassium permanganate. $KMnO_4$. (M_r 158.0). [7722-64-7]. Potassium permanganate.

Contains not less than 99,0% and not greater than 100.5% of $KMnO_4$.

Dark violet or brownish black granular powder or dark violet or off-black crystals, usually with a metallic lustre.

Soluble in cold water, freely soluble in boiling water.

Decomposes when interacting with certain organic substances.

Potassium permanganate solution in phosphoric acid.

Dissolve 3 g of *potassium permanganate R* in a mixture of 15 mL of *phosphoric acid R* and 70 mL of *water R*, dilute to 100 mL with *water R*.

Potassium permanganate solution.

A 30 g/L solution.

Potassium perrhenate. $KReO_4$. (M_r 289.3). [10466-65-6]. Potassium perrhenate.

White or almost white crystalline powder. Soluble in water, slightly soluble in 96% ethanol, methanol, and propylene glycol.

Potassium persulfate. $K_2S_2O_8$. (M_r 270.32). [7727-21-1]. Dipotassium peroxydisulfate.

Colourless crystals or white or almost white crystalline powder. Sparingly soluble in water, practically insoluble in 96% ethanol.

Water solutions decompose at room temperature and more rapidly on warming.

Potassium pyroantimonate. $KSb(OH)_6$. (M_r 262.90). [12208-13-8]. Potassium hexahydroxoantimoniate(V).

White or almost white crystals or crystalline powder. Sparingly soluble in water.

Potassium pyroantimonate solution.

Dissolve 2 g of *potassium pyroantimonate R* in 95 mL of hot *water R*, quickly cool, add a solution containing 2.5 g of *potassium hydroxide R* in 50 mL of *water R*, and 1 mL of a *dilute sodium hydroxide solution R*. Allow to stand for 24 h, filter, and dilute to 150 mL with *water R*.

Potassium plumbite solution.

Dissolve 1.7 g of *lead acetate R*, 3.4 g of *potassium citrate R* and 50 g of *potassium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Potassium sulfate. K_2SO_4. (M_r 174.26). [7778-80-5]. Dipotassium sulfate.

Colourless crystals. Soluble in water.

Potassium tartrate. $C_4H_4K_2O_6 \cdot {}^1/_2H_2O$. ($M_r$ 235.27). [921-53-9]. Dipotassium (*2R,3R*)-2,3-dihydroxybutane-1,4- dioate hemihydrate.

White or almost white granular powder or crystals. Very soluble in water, very slightly soluble in 96% ethanol.

Potassium tetraiodomercurate solution.

Dissolve 1.35 g of *mercuric chloride R* in 50 mL of *water R*, add 5 g of *potassium iodide R* and dilute to 100 mL with *water R*.

Potassium tetraiodomercurate alkaline solution.

Dissolve 11 g of *potassium iodide R* and 15 g of *mercuric iodide R* in *water R*, dilute to 100 mL with the same solvent.

Immediately before use, mix equal volumes of the resulting solution and a 250 g/L solution of *sodium hydroxide R*.

Potassium tetraoxalate. $C_4H_3KO_8 \cdot 2H_2O$. (M_r 254.19). [6100-20-5]. Potassium tetraoxalate dihydrate.

White or almost white crystalline powder. Sparingly soluble in water, soluble in boiling water, slightly soluble in 96% ethanol.

Potassium thiocyanate. KSCN. (M_r 97.18). [333-20-0]. Potassium thiocyanate.

Colourless crystals, deliquescent. Very soluble in water and 96% ethanol.

Store in an airtight container.

Potassium thiocyanate solution.

A 97 g/L solution.

Potassium ferriperiodate solution.

Dissolve 1 g of *potassium periodate R* in 5 mL of a freshly prepared 120 g/L solution of *potassium hydroxide R*, add 20 mL of *water R* and 1.5 mL of *ferric chloride R1*, dilute to 50 mL with the freshly prepared 120 g/L solution of *potassium hydroxide R*.

Potassium ferricyanide. $K_3[Fe(CN)_6]$. (M_r 329.26). [13746-66-2]. Tripotassium hexacyanoferrate (III).

Red crystals. Freely soluble in water.

Potassium ferricyanide solution.

Wash 5 g of *potassium ferricyanide R with a small amount of water R*, dissolve in *water R*, and dilute to 100 mL with the same solvent.

Prepare immediately before use.

Potassium ferrocyanide. $K_4[Fe(CN)_6] \cdot 3H_2O$. ($M_r$ 422.39). [14459-95-1]. Potassium hexacyanoferrate(II) trihydrate.

Clear yellow crystals. Freely soluble in water, practically insoluble in 96% ethanol.

Potassium ferrocyanide solution.

A 53 g/L solution.

Potassium chlorate. $KClO_3$. (M_r 122.55).

[3811-04-9]. Potassium chlorate.

White or almost white powder, or granules, or crystals. Soluble in water.

Potassium chloride. KCl. (M_r 74.6). [7447-40-7]. Potassium chloride.

Contains not less than 99.0% and not greater than 101.0% KCI equivalent to the dried substance.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, practically insoluble in anhydrous ethanol.

Potassium chloride used for infrared absorption spectrophotometry (*2.1.2.23*) *complies with the following additional requirement*.

The IR spectrum of a 2 mm thick disc of potassium chloride pre-dried at 250 °C for 1 h should have an almost even baseline in the wavelength range from 4000 cm^{-1} to 620 cm^{-1}. Must not have maxima with an absorption greater than 0.02 above the baseline, except for maxima for water at wavelengths of 3440 cm^{-1} and 1630 cm^{-1}.

0.1 M potassium chloride solution.

A solution of *potassium chloride R* contains 7.46 g of KCl equivalent to KCl per 1000 mL.

Potassium chromate. K_2CrO_4. (M_r 194.19). [7789-00-6]. Dipotassium chromate.

Yellow crystals. Freely soluble in water.

Potassium chromate solution.

A 50 g/L solution.

Potassium cyanide. KCN. (M_r 65.12). [151-50-8]. Potassium cyanide.

White or almost white crystalline powder, or mass, or granules. Freely soluble in water, slightly soluble in 96% ethanol.

Potassium cyanide solution.

A 100 g/l solution.

Potassium citrate. $C_6H_5K_3O_7 \cdot H_2O$. (M_r 324.4). [6100-05-6]. Tripotassium 2-hydroxypropane-1,2,3-tricarboxylate.

Potassium citrate contains not less than 99.0% and not greater than 101.0% $C_6H_5K_3O_7 \cdot H_2O$ calculated with reference to the anhydrous substance.

White or almost white granular powder or colorless crystals. Hygroscopic.

Very soluble in water, practically insoluble in 96% ethanol.

Calconecarboxylic acid. [3737-95-9].

See *Calconecarboxylic acid R.*

Calconecarboxylic acid indicator.

See *Calconecarboxylic acid indicator R.*

Calcium acetate. $C_4H_6CaO_4$. (M_r 158.2). [62-54-4]. Calcium diacetate.

Contains not less than 99.0% and not greater than 101.0% of $C_4H_6CaO_4$ calculated with reference to the anhydrous substance.

White or almost white hygroscopic powder.

Freely soluble in water, slightly soluble in 96% ethanol.

Calcium hydroxide. $Ca(OH)_2$. (M_r 74.09). [1305-62-0]. Calcium dihydroxide.

White or almost white powder. Almost completely soluble in 600 parts of water.

Calcium hydroxide solution.

A freshly prepared saturated solution.

Calcium carbonate. $CaCO_3$. (M_r 100.1). [471-34-1]. Calcium carbonate.

Contains not less than 98.5% and not greater than 100.5% of $CaCO_3$ calculated on a dry substance basis.

White or almost white powder. Practically insoluble in water.

Calcium carbonate R1.

Complies with the requirements prescribed for *calcium carbonate R* with the following additional requirement.

Chlorides (2.1.4.4). NMT 50 ppm.

Calcium lactate. $C_6H_{10}CaO_6 \cdot 5H_2O$. ($M_r$ 308.3). [41372-22-9]. Calcium lactate pentahydrate.

Contains NLT 98.0% and NMT 102.0% of calcium bis(2-hydroxypropanoate) or a mixture of calcium pentahydrates (2R)-, (2S)- and (2RS)-2-hydroxypropanoates calculated on a dry substance basis.

White or almost white crystalline or granular powder, slightly efflorescent in the air.

Soluble in water, freely soluble in boiling water, very slightly soluble in 96% ethanol.

Calcium sulfate. $CaSO_4 \cdot 1/_2H_2O$. (M_r 145.14). [10034-76-1]. Calcium sulfate hemihydrate.

White or almost white powder. Soluble in about 1500 parts of water, practically insoluble in 96% ethanol.

When mixed with water, the mass of which is equal to half the mass of calcium sulfate, the powder quickly solidifies, turning into a solid porous mass.

Calcium sulfate solution.

Shake 5 g of *calcium sulfate R* with 100 mL of *water R* for 1 h and filter.

Calcium chloride.$CaCl2{\cdot}2H_2O$. (M_r 147.0). [10035-04-8]. Calcium chloride dihydrate.

Calcium chloride dihydrate contains not less than 97.0% and not greater than 103.0% of $CaCl2{\cdot}2H_2O$.

White or almost white crystalline powder. Hygroscopic.

Freely soluble in water, soluble in 96% ethanol.

Calcium chloride solution.

A 73.5 g/L solution.

0.01 M calcium chloride solution.

Dissolve *0.147* g of *calcium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

0.02 M calcium chloride solution.

Dissolve 2.94 g of *calcium chloride R* in 900 mL of *water R*, adjust the pH of the solution to the range from 6.0 to 6.2, and dilute to 1000 mL with *water R*.

Store at a temperature between 2 °C and 8 °C.

0.025 M calcium chloride solution.

Dissolve 0.368 g of *calcium chloride R* in *water R* and dilute to 100.0 mL with *water R*.

Calcium chloride R1. $CaCl_2{\cdot}4H_2O$ (*M*. 18305). Calcium chloride tetrahydrate.

Contains no greater than 0.05 ppm of Fe.

Calcium chloride, anhydrous. $CaCl_2$.

(M_r 110.98). [10043-52-4]. Calcium chloride.

Contains not less than 98.0% $CaCl_2$ calculated on the dried basis.

White or almost white granules, deliquescent. Very soluble in water, freely soluble in 96% ethanol and methanol.

Loss on drying (*2.1.2.31*). NMT 5.0%. Determination is carried out in a drying cabinet at 200 °C.

Store in an airtight container in a dry place.

Carob bean gum.

The ground endosperm of the fruit kernels of *Ceratonia siliqua L.* Taub.

White or almost white powder containing 70-80% of water-soluble gum consisting mainly of galactomannoglycone.

Camphor. $C_{10}H_{16}O$. (M_r 152.2). [76-22-2].

Racemic camphor is (1*R*,4*S*)-1,7,7-trimethylbicyclo[2.2.1]heptane-2-one.

White or almost white crystalline powder or loose crystalline mass. Easily volatile even at room temperature.

Slightly soluble in water, very soluble in 96% ethanol and petroleum ether, freely soluble in fatty oils, sparingly soluble in glycerol.

Camphor used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as specified in the monograph *Lavender oil*.

Test solution. A 10 g/L solution of the test substance in *hexane R*.

The camphor content calculated by the internal normalisation procedure should be minimum 95.0%.

(1*S*)-(+) 10-Camphorosulfonic acid. $C_{10}H_{16}O_4S$. (M_r 232.30). [3144-16-9]. (1*S*,4*R*)-(+)- 2-Oxo-10-bornenesulfonic acid. [(1*S*)-7,7-dimethyl-2-oxobicyclo[2.2.1]heptane-1-yl]methane sulfonic acid. Reychler's acid.

Prismatic crystals. Hygroscopic, soluble in water.

Contains not less than 99,0% of (1*S*)-(+)-10-camphorsulfonic acid.

$[\alpha]_D^{20}$ — 18 ± 2. Determination is carried out using a 43 g/L solution in *water R*.

The melting point is about 194 °C with decomposition.

ΔL (*2.2.41*): $10.2{\cdot}10^3$. Determination is carried out at 290.5 nm, using a 1.0 g/L solution.

Kaolin, light. [1332-58-7].

Purified natural hydrated aluminosilicate. Contains a suitable dispersant.

White or almost white light powder, not containing solid caked particles, oily to the touch. Practically insoluble in water and mineral acids.

Large particles. NMT 0.5%.

Place 5.0 g of kaolin in a ground-glass-stoppered cylinder about 160 mm long and 35 mm in diameter, add 60 mL of a 10 g/L solution of *sodium pyrophosphate R*, shake vigorously, and allow to stand for 5 min. Using a pipette, take 50 mL of liquid at a level about 5 cm below the surface and discard. To the remaining liquid, add 50 mL of *water R*, shake, allow to stand for 5 min, and remove 50 mL, as described above. This operation is repeated until a total of 400 mL is removed. Transfer the remaining suspension to an evaporating dish, evaporate in a water bath to dryness and dry to a constant weight at a temperature from 100 °C to 105 °C. The weight of the residue should not exceed 25 mg.

Disperse *fine particles* of 5.0 g of kaolin in 250 mL of *water R* with vigorous shaking for 2 min and immediately pour into a glass cylinder with a diameter of 50 mm. Using a pipette, take 20 mL, place in a silica crucible, evaporate in a water bath to dryness, and dry to a constant mass at 100-105 °C. Allow the remaining suspension to stand at 20 °C for 4 h and remove 20 mL using a pipette at a level accurately 5 cm below the surface, without stirring the sediment. Place the residue in a silica crucible, evaporate to dryness, and dry to a constant mass at 100-105 °C. The mass of the second residue must be NLT 70% of the mass of the first residue.

Caprylic alcohol.

See Decanol R.

Carbazole. $C_{12}H_9N$. (M_r 167.19). [86-74-8]. Dibenzopyrrol.

Crystals. Practically insoluble in water, freely soluble in acetone, slightly soluble in anhydrous ethanol.

The melting point is about 245 °C.

Carbomer. [9007-20-9].

A cross-linked polymer of acrylic acid; contains a large number of carboxylic acid groups (CO_2H, between 56% and 68%) after drying at 80 °C for 1 h.

The average molecular weight is about 3 x 10^6.

pH (2.1.2.3). About 3.

Measure pH for a 10 g/L suspension.

Carbophenothion. $C_{11}H_{16}C1O_2PS_3$. (M_r 342.87). [786-19-6]. *O,O*-Diethyl-*S*-[[(4-chlorophenyl)thio]methyl]phosphorodithioate.

A yellowish liquid. Practically insoluble in water, miscible with organic solvents.

d_4^{25} is about 1.27.

For the monograph *Lanolin*, a certified reference solution can be used (10 ng/µL in isooctane).

Carvacrol. $C_{10}H_{14}O$. (M_r 150.22). [499-75-2]. 5-Isopropyl-2-methylphenol.

Brownish liquid. Practically insoluble in water, very soluble in 96% alcohol.

d_{20}^{20} is about 0.975.

n_D^{20} is about 1.523.

The boiling point is about 237 °C.

Carvacrol used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil.*

Test solution. Dissolve 0.1 g in 10 mL of acetone R.

The carvacrol content calculated by the internal normalisation procedure should be minimum 95.0%.

Carvone. $C_{10}H_{14}O$. (M_r 150.2). [2244-16-8]. (+)-*p*-meta-6,8-diene-2-one. (5*S*)-2-Methyl-5-(1-methylethenyl)cyclohex-2-enone.

Liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.965.

n_D^{20} of about 1,500.

$[\alpha]_D^{20}$ is about +61.

The boiling point is about 230 °C.

Carvone used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil*, using carvone as the test solution.

The carvon content calculated by the internal normalisation procedure should be minimum 98.0%.

Catechin. $C_{15}H_{14}O_6 \cdot H_2O$. ($M_r$ 290.3, for anhydrous substance). [154-23-4]. (+)-(2*R*,3*S*)-2-(3,4-Digcdroxyphenyl)3,4-dihydro-2*H*-chromene-3,5,7-triol.

Catechol. Cyanidanol. Cyanidol.

Cation-exchange resin.

Resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8% of divinylbenzene. Available in the form of spherical granules.

Cation-exchange resin R1.

Resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 4% of divinylbenzene.

Available in the form of spherical granules.

Cation-exchange resin (calcium form), strong.

Resin in calcium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8% of divinylbenzene.

Cation-exchange resin (sodium form), strong.

Resin in sodium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

Cetostearyl alcohol. [67762-27-0].

A mixture of solid aliphatic alcohols, mainly octadecane-1-ol (stearyl alcohol, $C_{18}H_{38}O$, M_r 270.5) and hexadecane-1-ol (cetyl alcohol, $C_{16}H_{34}O$, M_r 242.4), of animal or vegetable origin.

Contains NLT 40.0% of stearyl alcohol and in total at NLT 90.0% of stearyl alcohol and cetyl alcohol.

White or pale yellow waxy mass, plates, flakes, or granules.

Practically insoluble in water, soluble in 96% ethanol, and petroleum ether. When melting, miscible with fatty oils, with liquid paraffin and with melted wool fat.

Kieselguhr G.

Consists of kieselguhr treated with hydrochloric acid and calcined, to which about 15% of calcium sulfate hemihydrate is added.

A fine greyish-white powder; the grey colour becomes more pronounced on triturating with water. The average particle size is from 10 μm to 40 μm.

Calcium sulfate. Determination is carried out by the method prescribed for *silica gel G R.*

pH (*2.1.2.3*). 7 to 8. Measure the pH of the suspension obtained by shaking 1 g with 10 mL of *carbon dioxide-free water R* for 5 min.

Chromatographic separation capacity. The determination is carried out by thin-layer chromatography (*2.1.2.26).* Prepare plates using a slurry of the kieselguhr G with a 2.7 g/L solution of *sodium acetate R.* Apply 5 μL of a solution containing 0.1 g/L of lactose, sucrose, glucose and fructose in *pyridine R*. Develop using a mixture of 12 volumes of *water R*, 23 volumes of 2-*propanol R* and 65 volumes of *ethyl acetate R* (12:23:65). The migration time for the development over a path of 14 cm is about 40 min. Dry the plate in air, spray with a solution of *anisaldehyde R*, using about 10 mL, and heat at 100-105 °C for 5 min.

The chromatogram shows four well-defined spots without tailing and well separated from each other.

Kieselguhr for chromatography.

White or yellowish white light powder. Practically insoluble in water, dilute acids, and organic solvents.

Filtration rate. Use a chromatographic column 0.25 m long and 10 mm in internal diameter with a sintered-glass (100) plate and two marks at 0.10 m and 0.20 m above the plate. Place the substance to be examined in the column to reach the first mark and fill to the second mark with *water R*. When the first drops begin to flow from the column, fill to the second mark again with *water R* and measure the time required for the first 5 mL to flow from the column. The flow rate is not less than 1 mL/min.

Colour index (*2.1.2.2, Method I).* The eluate obtained in the test for filtration rate is colourless.

Acidity or alkalinity. To 1.00 g, add 10 mL of *water R*, shake vigorously, and allow to stand for 5 min. Filter the suspension through a filter previously washed with hot *water R* until a neutral reaction in the washing water. To 2.0 mL of the filtrate, add 0.05 mL of *methyl red solution R*; the solution is yellow. To 2.0 mL of the filtrate, add 0.05 mL of *phenolphthalein solution R1*; the solution is at most slightly pink.

Water-soluble substances. Place 10.0 g in a chromatographic column (0.25 m x 10 mm), elute with *water R*, collect the first 20 mL of the eluate, evaporate to dryness, dry the residue at 100-105 °C. The mass of the residue should not exceed 10 mg.

Iron (*2.1.4.9*). NMT 200 ppm.

To 0.50 g, add 10 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R*, shake vigorously, allow to stand for 5 min, and filter. 1.0 mL of the filtrate complies with the test for iron.

Loss on ignition. NMT 0.5%.

During heating to red heat (600 ± 50 °C), the substance does not become brown or black.

Oxygen. O_2. (M_r 32.00).

Contains minimum 99.99% (*V/V*) of O_2.

Nitrogen and *argon*. NMT 100 ppm.

Carbon dioxide. NMT 10 ppm.

Carbon monoxide. NMT 5 ppm.

Acid blue 83. $C_{45}H_{44}N_3NAO_7S_2$. (M_r 826). [6104-59-2].

colour index (C.I.) No. 42660.

Brilliant blue. Coomassie brilliant blue R 250.

Brown powder. Insoluble in cold water, slightly soluble in boiling water and anhydrous ethanol, soluble in sulfuric acid, glacial acetic acid, and dilute solutions of alkali metal hydroxides.

Acid blue 90. $C_{47}H_{48}N_3NaO_7S_2$. (M_r 854). [6104-58-1].

Colour index (C.I.) No. 42655.

Sodium [4-[[4-[(4-ethoxyphenyl)amino] phenyl] [[4-(ethyl)(3-sulfonatobenzyl)amino]phenyl] methylene]cyclohexa-2,5-diene-1-ylidene] (ethyl)- (3-sulfonatobenzyl)ammonium.

Dark brown powder with a violet lustre and interspersed with particles that have a metallic lustre. Soluble in water and anhydrous ethanol.

Loss on drying (*2.1.2.31*). NMT 5.0%. Dry 0.500 g in an oven at a temperature between 100 °C and 105 °C.

$E_{1cm}^{1\%}$ greater than 500 equivalent to dry substance. Determination is carried out at 577 nm, using a 0.01 g/L solution in a buffer solution pH 7.0.

Loss on drying (*2.1.2.31*). NMT 5.0%.

Dry 0.500 g in a drying cabinet at 100-105 °C.

Acid blue 92. $C_{26}H_{16}N_3Na_3O_{10}S_3$. ($M_r$ 695.6). [3861-73-2].

Colour index (C.I.) No. 13390.

Coomassie blue. Anazolen sodium. Trisodium 8-hydroxy-4'-(phenylamino)azonaphthalene-3,5', 6-trisulfonate.

Dark blue crystals. Slightly soluble in 96% ethanol, soluble in water, acetone, and ethylene glycol monoethyl ether.

Acid blue 92 solution.

Dissolve 0.5 g of acid blue 92 R *in a mixture of 10 mL of* glacial acetic acid R, *45 mL of 96%* ethanol R, *and 45 mL of* water R.

Clobetasol propionate. $C_{25}H_{32}ClFO_5$. (M_r 467.0). [25122-46-7].

21-Chloro-9-fluoro-11 β, 17-dihydroxy-16P-metilpregna-1,4 diene-3,20-dione-17-propionate.

White or almost white crystalline powder. Insoluble in water, soluble in 96% ethanol, and acetone.

$[\alpha]_D^{20}$ is about + 104 (in dioxane).

The melting point is about 196 °C.

Cobalt nitrate. $Co(NO_3)_2 \cdot 6H_2O$. (M_r 291.0). [10026-22-9]. Cobalt(II) nitrate hexahydrate.

Small garnet-red crystals. Very soluble in water.

Cobalt chloride. $CoCl_2 \cdot 6H_2O$. (*Mr* 237.93). [7791-13-1]. Cobalt(II) chloride hexahydrate.

Red crystalline powder or dark red crystals. Very soluble in water, soluble in 96% ethanol.

Codeine. $C_{18}H_{21}NO_3 \cdot H_2O$ (M_r 317.4). [6059-47-8]. 7,8-Didehydro-4,5α-epoxy-3-methoxy-17-methyl-morphinan-6α-ol.

Contains not less than 99.0% and not greater than 101.0% of $C_{18}H_{21}NO_3 \cdot H_2O$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Soluble in boiling water, freely soluble in 96% ethanol.

Codeine phosphate. $C_{18}H_{24}NO_7P \cdot 5H_2O$. ($M_r$ 406.4). [52-28-8]. 7,8-Didehydro-4,5 α-epoxy-3-methoxy-17-methylmorphinan-6α-ol phosphate hemihydrate.

Contains not less than 98.5% and not greater than 101.0% of $C_{18}H_{24}NO_7P \cdot SH_2O$ calculated on a dry substance basis.

White or almost white crystalline powder or small colorless crystals.

Freely soluble in water, slightly soluble or very slightly soluble in 96% ethanol.

Congo red. $C_{32}H_{22}N_6Na_2O_6S_2$. ($M_r$ 696.7). [573-58-0]. Disodium (biphenyl-4,4'-diyl-bis-2,2'azo)bis(1-aminonaphthalene-4-sulfonate).

Schultz No. 360.

Colour index (C.I.) No. 22120.

Brownish red powder. Soluble in water.

Congo red paper.

Immerse filter paper strips for a few minutes in a solution of *congo red R*. Dry.

Congo red solution.

Dissolve 0.1 g of *congo red R* in a mixture of 20 mL of *96% ethanol R* and *water R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.2 mL of a solution of congo red and 0.3 mL of *0.1 M hydrochloric acid*; a blue colour appears, which should turn pink when no greater than 0.3 mL of *0.1 M sodium hydroxide* is added.

Colour change. Blue to pink in the pH range of 3.0-5.0.

Cinnamic aldehyde. C_9H_8O. (M_r 132.16). [104-55-2]. 3-Phenylpropenal.

Oily yellowish to greenish-yellow liquid. Slightly soluble in water, very soluble in 96% ethanol.

n_D^{20} is about 1,620.

Store in a place protected from light.

Cortisone. $C_{21}H_{28}O_5$. (M_r 360.44). [53-06-5].

Contains not less than 95.0% of $C_{21}H_{28}O_5$.

The melting point is from 223 °C to 228 °C.

Cortisone acetate. $C_{23}H_{30}O_6$**.** (M_r 402.5). [50-04-4]. 17-Hidroxy-3,11,20-triooxopregn-4-en-21-yl acetate.

Contains not less than 97.0% and not greater than 103.0% of $C_{23}H_{30}O_6$ calculated on a dry substance basis.

White or almost white crystalline powder.

Practically insoluble in water, freely soluble in methylene chloride, soluble in dioxane, sparingly soluble in acetone, slightly soluble in 96% ethanol and methanol. Has polymorphism.

Caffeine. $C_8H_{10}N_4O_2$. (M_r 194.2). [58-08-2]. 1,3,7-TRIMETHYL-3,7-DIHYDRO-1*H*-PURINE-2,6-DIONE.

Contains not less than 98.5% and not greater than 101.5% of $C_8H_{10}N_4O_2$ calculated on a dry substance basis.

White or almost white crystalline powder or silky crystals. Easily sublimated.

Sparingly soluble in water, freely soluble in boiling water, slightly soluble in 96% ethanol.

Soluble in concentrated solutions of alkaline benzoates or salicylates.

Caffeic acid. $C_9H_8O_4$. (M_r 180.16). [331-39-5]. (E)-3-(3,4-dihydroxyphenyl) propanoic acid.

White or almost white crystals or plates. Freely soluble in hot water and 96% ethanol, sparingly soluble in cold water.

The melting point is about 225 °C with decomposition.

A freshly prepared solution (pH 7.6) has two absorption maxima (*2.1.2.24*) at wavelengths of 293 nm and 329 nm.

Soluble starch. [9005-84-9].

White or almost white powder.

Starch solution.

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R*, pour the resulting mixture slowly with constant stirring into 100 mL of boiling *water R* containing 10 mg of *mercuric iodide R*.

Each time the reagent is used, perform the test for sensitivity.

Test for sensitivity. To a mixture of 1 mL of starch solution and 20 mL of *water R*, add about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1*; the solution should have a blue colour.

Starch solution R1.

Mix 1 g of *soluble starch R* with a small volume of cold *water R*. Add the resulting mixture with stirring to 200 mL of boiling *water R*, add 250 mg of *salicylic acid R*, boil for 3 min, and immediately cool.

Shelf life. 2 to 3 weeks at 4 °C to 10 °C.

A fresh starch solution is prepared in the case of an indistinct colour transition from blue to colorless at the equivalence point.

Test for sensitivity. To 2 mL of *starch solution R1*, add 20 mL of *water R*, about 50 mg of *potassium iodide R*, and 0.05 mL of *iodine solution R1*; the resultant solution should have a blue colour.

Starch solution R2.

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R* and, with stirring, pour the mixture into 100 mL of boiling *water R*. Use a freshly prepared solution.

Test for sensitivity. To 1 mL of *starch solution R1*, add 20 mL of *water R*, about 50 mg of *potassium iodide R*, and 0.05 mL of *iodine solution R1*; the resulting solution should have a blue colour.

Starch solution, iodide-free.

Prepare the solution according to the instructions for the *starch solution R* but without mercury iodide. Prepare immediately before use.

Potassium iodide and starch solution.

Dissolve 0.75 g of *potassium iodide R* in 100 mL of *water R*, heat to boiling, and add with stirring a 0.5 g solution of *soluble starch R* in 35 mL of *water R*.

Boil for 2 min and cool.

Test for sensitivity.

A mixture consisting of 15 mL of potassium iodide and starch solution R, 0.05 mL of gl*acial acetic acid R*, and 0.3 mL of *iodine solution R2*; the mixture should have a blue colour.

Cresol. C_7H_8O. (M_r108,14). [95-48-7]. o-Cresol. 2-Methylphenol.

Crystals or a super-cooled liquid that darkens in light and air. Miscible with anhydrous ethanol, soluble in about 50 parts of water and solutions of alkali metal hydroxides.

d_{20}^{20} is about 1.05.

n_D^{20} from 1.540 to 1.550.

The boiling point is about 190 °C.

Freezing point (*2.1.2.17*). Not less than 30.5 °C.

Residue on evaporation. NMT 0.1% (*w/w*).

evaporate in a water bath and dry at 100-105 °C.

Store in a dark, dry place protected from oxygen and distill before use.

Cresol red. $C_{21}H_{18}O_5S$. (M_r382.4). [1733-12-6]. Cresolsulfonphthalein. 4,4'-(3*H*-2,1-Ben-zoxatiol-3-ylidene)bis-(2-methylphenol)S,S-dioxide.

Reddish brown crystalline powder. Slightly soluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Cresol red solution.

Dissolve 0.1 g of *cresol red R* in a mixture of 2.65 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R*, dilute to 100 mL with *water R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.1 mL of cresol red solution and 0.15 mL of *0.02 M sodium hydroxide*; a purple-red colour appears, which should turn yellow when no greater than 0.15 mL of *0.02 M hydrochloric acid* is added.

Colour change. Yellow to red in the pH range from 7.0 to 8.6.

m-Cresol purple. $C_{21}H_{18}O_5S$. (M_r382.43). [2303-01-7]. m-Cresolsulfonphthalein.

Olive green crystal powder. Slightly soluble in water, soluble in 96% ethanol, glacial acetic acid, and methanol.

m-Cresol purple solution.

Dissolve 0.1 g of *m-cresol purple R* in 13 mL of 0.*01 M sodium hydroxide*, dilute to 100 mL with *water R*, and mix.

Colour change. Red to yellow in the pH range from 1.2 to 2.8. Yellow to violet in the pH range from 7.4 to 9.0.

Silicotungstic acid. $H_4SiW_{12}O_{40}·H_2O$. [11130-20-4].

White or yellowish white crystals, deliquescent in the air. Very soluble in water and 96% ethanol.

Store in an airtight container.

Crystal violet. $C_{25}H_{30}ClN_3$. (M_r 408.0). [548-62-9]. Schultz No. 78.

Colour Index (C. I.) No. 42555. Hexamethylpropylene chloride.

Dark green crystals or powder. Soluble in water and 96% ethanol.

Crystal violet solution.

Dissolve 0.5 g of *crystalline violet R* in *anhydrous acetic acid R* and dilute to 100 mL with the same solvent.

Test for sensitivity. To 50 mL of *anhydrous acetic acid R*, add 0.1 mL of a solution of crystalline violet; a bluish-violet colour develops, which should turn bluish-green when 0.1 mL of *0.1 M solution of perchloric acid* is added.

Xanthydrol. $C_{13}H_{10}O_2$. (M_r 198.22). [90-46-0]. 9-Xanthenol.

Contains not less than 90.0% of $C_{13}H_{10}O_2$.

White to light yellow powder. Very slightly soluble in water, soluble in 96% ethanol and glacial acetic acid. Also available as a solution containing 90 g/L to 110 g/L of xanthydrol in methanol R.

The melting point is about 123 °C.

Quantitation. Place 0.300 g of xanthydrol in a 250 mL flask, dissolve in 3 mL of *methanol R*, or use 3.0 mL of the solution. Add 50 mL of *glacial acetic acid R* and dropwise, with shaking, 25 mL of a 20 g/L solution of *urea R*. Allow to stand for 12 h, then filter through a glass filter (16) (*2.1.1.2*). Wash the precipitate on the filter with 20 mL of *96% ethanol R*, dry at 100-105 °C, and weigh.

1 g of sediment is equivalent to 0.9429 g of xanthydrol.

Store in a place protected from light. The methanol solution should be stored in small hermetically sealed ampoules and filtered if necessary before use.

Xanthydrol R1.

Must meet the requirements for *xanthydrol R* and the following additional requirement.

Contains not less than 98% of $C_{13}H_{10}O_2$.

Xanthydrol solution.

To 100 mL of *anhydrous acetic acid R*, add 0.1 mL of a 100 g/L solution of *xanthydrol R* in *methanol R*, 1 mL of *hydrochloric acid R*, and allow to stand for 24 h before use.

Xylenol orange. $C_{31}H_{28}N_2Na4O_{13}S$. ($M_r$ 761). [3618-43-7]. Tetranatrium 3,3'-(3*H*-- 2,1-benzoxa-thiol-3-ylidene) bis[(6-hydroxy-5-methyl-3,1-phenylene) methyleniminobisacetate]S,S-dioxide.

Reddish brownish crystalline powder. Soluble in water.

Xylenol orange indicator.

Grind into powder 1 part of *xylenol orange R* with 99 parts of *potassium nitrate R*.

Test for sensitivity. To 50 mL of *water R*, add 1 mL of *dilute acetic acid R*, 50 mg of xylenol orange indicator and 0.05 mL of a solution of *lead(II) nitrate R*. Add *hexamethylenetetramine R* until the colour of the solution changes from yellow to violet-red; after adding 0.1 mL of a *0.1 M sodium edetate*, the colour changes to yellow.

Xylose. $C_5H_{10}O_5$. (M_r 150.1). [58-86-6]. D-Xylopyranose.

White or almost white crystalline powder or colorless needles.

Freely soluble in water, soluble in hot 96% ethanol.

Xylene. C_8H_{10}. (M_r 106.17). [1330-20-7].

A mixture of isomers. A clear, colorless, flammable liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.867.

n_D^{20} is about 1.497.

The boiling point is about 138 °C.

o-Xylene. C_8H_{10}. (M_r 106.17). [95-47-6]. 1,2-Dimethylbenzene.

A clear, colorless, flammable liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.881.

n_D^{20} is about 1.505.

The boiling point is about 144 °C.

The melting point is about -25 °C.

m-Xylene. C_8H_{10}. (M_r106.17). [108-38-3].

1,3-Dimethylbenzene.

A clear, colorless, flammable liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.884.

n_D^{20} is about 1.497.

The boiling point is about 139 °C.

The melting point is about 47 °C.

Corn oil.

An oily liquid obtained from the seeds of *Zea mays L.* by pressing or extraction.

Clear light yellow or yellow oil.

Practically insoluble in water and 96% ethanol, miscible with petroleum ether (40-60 °C) and methylene chloride.

d_{20}^{20} is about 0.920.

n_D^{20} is about 1.474.

Coomassie staining solution.

1.25 g/L solution of *acid blue 83 R* in a mixture of glacial *acetic acid R*/*methanol R*/*water R* (1:4: 5). Filter.

Coomassie blue. [3861-73-2]. See *Acid blue 92 R.*

Coomassie blue solution. See *Acid blue 92 solution R*

Curcumin. $C_{21}H_{20}C_6$. (M_r 368.38). [458-37-7]. 1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

Orange-brown crystal powder. Practically insoluble in water, soluble in glacial acetic acid.

The melting point is about 183 °C.

Lavandulol. $C_{10}H_{18}O$. (M_r 154.25). [498-16-8].

(*R*)-5-methyl-2-(1-methylethyl)-4-hexane-1-ol.

An oily liquid with a characteristic odour.

Lavandulol used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as specified in the monograph *Lavender oil.*

Test solution. Substance to be examined.

The content of lavandulol calculated by the internal normalisation procedure should be minimum 90.0%.

Lavandulyl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). [25905-14-0]. 2-Isopropenyl-5-methylhex-4-en-1-yl acetate.

Colourless liquid with a characteristic odour.

Lavandulyl acetate used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as specified in the monograph *Lavender oil.*

Test solution. Substance to be examined.

The content of lavandulyl acetate calculated by the internal normalisation procedure should be minimum 93.0%.

Litmus. [1393-92-6].

Schultz No. 1386.

A blue-violet pigment derived from various species of Rocella, Lecanora, or other lichens. Soluble in water, practically insoluble in 96% ethanol.

Colour change. Red to blue in the pH range 5-8.

Litmus paper, blue.

Boil 10 parts of roughly ground *litmus R* with 100 parts of *96% ethanol R* for 1 h. Decant alcohol, add a mixture of 45 volumes of *96% ethanol R* and 55 volumes of *water R* to the remainder.

After 2 days, decant the clear liquid, impregnate filter paper strips with the resulting solution, and dry.

Test for sensitivity. Immerse a strip of filter paper (10 mm x 60 mm) in a mixture of 10 mL of *0.02 M hydrochloric acid* and 90 mL of *water R*. When shaken, the paper should turn red within 45 s.

Litmus paper, red.

To the blue litmus extract, dropwise add *dilute hydrochloric acid R* until the blue colour changes to red. Strips of filter paper are impregnated with the resulting solution and dried.

Test for sensitivity. A 10 mm × 60 mm strip of filter paper is immersed in a mixture of 10 mL of *0.02 M sodium hydroxide* and 90 mL of *water R*. When shaken, the paper should turn blue within 45 s.

Lactobionic acid. $C_{12}H_{22}O_{12}$. (*Mr* 358.30). [96-82-2].

White or almost white crystalline powder. Freely soluble in water, practically insoluble in 96% ethanol.

The melting point is about 115 °C.

Lactose. $C_{12}H_{22}O_{11}·H_2O$. (*Mr*360.3). [5989-81-1]. Lactose monohydrate. O-β-b-Galactopyranosyl-(1→4)-α-d-glucopyranose.

White or almost white crystalline powder.

Freely soluble in water, practically insoluble in ethanol (96%).

Lanthanum(III) nitrate. $La(NO_3)_3·6H_2O$. (*Mr* 433.0). [10277-43-7].

Lanthanum(III) nitrate hexahydrate.

Colourless crystals, deliquescent.

Freely soluble in water.

Store in an airtight container.

Lanthanum(III) nitrate solution.

A 50 g/L solution.

Lanthanum trioxide. La_2O_3. (M_r 325.81). [1312-81-8]. Lanthanum(III) oxide.

White or almost white amorphous powder. Practically insoluble in water, soluble in dilute mineral acids, absorbs carbon dioxide from the air.

Calcium. NMT 5 ppm.

Lanthanum chloride solution.

To 58.65 g of *lanthanum trioxide R*, slowly add 100 mL of *hydrochloric acid R*, heat to boiling, allow to cool, and dilute to 1000.0 mL with *water R*.

Lauryl alcohol. $C_{12}H_{26}O$. (M_r 186.3). [112-53-8]. Dodecan-1-ol.

d220 is about 0.820.

The boiling point is from 24 °C to 27 °C.

Contains not less than 98.0% of $C_{12}H_{26}O$. Determined by gas chromatography.

Leucine. $C_6H_{13}NO_2$. (M_r 131.2). [61-90-5]. (2*S*)-2-Amino-4-methylpentanoic acid.

Contains not less than 98.5% and not greater than 101.0% of $C_6H_{13}NO_2$ calculated on a dry substance basis.

A fermentation product, extract, or protein hydrolysate.

White or almost white crystalline powder or shiny plates.

Sparingly soluble in water, practically insoluble in 96% ethanol.

Soluble in dilute mineral acids and dilute solutions of alkali metal hydroxides.

Limonene. $C_{10}H1_6$. (M_r 136.23). [5989-27-5]. D-Limonene. (+)- *p*-Menta-1,8-Dien. (*R*)-4-Iso-propenyl-1-methylcyclohex-1-ene.

A colorless liquid. Practically insoluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.84.

n_D^{20} from 1.471 to 1.474.

$[\alpha]_D^{20}$ is about + 124.

The boiling point is from 175 °C to 177 °C.

Limonene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil*, using limonene as the test solution.

The limonene content calculated by the internal normalisation procedure should be minimum 99%.

Citric acid monohydrate.

$C_6H_8O_7 \cdot H_2O$. (M_r 210.1). [5949-29-1]. 2-Hydroxypropane-1,2,3-tricarboxylic acid monohydrate.

Contains not less than 99.5% and not greater than 100.5% of $C_6H_8O_7 \cdot H_2O$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder, colorless crystals or granules. Efflorescent in air.

Very soluble in water, freely soluble in 96% ethanol.

When used in the test for iron, citric acid complies with the following additional requirement.

Dissolve 0.5 g of citric acid in 10 mL of *water R*, add 0.1 mL of *thioglycolic acid R*, stir, add *ammonia solution R* to an alkaline reaction, and dilute the resulting solution to 20 mL with *water R*. The solution should not be discoloured to pink.

Citric acid, anhydrous. $C_6H_8O_7$. (M_r 192.1). [77-92-9]. 2-Hydroxypropan-1,2,3-tricarboxylic acid.

Contains not less than 99.5% and not greater than 100.5% of $C_6H_8O_7$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder, colorless crystals or granules.

Very soluble in water, freely soluble in 96% ethanol.

The melting point is about 153 °C with decomposition.

Lemon oil.

Essential oil obtained using mechanical means without heat treatment, from the fresh peel of *Citrus limon (L.) Burman fil*.

Clear, mobile, pale yellow or greenish-yellow liquid. At low temperatures, becomes cloudy. It has a characteristic odor.

Linalyl acetate. $C_{12}H_{20}O_2$. (M_r 196,3). [115-95-7]. (*RS*)-1,5-dimethyl-1-vinylhex-4-enyl-acetate.

Colourless or slightly yellow liquid with a strong odour of bergamot and lavender.

d_{25}^{25} from 0.895 to 0.912.

n_D^{20} from 1.448 to 1.451.

The boiling point is about 215 °C.

Linalyl acetate used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Bitter-orange-flower oil*, using linalyl acetate as the test solution.

The linalyl acetate content calculated by the internal normalisation procedure should be minimum 95.0%.

Linalol. $C_{10}H_{18}O$. (M_r 154.25). [78-70-6]. (*RS*)-3,7-Dimethylocta-1,6-diene-3-ol.

Mixture of two stereoisomers (licareol and coriandrol)

Liquid. Practically insoluble in water.

d_{20}^{20} is about 0.860.

n_D^{20} is about 1.462.

The boiling point is about 200 °C.

Linalol used in gas chromatography complies with the following additional test.

Quantitation. Carry out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Anise oil*, using linalol as the test solution.

The linalol content calculated by the internal normalisation procedure should be minimum 98.0%.

Lithium. Li. (A_r 6.94). [7439-93-2].

Soft metal, silver-grey on a fresh cut, quickly becomes dull under exposure to air. Reacts violently with water to form hydrogen and a solution of lithium hydroxide; soluble in methanol to form hydrogen and a solution of lithium methoxide; practically insoluble in petroleum ether.

Store under petroleum ether or liquid paraffin.

Lithium hydroxide. $LiOH \cdot H_2O$. (M_r 41.96). [1310-66-3]. Lithium hydroxide monohydrate.

White or almost white granular powder. Strong alkali, quickly absorbs water and carbon dioxide, soluble in water, sparingly soluble in 96% ethanol.

Store in an airtight container.

Lithium carbonate. Li_2CO_3. (M_r 73.89). [554-13-2]. Dilithium carbonate.

White or almost white light powder. Sparingly soluble in water, very slightly soluble in 96% ethanol. A saturated solution at 20 °C contains about 13 g/L of Li2CO3.

Lithium metaborate, anhydrous. $LiBO_2$. (M_r 49.75). [13453-69-5]. Lithium metaborate.

Lithium sulfate. $Li_2SO_4 \cdot H_2O$. (M_r 127.96). [10102-25-7]. Dilithium sulfate monohydrate.

Colourless crystals. Freely soluble in water, practically insoluble in 96% ethanol.

Lithium chloride. LiCl. (M_r 42.39). [7447-41-8]. Lithium chloride.

Crystalline powder or granules, or cubic crystals; deliquescent. Freely soluble in water, soluble in acetone and 96% ethanol. Aqueous solutions have a neutral or slightly alkaline reaction.

Store in an airtight container.

Lugol's solution.

Dissolve 0.5 g of *iodine R* and 1 g of *potassium iodide R* in a small amount of *water R* and dilute to 100 mL with *water R*.

Magnesium. Mg. (A_r 24.31). [7439-95-4].

Silver-white tape or shavings or wire, or grey powder.

Magnesium acetate. $C_4H_6MgO_4 \cdot 4H_2O$ (M_r 214.45). [16674-78-5]. Magnesium acetate tetrahydrate.

Colourless crystals, deliquescent. Freely soluble in water and 96% ethanol.

Store in an airtight container.

Magnesium nitrate. $Mg(NO_3)_2 \cdot 6H_2O$. (M_r 256.41). [13446-18-9]. Magnesium nitrate hexahydrate.

colorless clear crystals, deliquescent. Very soluble in water, freely soluble in 96% ethanol.

Store in an airtight container.

Magnesium nitrate solution.

Under gentle heating, dissolve 17.3 g of *magnesium nitrate R* in 5 mL of *water R*, add 80 mL of *96% ethanol R*, allow to cool, and dilute to 100.0 mL with the same solvent.

Magnesium nitrate solution R1.

Under careful heating, dissolve 20 g of *magnesium nitrate R* ($Mg(NO_3)_2 \cdot 6H_2O$) in *distilled deionised water R* and dilute to 100 mL with the same solvent.

Immediately before use, dilute 10 mL of the resulting solution to 100 mL with *distilled deionised water R*. 5 µl of the solution contains 0.06 mg of $Mg(NO_3)_2$.

Magnesium oxide. MgO. (M_r 40.30). [1309-48-4].

Light magnesium oxide contains not less than 98.0% and not greater than 100.5% of MgO calculated with reference to the ignited substance.

White or almost white fine amorphous powder.

Practically insoluble in water. Soluble in dilute acids with a weak release of gas bubbles.

Magnesium oxide R1.

Must meet the requirements for *magnesium oxide R* with the following modifications.

Arsenic (*2.1.4.2, Method A*). Maximum 2 ppm.

Dissolve 0.5 g of magnesium oxide in a mixture of 5 mL of *water R* and 5 mL of *hydrochloric acid R1*.

Heavy metals (*2.1.4.8, Method A*). Maximum 10 ppm. Dissolve 1.0 g of magnesium oxide in a mixture of 3 mL of *water R* and 7 mL of *hydrochloric acid R1*, add 0.05 mL of *phenolphthalein solution R*, and *concentrated ammonia R* until pink colour develops. Neutralise the excess ammonia with *glacial acetic acid R*, add 0.5 mL of excess acid, dilute to 20 mL with *water R*, and filter if necessary. 12 mL of the solution must pass the test on heavy metals. Prepare the reference solution using 5 mL of *lead standard solution (1* ppm Pb^{2+}*) R* and 5 mL of *water R*.

Iron (*2.1.4.9*). Maximum 50 ppm. Dissolve 0.2 g of magnesium oxide in 6 mL of *diluted hydrochloric acid R* and dilute to 10 mL with *water R*.

Magnesium oxide, heavy. MgO. (M_r 40.30). [1309-48-4].

Contains not less than 98.0% and not greater than 100.5% of MgO calculated with reference to the ignited substance.

White or almost white fine powder.

Practically insoluble in water. Soluble in dilute acids with a weak release of gas bubbles.

Magnesium sulfate. $MgSO_4 \cdot 7H_2O$. (M_r 246.5). [10034-99-8]. Copper(II) sulfate heptahydrate.

Contains not less than 99.0% and not greater than 100.5% of $MgSO_4 \cdot 7H_2O$ calculated on a dry substance basis.

White or almost white crystalline powder or shiny colorless crystals.

Freely soluble in water, very soluble in boiling water, practically insoluble in 96% ethanol.

Magnesium chloride. $MgCI_2 \cdot 6H_2O$. (M_r 203.3). [7791-18-6]. Magnesium chloride hexahydrate.

Contains no less than 98.0% and no greater than 101.0% of MgCl2·6H2O

Colourless crystals. Hygroscopic.

Very soluble in water, freely soluble in 96% ethanol.

Macrogol 200. [25322-68-3]. Polyethylene glycol 200.

Clear colorless or almost colorless viscous liquid. Freely soluble in acetone and anhydrous ethanol, practically insoluble in fatty oils.

d_{20}^{20} is about 1.127.

n_D^{20} is about 1.450.

Macrogol 200 R1.

Introduce 500 mL of *macrogol 200 R* into a 1000 mL round bottom flask, using a rotation evaporator remove any volatile components applying for 6 h a temperature of 60 °C and a vacuum with a pressure of 1.5-2.5 kPa.

Macrogol 300. [25322-68-3]. Polyethylene glycol 300.

Clear, viscous, colorless or almost colorless hygroscopic liquid.

Miscible with water, very soluble in acetone, 96% ethanol and methylene chloride, practically insoluble in fatty and mineral oils.

Macrogol 400. [25322-68-3]. Polyethylene glycol 400.

Clear, viscous, colorless or almost colorless hygroscopic liquid.

Miscible with water, very soluble in acetone, 96% ethanol and methylene chloride, practically insoluble in fatty and mineral oils.

Macrogol 600. [25322-68-3]. Polyethylene glycol 600.

Clear, viscous, colorless or almost colorless hygroscopic liquid.

Miscible with water, very soluble in acetone, 96% ethanol and methylene chloride, practically insoluble in fatty and mineral oils.

Macrogol 1000. [25322-68-3]. Polyethylene glycol 1000.

White or off-white, hygroscopic, waxy, or paraffin-like mass.

Very soluble in water, freely soluble in 96% ethanol and methylene chloride, practically insoluble in fatty and mineral oils.

Macrogol 1500. [25322-68-3]. Polyethylene glycol 1500.

White or almost white waxy or paraffin-like mass.

Very soluble in water and methylene chloride, freely soluble in 96% ethanol, practically insoluble in fatty and mineral oils.

Macrogol 20 000. Polyethylene glycol 20 000.

White or almost white waxy or paraffin-like mass.

Very soluble in water, soluble in methylene chloride, practically insoluble in 96% ethanol, fatty and mineral oils.

Macrogol 20 000 2-nitroterephthalate.

Polyethylene glycol 20 000 2-nitroterephthalate.

Macrogol 20 000 R modified by treatment with 2-nitroterephthalic acid. White or almost white solid waxy mass. Soluble in acetone.

Malachite green. $C_{23}H_{25}ClN_2$. (M_r 364.91). [123333-61-9]. [4-[[4-(dimethylamino) phenyl)phenyl-methylene]-cyclohexa-2,5-diene-1-ylidene]dimethylammonium chloride.

Schultz No. 754.

Colour index (C.I.) No. 42000.

Green crystals with a metallic lustre. Very soluble in water to form a bluish-green solution, soluble in 96% ethanol and methanol.

A 0.01 g/L solution in *96% ethanol R* has an absorption maximum (*2.1.2.24*) at a wavelength of 617 nm.

Malachite green solution.

A 5 g/L solution in anhydrous acetic acid R.

Maleic acid. $C_4H_4O_4$. (M_r 116.1). [110-16-7]. (2)-Butenedionic acid.

Contains not less than 99.0% and not greater than 101.0% of $C_4H_4O_4$ calculated with reference to the anhydrous substance.

White crystalline powder.

Freely soluble in water and 96% ethanol.

Maleic anhydride. $C_4H_2O_3$. (M_r98.06). [108-31-6]. Butendionic anhydride. 2,5-Furandione.

White or almost white crystals. Soluble in water to form maleic acid, very soluble in acetone and ethyl acetate, freely soluble in toluene, soluble in 96% ethanol to form an ester, very slightly soluble in petroleum ether.

The melting point is about 52 °C.

Any residue that is insoluble in toluene should not exceed 5% (maleic acid).

Maleic anhydride solution.

Dissolve 5 g of *maleic anhydride R* in *toluene R* and dilute to 100 mL with the same solvent.

Use within 1 month. In case of turbidity, filter the solution.

Mannitol. $C_6H_{14}O_6$. (M_r 182.2). [69-65-8]. D-mannitol.

Contains not less than 97.0% and not greater than 102.0% of $C_6H_{14}O_6$ calculated with reference to the anhydrous substance.

White or almost white crystals or powder.

Freely soluble in water, practically insoluble in 96% ethanol.

Shows polymorphism.

Mannose. $C_6H_{12}O_6$. (M_r 180.16). [3458-28-4]. D-(+)-Mannose.

White or almost white crystalline or fine-grained powder. Very soluble in water, slightly soluble in anhydrous ethanol.

$[\alpha]_{20}^{20}$ from + 13.7 to + 14.7. Determination is carried out using a 200 g/L solution in *water R* containing about 0.05% of NH_3.

The melting point is about 132 °C with decomposition.

Manganese sulfate. $MnSO_4 \cdot H_2O$. (M_r 169.02). [10034-96-5]. Manganese(II) sulfate monohydrate.

Pale pink crystalline powder or crystals. Freely soluble in water, practically insoluble in 96% ethanol.

Loss on ignition. From 10.0% to 12.0%. Determination is carried out from 1.000 g at 500 ± 50 °C.

Butyric acid. $C_4H_8O_2$. (M_r 88.11). [107-92-6]. Butanoic acid.

Contains not less than 99.0% of $C_4H_8O_2$. Oily liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.96.

n_D^{20} is about 1.398.

The boiling point is about 163 °C.

Cupric acetate. $C_4H_{6Cu}O_4 \cdot H_2O$. (M_r 199.65). [6046-93-1]. Copper(II) acetate monohydrate.

Bluish green crystals or powder. Freely soluble in boiling water, soluble in water and 96% ethanol, slightly soluble in 85% glycerol.

Copper(II) nitrate. $Cu(NO_3)_2 \cdot 3H_2O$. (M_r 241.62). [10031-43-3]. Cupric nitrate trihydrate.

Blue crystals. Hygroscopic, very soluble in water, the aqueous solution has a strong acid reaction, freely soluble in 96% ethanol and dilute nitric acid.

Store in an airtight container.

Copper(II) sulfate pentahydrate. $CuSO_4 \cdot 5H_2O$. (M_r 249.67). [7758-99-8]. Copper(II) sulfate pentahydrate.

Blue powder or crystals. Slowly efflorescent in air, very soluble in water, slightly soluble in 96% ethanol.

Copper sulfate solution.

A 125 g/L solution.

Copper tetrammine, ammoniacal solution.

Dissolve 34.5 g of *copper sulfate R* in 100 mL of *water R*, add dropwise *concentrated ammonia solution R* to dissolve the resulting precipitate.

Maintaining the temperature below 20 °C, with continuous shaking, add 30 mL of *strong sodium hydroxide solution R* dropwise. Filter through a glass filter (*2.1.1.2*), wash with *water R* until a clear filtrate is obtained. Shake with 200 mL of *concentrated ammonia solution R* and filter through a glass filter (*2.1.1.2*), then filter again to minimise the precipitate.

Cupric chloride. $CuCl_2 \cdot 2H_2O$. (M_r 170.48). [10125-13-0]. Cupric chloride dihydrate.

Greenish blue powder or crystals, deliquescent, and efflorescent in dry air. Freely soluble in water, 96% ethanol and methanol, sparingly soluble in acetone.

Store in an airtight container.

Copper edetate solution.

To 2 mL of a 20 g/L solution of *copper acetate R*, add 2 mL of *0.1 M sodium edetate* and dilute to 50 mL with *water R*.

Cupric-tartaric solution.

Solution A. Dissolve 34.6 g of *copper sulfate R* in *water R*, dilute to 500 mL with the same solvent.

Solution B. Dissolve 173 g of *potassium-sodium tartrate R* and 50 g of *sodium hydroxide R* in 400 mL of *water R*. Heat to boiling, cool, dilute the resulting solution to 500 mL with *carbon dioxide-free water R*.

Mix equal volumes of solutions A and B immediately before use.

Copper tartrate solution R2.

Mix 1 mL of a solution containing 5 g/L of *copper sulfate R* and 10 g/L of *potassium tartrate R* with 50 mL of *sodium carbonate solution R1*.

Prepare immediately before use.

Copper-tartrate solution R3.

Mix equal volumes of a 10 g/L solution of *copper sulfate pentahydrate R* and a 20 g/L solution of *sodium tartrate R*.

To 1.0 mL of the resulting solution, add 50 mL of *sodium carbonate solution R2*. Prepare immediately before use.

Copper tartrate solution R4.

Solution A. A 150 g/L solution of *copper sulfate R*.

Solution B. Dissolve 2.5 g of anhydrous sodium carbonate R, *2.5 g of* potassium-sodium tartrate R, *2.0 g of* sodium bicarbonate R, *and 20.0 g of* anhydrous sodium sulfate R *in* water R, dilute the resulting solution to 100 mL with the same solvent.

Mix solutions A and B in a ratio of 1: 25 Immediately before use.

Cupric-citrate solution.

Dissolve 25 g of *copper sulfate R*, 50 g of *citric acid R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

Copper citrate solution R1.

Dissolve 25 g of *copper sulfate R*, 50 g of *citric acid R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent (test solution).

Adjust the solution to meet the following requirements:

a) To 25.0 mL of the test solution, add 3 g of *potassium iodide R*, then carefully add 25 mL of 25% (*m*/*m*) solution of *sulfuric acid R* in small portions, and titrate with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R* as indicator, which is added at the end of the titration.

24.5 mL to 25.5 mL of *0.1 M sodium thiosulfate* should be used for titration.

b) Dilute 10.0 mL of the test solution to 100.0 mL with *water R* and stir. To 10.0 mL of the resulting solution, add 25.0 mL of *0.1 M hydrochloric acid*, heat in a water bath for 1 h, cool, dilute to the initial volume with *water R*, and titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

5.7 mL to 6.3 mL of *0.1 M sodium hydroxide* should be used for titration.

c) Dilute 10.0 mL of the test solution to 100.0 mL with *water R* and stir. Titrate 10.0 mL of the resulting solution with *0.1 M hydrochloric acid*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

6.0 mL to 7.5 mL of *0.1 M hydrochloric acid* should be used for titration.

Copper. Cu. (A_r 63.55). [7440-50-8].

Cleaned foil, shavings, wire, or metal powder of electrolytic purity.

Mesityloxide. $C_6H_{10}O$. (M_r 98.14). [141-79-7]. 4-Methylpent-3-en-2-one.

A colorless oily liquid. Soluble in 30 parts of water, miscible with most organic solvents.

d_{20}^{20} is about 0.858.

The boiling point is from 129 °C to 130 °C.

Meclosin hydrochloride. $C_{25}H_{27}Cl_2N_2 \cdot 2HCl$. ($M_r$ 463.9). [1104-22-9]. 1-[(RS)-(4-Chlorophenyl) phenylmethyl]-4-[(3-methylphenyl)methyl] piperazine dihydrochloride.

Contains not less than 98.0% and not greater than 102.0% of $C_{25}H_{27}C1_2N_2 \cdot 2HCl$ calculated with reference to the anhydrous substance.

White or yellowish white crystalline powder, slightly hygroscopic.

Slightly soluble in water, soluble in 96% ethanol in methylene chloride.

Melamine. $C_3H_6N_6$. (M_r 126.14). [108-78-1].

1,3,5-Triazine-2,4,6-triamine.

Amorphous white or almost white powder. Very slightly soluble in water and 96% ethanol.

Menadione. $C_{11}H_8O_2$. (M_r 172.2). [58-27-5]. 2-Methylnaphthalene-1,4-dion.

Menadione contains not less than 98.5% and not greater than 101.0% of $C_{11}H_8O_2$ calculated on a dry substance basis.

Pale yellow crystalline powder.

Practically insoluble in water, freely soluble in toluene, sparingly soluble in alcohol and methanol. Photosensitive.

Menthyl acetate. $C_{12}H_{22}O_2$. (M_r 198.3). [2623-23-6]. 2-Isopropyl-5-methylcyclohexyl-acetate.

A colorless liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.92.

n_D^{20} is about 1.447.

The boiling point is about 228 °C.

Menthyl acetate used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Peppermint oil*, using menthyl acetate as the test solution.

The menthyl acetate content calculated by the internal normalisation procedure should be minimum 97.0%.

Menthol. $C_{10}H_{20}O$. (M_r 156.3). [2216-51-5].

Levomenthol is a (1*R*,2*S*,5*R*)-5-methyl-2-(1-methylethyl)cyclohexanol.

Prismatic or needle-like, colourless, shiny crystals.

Practically insoluble in water, very soluble in 96% ethanol and petroleum ether, freely soluble in fatty oils and vaseline oil, very slightly soluble in glycerol.

The melting point is about 43 °C.

Racemic menthol is a mixture of equal parts (1*RS*,2*SR*,5*RS*)-5-methyl-2-(1-methylethyl)cyclohexanol.

Free-flowing crystalline powder or in the form of agglomerates or prismatic or needle-like colorless shiny crystals.

Practically insoluble in water, very soluble in 96% ethanol and petroleum ether, freely soluble in fatty oils and liquid paraffin, very slightly soluble in glycerol.

The melting point is about 34 °C.

Menthol used in gas chromatography complies with the following additional test.

Quantitation. Conduct by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Menthol racemic* in the *Related substances* test.

The menthol content calculated by the internal normalisation procedure should be minimum 98.0%.

Menthone. $C_{10}H_{18}O$. (M_r 154.25). [14073-97-3]. (2*S*,5*R*)-2-Isopropyl-5-methyl-cyclohexanone. (-)-*trans*-p-mentan-3-one. Contains various amounts of isomenthone.

A colorless liquid. Very slightly soluble in water, very soluble in 96% ethanol.

d_{20}^{20} is about 0.897.

n_D^{20} is about 1.450.

Menthone used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Peppermint oil*, using menthone as the test solution.

The menthone content calculated by the internal normalisation procedure must be minimum 90.0%.

Menthofuran. $C_{10}H_{14}O$. (M_r 150.22). [17957-94-7]. 3,9-Epoxy-*p*-menta-3,8-diene. 3,6-dimethyl-4,5,6,7-tetrahydrobenzofuran.

Slightly bluish liquid. Very slightly soluble in water, soluble in 96% ethanol.

d_{15}^{20} is about 0.965.

n_D^{20} is about 1.480.

$[\alpha]_D^{20}$ is about + 93.

The boiling point is 196 °C.

Menthofuran used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Peppermint oil*, using menthofuran as the test solution.

The menthofuran content calculated by internal normalisation procedure should be minimum 97.0%.

Mercaptopurine. $C_5H_4N_4S{\cdot}H_2O$. (M_r 170.2). [6112-76-1]. 7*H*-Purine-6-thiol monohydrate.

Contains not less than 98.5% and not greater than 101.0% of $C_5H_4N_4S{\cdot}H_2O$ calculated with reference to the anhydrous substance.

Yellow crystalline powder.

Practically insoluble in water, slightly soluble in 96% ethanol.

Soluble in solutions of alkali metal hydroxides.

2-Mercaptoethanol. C_2H_6OS. (M_r 78.13). [60-24-2].

Liquid miscible with water.

d_{20}^{20} is about 1.116.

The boiling point is about 157 °C.

Methacrylic acid. $C_4H_6O_2$. (M_r 86.09). [79-41-4]. 2-Methylprop-2-enoic acid.

A colorless liquid.

n_D^{20} is about 1.431.

The boiling point is about 160 °C.

The melting point is about 16 °C.

Metanil yellow. $C_{18}H_{14}N_3NaO_3S$. (M_r 375.38). [587-98-4]. Sodium 3-[4-(phenylamino) phenylazo]benzenesulfonate.

Schultz No. 169.

Colour index (C.I.) No. 13065.

Brownish yellow powder. Soluble in water and 96% ethanol.

Metanil yellow solution.

A 1 g/L solution in *methanol R.*

Test for sensitivity.

To 50 mL of *anhydrous acetic acid R*, add 0.1 mL of a solution of metanil yellow; a pinkish-red colour appears, which should turn violet when 0.05 mL of *0.1 M perchloric acid* is added.

Colour change. Red to orange-yellow in the pH range of 1.2-2.3.

Methanol. CH_4O. (M_r 32.04). [67-56-1].

A clear, colorless, flammable liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is from 0.791 to 0.793.

The boiling point is from 64 °C to 65 °C.

Methanol R1.

Complies with the requirements for *methanol R* and the following additional test.

The *absorbance* (*2.1.2.24*) is determined using *water R* as the compensation liquid:

maximum 0.70 at a wavelength of 210 nm, maximum 0.30 at a wavelength of 220 nm, maximum 0.13 at a wavelength of 230 nm, maximum 0.02 at a wavelength of 250 nm, maximum 0.01 at a wavelength of 260 nm or greater.

Methanol R2.

Methanol R2 used in liquid chromatography complies with and the following additional requirement.

Contains not less than 99.8% of CH_4O.

Absorbance (*2.1.2.24*). NMT 0.17. Measure at a wavelength of 225 nm, using *water R* as compensation liquid.

Acidified methanol.

Dilute 1.0 mL of *hydrochloric acid R1* to 100.0 mL with *methanol R*.

Methanol, anhydrous. [67-56-1].

Treat 1000 mL of *methanol R* with 5 g of *magnesium R*. If necessary, initiate the reaction by adding 0.1 mL of a solution of *mercuric chloride R*. After stopping the gas release, distill the liquid, collect the distillate in a dry container protected from moisture.

Water (*2.1.5.12*). NMT 0.3 g/l.

Methanol, aldehyde-free.

Dissolve 25 g of *iodine R* in 1 L of *methanol R*, add the resulting solution with constant stirring to 400 mL of *1 M sodium hydroxide*, then add 150 mL of *water R*, and allow to stand for 16 h. Filter and boil under reflux until the odour of iodoform disappears. Distill the solution by fractional distillation.

Contains no greater than 10^{-3}% of aldehydes and ketones.

Methanesulfonic acid. CH_4O_3S. (M_r 96.1). [75-75-2].

Clear colorless liquid that solidifies at a temperature of about 20 °C.

Miscible with water, slightly soluble in toluene, practically insoluble in hexane.

d_{20}^{20} is about 1.48.

n_D^{20} is about 1.430.

Metaphosphoric acid. $(HPO_3)_x$. [37267-86-0].

Glassy lumps or sticks containing a certain amount of sodium metaphosphate. Hygroscopic, very soluble in water.

Nitrates. Boil 1.0 g with 10 mL of *water R*, cool, add 1 mL of a solution of *indigocarmine R*, 10 mL of *nitrogen-free sulfuric acid R*, and heat to boiling. The blue colour should not completely disappear.

Reducing substances. NMT 0.01% equivalent to H_3PO_3.

Dissolve 35,0 g in 50 mL of *water R*, add 5 mL of a 200 g/L solution of *sulfuric acid R*, 50 mg of *potassium bromide R*, and 5.0 mL of *0.02 M potassium bromate*, and heat on a water bath for 30 min; cool, add 0.5 g of *potassium iodide R* and titrate the liberated iodine with *0,1 M sodium thiosulfate*, using as indicator 1 mL of *starch solution R*. Conduct a blank titration.

1 mL of *0.02 M potassium bromate solution* is equivalent to 4.10 mg of H_3PO_3.

Store in an airtight container.

4-Methylaminophenol sulfate.

$C_{14}H_{20}N_2O_6S$. (M_r 344.38). [55-55-0]. Methol.

Colourless crystals. Very soluble in water, slightly soluble in 96% ethanol.

The melting point is about 260 °C.

Methyl anthranilate. $C_8H_9NO_2$. (M_r 151.16). [134-20-3]. Methyl-2-aminobenzoate.

Colourless crystals or colorless to yellowish liquid. Soluble in water, freely soluble in 96% ethanol.

The melting point is from 24 °C to 25 °C.

Methyl anthranilate used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Bitter-orange-flower oil*, using methyl anthranilate as the test solution.

The methyl anthranilate content calculated by the internal normalisation procedure should be minimum 95.0%.

Methyl arachidate. $C_{21}H_{42}O_2$. (M_r 326.56). [1120-28-1]. Methyl eicosanoate.

Contains not less than 98.0% of $C_{21}H_{42}O_2$.

Determination is performed by gas chromatography (*2.4.22)*.

White to yellow crystalline mass. Soluble in 96% ethanol and petroleum ether.

The melting point is about 46 °C.

Methyl acetate. $C_3H_6O_2$. (M_r 74.08). [79-20-9].

A clear, colorless liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.933.

n_D^{20} is about 1.361.

The boiling point is from 56 °C to 58 °C.

Methyl behenate $C_{23}H_{46}O_2$. (M_r 354.61). [929-77-1]. Methyl docosanoate.

The melting point is from 54 °C to 55 °C.

Methylbenzothiazolone hydrazone hydrochloride. C8H10C1N3S-H2O. (M_r233.72).

[38894-11-0]. 3-Methylbenzothiazole-2(3*H*)-one hydrazone hydrochloride monohydrate.

Almost white or yellowish crystalline powder.

The melting point is about 270 °C.

Suitability test for the determination of aldehydes. To 2 mL of *aldehyde-free methanol R*, add 60 mL of a 1 g/L solution of *propane aldehyde R* in *aldehyde-free methanol R* and 5 mL of a 4 g/L solution of methylbenzothiazolone hydrazone hydrochloride, mix, and allow to stand for 30 min. Prepare a blank solution that does not contain propane aldehyde. To the test and blank solutions, add 25.0 mL of a 2 g/L solution of *ferric chloride R*, dilute each solution to 100.0 mL with *acetone R*, and stir. The absorbance (*2.1.2.24*) of the test solution measured at a wavelength of 660 nm using the blank solution as compensation liquid is not less than 0.62.

2-Methylbutane. C_5H_{12}. (M_r 72.15). [78-78-4]. Isopentane.

Contains less than 99.5% of C_5H_{12}.

Colourless, highly flammable liquid.

d_{20}^{20} is about 0.621.

n_D^{20} is about 1.354.

The boiling point is about 29 °C.

Water (*2.1.5.12*). NMT 0.02%.

Residue on evaporation. NMT 3· 10^-4%.

Determine the *absorbance* (*2.1.2.24*) using *water R* as compensation liquid: a maximum 0.30 at 210 nm, a maximum 0.07 at 220 nm, and a maximum 0.01 at 240 nm or greater.

2-Methylbut-2-ene. C_5H_{10}. (M_r 70.13). [513-35-9].

Highly flammable liquid. Practically insoluble in water, miscible with 96% ethanol.

The boiling point is from 37.5 °C to 38.5 °C.

Methyldecanoate. $C11H_{22}O_2$. (M_r 186.29). [110-42-9].

Contains not less than 99.0% of $C_{11}H_{22}O_2$.

Clear, colorless or yellow liquid. Soluble in petroleum ether.

d_{20}^{20} is from 0.871 to 0.876.

n_D^{20} from 1.425 to 1.426.

Foreign matter. Determination is carried out by gas chromatography (*2.1.2.27*), testing equal volumes of each of the following solutions of substances:

A 0.02 g/L solution of *methyl decanoate in carbon disulfide R* (solution A), a 2 g/L solution of methyl decanoate in *carbon disulfide R* (solution B), *carbon disulfide R* (solution C).

Perform the chromatography under test conditions for butylhydroxytoluene as prescribed in the monograph *Wool fat*.

On the chromatogram of solution (B), the sum of the areas of all peaks except the main peak and the solvent peak must be less than the area of the main peak on the chromatogram of the solution (A).

3-O-Methyldopamine hydrochloride. $C_9H_{14}ClNO_2$. (M_r 203.67). [1477-68-5]. 4-(2-Aminoethyl)-2-methoxy-phenol hydrochloride.

The melting point is from 213 °C to 215 °C.

4-O-Methyldopamine hydrochloride. $C_9H_{14}ClNO_2$. (M_r 203.67). [645-33-0]. 5-(2-Amino-ethyl)-2-methoxy-phenol hydrochloride.

The melting point is from 207 °C to 208 °C.

Methylene bisacrylamide. $C_7H_{10}K_2O_2$. (M_r 154.17). [110-26-9]. *N,N'*-Methylenebisacrylamide.

Very fine white or almost white powder. Slightly soluble in water, soluble in 96% ethanol.

The melting point is 300 °C with decomposition.

Methylene blue. $C_{16}H_{18}C1N_3S{\cdot}H_2O$. ($M_r$ 319.90, anhydrous). [122965-43-9]. 3,7-Dimethylaminophenothiazine-5 chloride.

Schultz No. 1038.

Colour index (C.I.) No. 52015.

Exists in various hydrated forms and can contain up to 22% of water.

Dark green or bronze crystalline powder. Freely soluble in water, soluble in 96% ethanol.

Methylene chloride. CH_2Cl_2. (M_r 84.93). [75-09-2]. Dichloromethane.

A colorless liquid. Sparingly soluble in water, miscible with 96% ethanol. The boiling point is from 39 °C to 42 °C.

Methylene chloride used in fluorimetry complies with the following additional test.

Fluorescence. When irradiated with light at 365 nm, the absorption (*2.1.2.20*) measured at a wavelength of 460 nm in a cell with a layer thickness of 1 cm should not be more intense than the absorption of a solution containing $2\cdot 10^{-3}$ ppm of *quinine R* in a *0.5 M sulfuric acid* measured under the same conditions.

Acidified methylene chloride.

To 100 mL of *methylene chloride R* add 10 mL of *hydrochloric acid R*, shake. After separating the layers, use the bottom layer.

Methyl isobutyl ketone. $C_6H_{12}O$. (M_r 100.16). [108-10-1]. 4-Methyl-2-pentanone.

A clear, colorless liquid. Slightly soluble in water, miscible with most organic solvents.

d_{20}^{20} is about 0.80.

The boiling point is about 115 °C.

Distillation range (*2.1.2.11*). Distill 100 mL. The distillation temperature range should not exceed 4.0 °C; it should be distilled from 1 mL to 95 mL.

Residue on evaporation. NMT 0.01%. evaporate in a water bath, dry the residue at 100-105 °C.

Methyl isobutyl ketone R1.

Shake 50 mL of freshly distilled *methyl butyl ketone R* with 0.5 mL of *hydrochloric acid R1* for 1 min. After separating the layers, discard the lower layer. Prepare immediately before use.

Methyl caprate.

See *Methyl decanoate R*.

Methyl caprylate. $C_9H_{18}O_2$. (M_r 158.24). [111-11-5]. Methyl octanoate.

d_{20}^{20} is about 0.876.

n_D^{20} is about 1.417.

The boiling point is from 193 °C to 194 °C.

Methyl caproate. $C_7H_{14}O_2$. (M_r 130.18). [106-70-7]. Methyl hexanoate.

d_{20}^{20} is about 0.885.

n_D^{20} is about 1.405.

The boiling point is from 150 °C to 151 °C.

Methyl laurate. $C_{13}H_{26}O_2$. (M_r 214.34). [111-82-0]. Methyl dodecanoate.

Contains less than 98.0% of $C_{13}H_{26}O_2$. The determination is carried out by gas chromatography (*2.1.2.27*).

Colourless or yellow liquid. Soluble in 96% ethanol and petroleum ether.

d_{20}^{20} is about 0.87.

n_D^{20} is about 1.431.

The melting point is about 5 °C.

Methyl lignocerate. $C_{25}H_{50}O_2$. (M_r 382.66). [2442-49-1]. Methyl tetracosanoate.

Flakes.

The melting point is about 58 °C.

Methyl linoleate. $C_{19}H_{34}O_2$. (M_r 294.47). [112-63-0]. Methyl-(9Z,12Z)-octadeca-9,12-dienoate.

d_{20}^{20} is about 0.888.

n_D^{20} is about 1.466.

The boiling point is from 207 °C to 208 °C.

Methyl linolenate. $C_{19}H_{32}O_2$. (M_r 292.46). [301-00-8]. Methyl-(9Z,12Z,15Z)-octadeca-9,12,15-trienoate. Methyl α-linolenate.

d_{20}^{20} is about 0.901.

n_D^{20} is about 1.471.

The boiling point is about 207 °C.

Methyl margarate. $C_{18}H_{36}O_2$. (M_r 284.48). [1731-92-6]. Methyl heptadecanoate.

White or almost white powder.

The melting point is from 32 °C to 34 °C.

Methyl margarate used in the quantitation of the number of fatty acids in the monograph "Saw Palmetto fruit" complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Saw Palmetto fruit.*

The methyl margarate content calculated using the normalisation procedure should be minimum 97%.

Methyl methacrylate. $C_5H_8O_2$. (M_r 100.12). [80-62-6]. Methyl-2-methylprop-2-enoate.

A colorless liquid.

n_D^{20} is about 1.414.

The boiling point is about 100 °C.

The melting point is about -48 °C.

Contains a suitable stabiliser.

Methyl myristate. $C_{15}H_{30}O_2$. (M_r 242.40). [124-10-7]. Methyl tetradecanoate.

Contains not less than 98.0% of $C_{15}H_{30}O_2$. The determination is carried out by gas chromatography (*2.1.2.27*).

A colorless or light yellow liquid. Soluble in 96% alcohol and petroleum ether.

d_{20}^{20} is about 0.87.

n_D^{20} is about 1.437.

The melting point is about 20 °C.

Methyl green. $C_{26}H_{33}C1_2N_3$. (M_r 458.5). [7114-03-6].

4-[[4-(Dimethylamino)phenyl][4-(dimethyliminio)cyclohexa-2,5-dienylidene]-methylphenyl]trimethylammmonium dichloride.

Schultz No. 788.

Colour Index (C. I.) No. 42585.

Green powder. Soluble in water, sulfuric acid to form a yellow discolouration that turns green when diluted with water.

Methyl green-iodomercurate paper.

Immerse thin strips of suitable filter paper in a 40 g/L solution of *methyl green R*, dry in air, then immerse for 1 h in a solution containing 140 g/L of *potassium iodide R* and 200 g/L of *mercuric iodide R*. Wash the strips with *distilled water R* until the washing water becomes almost colourless, and dry in air.

Store in a dark place. Use within 48 h.

Methyl red. $C_{15}H_{15}N_3O_2$. (M_r 269.30). [493-52-7].

2-(4-Dimethylaminophenylazo)benzoic acid.

Schultz No. 250.

Colour index (C.I.) No. 13020.

Dark red powder or violet crystals.

Practically insoluble in water, soluble in 96% ethanol.

Methyl red mixed solution.

Dissolve 0.1 g of *methyl red R* and 50 mg of *methylene blue R* in 100 mL of

96% ethanol R.

Colour change. Red-violet to green in the pH range of 5.2-5.6.

Methyl red solution.

Dissolve 50 mg of *methyl red R* in a mixture of 1.86 mL of *0.1 M sodium hydroxide* and 50 mL of *96% ethanol R*, dilute to 100 mL with *water R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.1 mL of a solution of methyl red and 0.05 mL of *0.02 M hydrochloric acid;* red colour develops, which should turn yellow when no greater than 0.1 mL of *0.02 M hydrochloric acid* is added.

Colour change. Red to yellow in the pH range of 4.4-6.0.

Methyl orange. $C_{14}H_{14}N_{3N}aO_3S$. (M_r 327.33). [547-58-0]. Sodium 4'-(dimethylamino)azobenzene-4-sulfonate.

Schultz No. 176.

Colour index (C.I.) No. 13025.

Orange-yellow crystalline powder. Slightly soluble in water, practically insoluble in 96% ethanol.

Methyl orange mixed solution.

Dissolve 20 mg of *methyl orange R* and 0.1 g of *bromocresol green R* in 1 mL of *0.2 M sodium hydroxide* and dilute to 100 mL with *water R*.

Colour change. From orange to yellowish green in the pH range 3.0 - 4.4.

Methyl orange solution.

Dissolve 0.1 g of *methyl orange R* in 80 mL of *water R* and dilute to 100 mL with *96% ethanol R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.1 mL of a methyl orange solution; yellow colouration develops, which should turn red when no greater than 0.1 mL of *0.1 M hydrochloric acid* is added.

Colour change. Red to yellow in the pH range of 3.0-4.4.

Methyl oleate. $C_{19}H_{36}O_2$. (*Mr* 296.49). [112-62-9]. Methyl-(9Z)-octadec-9-enate.

Contains not less than 98.0% of $C_{19}H_{36}O_2$. The determination is carried out by gas chromatography (*2.1.2.27*).

Colourless or slightly yellow liquid. Soluble in 96% ethanol and petroleum ether.

d_{20}^{20} is about 0.88.

n_D^{20} is about 1.452.

Methyl palmitate. $C_{17}H_{34}O_2$. (M_r 270.45). [112-39-0]. Methyl hexadecanoate.

Contains not less than 98.0% of $C_{17}H_{34}O_2$. The determination is carried out by gas chromatography (*2.1.2.27*).

White or yellow crystalline mass. Soluble in 96% ethanol and petroleum ether.

The melting point is about 30 °C.

Methyl palmitoleate. $C_{17}H_{32}O_2$. (M_r 268.43). [1120-25-8]. Methyl-(9Z)-hexadec-9-enoate.

d_{20}^{20} is about 0.876.

n_D^{20} is about 1.451.

Methyl parahydroxybenzoate. $C_8H_8O_3$. (M_r 152.1). [99-76-3]. Methyl-4-hydroxybenzoate.

Contains no less than 98.0% and no greater than 102.0% of $C_8H_8O_3$.

White or almost white crystalline powder or colorless crystals.

Very slightly soluble in water, freely soluble in 96% ethanol and methanol.

4-Methylpentan-2-ol. $C_6H_{14}O$. (M_r 102.17). [108-11-2].

A clear, colorless, volatile liquid.

d_4^{20} is about 0.802.

n_D^{20} is about 1.411.

The boiling point is about 132 °C.

Methylpiperazine. $C_5H_{12}N_2$. (M_r 100.16). [109-01-3]. 1-Methylpiperazine.

A colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.90.

n_D^{20} is about 1.466.

The boiling point is about 138 °C.

4-(4-Methylpiperidane-1-yl)pyridine. $C_{11}H_{16}N_2$. (M_r 176.26). [80965-30-6].

Clear liquid.

n_D^{20} is about 1.565.

2-Methylpropanol. $C_4H_{10}O$. (M_r 74.12). [78-83-1]. Isobutyl alcohol. 2-Methylpro-pan-1-ol.

A clear, colorless liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.80.

n_D^{20} from 1.397 to 1.399.

The boiling point is about 107 °C.

Distillation range (*2.1.2.11*). From 107 °C to 109 °C; not less than 95% must be distilled.

2-Methyl-2-propanol. $C_4H_{10}O$. (M_r 74.12). [75-65-0]. 1,1-Dimethylethyl alcohol. *tert*-Butyl alcohol.

Clear colorless liquid or crystalline mass. Soluble in water, miscible with 96% ethanol.

Freezing point (*2.1.2.17*). About 25 °C.

Distillation range (*2.1.2.11*). From 81 °C to 83 °C; not less than 95% must be distilled.

Methyl stearate. $C_{19}H_{38}O_2$. (M_r 298.5). [112-61-8]. Methyl octadecanoate.

Contains not less than 98.0% of $C_{19}H_{38}O_2$.

Determination is performed by gas chromatography (*2.4.22)*.

White or yellow crystalline mass. Soluble in 96% ethanol and petroleum ether.

The melting point is about 38 °C.

Methyl tridecanoate. $C_{14}H_{28}O_2$. (M_r 228.37). [1731-88-0].

A colorless or slightly yellowish liquid. Soluble in 96% ethanol and petroleum ether.

d_{20}^{20} is about 0.86.

n_D^{20} is about 1.441.

The melting point is about 6 °C.

Methyl tricosanoate. $C_{24}H_{48}O_2$. (M_r 368.64). [2433-97-8]. Tricosanoic acid methyl ether.

Contains not less than 99.0% of $C_{24}H_{48}O_2$.

White or almost white crystals. Practically insoluble in water, soluble in hexane.

The melting point is from 55 °C to 56 °C.

Methylphenyloxazolylbenzene. $C_{26}H_{20}N_2O_2$. (M_r 392.45). [3073-87-8]. 1,4-Bis[2-(4-methyl-5-phenyl)oxazolyl]-benzene.

Greenish yellow fine powder with blue fluorescence or small crystals. Soluble in 96% ethanol, sparingly soluble in xylene.

The melting point is about 233 °C.

Methylphenyloxazolylbenzene used for liquid scintillation must be of an appropriate grade.

Methylcellulose 450. [9004-67-5]. Methyl ether of cellulose.

Partially *O*-methylated cellulose contains not less than 26.0% and not greater than 33.0% of methoxy groups (- OSN_3, M_r 31.03) calculated on a dry substance basis.

White, yellowish white, or greyish white powder or granules. Hygroscopic after drying.

Practically insoluble in hot water, acetone, anhydrous ethanol, and toluene. Soluble in cold water to form a colloidal solution.

Rated viscosity: 450 MPa· s.

Methyl cinnamate. $C_{10}H_{10}O_2$. (M_r 162.19). [103-26-4].

Colourless crystals. Practically insoluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 1.56.

The boiling point is about 260 °C.

The melting point is from 34 °C to 36 °C.

Methyl ethyl ketone. C_4H_8O. (M_r 72.11). [78-93-3].

Ethyl methyl ketone. 2-Butanone.

A clear, colorless, flammable liquid. Very soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.81.

The boiling point is from 79 °C to 80 °C.

Methyl eicosenoate. $C_{21}H_{40}O_2$. (M_r 324.54). [2390-09-2]. Methyl-(11Z)-eicose-11-enoate.

L-Methionine. $C_5H_{11}NO_2S$. (M_r 149.2). [63-68-3]. (2S)-2-Amino-4-(methylsulfanyl)butanoic acid.

Contains not less than 99.0% and not greater than 101.0% of $C_5H_{11}NO_2S$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Soluble in water, very slightly soluble in 96% ethanol.

Methoxyphenylacetic acid. $C_9H_{10}O_3$. (M_r 166.17). [7021-09-2]. (*RS*)-2-Methoxy-2-phenylacetic acid.

White crystalline powder or white or almost white crystals. Sparingly soluble in water, freely soluble in 96% ethanol.

The melting point is about 70 °C.

Methoxyphenylacetic acid reagent.

Dissolve 2.7 g of *methoxyphenylacetic acid R* in 6 mL of a solution of *tetramethylammonium hydroxide R* and add 20 mL of *anhydrous ethanol R.*

Store in a polyethylene container.

(RS)-Methotrexate. $C_{20}H_{22}N_8O_5$. (M_r 454.4).

[60388-53-6]. (*RS*)-2-[4-[[(2,4-diaminopteridine-6-yl)methyl]-methylamino]benzoylamino] pentanedionic acid.

Contains not less than 96.0% of $C_{20}H_{22}N_8O_5$.

The melting point is about 195 °C.

Myosmin. $C_9H_{10}N_2$. (M_r 146.19). [532-12-7].

3-(4,5-Dihydro-3*H*-pyrrole-2-yl)pyridine.

Colourless crystals.

The melting point is about 45 °C.

Myristyl alcohol. $C_{14}H_{30}O$. (M_r 214.39). [112-72-1]. Tetradecan-1-ol.

d_{20}^{20} is about 0.823.

The melting point is from 38 °C to 40 °C.

Myristicine. $C_{11}H_{12}O_3$. (M_r 192.21). [607-91-0]. 5-Allyl-1-methoxy-2,3-methylenedioxybenzene. 4-Methoxy-6-(prop-2-enyl)-1,3-benzodioxol.

A colorless oily liquid. Practically insoluble in water, slightly soluble in anhydrous ethanol, miscible with toluene and xylene.

d_{20}^{20} is about 1.144.

n_D^{20} is about 1.540.

The boiling point is from 276 °C to 277 °C.

The melting point is about 173 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) in accordance with the instructions of the monograph of *Star anise fruit*; the resulting chromatogram shows only one principal spot.

Store in a place protected from light.

Myristicine used in gas chromatography complies with the following additional requirement.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the monograph *Nutmeg oil.*

The miristicin content calculated by internal normalisation procedure should be minimum 95.0%.

β-Myrcene. C10H16. (*Mr* 136.23). [123-35-3]. 7-Methyl-3-methylenocta-1,6-diene.

An oily liquid with a pleasant odour. Practically insoluble in water, miscible with 96% ethanol, soluble in glacial acetic acid and solutions of alkali metal hydroxides.

d_4^{20} is about 0.794.

n_D^{20} is about 1.470.

β-Myrcene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Peppermint oil*, using β-myrcene as the test solution.

The β-myrcene content calculated by the internal normalisation procedure should be minimum 90.0%.

Molecular sieve.

The molecular sieve consists of sodium aluminosilicate. It has the form of balls with a pore size of 0.4 nm and a diameter of 2 mm.

Molybdovanadic reagent.

In a 150 mL beaker, mix 4 g of powdered *ammonium molybdate R* and 0.1 g of powdered *ammonium vanadate R*, add 70 mL of *water R*, and stir using a glass rod until dissolved. After a few minutes, a clear solution should be formed; add 20 mL of *nitric acid R* to this solution and dilute to 100 mL with *water R*.

Lactic acid. $C_3H_6O_3$. (M_r 90.1). [50-21-5].

A mixture of 2-hydroxypropanoic acid, its condensation products such as lactoyl-lactic acid and polylactic acid, and water. The balance between lactic acid and polylactic acid depends on the concentration and temperature. This is usually racemate ((RS) — lactic acid).

Contains not less than 88.0% (m/m) and not greater than 92.0% (m/m) of $C_3H_6O_3$.

Colourless or light yellow syrupy liquid.

Miscible with water and 96% ethanol.

Lactic acid reagent.

Solution A. To 60 mL of *lactic acid R*, add 45 mL of a previously filtered solution of *lactic acid R*, saturated without heating with *Sudan red G R*. Lactic acid is saturated slowly without heating, so an excess of dye is always necessary.

Solution B. Prepare 10 mL of a saturated solution of *aniline R* and filter.

Solution C. Dissolve 75 mg of *potassium iodide R* in *water R* and dilute to 70 mL with the same solvent. To the resulting solution, add 10 mL of *96% ethanol R* and 0.1 g of *iodine R*, shake.

Mix solutions A and B, add solution C.

Morphine hydrochloride. $C_{17}H_{20}ClNO_3 \cdot 3H_2O$. ($M_r$ 375.8). [6055-06-7]. 7,8-Didehydro-4,5α-epoxy-17-methylmorphinan-3,6 α-diolhydrohydrate.

Contains not less than 98.0% and not greater than 102.0% of $C_{17}H_{20}ClNO_3$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder or colorless silky needles or cubic masses, efflorescent in dry air.

Soluble in water, slightly soluble in 96% ethanol, practically insoluble in toluene.

Morpholine. C_4H_9NO. (M_r 87.12). [110-91-8]. Tetrahydro-1,4-oxazine.

Colourless, hygroscopic, flammable liquid. Soluble in water and 96% ethanol.

d_{20}^{20} is about 1.01.

Distillation range (*2.1.2.11*). From 126 °C to 130 °C; not less than 95% must be distilled.

Store in an airtight container.

Urea. CH_4N_2O. (M_r 60.1). [57-13-6].

Contains not less than 98.5% and not greater than 101.5% of urea calculated with reference to the anhydrous substance.

White or almost white crystalline powder or clear crystals. Slightly hygroscopic.

Very soluble in water, soluble in 96% ethanol, practically insoluble in methylene chloride.

Formic acid, anhydrous. CH_2O_2 (M_r 46.03). [64-18-6].

Contains not less than 98.0% (*w/w*) of CH_2O_2.

A colorless liquid. Corrosive and miscible with water and 96% ethanol.

d_{20}^{20} is about 1.22.

Quantitation. Place 10 mL of *water R* in a conical flask, accurately weigh, quickly add about 1 mL of anhydrous formic acid, and weigh again. Add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide solution* is equivalent to 46.03 mg of CH_2O_2.

Arsenic trioxide. As_2O_3. (M_r197.8). [1327-53-3]. Arsenic(III) oxide.

White or almost white crystalline powder or mass. Slightly soluble in water, soluble in boiling water.

Sodium. Na. (A_r 22.99). [7440-23-5].

Metal, on a fresh cut, has a shiny silver-grey surface. In the air, it quickly fades and is completely oxidised to sodium oxide, which turns into sodium carbonate. Reacts violently with water to form hydrogen and sodium hydroxide; soluble in anhydrous methanol to form hydrogen and sodium methylate; practically insoluble in petroleum ether. Store in petroleum ether or liquid paraffin.

Sodium azide. NaN_3. (M_r 65.02). [26628-22-8]. Sodium azide.

White or almost white crystalline powder or white crystals. Freely soluble in water, slightly soluble in 96% ethanol.

Sodium arsenite. $NaAsO_2$. (M_r 129.91). [7784-46-5]. Sodium metaarsenite.

Sodium arsenite solution.

Dissolve 5.0 g of *sodium arsenite R* in 30 mL of a *1 M sodium hydroxide solution*, cool to a temperature of 0 °C, and add with stirring 65 mL of *dilute hydrochloric acid R*.

Sodium ascorbate solution. [134-03-2].

Dissolve 3.5 g of *ascorbic acid R* in 20 mL of *1 M sodium hydroxide*. Prepare immediately before use.

Sodium acetate. $C_2H_3NaO_2 \cdot 3H_2O$. (M_t 136.1). [6131-90-4]. Sodium acetate trihydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_2H_3NaO_2 \cdot 3H_2O$ calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Very soluble in water, soluble in 96% ethanol.

Sodium acetate, anhydrous. $C_2H_3NaO_2$. (*Mr* 82.03). [127-09-3].

Colourless crystals or granules. Very soluble in water, sparingly soluble in 96% ethanol.

Loss on drying (*2.1.2.31*). NMT 2.0%. Determination is carried out at 105 °C.

Sodium bicarbonate. [144-55-8].

See *Sodium bicarbonate R.*

Sodium butanesulfonate. $C_4H_9NaO_3S$. (M_r 160.16). [2386-54-1]. Sodium butanesulfonate.

White or almost white crystalline powder. Soluble in water.

The melting point is greater than 300 °C.

Sodium bismuthate. $NaBiO_3$. (M_r 279.97). [12232-99-4]. Sodium bismuthate.

Contains not less than 85.0% of $NaBiO_3$.

Yellow or yellowish brown powder. Slowly decomposes under the influence of moisture or high temperature, practically insoluble in cold water.

Quantitation. Suspend 0.200 g in 10 mL of a 200 g/L solution of *potassium iodide R*, add 20 mL of *dilute sulfuric acid R*, and titrate with *0.1 M sodium thiosulfate* to obtain an orange colour, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 14.00 mg of $NaBiO_3$.

Sodium tungstate. $NaWO_4 \cdot 2H_2O$. (M_r 329.9). [10213-10-2]. Sodium tungstate dihydrate.

White or almost white crystalline powder or colorless crystals. Freely soluble in water to form a clear solution, practically insoluble in 96% ethanol.

Sodium hexanesulfonate. $C_6H_{13}NaO_3S$. (M_r 188.2). [2832-45-3]. Sodium hexanesulfonate.

White or almost white powder. Freely soluble in water.

Sodium heptanesulfonate. $C_7H_{15}NaO_3S$. (M_r 202.3). [22767-50-6]. Sodium heptanesulfonate.

White or almost white crystalline mass. Freely soluble in water, soluble in methanol.

Sodium heptanesulfonate monohydrate. $C_7H_{15}NaO_3S \cdot H_2O$. (*Mr* 220.3).

Contains not less than 96% of $C_7H_{15}NaO_3S$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder. Soluble in water, very slightly soluble in anhydrous ethanol.

Water (2.1.5.12). NMT 8%. Determination is carried out from 0.300 g.

Quantitation. Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R* and titrate with *0.1 M perchloric acid* potentiometrically (*2.1.2.19*).

1 mL of *0.1 M perchloric acid solution* is equivalent to 20.22 mg of $C_7H_{15}NaO_3S$.

Sodium bicarbonate. $NaHCO_3$. (M_r 84.0). [144-55-8]. Sodium bicarbonate.

Contains no less than 99.0% and no greater than 101.0% of $NaHCO_3$.

White or almost white crystalline powder.

Soluble in water, practically insoluble in 96% ethanol.

Heating a dry substance or its solution leads to the gradual conversion of sodium bicarbonate to sodium carbonate.

Sodium bicarbonate solution.

A 42 g/L solution.

Sodium hydroxide. NaOH. (M_r 40,00). [1310-73-2]. Sodium hydroxide.

Contains not less than 97.0% and not greater than 100.5% of the amount of alkalis calculated with reference to NaOH.

White or almost white crystalline mass in the form of granules, sticks, or plates. Deliquescent in the air, easily absorbs atmospheric carbon dioxide.

Very soluble in water, freely soluble in 96% ethanol.

Sodium hydroxide solution.

Dissolve 20.0 g of *sodium hydroxide R* in *water R* and dilute to 100.0 mL with the same solvent. Determine the concentration of the solution by titration with *1 M hydrochloric acid*, using a *methyl orange R* solution as indicator; if necessary, strengthen the solution or dilute to a concentration of 200 g/l.

Sodium hydroxide 2 M solution.

Dissolve 84 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Diluted sodium hydroxide solution.

Dissolve 8.5 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Sodium hydroxide methanol solution.

Dissolve 40 mg of *sodium hydroxide R* in 50 mL of *water R*, cool the resulting solution, and add 50 mL of *methanol R*.

Methanolic sodium hydroxide solution R1.

Dissolve 200 mg of *sodium hydroxide R* in 50 mL of *water R*, cool the resulting solution, and add 50 mL of *methanol R*.

Sodium hydroxide solution, strong.

Dissolve 42 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Sodium hydrogen sulfate. $NaHSO_4$. (M_r120.1). [7681-38-1]. Sodium hydrogen sulfate.

Freely soluble in water, very soluble in boiling water. Decomposes in 96% ethanol to sodium sulfate and free sulfuric acid.

The melting point is about 315 °C.

Sodium hydrosulfite. $NaHSO_3$. (M_r 104.1). [7631-90-5]. Sodium hydrosulfite.

White or almost white crystalline powder.

Freely soluble in water, sparingly soluble in 96% ethanol.

In the air, it partially loses sulfur dioxide and gradually oxidises to sulfate.

Sodium hypobromite solution.

Mix 20 mL of *strong sodium hydroxide solution R* and 500 mL of *water R* in an ice bath, add 5 mL of *bromine solution R*, and mix gently until dissolved. Prepare immediately before use.

Sodium hypophosphite. $NaH_2PO_2{\cdot}H_2O$. (M_r 106.0). [10039-56-2]. Sodium phosphinate monohydrate.

White or almost white crystalline powder or colorless crystals. Hygroscopic, freely soluble in water, soluble in 96% ethanol.

Store in an airtight container.

Sodium hypochlorite solution, strong.

Contains between 25 g/l and 30 g/l of active chlorine.

Yellowish liquid with an alkaline reaction.

Quantitation. In a flask with 50 mL of *water R*, successively place 1 g of *potassium iodide R* and 12.5 mL of *dilute acetic acid R*. Dilute 10.0 mL of strong sodium hypochlorite solution to 100.0 mL with *water R*. Place 10.0 mL of the resulting solution in a flask with reagents and titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 3.546 mg of active chlorine.

Store in a place protected from light.

Sodium glucuronate. $C_6H_9NaO_7 \cdot H_2O$. (M_r 234.1).

Sodium D-glucuronate monohydrate.

$[\alpha]_{20}^{20}$ about +21.5. Determination is carried out using a 20 g/L solution.

Sodium deoxyribonucleate. (About 85% has a relative molecular mass of 2 x 10^7 or greater). [73049-39-5].

White or almost white fibrous substance; obtained from calf thymus.

Suitability test. Dissolve 10 mg in *imidazole buffer solution pH 6.5 R* and dilute to 10.0 mL with the same buffer solution (solution A). Dilute 2.0 mL of solution A to 50.0 mL with *imidazole buffer solution pH 6,5 R*. The absorbance (*2.1.2.24*) of the resulting solution, measured at a wavelength of 260 nm, should be from 0.4 to 0.8.

To 0.5 mL of solution A, add 0.5 mL of *imidazole buffer solution pH 6.5 R*, 3 mL of a 25 g/L solution of perchloric acid; a precipitate is formed, which is centrifuged. The absorbance of the supernatant is measured at 260 nm using a mixture consisting of 1 mL of *imidazole buffer solution pH 6.5 R* and 3 mL of a 25 g/L solution of perchloric acid ($HClO_4$) as compensation liquid. The optical density should not exceed 0.3.

In each of the two test tubes, place 0.5 mL of a solution and 0.5 mL of a streptodornase reference solution containing 10 IU/mL in an *imidazole buffer solution pH 6.5 R*. In one test tube, immediately add 3 mL of a 25 g/L solution of perchloric acid ($HClO_4$); a precipitate is formed, which is centrifuged and the supernatant (A) is collected.

Heat another test tube at 37 °C for 15 min, add 3 mL of a 25 g/L solution of perchloric acid, centrifuge, and collect the supernatant (B). The absorbance of the supernatant (B) is measured at a wavelength of 260 nm, using the supernatant (A) as compensation solution. The absorbance must be NLT 0.15.

Sodium decanesulfonate. $C_{10}H_{21}Na_3S$. (*Mr* 244.3). [13419-61-9].

White or almost white crystalline powder or flakes. Freely soluble in water, soluble in methanol.

Sodium dihydrogen phosphate. $NaH_2PO_4 \cdot 2H_2O$. (M_r 156.0). [13472-35-0]. Sodium dihydrogen phosphate dihydrate.

Contains not less than 98.0% and not greater than 100.5% of NaH_2PO_4 calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Very soluble in water, very slightly soluble in 96% ethanol.

Sodium dihydrogen phosphate, anhydrous. $NaH2PO_4$. (*Mr* 120.0). [7558-80-7].

White or almost white powder, hygroscopic.

Store in an airtight container.

Sodium dihydrogen phosphate monohydrate. $NaH2PO_4 \cdot H_2O$. (*Mr* 138.0). [10049-21-5].

White or almost white crystals or granules, slightly deliquescent. Freely soluble in water, practically insoluble in 96% ethanol.

Store in an airtight container.

Sodium dithionite. $Na_2S_2O_4$. (M_r 174.1). [7775-14-6]. Sodium dithionite.

White or greyish white crystalline powder; oxidises in air. Very soluble in water, slightly soluble in 96% ethanol.

Store in an airtight container.

Sodium diethyldithiocarbamate. $C_5H_{10}NNaS_2 \cdot 3H_2O$. (*Mr* 225.3). [20624-25-3].

Colourless or white crystals. Freely soluble in water, soluble in 96% ethanol. Colorless aqueous solution.

Sodium dodecyl sulfate. [151-21-3].

See *Sodium lauryl sulfate.*

Contains not less than 99.0% of sodium dodecyl sulfate.

Натрия йодид. NaI. (*Mr* 149.9). [7681-82-5]. Sodium iodide.

Contains NLT 99.0% and NMT 100.5% of NaI calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals. Hygroscopic.

Very soluble in water, freely soluble in 96% ethanol.

Sodium carbonate. $Na_2CO_3 \cdot 10H_2O$. (M_r 286.1). [6132-02-1]. Sodium carbonate decahydrate.

Contains no less than 36.7% and no greater than 40.0% of Na2CO_3.

White or almost white crystalline powder or colorless clear crystals. Efflorescent in air.

Freely soluble in water, practically insoluble in 96% ethanol.

Sodium carbonate, anhydrous. Na_2CQ_3. (M_r 106.0). [497-19-8]. Disodium carbonate.

White or almost white powder, hygroscopic. Freely soluble in water.

The loss on drying at a temperature of about 300 °C should not exceed 1%.

Store in an airtight container.

Sodium carbonate solution.

A 106 g/L solution of *anhydrous sodium carbonate R.*

Sodium carbonate solution R1.

A 20 g/L solution of *anhydrous sodium carbonate R* in *0.1 M sodium hydroxide*.

Sodium cobaltinitrite. $Na_3[Co(NO_2)_6]$. (M_r 403.9). [13600-98-1]. Sodium hexanitrocobaltate(III).

Orange-yellow powder. Freely soluble in water, slightly soluble in 96% ethanol.

Sodium cobaltinitrite solution.

A 100 g/l solution.

Prepare immediately before use.

Sodium lauryl sulfate. [151-21-3]. Sodium lauryl sulfate.

A mixture of sodium alkyl sulfates consisting primarily of sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$, M_r 288.4).

The substance contains not less than 85.0% of sodium alkyl sulfates calculated with reference to $C_{12}H_{25}NaO_4S$.

White or pale yellow powder or crystals.

Freely soluble in water to form an opalescent solution, partially soluble in 96% ethanol.

Sodium metabisulfite. $Na_2S_2O_5$. (M_r 190.1). [7681-57-4]. Sodium disulfite.

Contains no less than 95.0% and no greater than 100.5% of $Na_2S_2O_5$.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, slightly soluble in 96% ethanol.

Sodium methanesulfonate. CH_3SO_3Na. (*Mr* 118.1). [2386-57-4].

White or almost white crystalline powder, hygroscopic.

Store in an airtight container.

Sodium molybdate. Na2$MoO_4 \cdot 2H_2O$. (M_r 242.0). [10102-40-6]. Disodium molybdate dihydrate.

White or almost white crystalline powder or colorless crystals. Freely soluble in water.

Sodium naphthoquinone sulfonate. $C_{10}H_5NaO_5S$. (M_r 260.2). [521-24-4]. Sodium 1,2-naphthoquinone-4-sulfonate.

Yellow to orange-yellow crystalline powder. Freely soluble in water, practically insoluble in 96% ethanol.

Sodium nitrate. $NaNO_3$. (M_r 85.0). [7631-99-4]. Sodium nitrate.

White or almost white powder or granules or colorless clear crystals, deliquescent in the air.

Freely soluble in water, slightly soluble in 96% ethanol.

Store in an airtight container.

Sodium nitrite. $NaNO_2$. (M_r 69.0). [7632-00-0]. Sodium nitrite.

Contains not less than 97.0% of HNO_2.

White or almost white granular powder or slightly yellowish crystalline powder. Freely soluble in water.

Quantitation. Dissolve 0.100 g of sodium nitrite R *in 50 mL of* water R*, add 50.0 mL of* 0.02 M potassium permanganate, *15 mL of* dilute sulfuric acid R, *3 g of* potassium iodide R, *and titrate with* 0.1 M sodium thiosulfate, *using 1 mL of* starch solution R *as indicator at the end of titration.*

1 mL of *0.02 potassium permanganate solution* is equivalent to 3.450 mg of $NaNO_2$.

Sodium nitrite solution.

A 100 g/l solution.

Prepare immediately before use.

Sodium nitroprusside. $Na_2[Fe(CN)_5(NO)]\cdot 2H_2O$. ($M_r$ 298.0). [13755-38-9]. Sodium pentacyanonitrosylferrate(III) dihydrate.

Reddish brown powder or crystals. Freely soluble in water, slightly soluble in 96% ethanol.

Sodium oxalate. $C_2Na_2O_4$. (M_r 134.0). [62-76-0]. Sodium oxalate.

White or almost white crystalline powder. Soluble in water, practically insoluble in 96% ethanol.

Sodium octanesulfonate. $C_8H_{17}NaO_3S$. (*Mr* 216.3). [5324-84-5].

Contains not less than 98.0% of $C_8H_{17}NaO_3S$.

White or almost white crystalline powder or flakes. Freely soluble in water, soluble in methanol.

Absorbance (*2.1.2.24*). The absorbance of the 54 g/l solution at a wavelength of 200 nm should be no greater than 0.10, and at a wavelength of 250 nm – no greater than 0.01.

Sodium octyl sulfate. $C_8H_{17}NaO_4S$. (*Mr* 232.3). [142-31-4].

White or almost white crystalline powder or flakes. Freely soluble in water, soluble in methanol.

Sodium pentanesulfonate. $C_5H_{11}NaO_3S$. (*Mr* 174.2). [22767-49-3].

White or almost white solid crystalline substance. Soluble in water.

Sodium periodate. $NaIO_4$. (M_r 213.9). [7790-28-5]. Sodium metaperiodate.

Contains not less than 99.0% of $NaIO_4$.

White or almost white crystalline powder or crystals. Soluble in water and mineral acids.

Sodium periodate solution.

Dissolve 1.07 g of *sodium periodate R* in *water R*, add 5 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*. Prepare immediately before use.

Sodium perchlorate. $NaClO_4\cdot H_2O$. (M_r 140.5). [7791-07-3]. Sodium perchlorate.

Contains not less than 99.0% of $NaClO_4\cdot H_2O$.

White or almost white crystals, deliquescent. Very soluble in water.

Store in a airight container.

Sodium picrate, alkaline solution.

Mix 20 mL of a solution of *picric acid R* and 10 mL of a 50 g/L solution of *sodium hydroxide R*, dilute to 100 mL with *water R*.

Use within 2 days.

Sodium pyrophosphate. $Na_4P_2O_7\cdot 10H_2O$. (M_r 446.1). [13472-36-1]. Tetrasodium diphosphate decahydrate.

Colourless, slightly efflorescent crystals. Freely soluble in water.

Sodium rhodizonate. $C_6Na_2O_6$. (M_r 214.0). [523-21-7]. [(3,4,5,6-tetraoxo-cyclohex-1-en-1,2-ylene-dioxy]disodium.

Violet crystals. Soluble in water to form an orange-yellow solution.

Solutions are unstable and must be prepared on the day of use.

Sodium salicylate. $C_7H_5NaO_3$. (M_r 160.1). [54-21-7]. Sodium 2-hydroxybenzenecarboxylate.

Contains not less than 99.0% and not greater than 101.0% of $C_7H_5NaO_3$ calculated on a dry substance basis.

White or almost white crystalline powder, or small colorless crystals, or shiny plates.

Freely soluble in water, sparingly soluble in 96% ethanol.

Sodium sulfate, anhydrous. [7757-82-6].

Ignited at 600-700 °C, anhydrous sodium sulfate must meet the requirements specified in the monograph *Sodium sulfate, anhydrous*.

Loss on drying (*2.1.2.31*). NMT 0.5%. Determination is carried out at 130 °C.

Sodium sulfide. $Na_2S·9H_2O$. (M_r 240.2). [1313-84-4]. Disodium sulfide nonahydrate.

Colourless, rapidly yellowing crystals, deliquescent. Very soluble in water.

Store in an airtight container.

Sodium sulfide solution.

Dissolve 12 g of *sodium sulfide R* by heating in 45 mL of a mixture of solvents *water R/glycerol (85%) R* (10:29), then allow to cool, and dilute to 100 mL with the same mixture of solvents. The solution should be colourless.

Sodium sulfite. $Na_2SO_3·7H_2O$. (M_r 252.2). [10102-15-5]. Sodium sulfite heptahydrate.

Contains not less than 48.0% and not greater than 52.5% of $Na_2SO_3·7H_2O$.

Colourless crystals.

Freely soluble in water, very slightly soluble in 96% ethanol.

Sodium sulfite, anhydrous. Na_2SO_3. (M_r 126.0). [7757-83-7]. Sodium sulfite.

Contains no less than 95.0% and no greater than 100.5% of Na2SO3.

White or almost white powder.

Freely soluble in water, very slightly soluble in 96% ethanol.

Sodium tartrate. $C_4H_4Na_2O_6·2H_2O$. (M_r 230.1). [6106-24-7]. Disodium (*2R,3R*)-2,3-dihydroxy-butanedioate dihydrate.

White or almost white crystals or granules. Very soluble in water, practically insoluble in 96% ethanol.

Sodium tetradeuterodimethyl silapentanate. $C_6H_9{}^2H_4NaO_2Si$. (M_r 172.3). Sodium (2,2,3,3-tetradeutero)-4,4-dimethyl-4-silapentanate.

The degree of deuterisation is not less than 99%.

White crystalline powder. Freely soluble in water, ethanol, and methanol.

The melting point is about 300 °C.

Water and deuterium oxide. NMT 0.5%.

Sodium tetraphenylborate. $NaB(C_6H_5)_4$. (M_r 342.2). [143-66-8].

White or slightly yellowish loose powder. Freely soluble in water and acetone.

Sodium tetraphenylborate solution.

A 10 g/l solution. If necessary, filter before use.

Storage: use within 7 days.

Sodium thioglycolate. $C_2H_3NaO_2S$. (M_r 114.1). [367-51-1]. Sodium mercaptoacetate.

White or almost white granular powder or crystals. Hygroscopic, freely soluble in water and methanol, slightly soluble in 96% ethanol.

Store in an airtight container.

Sodium thiosulfate. $Na_2S_2O_3·5H_2O$. (M_r 248.2). [10102-17-7]. Thiosulfonate.

Contains not less than 99.0% and not greater than 101.0% of $Na_2S_2O_3·5H_2O$.

Clear, colorless crystals. Efflorescent in dry air.

Very soluble in water, practically insoluble in 96% ethanol. Soluble in crystallisation water at about 49 °C.

Sodium fluoresceinate. $C_{20}H_{10}Na_2O_5$. (M_r 376.3). [518-47-8]. Sodium fluorescein. Disodium 2-(3-oxo-6-oxido-3*H*-xanthene-9-yl)benzoate.

Schultz No. 880.

Colour index (C.I.) No. 45350.

Orange-red powder. Freely soluble in water. Aqueous solutions have an intense yellowish green fluorescence.

Sodium formate. $CHNaO_2$. (M_r 68.0). [141-53-7]. Sodium methanoate.

White or almost white crystalline powder or deliquescent granules. Soluble in water and glycerin, slightly soluble in 96% ethanol.

The melting point is about 253 °C.

Sodium fluoride. NaF. (M_r 41.99). [7681-49-4]. Sodium fluoride.

Contains not less than 98.5% and not greater than 100.5% of NaF calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Soluble in water, practically insoluble in 96% ethanol.

Sodium chloride. NaCl. (M_r 58.44). [7647-14-5]. Sodium chloride.

Contains not less than 99.0% and not greater than 100.5% of NaCl calculated on a dry substance basis.

White or almost white crystalline powder, or colorless crystals, or white grains.

Freely soluble in water, practically insoluble in anhydrous ethanol.

Sodium chloride solution.

A 20% solution (*m/m*).

Sodium chloride saturated solution.

Mix 1 part of *sodium chloride R* with 2 parts of *water R*, periodically shake and allow to settle. If necessary, decant the solution and filter before use.

Sodium cetostearyl sulfate.

A mixture of sodium cetyl sulfate ($C_{16}H_{33}NaO_4S$; M_r 344.5) and sodium stearyl sulfate ($C_{18}H_{37}NaO_4S$; M_r 372.5). Contains not less than 99.0% of sodium cetostearyl sulfate and at least 40.0% of sodium cetyl sulfate, both calculated with reference to the anhydrous substance. A suitable buffer solution may be added.

White or pale yellow amorphous or crystalline powder.

Soluble in hot water to form a turbid solution, practically insoluble in cold water, partially soluble in 96% ethanol.

Sodium citrate. $C_6H_5Na_3O_7 \cdot 2H_2O$. ($M_r$ 294.1). [6132-04-3]. Trisodium 2-hydroxypropan-1,2,3-tricarboxylate dihydrate.

Contains not less than 99.0% and not greater than 101.0% calculated with reference to the anhydrous substance.

White or almost white crystalline powder or granular crystals, slightly deliquescent in moist air.

Freely soluble in water, practically insoluble in 96% anhydrous ethanol.

Sodium edetate. $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$. ($M_r$ 372.2). [6381-92-6]. Disodium diester (ethylenedinitrile)tetraacetate dihydrate.

Contains not less than 98.5% and not greater than 101.0% of $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$.

White or almost white crystalline powder.

Soluble in water, practically insoluble in 96% ethanol.

Naphthalene. $C_{10}H_8$. (M_r 128.2). [91-20-3].

White or almost white crystals. Practically insoluble in water, freely soluble in ether, soluble in 96% alcohol.

The melting point is about 80 °C.

Naphthalene used for liquid scintillation must be of an appropriate grade.

Naphtharson. $C_{16}H_{11}AsN_2Na_2O_{10}S_2$. ($M_r$ 576.3). [3688-92-4]. Thorin. Disodium 4-[(2-arsonophenyl)azo]-3- hydroxynaphthalene-2,7-disulfonate.

Red powder. Soluble in water.

Naphtharson solution.

A 0.58 g/L solution.

Test for sensitivity. To 50 mL of *96% ethanol R*, add 20 mL of *water R*, 1 mL of *0.05 M sulfuric acid*, 1 mL of naftharzon solution, and titrate *0.025 M barium perchlorate* until the colour of the solution changes from orange-yellow to orange-pink.

Store in a place protected from light.

Storage: use within 7 days.

Naphthylamine. $C_{10}H_9N$. (*Mr* 143.2). [134-32-7]. 1-Naphthylamine.

White or almost white crystalline powder that turns pink under exposure to light and air. Slightly soluble in water, freely soluble in 96% ethanol.

The melting point is about 51 °C.

Store in a place protected from light.

Naphthylethylenediamine dihydrochloride. $C_{12}H_{16}Cl_2N_2$ (M_r259.2). [1465-25-4]. N-(1-Naphthyl)ethylenediamine dihydrochloride.

May contain crystallisation methanol.

White or yellowish white powder. Soluble in water, slightly soluble in 96% ethanol.

α-Naphthol. $C_{10}H_8O$. (M_r 144.2). [90-15-3]. 1-Naphthol.

White or almost white crystalline powder or colorless or white crystals that darken under exposure to light. Slightly soluble in water, freely soluble in 96% ethanol.

The melting point is about 95 °C.

Store in a place protected from light.

α-Naphthol solution.

Dissolve 0.10 g of *α-naphthol R* in 3 mL of a 150 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*. Prepare immediately before use.

β-Naphthol. $C_{10}H_8O$. (M_r 144.2). [135-19-3]. 2-Naphthol.

White or faintly pink plates or crystals. Very slightly soluble in water, very soluble in 96% ethanol.

The melting point is about 122 °C. Store in a place protected from light.

β-Naphthol solution.

Dissolve 5 g of freshly crystallised *β-naphthol R* in 40 mL of *dilute sodium hydroxide R* and dilute to 100 mL with *water R*.

Prepare immediately before use.

β-Naphthol solution R1.

Dissolve 3.0 mg of *β-naphthol R* in 50 mL of *sulfuric acid R* and dilute to 100.0 mL with the same acid.

Prepare immediately before use.

Naphtholbenzein. $C_{27}H_{18}O_2$. (M_r 374.4). [145-50-6]. α-Naphtholbenzein. 4-[(4-hydroxynaphthalen-1-yl)(phenyl)methylidene]naphthalene-1(4*H*)-one.

Brownish red powder or shiny brownish black crystals. Practically insoluble in water, soluble in 96% ethanol and glacial acetic acid.

Naphtholbenzein solution.

A 2 g/L solution in *anhydrous acetic acid R*.

Test for sensitivity. To 50 mL of *glacial acetic acid R*, add 0.25 mL of naphtholbenzein solution; a brownish-yellow colouration appears, which should turn green when no greater than 0.05 mL of *0.1 M perchloric acid solution* is added.

Neryl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). [141-12-8]. (*Z*)-3,7-Dimethylocta-2,6-dienyl acetate. A colorless oily liquid.

d_{20}^{20} *is about* 0.907.

n_D^{20} is about 1,460.

The boiling point$_{25}$ is 134 °C.

Neryl acetate used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography *(2.1.2.27)* in accordance with the instructions of the monograph *Bitter-orange-flower oil*, using neryl acetate as the test solution.

The neryl acetate content calculated by the normalisation procedure should be minimum 93.0%.

trans-Nerolidol. $C_{15}H_{26}O$. (M_r 222.4). [40716-66-3]. 3,7,11-Trimethyldodeca-1,6,10-triene-3-ol.

Slightly yellow liquid with a slight odour of lily or lily-of-the-valley. Practically insoluble in water and glycerol, miscible with 96% ethanol.

d_{20}^{20} is about 0.876.

n_D^{20} is about 1.479.

The boiling point$_{12}$ is from 145 °C to 146 °C.

trans-Nerolidol used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27)* in accordance with the instructions of the monograph *Bitter-orange-flower oil*, using *trans*-nerolidol as the test solution.

The trans-nerolidol content calculated by the normalisation procedure should be minimum 90.0%.

Nickel-aluminum alloy.

It contains from 48% to 52% of aluminum (Al, A_r 26.98) and from 48% to 52% Nickel (Ni, W 58.70).

Before use, grind to a fine powder (180) (*2.9.12*).

Practically insoluble in water, soluble in mineral acids.

Nickel-aluminum alloy, halogen-free.

It contains 48% to 52% of aluminum (Al, A_rAG 26.98) and from 48% to 52% of nickel (Ni, A_r 58.71).

Fine grey powder. Practically insoluble in water, soluble in mineral acids to form salts.

Chlorides. NMT 10 ppm.

Dissolve 0.400 g of the alloy in 40 mL of a mixture of *sulfuric acid R*/*dilute nitric acid R* (67:33). Evaporate the solution almost to dryness, dissolve the remainder in *water R* and dilute to 20.0 mL with the same solvent. To 10.0 mL of the test solution, add 1.0 mL of *0.1 M silver nitrate*, filter after 15 min, and add 0.2 mL of sodium chloride solution (10 µg/mL Cl^-) to the filtrate. After 5 min, the opalescence of the resulting solution should not exceed the opalescence of the second half of the test solution with 1.0 mL of *0.1 M silver nitrate*.

Nickel(II) sulfate. $NiSO_4 \cdot 7H_2O$. (*Mr* 280.9). [10101-98-1]. Nickel sulfate heptahydrate.

Crystalline powder or green crystals. Freely soluble in water, slightly soluble in 96% ethanol.

Nickel (II) chloride. $NiCl_2$. (*Mr* 129.6). [7718-54-9]. Nickel chloride, anhydrous.

Yellow crystalline powder. Very soluble in water, soluble in 96% ethanol. Sublimates in the absence of air and easily absorbs ammonia. An aqueous solution has an acidic reaction.

Nicotinamide-adenine dinucleotide.

$C_{21}H_{27}N_7O_{14}P_2$. (M_r 663). [53-84-9].

White or almost white powder, highly hygroscopic. Freely soluble in water.

Nicotinamide-adenine dinucleotide solution.

Dissolve 40 mg of *nicotinamide-adenin dinucleotide R* in *water R* and dilute to 10 mL with the same solvent.

Prepare immediately before use.

Nile blue A. $C_{20}H_{21}N_3O_5S$. (M_r 415.5). [3625-57-8].

Schultz No. 1029.

Colour index (C.I.) No. 51180.

5-Amino-9-(diethylamino)benzo[α]of phenoxsenile hydrogen sulfate.

Green crystalline powder with a bronze lustre. Sparingly soluble in 96% ethanol, glacial acetic acid, and pyridine.

A 0.005 g/L solution in *ethanol (50%, V/V) R* has an absorption maximum (*2.1.2.24*) at a wavelength of 640 nm.

Nile blue A solution.

A 10 g/L solution in *anhydrous acetic acid R.*

Test for sensitivity. To 50 mL of *anhydrous acetic acid R*, add 0.25 mL of a solution of Nile blue A; a blue colour appears, which turns blue-green when 0.1 mL of *0.1 M perchloric acid* is added.

Colour change. Blue to red in the *pH* range of 9.0-13.0.

Ninhydrin. $C_9H_4O_3,H_2O$. (M_r 178.1). [485-47-2]. 1,2,3-Indantrione monohydrate.

White or slightly yellow crystalline powder. Soluble in water and 96% ethanol.

Store in a place protected from light.

Ninhydrin and stannous chloride reagent.

Dissolve 0.2 g of *ninhydrin R* in 4 mL of hot *water R*, add 5 mL of a 1.6 g/L solution of *stannous chloride R*, allow to stand for 30 min, filter, and store at a temperature of 2 °C to 8 °C. Immediately before use, add 5 mL of *water R* and 45 mL of *2-propanol R* to 2.5 mL of the resulting solution.

Ninhydrin and stannous chloride reagent R1.

Dissolve 4 g of *ninhydrin R* in 100 mL of *ethylene glycol monoethyl ether R*. Gently shake with 1 g of *cation exchange resin R* (300 µm to 840 µm) and filter (solution A). Dissolve 0.16 g of *stannous chloride R* in 100 mL of *buffer solution pH 5.5 R* (solution B). Mix equal volumes of solutions A and B immediately before use.

Ninhydrin solution.

2 g/l *ninhydrin R solution* in a mixture of solvents: *dilute acetic acid R – butanol R* (5:95).

Ninhydrin solution R1.

Dissolve 1.0 g of *ninhydrin R* in 50 mL of *96% ethanol R* and add 10 mL of *glacial acetic acid R.*

Ninhydrin solution R2.

Dissolve 3 g of *ninhydrin R* in 100 mL of a 45.5 g/L solution of *sodium metabisulfite R.*

Ninhydrin solution R3.

A 4 g/L solution of *ninhydrin R* in a mixture of solvents *anhydrous acetic acid R*/*butanol R* (5:95).

Ninhydrin solution R4.

A 3 g/L solution of *ninhydrin R* in a mixture of solvents *anhydrous acetic acid R*/*2-propanol R* (5:95).

Nitroaniline. $C_6H_6N_2O_2$. (M_r 138.1). [100-01-6]. 4-Nitroaniline.

Bright yellow crystalline powder. Very slightly soluble in water, sparingly soluble in boiling water, soluble in 96% ethanol, forms water-soluble salts with strong mineral acids.

The melting point is about 147 °C.

Nitrobenzaldehyde. $C_7H_5NO_3$. (M_r 151.1). [552-89-6]. 2-Nitrobenzaldehyde.

Needle-like yellow crystals. Slightly soluble in water, freely soluble in 96% ethanol, sublimated by steam.

The melting point is about 42 °C.

Nitrobenzaldehyde paper.

Dissolve 0.2 g of *nitrobenzaldehyde R* in 10 mL of a 200 g/L solution of *sodium hydroxide R*. Use the solution within 1 h. Immerse the lower half of a strip of slowly filtering paper 10 cm long and 0.8 - 1 cm wide in the resulting solution. Remove excess reagent by blotting the strip between two sheets of filter paper. Use within a few minutes of preparation.

Nitrobenzaldehyde solution.

Add 0.12 g of *nitrobenzaldehyde R* in the form of powder to 10 mL of *dilute sodium hydroxide solution R*, shake for 10 min, and filter. Prepare immediately before use.

Nitrobenzyl chloride. $C_7H_6ClNO_2$. (M_r 171.6). [100-14-1]. 4-Nitrobenzyl Chloride.

Pale-yellow crystals. Lachrymatory. Practically insoluble in water, very soluble in 96% ethanol.

Nitrobenzoyl chloride. $C_7H_4ClNO_3$. (M_r 185.6). [122-04-3]. 4-nitrobenzoyl chloride.

Yellow crystals or crystalline mass, deliquescent. Soluble in a sodium hydroxide solution with yellowish orange discolouration.

The melting point is about 72 °C.

4-(4-Nitrobenzyl)pyridine. $C_{12}H_{10}N_2O_2$. (M_r 214.2). [1083-48-3].

Yellow powder.

The melting point is about 70 °C.

4-Nitrobenzoic acid. $C_7H_5NO_4$. (M_r 167.1). [62-23-7].

Yellow crystals.

The melting point is about 240 °C.

Nitrobenzene. $C_6H_5NO_2$. (M_r 123.1). [98-95-3].

A colorless or slightly yellowish liquid. Practically insoluble in water, miscible with 96% ethanol.

The boiling point is about 211 °C.

Dinitrobenzene. To 0.1 mL of nitrobenzene, add 5 mL of *acetone R*, 5 mL of *water R*, and 5 mL of *strong sodium hydroxide solution R* and shake; after separating the layers, the top layer should be almost colourless.

Nitrosodipropylamine. $C_6H_{14}N_2O$. (M_r 130.2). [621-64-7]. Dipropylnitrosamine.

Liquid. Soluble in ethanol and concentrated acids.

d_{20}^{20} is about 0.915.

The boiling point is about 78 °C.

The purity level is suitable for determining chemiluminescence.

Nitrosodipropylamine solution.

Enter 78.62 g of *ethanol R*, piercing the stopper of a vessel containing *nitrosodipropylamine R* with an injection needle, dilute with *anhydrous ethanol R* in a ratio of 1:100, and place portions, each of 0.5 mL, in vials with flip-off caps.

Store in a dark place at a temperature of 5 °C.

Nitromethane. CH_3NO_2. (M_r 61.0). [75-52-5].

A clear, colourless, oily liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is from 1,132 to 1,134.

n_D^{20} from 1.381 to 1.383.

Distillation range (*2.1.2.11*). From 100 °C to 103 °C; not less than 95% must be distilled.

Nitro-molybdovanadic reagent.

Solution A. Dissolve 10 g of *ammonium molybdate R* in *water R*, add 1 mL of *ammonia solution R* and dilute to 100 mL with *water R*.

Solution B. Dissolve 2.5 g of *ammonium vanadate R* in hot *water R*, add 14 mL of *nitric acid R*, and dilute to 500 mL with *water R*.

To 96 mL of *nitric acid R*, add 100 mL of solution A, 100 mL of solution B, and dilute to 500 mL with *water R*.

Nitrotetrazolium blue. $C_{40}H_{30}C1_2N_{10}O_6$. ($M_r$ 818). [298-83-9]. 3,3'-(3,3'-Dimethoxy-4,4'-diphenylene) di[2-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium]dichloride. *p*-Nitrotetrazolium blue.

Crystals. Soluble in methanol to form a clear yellow solution.

The melting point is about 189 °C with decomposition.

Nitrofurantoin. $C_8H_6N_4O_5$. (M_r 238.2). [67-20-9]. 1-[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione.

Contains not less than 98.0% and not greater than 102.0% of $_8H_6N_4O_5$calculated on a dry substance basis.

Crystalline powder or yellow crystals, very slightly soluble in water and 96% ethanol, soluble in dimethylformamide.

(5-Nitro-2-furyl)methylene diacetate. $C_9H_9NO_7$. (M_r 243.2). [92-55-7]. Nitrofurfural diacetate. 5-Nitrofurfurylidene diacetate.

Yellow crystals.

The melting point is about 90 °C.

Nitroaromatic reagent.

Dissolve 0.7 g of p*otassium dichromate R* in *nitric acid R* and dilute to 100 mL with the same acid.

Nitroethane. $C_2H_5NO_2$. (M_r 75.1). [79-24-3].

A clear, colourless, oily liquid.

The boiling point is about 114 °C.

Nordazepam. $C_{15}H_{11}CIN_{20}$. (M_r 270.7). [1088-11-5]. 7-Chloro-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepine-2-one.

White or light yellow crystalline powder. Practically insoluble in water, slightly soluble in 96% ethanol.

The melting point is about 216 °C.

D, L-Norleucine. $C_6H_{13}NO_2$. (M_r 131.2). [616-06-8]. (*RS*)-2-Amino-hexanoic acid.

Shiny crystals. Sparingly soluble in water and 96% ethanol, soluble in acids.

Norpseudoephedrine hydrochloride. C9H13NO. (*Mr* 187.7). [53643-20-2]. (*1R,2R*) or (*1S,2S)*-2-Amino-1-phenylpropanol hydrochloride. Crystalline powder. Soluble in water.

The melting point is from 180 °C to 181 °C.

Noscapine hydrochloride. $C_{22}H_{24}ClNO_7 \cdot H_2O$. (*Mr* 467.9). [912-60-7]. (3S)-6,7-Dimethoxy-3-[(5R)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*H*]isochinoline-5-yl]-2-benzo-furan-1(3H)-one hydrochloride hydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_{22}H_{24}ClNO_7$calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals. Hygroscopic.

Freely soluble in water and 96% ethanol. Aqueous solutions are slightly acidic, and the base may precipitate when the solutions are standing.

The melting point is about 200 °C with decomposition.

Destaining solution.

A mixture of *glacial acetic acid R*/*methanol R*/*water R* (1:4:5).

Octadecyl[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propionate]. $C_{35}H_{62}O_3$. (*M_r* 530.9). [2082-79-3]. Octadecyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate.

White or slightly yellowish crystalline powder. Practically insoluble in water, very soluble in acetone and hexane, slightly soluble in methanol.

The melting point is from 49 °C to 55 °C.

Octanol. $C_8H_{18}O$. (M_r 130.2). [111-87-5]. Octanol-1. Caprylic alcohol.

A colorless liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.828.

The boiling point is about 195 °C.

3-Octanone. C8H16O. (*Mr* 128.2). [106-68-3]. Octan-3-one. Ethylpentylketone.

Colourless liquid with a characteristic odour.

d_{20}^{20} is about 0.822.

n_D^{20} is about 1.415.

The boiling point is about 167 °C.

*3-Octanone used in gas chromatography complies with the following additional tes*t.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Lavender oil*, using 3-octanone as the test solution.

3-Octanone content calculated by the internal normalisation procedure should be minimum 98.0%.

Octoxinol 10. $C_{34}H_{62}O_{11}$ (average). (M_r 647). [9002-93-1]. α-[4-(1,1,3,3-Tetramethyl-butyl)phenyl]-ω-hydroxypoly-(oxyethylene).

Clear light yellow viscous liquid. Miscible with water, acetone, and 96% ethanol, soluble in toluene.

Store in an airtight container.

Oleamide. $C_{18}H_{35}NO$. (M_r 281.5). (9*Z*)-Octadec-9-enoamide

White to yellowish powder or granules. Practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

The melting point is about 80 °C.

Olive oil. [8001-25-0].

An oily liquid obtained by cold pressing or other mechanical methods from mellow fruits of *Olea europaea L.*

Clear yellow or greenish-yellow liquid.

Practically insoluble in 96% ethanol, miscible with petroleum ether (50-70 °C).

When cooled to 10 °C, it becomes cloudy and oily mass at about 0 °C.

d_{20}^{20} is about 0.913.

Stannous chloride. $SnCl_2 \cdot 2H_2O$. (M_r 225.6). [10025-69-1]. Stannous dichloride dihydrate.

Contains not less than 97.0% of $SnCl_2 \cdot 2H_2O$.

Colourless crystals. Very soluble in water, freely soluble in 96% ethanol, glacial acetic acid, dilute and concentrated hydrochloric acid.

Quantitation. Place 0.500 g in a flask fitted with a ground-glass stopper, dissolve in 15 mL of *hydrochloric acid R*, add 10 mL of *water R* and 5 mL of *chloroform R*. Quickly titrate with *0.05 M potassium Iodate* until the chloroform layer is discharged.

1 mL of *0.05 M potassium Iodate solution* is equivalent to 22.56 mg of $SnCl_2 \cdot 2H_2O$.

Stannous chloride solution.

Heat 20 g of *tin R* with 85 mL of *hydrochloric acid R* until hydrogen stops to liberate, and cool. Store the solution over the excess of *tin R*, in an airtight container.

Stannous chloride solution R1.

Immediately before use, dilute a solution of *stannous chloride R* with *dilute hydrochloric acid R* (1:10).

Stannous chloride solution R2.

To 8 g of *stannous chloride R*, add 100 mL of 20% (*V/V*) solution of *hydrochloric acid R*, shake until dissolved, if necessary, heat in a water bath at 50 °C and pass *nitrogen R* for 15 min. Prepare immediately before use.

Tin. Sn. (A_r 118.7). [7440-31-5].

Silver-white granules. Soluble in hydrochloric acid with the release of hydrogen.

Arsenic (*2.1.4.2, Method A*). NMT 10 ppm. 0.1 g complies with the test for arsenic.

Oracet blue 2R. $C_{20}H_{14}N_2O_2$. (M_r 314.3). [4395-65-7].

Colour Index No. 61110. 1-Amino-4-(phenylamino)-anthracene-9,10-dione.

The melting point is about 194 °C.

Orcinol. $C_7H_8O_2 \cdot H_2O$. (M_r 142.2). [6153-39-5]. 5-Methylbenzene-1,3-diol monohydrate.

Light-sensitive crystalline powder.

The boiling point is about 290 °C.

The melting point is from 58 °C to 61 °C.

Osmium (VIII) oxide.OsO_4. (M_r 254.2). [20816-12-0].

Needle-like light yellow crystals or yellow crystalline mass.

Hygroscopic, light-sensitive, soluble in water, 96% ethanol.

Store in an airtight container.

Osmium (VIII) oxide solution.

A 2.5 g/L solution of in *0.05 M sulfuric acid*.

Palladium. Pd. (A_r 106.4). [7440-05-3].

Greyish white metal. Soluble in hydrochloric acid.

Palladium chloride. $PdCl_2$. (M_r 177.3). [7647-10-1].

Red crystals.

The melting point is from 678 °C to 680 °C.

Palladium chloride solution.

Dissolve 1 g of *palladium chloride R* in 10 mL of warm *hydrochloric acid R*, dilute the resulting solution to 250 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Immediately before use, dilute the solution with two volumes of *water R*.

Palmitic acid. $C_{16}H_{32}O_2$. (M_r 256.4). [57-10-3]. Hexadecanoic acid.

White or almost white crystal scales. Practically insoluble in water, freely soluble in hot 96% ethanol.

The melting point is about 63 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Chloramphenicol palmitate*; the resulting chromatogram shows only one principal spot.

Palmitic acid used in the quantitation of the number of fatty acids in the monograph Saw Palmetto fruit must comply with the following additional requirement.

Quantitation. Perform gas chromatography (*2.1.2.27*) as prescribed in the monograph *Saw Palmetto fruit*.

The content of palmitic acid calculated by the internal normalisation procedure should be minimum 98%.

Pancreatin powder.

It is obtained from the fresh or frozen mammalian pancreas. Contains various enzymes with proteolytic, lipolytic, and amylolytic activity.

1 mg of pancreatin contains not less than 1.0 PEU of total proteolytic activity, 15 PEU of lipolytic activity, and 12 PEU of amylolytic activity.

Slightly brown amorphous powder.

Partially soluble in water, practically insoluble in 96% ethanol.

Papaverine hydrochloride. $C_{20}H_{22}ClNO_4$. (M_r 375.9). [61-25-6]. 1-(3,4-Dimethoxybenzyl)-6,7-dimethoxyisoquinoline hydrochloride.

Contains not less than 99.0% and not greater than 101.0% of $C_{20}H_{22}ClXO_4$ calculated on a dry substance basis.

White or almost white crystalline powder or crystals.

Sparingly soluble in water, slightly soluble in 96% ethanol.

Pararosaniline hydrochloride. $C_{19}H_{18}ClK_3$. (M_r 323,8). [569-61-9]. 4-[Bis(4-aminophenyl)methylene]cyclo-hexa-2,5-dieniminium chloride.

Schultz No. 779.

Colour Index (C.I.) No. 42500.

Bluish red crystalline powder. Slightly soluble in water, soluble in anhydrous ethanol.

Solutions in water and ethanol have an intense red colour, solutions in sulfuric acid and hydrochloric acid have yellow colour.

The melting point is about 270 °C with decomposition.

Decolourised pararosaniline solution.

To 0.1 g of *pararosaniline hydrochloride R* in a ground-glass-stoppered flask, add 60 mL of *water R* and a solution of 1.0 g of *anhydrous sodium sulfite R* or 2.0 g of *sodium sulfite R* or 0.75 g of *sodium metabisulfite R* in 10 mL of *water R*, slowly and with stirring add 6 mL of *dilute hydrochloric acid R*, stopper the flask, and continue stirring until dissolution is complete, then dilute to 100 mL with *water R*.

The solution is used 12 h after preparation.

Store in a place protected from light.

Liquid paraffin. [8042-47-5].

Purified mixture of liquid saturated hydrocarbons obtained from oil.

Colourless, clear, oily liquid; does not fluoresce in daylight.

Practically insoluble in water, slightly soluble in 96% ethanol, miscible with hydrocarbons.

Paraffin, white soft.

Semi-liquid mixture of hydrocarbons obtained from oil and discoloured. Practically insoluble in water and 96% ethanol, soluble in *petroleum ether R1*; the solution sometimes shows a weak opalescence.

Paracetamol. $C_8H_9NO_2$. (M_r 151.2). [103-90-2]. *N*-(4-hydroxyphenyl)acetamide.

Contains not less than 99.0% and not greater than 101.0% of $C_8H_9NO_2$ calculated on a dry substance basis.

White or almost white crystalline powder.

Sparingly soluble in water, freely soluble in 96% ethanol, and very slightly soluble in methylene chloride.

4-aminophenol-free paracetamol.

Recrystallise *paracetamol R* from *water R* and dry *in vacuo* at 70 °C; repeat the procedure until paracetamol passes the following test: Dissolve 5 grams of dried paracetamol in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same solvent mixture.

Add 1 mL of a freshly prepared solution containing 10 g/L of *sodium nitroprusside R* and 10 g/L of *anhydrous sodium carbonate R*, mix and allow to stand for 30 min, protected from light. No blue or green colouring should appear.

Penicillinase solution.

Dissolve 10 g of casein hydrolysate, 2.72 g of *potassium dihydrogen phosphate R* and 5.88 g of *sodium citrate R* in 200 mL of *water R*, adjust the *pH* to 7.2 with a 200 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*. Dissolve 0.41 g of *magnesium sulfate R* in 5 mL of *water R*, add 1 mL of a 1.6 g/L solution of *ferrous ammonium sulfate R* and dilute to 10 mL with *water R*. Sterilise both solutions by heating in an autoclave, cool, mix, distribute in thin layers in conical flasks, and cultivate with *Bacillus cereus* (NOTC 9946). Maintain the flasks at a temperature of 18 °C to 37 °C until obvious signs of growth, and then maintain at a temperature of 35 °C to 37 °C for 16 h, constantly shaking to ensure maximum aeration. Centrifuge, sterilise the supernatant by membrane filtration. 1.0 mL of penicillinase solution contains not less than 0.4 microkatals (which corresponds to the hydrolysis of at least 500 mg of benzylpenicillin to benzylpenicillic acid per hour) at a temperature of 30 °C and *pH* 7, provided that the concentration of benzylpenicillin does not fall below the level required for enzyme saturation.

The Michaelis constant for penicillinase per benzylpenicillin in penicillinase solution is about 12 µg/mL.

Sterility (*2.1.6.1*). Must withstand the test for sterility. Store at a temperature from 0 °C to 2 °C and use within 2-3 days. Store the lyophilised drug in sealed ampoules for several months.

Pentane. C_5H_{12}. (M_r 72.2). [109-66-0].

A clear, colorless, flammable liquid. Very slightly soluble in water, miscible with acetone and with anhydrous ethanol.

d_{20}^{20} is about 0.63.

n_D^{20} is about 1.359.

The boiling point is about 36 °C.

Pentane used in spectrophotometry complies with the following additional test.

Determine the *minimum transmission* (*2.1.2.24*) using *water R* as compensation liquid.

0.70 at a wavelength of 200 nm,
0.30 at a wavelength of 210 nm,
0.07 at a wavelength of 220 nm,
0.03 at a wavelength of 230 nm,
0.01 at a wavelength of 240 nm.

1,2-Pentanediol. $C_5H_{12}O_2$. (M_r 104.2). [5343-92-0]. (2*RS*)-Pentane-1,2-diol.

d_4^{20} is about 0.971.

n_D^{20} is about 1.439.

The boiling point is about 201 °C.

Pentanol. $C_5H_{12}O$. (M_r 88.1). [71-41-0]. 1-Pentanol.

A colorless liquid. Sparingly soluble in water, miscible with 96% ethanol.

n_D^{20} is about 1.410.

The boiling point is about 137 °C.

Pentaerythrityltetrakis[3-(3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl)propionate]. $C_{73}H_{108}O_{12}$. (M_r 1178). [6683-19-8]. Pentaerythritiltetrakis[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate]. 2,2'-bis(Hydroxymethyl)Pro-pan-1,3-dioltetrakis[3-[3,5-di(1,1-dimethyl-ethyl)-4-hydroxyphenyl]]propionate.

White to slightly yellow crystalline powder. Practically insoluble in water, very soluble in acetone, soluble in methanol, slightly soluble in hexane.

The melting point is from 110 °C to 125 °C.

α-form from 120 °C to 125 °C.

β-form from 110 °C to 115 °C.

***tert*-Pentyl alcohol.** $C_5H_{12}O$. (M_r 88.1). [75-85-4]. *tert*-Amyl alcohol. 2-Methyl-2-butanol.

Volatile, flammable liquid. Freely soluble in water, miscible with 96% ethanol and glycerin.

d_{20}^{20} is about 0.81.

Distillation range (*2.1.2.11*). From 100 °C to 104 °C; not less than 95% must be distilled.

Store in a dark place.

Pepsin powder. [9001-75-6].

The powder is obtained from the gastric mucosa of pigs, cattle or sheep. Contains gastric proteinases active in acidic conditions (pH 1-5).

It has an activity of at least 0.5 PEU/mg calculated on a dry substance basis.

White or slightly yellow crystalline or amorphous powder. Hygroscopic.

Soluble in water, practically insoluble in 96% ethanol.

Aqueous solutions have a weak opalescence and a weak acid reaction.

Sand.

White or slightly greyish grains of silicon dioxide with a particle size between 150 µm and 300 µm.

Petroleum ether. [8032-32-4]. Petroleum ether 50-70 °C.

Clear colorless flammable liquid, does not fluoresce. Practically insoluble in water, miscible with 96% alcohol.

d_{20}^{20} is from 0.661 to 0.664.

Distillation range (*2.1.2.11*). 50 °C to 70 °C.

Petroleum ether R1. Petroleum ether 40-60 °C.

Complies with the requirements prescribed for *petroleum ether R*, with the following modifications:

d_{20}^{20} is from 0.630 to 0.656.

Distillation range (2.1.2.11). 40 °C to 60 °C.

Should not become cloudy at 0 °C.

Petroleum ether R2. Petroleum ether 30-40 °C.

Complies with the requirements prescribed for *petroleum ether R*, with the following modifications:

d2020 from 0.620 to 0.630.

Distillation range (*2.1.2.11*). 30 °C to 40 °C.

Should not become cloudy at 0 °C.

Petroleum ether R3. Petroleum ether 100-120 °C.

Complies with the requirements prescribed for *petroleum ether R*, with the following modifications:

d_{20}^{20} is from 0.720.

Distillation range (*2.1.2.11*). 100 °C to 120 °C.

Water (*2.1.5.12*). NMT 0.03%.

Picric acid. $C_6H_3N_3O_7$. (M_r 229.1). [88-89-1]. 2,4,6-Trinitrophenol.

Yellow prisms or plates. Soluble in water and 96% ethanol.

Store moistened with *water R*.

Picric acid solution.

A 10 g/l solution.

Picric acid solution R1.

Prepare 100 mL of a saturated solution of picric acid R *and add 0.25 mL of* strong sodium hydroxide solution R.

α-Pinene. $C_{10}H_{16}$. (M_r 136.2). [7785-70-8]. (1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene.

Liquid. Not miscible with water.

d_{20}^{20} is about 0.859.

n_D^{20} is about 1.466.

The boiling point of 154 °C to 156 °C.

α-Pinene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Bitter-orange-flower oil*, using α-pinene as the test solution.

The α-pinene content calculated by the internal normalisation procedure should be minimum 99%.

β-**Pinene**. $C_{10}H_{16}$. (M_r 136.2). [127-91-3]. 6,6-Dimethyl-2-methylenbicyclo[3.1.1] heptane.

colourless, oily liquid with a turpentine-like odour. Practically insoluble in water, miscible with 96% ethanol.

β-Pinene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) in accordance with the instructions of the monograph *Bitter-orange-flower oil*, using β-pinene as the test solution.

β-pinene content must be minimum 95.0%.

Piperazine hydrate. $C_4H_{10}N_2 \cdot 6H_2O$. (M_r 194.2). [142-63-2]. Hexagonal piperazine.

Contains not less than 98.0% and not greater than 101.0% of $C_4H_{10}N_2 \cdot 6H_2O$.

Colourless crystals.

Freely soluble in water and 96% ethanol.

The melting point is about 43 °C.

Piperidine. $C_5H_{11}N$. (M_r 85.2). [110-89-4]. Hexahydropyridine.

Colourless or slightly yellow, alkaline liquid. Miscible with water, 96% ethanol, and petroleum ether.

The boiling point is about 106 °C.

Pyridine-2-amine. C_5H6N_2. (*Mr* 94.1). [504-29-0]. 2-Aminopyridine.

Large crystals. Soluble in water and 96% ethanol.

The boiling point is about 210 °C.

The melting point is about 58 °C.

Pyridylazonaphthol. $C_{15}H_{11}N_3O$. (M_r 249.3). [85-85-8]. 1-(2-Pyridylazo)-2-naphthol.

Brick-red powder. Practically insoluble in water, soluble in 96% ethanol, methanol, and hot dilute solutions of alkali metal hydroxides.

The melting point is about 138 °C.

Pyridylazonaphthol solution.

A 1 g/L solution in *anhydrous ethanol R.*

Test for sensitivity. To 50 mL of water R, add 10 mL of acetate buffer solution pH 4.4 R, 0.10 mL of *0.02 M sodium edetate*, and 0.25 mL of pyridylazonaphthol solution; after adding 0.15 mL of a 5 g/L solution of *copper sulfate R*, the colour should change from light yellow to violet.

Pyridine. C_5H_5N. (M_r 79.1). [110-86-1].

Clear colorless hygroscopic liquid. Miscible with water and 96% ethanol.

The boiling point is about 115 °C.

Store in an airtight container.

Pyridine, anhydrous.

Dry *pyridine R over anhydrous sodium carbonate R*, filter, and distill.

Water (*2.1.5.12*). NMT 0.01% (*w/w*).

Pyridinium hydrobromide perbromide. $C_5H_6Br_3N$. (M_r 319.8). [39416-48-3]. Pyridinium tribromide(1-).

Red crystals.

Pyruvic acid. C_3H4O_3. (*Mr* 88.1). [127-17-3]. 2-Oxopropanoic acid.

A yellowish liquid. Miscible with water and anhydrous ethanol.

d_{20}^{20} is about 1.267.

n_D^{20} is about 1.413.

The boiling point is about 165 °C.

Pyrogallol. C_6H_6O3. (M_r 126.1). [87-66-1]. Benzene-1,2,3-triol.

White or almost white crystals that turn brown under exposure to light and air. Very soluble in water, 96% ethanol, slightly soluble in carbon disulfide. On exposure to air, aqueous solutions, and more rapidly alkaline solutions, become brown owing to the absorption of oxygen.

The melting point is about 131 °C.

Store in a place protected from light.

Pyrogallol, alkaline.

Dissolve 0.5 g of pyrogallol R *in 2 mL of* carbon dioxide-free water R. *Dissolve 12 g of* potassium hydroxide R *in 8 mL of* carbon dioxide-free water R. *Mix the two solutions immediately before use.*

Pyrocatechol. $C_6H_6O_2$. (M_r 110.1). [120-80-9]. Benzene-1,2-diol.

Colourless or slightly yellow crystals. Soluble in water, acetone, 96% ethanol.

The melting point is about 102 °C.

Store in a place protected from light.

Plasma with a low platelet count.

Withdraw 45 mL of human blood into a 50 mL plastic syringe containing 5 mL of a sterile 38 g/L solution of *sodium citrate R*, and without delay, centrifuge at 1500 *g* at 4 °C for 30 min. Remove the upper 2/3 of the supernatant plasma using a plastic syringe and without delay centrifuge at 3500 *g* at 4 °C for 30 min. Remove the upper 2/3 of the liquid and freeze it rapidly in suitable amounts in plastic tubes at or below − 40 °C. Use plastic equipment or equipment treated with silicone.

Plasminogen, human. [9001-91-6].

A substance present in blood that may be activated to plasmin, an enzyme that lyses fibrin in blood clots.

Plasma substrate.

Separate the plasma from human or bovine blood collected into one-ninth its volume of a 38 g/L solution of *sodium citrate R*, or into two-sevenths its volume of a solution containing 20 g/L of *disodium hydrogen citrate R* and 25 g/L of *glucose R*. With the former, prepare the substrate on the day of collection of the blood; with the latter, prepare within 2 days of collection of the blood.

Store at -20 °C.

Plasma substrate R1.

Water-repellent equipment (made of suitable plastics or glass treated with silicone) is used for blood collection and processing. Collect a suitable volume of blood from each of at least five sheep. A 285 mL volume of blood collected into 15 mL of anticoagulant solution is suitable but smaller volumes may be collected. Blood is taken from a live animal or during slaughter, using a needle attached to a suitable cannula with a length sufficient to reach the bottom of the collection vessel. Discard the first few millilitres and collect only free-flowing blood. Collect the blood in a sufficient quantity of an anticoagulant solution containing 8.7 g of *sodium citrate R* and 4 mg of *aprotinin R* per 100 mL of *water R* to give a final ratio of blood to the anticoagulant solution of 19 to 1. During collection and immediately after collection, mix the blood gently, preventing foaming. At the end of the collection, close the vessel and cool to 10-15 °C. After cooling, the contents of all flasks are combined with the exception of those in which there is obvious hemolysis or clot formation, and the collected blood is stored at a temperature of 10 °C to 15 °C. If possible, within 4 h after collection, centrifuge the pooled blood at acceleration from1000 *g* to 2000 *g* at a temperature from 10 °C to 15 °C for 30 min. Separate and centrifuge the supernatant at an acceleration of 5000 *g* for 30 min. (If necessary, to obtain a clear plasma, it can be centrifuged with high acceleration, for example, at 20000 *g* for 30 min, but filtration is not allowed.) Separate the supernatant, immediately mix thoroughly and place the plasma substrate in small containers fitted with stoppers in portions sufficient to perform a complete quantitation of heparin (e.g., 10 mL to 30 mL). Immediately cool to a temperature below -70 °C (for example, by immersing containers in liquid nitrogen) and store at a temperature below -30 °C. Plasma is suitable as a plasma substrate for the quantitation of heparin if, under assay conditions, it provides a clot formation time corresponding to the determination method used and provides steep logarithmic dose-response curves. Before use, thaw the required portion of the plasma in a water bath at 37 °C, stirring gently until it is completely thawed. Allow thawed plasma to stand at 10-20 °C and immediately use it. If necessary, slightly centrifuge the thawed plasma substrate but not filter.

Plasma substrate R2.

Prepare from human blood containing less than 1% of the usual amount of factor IX. Blood is collected into a 38 g/l *sodium citrate R* solution, the volume of which is 1/9 of the plasma volume.

Store in small amounts in plastic containers at a temperature of -30 °C or lower.

Plasma substrate deficient in factor V.

Preferably, use plasma obtained from a donor with congenital insufficiency, or prepare it as follows: separate the plasma from human blood collected in a 13.4 g/L solution of *sodium oxalate R*, the volume of which is 1/10 of the blood volume. Incubate at 37 °C for 24-36 h. The clotting time determined by the method, in accordance with the descriptions for the solution of *blood clotting factor V R*, should be from 70 s to 100 s. If the clotting time is less than 70 s, then incubate again for 12-24 h.

Store in small amounts at -20 °C or lower.

Povidone. $C_{6n}H_{9n+2}N_nO_n$. [9003-39-8]. *a*-Hydro-*ω*-hydropoly[1-(2-oxopyrrolidine-1-yl)ethylene].

Consists of linear polymers of 1-ethenyl-pyrrolidine-2-one. Contains not less than 11.5% and not greater than 12.8% of nitrogen (N; A_r14.01) calculated with reference to the anhydrous substance.

Various types of povidone are characterised by their viscosity in solution, expressed as the *K* value. The nominal K value is from 10 to 120.

White or yellowish white powder or plates. Hygroscopic.

Freely soluble in water, 9b% ethanol and methanol, very slightly soluble in acetone.

Sunflower oil.

An oily liquid obtained from the seeds of *Helianthus annuus L.* by mechanical pressing or extraction. A suitable antioxidant may be added.

A clear light yellow liquid.

Practically insoluble in water and 96% ethanol, miscible with petroleum ether (40-60 °C).

d_{20}^{20} is equal to 0.921.

n_D^{20} is about 1.474.

Poly(dimethyl)(diphenyl)siloxane. DB-5, SE52.

Stationary phase for gas chromatography.

Contains 95% of methyl groups and 5% of phenyl groups DB-5, SE52.

Poly(dimethyl)(75)(diphenyl)(25)siloxane.

Stationary phase for gas chromatography.

Contains 75% of methyl groups and 25% of phenyl groups.

Poly(dimethyl)(85)(diphenyl)(15)siloxane.

PS086.

Stationary phase for gas chromatography. Contains 85% of methyl groups and 15% of the phenyl groups.

Poly(dimethyl)(diphenyl)(divinyl)siloxane. SE54.

Stationary phase for gas chromatography.

Contains 94% of methyl groups, 5% of phenyl groups, and 1% of vinyl groups.

Poly(dimethyl)siloxane.

Silicone rubber (methyl). Organosilicon polymer that looks like a semi-liquid colorless resin.

The intrinsic viscosity determined as indicated below should be about 115 mL/g. Weigh 1.5 g, 1 g and 0.3 g of poly(dimethyl)siloxane to an accuracy of 0.1 mg into 100 mL volumetric flasks, add 40 mL to 50 mL of *toluene R*, shake until dissolved, and dilute to 100.0 mL with the same solvent. Determine the viscosity (*2.1.2.9*) of each solution and the viscosity of *toluene R* under the same conditions. Halve the concentration of each solution by diluting with *toluene R* and determine the viscosity of the resulting solutions.

c is the concentration, g/100 mL; t_1 is the flow time of the test solution; t_2 is the toluene flow time; η_1 is the viscosity of the test solution, MPa·s; η_2 is the toluene viscosity, MPa· s; d_1 is the relative density of the test solution; d_2 is the relative density of toluene.

To obtain the values of the relative density, use the following data:

Concentration (*c*), g/100 mL	Relative density(*d*)
0 0.5	1.000
0.5 - 1.25	1.001
1.25-2.20	1.002
2.20-2.75	1.003
2.75-3.20	1.004
3.20-3.75	1.005
3.75-450	1.00

The specific viscosity (η_{sp}) is determined by the equation:

$$\eta_{уд} = \frac{\eta_1 - \eta_2}{\eta_2} = \frac{t_1 d_1}{t_2 d_2} - 1.$$

The reduced viscosity (η_{red}) is determined by the equation:

$$\eta_{пр} = \frac{\eta_{уд}}{c}.$$

The Intrinsic viscosity (η) is obtained by extrapolating the previous equation to $c = 0$.

To do this, plot the η_{sp}/s curve or log η_{sp}/s as a function of C. Extrapolation to c = 0 yields n. The intrinsic viscosity is expressed in mL/g, so the resulting value must be multiplied by 100. The infrared absorption spectrum (*2.1.2.23*) obtained by applying a substance, if necessary dispersed in a few drops of *carbon tetrachloride R*, to a disk of sodium chloride, should not have absorption at a wavelength of 3053 cm^{-1} correspondings to the vinyl groups.

Loss on drying (*2.1.2.31*). NMT 2.0%. Determination is carried out from 1.000 g, dried in vacuo at 350 °C for 15 min. NMT 0.8%. Determination is carried out from 2.000 g, dried at 200 °C for 2 h.

Polymethylphenylsiloxane.

Stationary phase for gas chromatography.

Contains 50% of methyl groups and 50% of phenyl groups. (The average molecular weight is 4000.) Very viscous liquid (the viscosity is about 1300 MPa·s).

d_{25}^{25} is about 1.09.

n_D^{20} is about 1.540.

Poly[methyl(95)phenyl(5)]siloxane.

See *Poly(dimethyl)(diphenyl)siloxane R.*

Poly[methyl(94)phenyl(5)vinyl(1)]siloxane.

See *Poly(dimethyl)-(diphenyl)(divinyl)siloxane R.*

Polyoxyethylated castor oil.

Light yellow liquid that becomes clear at a temperature of about 26 °C.

Polysorbate 20. [9005-64-5].

A mixture of partially esterified fatty acids, mainly lauric acid (dodecanoic), with sorbitol and its ethoxylated anhydrides with approximately 20 mol of ethylene oxide for each mole of sorbitol or its anhydrides.

Oily yellow or brownish-yellow, clear or slightly opalescent liquid.

Soluble in water, anhydrous ethanol, ethyl acetate, and methanol, practically insoluble in fatty oils and liquid paraffin.

d_{20}^{20} is about 1.10.

The viscosity is about 400 mPa· s at a temperature of 25 °C.

Polysorbate 80. Tween 80. [9005-65-6].

A mixture of partially esterified fatty acids, mainly *Oleic acid*, with sorbitol and its ethoxylated anhydrides, with approximately 20 mol of ethylene oxide for each mole of sorbitol and its anhydrides.

Oily, colorless or brownish-yellow, clear or slightly opalescent liquid.

Miscible with water, anhydrous ethanol, ethyl acetate, and methanol, practically insoluble in fatty oils and liquid paraffin.

d_{20}^{20} is about 1.10.

The viscosity is about 400 mPa· s at a temperature of 25 °C.

Polystyrene 900-1000. [9003-53-6].

Organic standard used for calibration in gas chromatography. M_m about 950. M_m/M_n 1.10.

Poly (cyanopropyl)siloxane.

Polysiloxane replaced by 100% of cyanopropyl groups.

Poly[(cyanopropyl)(phenyl)][dimethyl]-siloxane.

Stationary phase for gas chromatography.

Contains 6% of cyanopropylphenyl groups and 94% of dimethyl groups.

Poly(cyanopropyl)(7)(phenyl)(7)(methyl) (86)-siloxane.

Stationary phase for gas chromatography.

Polysiloxane replaced by 7% of cyanopropyl groups, 7% phenyl groups, and 86% of dimethyl groups.

Poly(cyanopropyl)(phenylmethyl)siloxane.

Stationary phase for gas chromatography.

Contains 90% of cyanopropyl groups and 10% of phenylmethyl groups.

Poly[(cyanopropyl)(methyl)][(phenyl)(me-til)]siloxane.

Contains 25% of cyanopropyl groups, 25% of phenyl groups, and 50% of methyl groups. (The average molecular weight is 8000.)

Very viscous liquid (the viscosity is about 9000 MPa·s).

d_{25}^{25} is about 1.10.

d_{20}^{20} is about 1,502.

Polyethyleneglycol adipate. $(C_8H_{12}O_4)_n$. [M_r $(172.2)_n$].

White or almost white waxy mass. Practically insoluble in water. The melting point is about 43 °C.

Polyethyleneglycol succinate. $(C_6H_8O_4)_n$. [M_r $(144.1)_n$].

White or almost white crystalline powder. Practically insoluble in water.

The melting point is about 102 °C.

Chromatographic grade polyester hydroxylated gel.

A gel with a small particle size that has a hydrophilic surface to hydroxyl groups. It has a dextran exclusion limit with a molecular mass from 2 x 10^5 to 2.5 x 10^6.

Procaine hydrochloride. $C_{13}H_{21}ClN_2O_2$. (M_r 272.8). 2-(Diethylamino)ethyl-4-aminobenzoate hydrochloride.

Contains not less than 99.0% and not greater than 101.0% of $C_{13}H_{21}ClN_2O_2$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Very soluble in water, soluble in 96% ethanol.

D-Prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride. $C_{26}H_{36}N_8O_5$. (M_r 612).

Propanol. C_3H_8O. (M_r 60.1). [71-23-8]. 1-Propanol.

A clear, colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is from 0.802 to 0.806.

The boiling point is about 97.2 °C.

Distillation range (2.1.2.11). From 96 °C to 99 °C; not less than 95% must be distilled.

Propanol R1. [71-23-8].

See *Propanol R.*

2-Propanol. C_3H_8O. (M_r 60.1). [67-63-0]. Isopropyl alcohol.

A clear, colorless, flammable liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.785.

The boiling point is from 81 °C to 83 °C.

2-Propanol R1.

Complies with the requirements for *2-propanol R* and the following additional requirements:

n_D^{20} is about 1.378.

Water (2.1.5.12) NMT 0.05%. Determination is carried out from 10 g.

Determine the *minimum transmission* (*2.1.2.24*) using *water R* as compensation liquid.

25% at a wavelength of 210 nm

55% at a wavelength of 220 nm

75% at a wavelength of 230 nm

95% at a wavelength of 250 nm

98% at a wavelength of 260 nm.

2-Propanol R2. [67-63-0].

See *Isopropyl alcohol.*

Propanolamine. C_3H_9NO. (M_r 75.1). [156-87-6]. 3-Amino-1propanol.

Transparent colorless viscous liquid.

d_{20}^{20} is about 0.99.

n_D^{20} is about 1.461.

The melting point is about 11 °C.

Propyl acetate. $C_5H_{10}O_2$. (M_r 102.1). [109-60-4].

d_{20}^{20} is about 0.888.

The boiling point is about 102 °C.

The melting point is about -95 °C.

Propylene glycol. $C_3H_8O_2$. (M_r 76.1). [57-55-6]. (*RS*)-Propane-1,2-diol.

Viscous, clear, colourless, hygroscopic liquid. Miscible with water and 96% ethanol.

Propylene oxide. C_3H_6O. (M_r 58.1). [75-56-9].

A colorless liquid. Miscible with 96% ethanol.

Propyl parahydroxybenzoate. $C_{10}H_{12}O_3$. (M_r 180.2). [94-13-3]. Propyl-4-hydroxybenzoate.

Contains not less than 98.0% and not greater than 102.0% of $C_{10}H_{12}O_3$.

White or almost white crystalline powder.

Very slightly soluble in water, freely soluble in 96% ethanol and methanol.

Propanoic acid. $C_3H_6O_2$. (M_r 74.1). [79-09-4].

Oily liquid. Soluble in 96% ethanol, miscible with water.

d_{20}^{20} is about 0.993.

n_D^{20} is about 1.387.

The boiling point is about 141 °C

The melting point is about -21 °C

Propane aldehyde. C_3H_6O. (M_r 58.1). [123-38-6]. Propanal.

Liquid. Freely soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.81.

n_D^{20} is about 1,365

The boiling point is about 49 °C.

The melting point is about -81 °C.

Propanoic anhydride. $C_6H_{10}O_3$. (M_r 130,1). [123-62-6].

A clear, colorless liquid. Soluble in 96% ethanol.

d_{20}^{20} is about 1.01. The boiling point is about 167 °C.

Propanoic anhydride reagent.

Dissolve 1 g of *toluenesulfonic acid R* in 30 mL of *glacial acetic acid R* and add 5 mL of *propane anhydride R*. Use 15 min after preparing. Use within 1 day.

Protamine sulfate. [9009-65-8].

Consists of sulfates of the basic peptides extracted from the sperm or eggs of fish, usually the species *Salmonidae* and *Clupeidae*. Binds with heparin in solution, inhibiting its anticoagulant activity; precipitated under conditions of the analysis. 1 mg of protamine sulfate precipitates not less than 100 IU of heparin calculated on a dry substance basis.

White or almost white powder. Hygroscopic.

Sparingly soluble in water, practically insoluble in 96% ethanol.

Staphylococcus aureus strain V8 protease. Type XVII-B. [66676-43-5].

Microbial extracellular proteolytic enzyme. The lyophilised powder contains from 500 units to 1000 units in 1 mg of solution.

Mordant black 11. $C_{20}H_{12}N_3NaO_7S$. (M_r 461.4). [1787-61-7]. Sodium 2-hydroxy-1-[(1-hydroxynaft2-yl)azo]-6-nitronaphthalene-4-sulfonate. Eriochrome black.

Schultz No. 241.

Colour Index (C. I.) No. 14645.

Brownish black powder. Soluble in water and 96% ethanol.

Store in an airtight container, protected from light.

Mordant black 11 indicator.

Mix 1 g of *mordant black 11 R* with 99 g of *sodium chloride R*.

Test for sensitivity. Dissolve 50 mg of the indicator in 100 mL of *water R*; a brownish-violet colour appears, which should turn blue when 0.3 mL of a *dilute ammonia solution R1* is added. With the subsequent addition of 0.1 mL of a 10 g/L solution of *magnesium sulfate R*, the colour should change to violet. Store in an airtight container, protected from light.

Mordant black 11 indicator R1.

Mix 1 g of *mordant black 11 R* with 0.4 g of *methyl orange R* and 100 g of *sodium chloride R*.

Fast red B salt. C_{17} H_{13} $N_3O_9S_2$. (M_r 467.4). [49735-71-9]. 2-Methoxy-4-nitrobenzenediazonium hydronaphthalene-1,5-disulfonate.

Schultz No. 155.

Colour Index (C. I.) No. 37125.

Orange-yellow powder. Soluble in water, slightly soluble in 96% ethanol.

Store in an airtight container in a dark place at a temperature from 2 °C to 8 °C.

Fast blue B salt. $C_{14}H_{12}Cl_2N_4O_2$. (*Mr* 339.2). [84633-94-3]. 3,3'-Dimethoxy(biphenyl)-4,4'-bisdiazonium dichloride.

Schultz No. 490.

Colour Index (C. I.) No. 37235.

Dark green powder. Soluble in water. Stabilised with zinc chloride.

Store in an airtight container at a temperature from 2 °C to 8 °C.

Developer solution.

To 2.5 mL of a 20 g/L solution of *citric acid R*, add 0.27 mL of *formaldehyde R* and dilute to 500.0 mL with *water R*.

Pulegone. $C_{10}H_{16}O$. (M_r 152.2). [89-82-7]. (*R*)-2-Isopropylidene-5-methylcyclohexanone. (+)-*p*-Menth-4-en-3-one.

A colorless oily liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{15}^{20} is about 0.936.

n_D^{20} is from 1.485 to 1.489.

$[\alpha]_D^{20}$ is between + 19.5 and + 22.5.

The boiling point is from 222 °C to 224 °C.

Pulegone used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil*, using pulegone as the test solution.

The content of pulegone calculated by the internal normalisation procedure should be minimum 98.0%.

Working solution for electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate [SDS-PAGE].

Dissolve 151.4 g of *tris(hydroxymethyl)aminomethane R*, 721.0 g of *glycine R*, 50.0 g of *sodium lauryl sulfate R* in *water R* and dilute to 5000 mL with the same solvent. Immediately before use, dilute with *water R* 10 times and mix. Measure the pH (*2.1.2.3*) of the dilute solution. The pH should be between 8.1 and 8.8.

Raclopride tartrate. $C_{19}H_{26}Cl_2N_2O_9$. (M_r 497.3). [98185-20-7].

Raclopride L-tartrate.

Solid substance of white or almost white colour, light-sensitive, soluble in water.

$[\alpha]_D^{20}$ + 0.3. Determination is carried out using a 3 g/L solution.

The melting point is about 141 °C.

Rhamnose. $C_6H_{12}O_5 \cdot H_2O$. (M_r 182.2). [6155-35-7]. (2*R*,3*R*,4*R*,5*R*,6*S*)-6-Methyltetrahydro-2*H*-pyran-2,3,4,5-of tetrol monohydrate. 6-Deoxy-α L-mannopyranose monohydrate. α-L-Rhamnopyranose monohydrate L-(+)-Rhamnose monohydrate.

White or almost white crystalline powder. Freely soluble in water.

$[\alpha]_D^{20}$ is between +7.8 and +8.3. Perform determination using a 50 g/l solution in *water R* containing about 0.05% of NH_3.

Rhaponticin. $C_{21}H_{24}O_9$. (M_r 420.4). [155-58-8].

3-Hydroxy-5-[2-(3-hydroxy-4-methoxyphenyl)ethenyl]phenyl β-D-glucopyranoside.

Yellowish grey crystalline powder. Soluble in 96% ethanol and methanol.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Rhubarb*; the chromatogram shows only one principal spot.

Rapeseed oil.

An oily liquid obtained from the seeds of *Brassica napus L.* and *Brassica campestris L.* by pressing or extraction. A suitable antioxidant may be added.

A clear light yellow liquid.

Practically insoluble in water and 96% ethanol, miscible with petroleum ether (40-60 °C).

d_{20}^{20} is about 0.917.

n_D^{20} is about 1.473.

Solution for the suitability test of TLC plates.

Mix 1.0 mL of a 0.5 g/L solution of *sudan red G R* in *toluene R*, a freshly prepared 0.5 g/L solution of *methyl orange R* in *anhydrous alcohol R*, a 0.5 g/L solution of *bromocresol green R* in *acetone R*, a 0.25 g/L solution of *methyl red R* in *acetone R* and dilute the resulting solution to 10.0 mL with *acetone R*.

Electrolyte solution for micro-quantitation of water.

A commercially available anhydrous reagent or a combination of anhydrous reagents for colourimetric titration of water, consisting of organic bases, sulfur dioxide, and iodine dissolved in a suitable solvent.

Resorcinol. $C_6H_6O_2$. (M_r 110.1). [108-46-3]. Benzene-1,3-diol.

Contains not less than 98.5% and not greater than 101.0% of $C_6H_6O_2$ calculated on a dry substance basis.

Colourless or slightly pinkish grey crystalline powder or crystals that redden under exposure to light and air. Very soluble in water and 96% ethanol.

Resorcinol reagent.

To 80 mL of *hydrochloric acid R*, add 10 mL of a 20 g/L solution of *resorcinol R*, 0.25 mL of a 25 g/L solution of *copper sulfate R*, and dilute to 100.0 mL with *water R*.

Use 4 h after preparation.

Store at a temperature between 2 °C and 8 °C.

Storage: use within 7 days.

Ribose. $C_5H_{10}O_5$. (M_r 150.1). [50-69-1]. D-Ribose.

Soluble in water, slightly soluble in 96% ethanol.

The melting point is from 88 °C to 92 °C.

Ricinoleic acid. $C_{18}H_{34}O_3$. (*M*r 298.5). [141-22-0].

(9*Z*,12*R*)-12-hydroxyoctadec-9-enoic acid. 12-Hydroxyoleic acid.

Yellow to yellowish-brown viscous liquid. Contains a mixture of fatty acids obtained by hydrolysis of castor oil. Practically insoluble in water, very soluble in anhydrous ethanol.

d_{20}^{20} is about 0.942.

n_D^{20} is about 1.472.

The melting point is about 285 °C with decomposition.

Rhodamine B. $C_{28}H_{31}C1N_2O_3$. (M_r 479.0). [81-88-9]. [9-(2-Carboxyphenyl)-6-(diethylamino)-3*H*-xanthene-3-ylidene]diethylammonium chloride.

Schultz No. 864.

Colour Index (C.I.) No. 45170.

Green crystals or reddish-violet powder. Very soluble in water and 96% ethanol.

Rhodamine 6 G. $C_{28}H_{31}ClN_2O_3$. (M_r 479.0). [989-38-8]. 9-[2-(ethoxycarbonyl)phenyl]-3,6-bis (ethylamino)-2,7-dimethylxanthene chloride.

Colour Index (C.I.) No. 45160. Brownish red powder.

Rosmarinic acid. $C_{18}H_{16}O_8$. (M_r 360.3). [20283-92-5].

The melting point is from 170 °C to 174 °C.

Mercury. Hg. (A_r 200.6). [7439-97-6].

Silver-white liquid, breaking into spherical globules that do not leave a metallic trace when rubbed on paper.

d_{20}^{20} is about 13.5.

The boiling point is about 357 °C.

Mercuric acetate. $C_4H_6HgO_4$. (M_r 318.7). [1600-27-7]. Mercuric diacetate.

White or almost white crystals. Very soluble in water, soluble in 96% ethanol.

Mercuric acetate solution.

Dissolve 3.19 g of *mercuric acetate R* in *anhydrous acetic acid R* and dilute with the same acid to 100 mL. If necessary, neutralise the resulting solution with a *0.1 M perchloric acid*, using 0.05 mL of *crystalline violet solution R* as indicator.

Mercuric bromide. $HgBr_2$. (M_r 360.4). [7789-47-1]. Mercuric dibromide.

White or light yellow crystals or crystalline powder. Slightly soluble in water, soluble in 96% ethanol.

Mercuric bromide paper.

In a rectangular cup, place a 50 g/L solution of *mercuric bromide R* in *anhydrous ethanol R* and immerse in the solution pieces of white filter paper with a density of 80 g/m^2 (the filtration rate is equal to the time of filtration, expressed in seconds, during the filtration of 100 mL of water at 20 °C through a filter surface of 10 cm^2, and at the constant pressure of 6.7 kPa: from 40 *s* to 60 s), with a size of 1.5 cm × 20 cm, folded twice. Hang the paper on a non-metallic thread, allowing excess liquid to flow down, and dry in a dark place. Cut 1 cm from each end of each strip and cut the rest of the paper into squares with a side of 1.5 cm or disks with a diameter of 1.5 cm.

Store in a container with a glass stopper, wrapped in black paper.

Mercuric iodide. HgI_2. (M_r 454.4). [7774-29-0]. Mercuric diiodide.

Bright red dense crystalline powder. Slightly soluble in water, sparingly soluble in acetone, 96% ethanol, soluble in excess of *potassium iodide R* solution.

Store in a place protected from light.

Mercuric nitrate. $Hg(NO_3)_2 \cdot H_2O$. (M_r 342.6). [7783-34-8]. Mercuric dinitrate monohydrate.

Colourless or slightly coloured crystals. Hygroscopic, soluble in water in the presence of a small amount of nitric acid.

Store in an airtight container, protected from light.

Mercuric oxide. HgO. (M_r 216.6). [21908-53-2]. Mercuric oxide is yellow. Oxide of mercury.

Yellow to orange-yellow powder. Practically insoluble in water and 96% ethanol.

Store in a place protected from light.

Mercuric sulfate solution. [7783-35-9].

Dissolve 1 g of *mercuric oxide R* in a mixture of 20 mL of *water R* and 4 mL of *sulfuric acid R*

Mercuric thiocyanate. $Hg(SCN)_2$. (M_r 316.7). [592-85-8]. Mercuric di(thiocyanate).

White or almost white crystalline powder. Very slightly soluble in water, slightly soluble in 96% ethanol, soluble in sodium chloride solutions.

Mercuric thiocyanate solution.

Dissolve 0.3 g of *mercuric thiocyanate R* in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

Storage: use within 7 days.

Mercury (II) chloride. $HgCl_2$. (M_r 271.5). [7487-94-7]. Mercuric chloride.

Contains not less than 99.5% and not greater than 100.5% of $HgCl_2$ calculated on a dry substance basis.

White or almost white crystalline powder, or colorless or white or almost white crystals, or a heavy crystalline mass.

Soluble in water and glycerin, freely soluble in 96% ethanol.

Mercuric chloride solution.

A 54 g/L solution.

Ruthenium red.

$[(NH_3)_5RuORu(NH_3)_4ORu(NH_3)_5]C1_6 \cdot 4H_2O$. ($M_r$ 858). [11103-72-3].

Brownish red powder. Soluble in water.

Red ruthenium solution.

A 0.8 g/L solution in *lead(II) acetate solution R.*

Ruthin. [250249-75-3].

See *Rutoside trihydrate R.*

Rutoside trihydrate. $C_{27}H_{30}O_{16} \cdot 3H_2O$. ($M_r$ 665). [250249-75-3]. Routine trihydrate.

2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromene-3-yl-6-*O*-(6deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside trihydrate.

Yellow crystalline powder that darkens under exposure to light. Very slightly soluble in water, soluble in about 400 parts of boiling water, slightly soluble in 96% ethanol, soluble in solutions of alkali metal hydroxides and ammonia.

The melting point is about 210 °C with decomposition.

Rutoside solution in *96% ethanol R* has two absorption maxima (*2.1.2.24*) at wavelengths of 259 nm and 362 nm.

Store in a place protected from light.

Sabinene. $C_{10}H_{16}$. (M_r 136.2). [3387-41-5]. Tui-4(10)-en.

4-Methylene-1-isopropylbicyclo[3.1.0]hexane.

A colorless oily liquid.

Sabinene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Bitter-orange-flower oil*, using sabinene as the test solution.

The sabinene content calculated by the internal normalisation procedure should be minimum 95.0%.

Salicylic acid. $C_7H_6O_3$. (M_r 138.1). [69-72-7]. 2-Hydroxybenzene carboxylic acid.

Contains not less than 99.0% and not greater than 100.5% of $C_7H_6O_3$ calculated on a dry substance basis.

White or almost white crystalline powder, white or colorless needle-like crystals.

Slightly soluble in water, freely soluble in 96% ethanol, sparingly soluble in methylene chloride.

Salicylaldehyde. $C_7H_6O_2$. (M_r 122.1). [90-02-8]. 2-Hydroxybenzaldehyde.

A clear, colourless, oily liquid.

d_{20}^{20} is about 1.167.

n_D^{20} is about 1.574.

The boiling point is about 196 °C.

The melting point is about -7 °C.

Salicylaldehyde azine. $C_{14}H_{12}N_2O_2$. (M_r 240.3). [959-36-4]. 2,2'-Azino-dimethyldiphenol.

Dissolve 0.30 g of *hydrazine sulfate R* in 5 mL of *water R*, add 1 mL of *glacial acetic acid R* and 2 mL of a freshly prepared 20% (*V/V*) solution of *salicylic aldehyde R* in *2-propanol R*. Stir, allow to stand until a yellow precipitate is formed, then shake with two portions of *methylene chloride R*, each of 15 mL. Decant or filter the combined organic extracts, dried over *anhydrous sodium sulfate R*, and evaporate to dryness. The precipitate is recrystallised by cooling from a mixture of *methanol R*/*toluene R* (40:60). The crystals are dried in vacuo.

The melting point is about 213 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Povidone* in the hydrazine test; the chromatogram shows only one principal spot.

Santonin. $C_{15}H_{15}O_3$. (M_r 246.3). (-)-α-Santonin. 3,5α,9-Trimethyl-3α,5,5α,9β-tetrahydro-*3H,4H*-naphtho[1,2]-furan-2,8-dione.

Colourless shiny crystals that turn yellow under the exposure to light. Very slightly soluble in water, freely soluble in hot 96% alcohol, sparingly soluble in ethanol.

The melting point is from 174 °C to 176 °C.

$[\alpha]_D^{18}$ - 173 in ethanol.

Chromatography. Determination is carried out in accordance with the instructions in the test.

Identification C in the monograph *Arnica flower*; a dark spot with an R_f of about 0.5 should be detected on the chromatogram obtained with 10 µl of the solution. Spray the chromatogram with a solution of *anisaldehyde R*, heat at a temperature of 105 °C for 5-10 min. On the chromatogram in daylight, a spot of initially yellow colour is observed, which then quickly turns violet-red.

Sucrose. $C_{12}H_{22}O_{11}$. (M_r 342.3). [57-50-1].

β-D-Fructofuranosyl α-d-glucopyranoside.

The substance does not contain additives.

White or almost white crystalline powder or shiny, colourless, or white or almost white crystals.

Very soluble in water, slightly soluble in 96% ethanol, practically insoluble in anhydrous ethanol.

Lead (II) acetate. $C_4H_6O_4Pb{\cdot}3H_2O$. (M_r 379.3). [6080-56-4]. Lead diacetate trihydrate.

Colourless crystals, efflorescent in the air. Freely soluble in water, soluble in 96% ethanol.

Lead-acetate paper.

Immerse a filter paper with a density of 80 g/m^2 in a mixture of *dilute acetic acid R – lead (II) acetate R solution* (1:10), then remove it, dry, and cut into strips measuring 15 mm x 40 mm.

Lead acetate cotton wool.

Immerse absorbent cotton wool in the mixture of solvents: *dilute acetic acid R – lead (II) acetate R solution* (1:10). Without pressing the cotton wool, remove the excess liquid, then place it on several layers of filter paper, and dry in air.

Store in an airtight container.

Lead (II) acetate solution.

A 95 g/L solution in *carbon dioxide-free water R.*

Lead (II) acetate basic solution. [1335-32-6]. Lead vinegar.

Contains 16.7% (*m/m*) to 17.4% (m/m) of Pb (A_r 207.2) as a compound approximately corresponding to the formula $C_8H_{14}O_{10}Pb_3$.

Dissolve 40.0 g of *lead(II) acetate R* in 90 mL of *carbon dioxide-free water R*. Adjust the pH to 7.5 with *strong sodium hydroxide solution R*, centrifuge and use a clear, colorless supernatant.

When stored in a well-closed container, the solution should be clear.

Lead dioxide. PbO_2. (M_r 239.2). [1309-60-0]. Lead dioxide.

Dark brown powder, oxygen-evolving when heated.

Practically insoluble in water, soluble in hydrochloric acid with the release of chlorine, nitric acid diluted in the presence of hydrogen peroxide, oxalic acid, or other reducing reagents, hot concentrated solutions of alkali metal hydroxides.

Lead(II) nitrate. $Pb(NO_3)_2$. (M_r 331.2). [10099-74-8]. Lead dinitrate.

White or almost white crystalline powder or colorless crystals. Freely soluble in water.

Lead(II) nitrate solution.

A 33 g/L solution.

Selenium. Se. (*Ar* 79.0). [7782-49-2].

Brownish red to black powder or granules. Practically insoluble in water and 96% ethanol, soluble in nitric acid.

The melting point is about 220 °C.

Selenic acid. H_2SeO_3. (M_r 129.0). [7783-00-8].

Crystals, deliquescent. Freely soluble in water.

Store in an airtight container.

Sulfur. S. (A_r 32.07). [7704-34-9].

Contains not less than 99.0% and not greater than 101.0% of S.

Yellow powder.

Practically insoluble in water, soluble in carbon disulfide, slightly soluble in vegetable oils.

The melting point is about 120 °C.

The size of most particles does not exceed 20 μm, the particle size should not exceed 40 μm.

Silver diethyldithiocarbamate. $C_5H_{10}AgNS_2$. (M_r 256.1). [1470-61-7].

Pale yellow to greyish yellow powder. Practically insoluble in water, soluble in pyridine.

Prepare as follows. Dissolve 1.7 g of *silver nitrate R* in 100 mL of *water R*. Separately dissolve 2.3 g of *sodium diethyldithiocarbamate R* in 100 mL of *water R*. Cool both solutions to 10 °C, then mix, and, with stirring, collect a yellow precipitate on a glass filter (*2.1.2*), wash with 200 mL of cold *water R*, and dry in vacuo for 2-3 h.

Silver diethyldithiocarbamate should not change colour or have a strong odor.

Silver nitrate. $AgNO_3$. (M_r 169.9). [7761-88-8]. Silver nitrate(1).

Contains not less than 99.0% and not greater than 100.5% of $AgNO_3$.

White or almost white crystalline powder or clear colorless crystals.

Very soluble in water, soluble in 96% ethanol.

Silver nitrate ammonia solution.

Dissolve 2.5 g of *silver nitrate R* in 80 mL of *water R*, add a *diluted ammonia solution R1* dropwise until the precipitate dissolves, and dilute to 100 mL with *water R*. Prepare immediately before use.

Silver nitrate solution R1.

A 42.5 g/L solution.

Store in a place protected from light.

Silver nitrate solution R2.

A 17 g/L solution.

Store in a place protected from light.

Silver nitrate solution in pyridine.

A 85 g/L solution in *pyridine R*.

Store in a place protected from light.

Silver nitrate reagent.

Prepare immediately before use.

To the mixture of 3 mL of *concentrated ammonia solution R* and 40 mL of *1 M sodium hydroxide*, add 8 mL of 200 g/L solution of *silver nitrate R* dropwise, and dilute to 200 mL with *water R*.

Silver oxide. Ag_2O. (M_r 231.7). [20667-12-3]. Silver(I) oxide.

Brownish black powder. Practically insoluble in water and 96% ethanol, freely soluble in dilute nitric acid and ammonia solutions.

Store in a place protected from light.

Silver manganese paper.

Immerse strips of slowly filtering paper in a solution containing 8.5 g/L of *manganese sulfate R* and 8.5 g/L of *silver nitrate R.* Allow to stand for several minutes, dry over *phosphorus pentoxide R*, protecting from the effects of acid and alkali vapours.

Serine. $C_3H_7NO_3$. (M_r 105.1). [56-45-1]. (2*S*)-2-Amino-3-hydroxypropanoic acid.

Contains not less than 98.5% and not greater than 101.0% of $C_3H_7NO_3$ calculated on a dry substance basis.

It is a fermentation product or protein hydrolysate.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, practically insoluble in 96% ethanol.

Sulfuric acid. H_2SO_4. (M_r 98.1). [7664-93-9].

Contains from 95.0% (*m/m*) to 97.0% (*m/m*) of H_2SO_4.

colourless, caustic liquid with an oily consistency, highly hygroscopic. Miscible with water and with 96% ethanol producing intense heat.

d_{20}^{20} is from 1,834 to 1,837.

A 10 g/L solution is a strong acid and reacts to sulfates (*2.1.3.1*).

Clarity (*2.1.2.1*). Sulfuric acid is clear.

Colour Index (*2.1.2.2, Method II).* Sulfuric acid is colourless.

Oxidisable substances. Cautiously with cooling, add 20 g of sulfuric acid to 40 mL of *water R*, then 0.5 mL of 0.002 M potassium permanganate. The violet colour persists for at least 5 min.

Chlorides. Maximum 0.5 ppm.

Under cooling, carefully add 10 g of sulfuric acid to 10 mL of *water R*, allow to cool and dilute to 20 mL with the same solvent. Add 0.5 mL of *silver nitrate R2* solution and keep for 2 min, protected from light. The resulting solution must withstand the test for chlorides. Prepare the reference solution using 1 mL of *chloride standard solution (5 ppm Cl^-) R*, 19 mL of *water R*, and 0.5 mL of *silver nitrate solution R2.*

Nitrates. Maximum 0.5 ppm.

Under cooling, carefully add 50 g or 27,2 mL of sulfuric acid to 15 mL of *water R*, then add 0.2 mL of a freshly prepared 50 g/L solution of *brucine R* in *glacial acetic acid R*. After 5 min, any colour is less intense than that of a reference mixture prepared in the same manner and containing 12.5 mL of *water R*, 50 g of *nitrogen-free sulfuric acid R*, 2.5 mL of *nitrate standard solution (10 ppm NO_3^-) R*, and 0.2 mL of a 50 g/L solution of *brucine R* in *glacial acetic acid R.*

Ammonium. Maximum 2 ppm.

Under cooling, carefully pour 2.5 g of sulfuric acid to *water R*, dilute to 20 mL with the same solvent, allow to cool, and add dropwise 10 mL of a 200 g/L solution of *sodium hydroxide R*, followed by 1 mL of *alkaline potassium tetraiodomercurate solution R*. The colour of the solution is less intense than that of a mixture of 5mL of *ammonium standard solution (1 ppm NH4) R*, 15 mL of *water R*, 10 mL of a 200 g/L solution of *sodium hydroxide R*, and 1 mL of *alkaline potassium tetraiodomercurate solution R.*

Arsenic (*2.1.4.2, Method A*). Maximum 0.02 ppm.

Cautiously with cooling, add 3 mL of *nitric acid R* to 50 g of sulfuric acid, carefully evaporate to 10 mL, cool, add 20 mL of *water R* to the resulting residue, and evaporate to 5 mL. The solution must withstand the test for arsenic. Prepare the reference solution using 1.0 mL of *arsenic standard solution (1 ppm As^+) R.*

Iron (*2.1.4.9*). Maximum 1 ppm.

Under low heating, dissolve the ash residue obtained after determining the loss on ignition in 1 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*. Dilute 5 mL of the resulting solution to 10 mL with *water R*. The solution must withstand the test for iron.

Heavy metals (*2.1.4.8, Method A*). Maximum 2 ppm.

Dilute 10 mL of the solution obtained in the test for iron to 20 mL with *water R*. 12 mL of the solution complies with the test on heavy metals.

Prepare the reference solution using *lead standard solution (2 ppm Pb^{2+}) R.*

Loss on ignition. Maximum 10^{-3}%. Determination is carried out from 100 g of sulfuric acid by carefully evaporating in a small crucible over an open flame and heating the residue to redness.

Quantitation. In a ground-glass-stoppered flask, place 30 mL of *water R*, accurately weigh, add 0.8 mL of sulfuric acid, cool and weigh again. Titrate with *1 M sodium hydroxide* using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide solution* is equivalent to 49.04 mg of H_2SO_4.

Store in a ground-glass-stoppered container made of glass or other inert material.

5 M sulfuric acid solution.

Dilute 28 mL of *sulfuric acid R* to 100 mL with *water R*.

Sulfuric acid, alcoholic, 2.5 M.

With continuous cooling and stirring, carefully add 14 mL of *sulfuric acid R* to 60 mL of *anhydrous ethanol R*, allow to cool, and dilute to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

Sulfuric acid, alcoholic, 0.25 M.

Dilute 10 mL of *2.5 M alcoholic sulfuric acid solution R* to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

Sulfuric acid, alcoholic solution.

With continuous cooling and stirring, carefully add 20 mL of *sulfuric acid R* to 60 mL of *96% ethanol R*, allow to cool, and dilute to 100 mL with *96% ethanol R*.

Prepare immediately before use.

Sulfuric acid, dilute.

Contains 98 g/L of H_2SO_4.

Add 5.5 mL of *sulfuric acid R* to 60 mL of *water R*, allow to cool, and dilute to 100 mL with the same solvent.

Quantitation. In a ground-glass-stoppered flask, place 30 mL of *water R*, add 10.0 mL of dilute sulfuric acid, and titrate with *1 M sodium hydroxide*, using 0.1 mL of a solution of *methyl red R* as indicator.

1 mL of *1 M sodium hydroxide solution* is equivalent to 49.04 mg of H_2SO_4.

Sulfuric acid-formaldehyde reagent.

A mixture of 2 mL of *formaldehyde solution R* and 100 mL of *sulfuric acid R*.

Sulfuric acid, nitrogen-free.

Complies with the requirements prescribed for *sulfuric acid R* and the following additional test.

Nitrates. To 5 mL of *water R*, carefully add 45 mL of sulfuric acid, cool to 40 °C, and add 8 mg of *diphenyl benzidine R*; the resulting solution should be colorless or slightly pale blue.

Sulfuric acid, nitrogen-free R1.

Complies with the requirements prescribed for *nitrogen-free sulfuric acid R*. Content from 95.0% (*m/m*) to 95.5% (*m/m*).

Sulfuric acid, heavy metal-free.

Complies with the requirements prescribed for *sulfuric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.
Cd: 0.002 ppm.
Cu: 0.001 ppm.
Fe: 0.05 ppm.
Hg: 0.005 ppm.
Ni: 0.002 ppm.
Pb: 0.001 ppm.
Zn: 0.005 ppm.

Sulfuric acid R1. H_2SO_4. (M_r 98.1). [7664-93-9].
Contains 75% (*V/V*) of H_2SO_4.

Hydrogen sulfide. H2S (M_r 34.08). [7783-06-4].
Gas. Slightly soluble in water.

Hydrogen sulfide R1. H_2S. (M_r 34.08). [7783-06-4]. Contains not less than 99.7% (*V/V*) of H_2S.

Sulfur dioxide. SO_2. (M_r 64.1). [7446-09-5]. Sulfurous anhydride.

Colorless gas. When compressed, it turns into a colorless liquid.

Sulfur dioxide R1. SO_2. (*Mr* 64.1). [7446-09-5]

Contains not less than 99.9% (*V/V*) of SO_2.

Silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases.

Silica gel, finely divided, with bonded alkyl groups suitable for use with highly aqueous mobile phases.

Silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases, end-capped.

Silica gel, finely divided, with bonded alkyl groups suitable for use with highly aqueous mobile phases. To minimise interaction with basic compounds, most of the remaining silanol groups are carefully protected.

Silica gel G. [112926-00-8].

Contains about 13% of calcium sulfate hexahydrate. The particle size is about 15 µm.

Calcium sulfate. Place 0.25 g in a ground-glass-stoppered flask, add 3 mL of *dilute hydrochloric acid R* and 100 mL of *water R*, shake vigorously for 30 min, filter through a glass filter (*2.1.1.2*), and wash the residue. Combine the filtrate and washing water and determine the calcium content by complexometry (*2.1.5.11*).

1 mL of *0.1 M sodium edetate solution* is equivalent to 14.51 mg of $CaSO_4 \cdot 1/2H_2O$.

pH (*2.1.2.3*). Shake 1 g with 10 mL of carbon *dioxide-free water R* for 5 min. The pH of the suspension is about 7.

Measure the pH of the suspension obtained by stirring 1 g with 10 mL of *dioxide-free water R* for 5 min.

Silica gel GF_{254}. [112926-00-8].

Contains about 13% of calcium sulfate hemihydrate and about 1.5% of a fluorescent indicator that has an optimal absorption intensity at a wavelength of 254 nm.

The particle size is about 15 µm.

Calcium sulfate. Determination is carried out by the method specified for *silica gel G R.*

pH (*2.1.2.3*). Complies with the requirements for *silica gel G R.*

Fluorescence. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel GF_{254} R thin layer.* On the chromatographic plate, apply ten spots of a 1 g/L solution of *benzoic acid R* in a mixture of *anhydrous formic acid R*/*2-propanol R* (10: 90), successively increasing volumes from 1 µl to 10 µl. Chromatograph in the same mixture of solvents. When the solvent front passes about 10 cm, dry the plate and examine in UV light at 254 nm. On the upper third of the chromatogram on a fluorescent background, dark spots of benzoic acid should be detected, starting from 2 µg or greater.

Silica gel H. [112926-00-8].

The particle size is about 15 µm.

pH (*2.1.2.3*). Complies with the requirements for *silica gel G R.*

Silica gel H, silanised.

Preparation of a thin layer. See *Silica gel HF_{254}, salinised R.*

Chromatographic separation capacity. complies with the test for *Silica gel HF_{254}, salinised R.*

Silica gel HF_{254}.

Contains about 1.5% of a fluorescent indicator having an optimal intensity at 254 nm. The particle size is about 15 µm.

pH (*2.1.2.3*). Complies with the test prescribed for *silica gel G R.*

Fluorescence. Complies with the test prescribed for *silica gel GF_{254} R.*

Silica gel HF_{254}, silanised.

Contains about 1.5% of a fluorescent indicator having an optimal intensity at 254 nm.

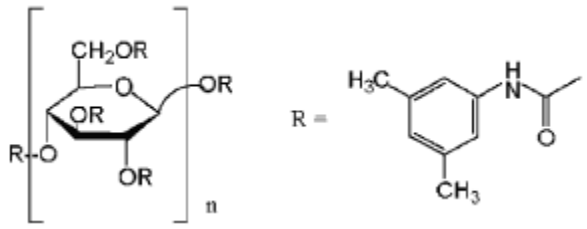
Preparation of a thin layer. Shake 30 g vigorously with 60 mL of a mixture of solvents *methanol R*/*water R* (1:2) for 2 min. Coat carefully cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air and then heat at 100 °C to 105 °C for 30 min.

Chromatographic separation capacity. Introduce 0.1 g each of *methyl laurate R, methyl myristate R, methyl palmitate R*, and *methyl stearate R* into a 250 mL conical flask, add 40 mL of *alcoholic potassium hydroxide solution R*, and heat under a reflux condenser on a water bath for 1 h. Cool down, transfer the solution to the separation funnel with 100 mL of *water R*, acidify (pH between 2 and 3) with *dilute hydrochloric acid R*, and shake with three quantities of *chloroform R*, each of 10 mL. Dry the combined chloroform extracts over *anhydrous sodium sulfate R*, filter, and evaporate to dryness on a water bath. Dissolve the residue in 50 mL of *chloroform R*. Examine by thin-layer chromatography (*2.1.2.26*), using silanised silica gel HF_{254}as the coating substance.

On a chromatographic plate, apply 10 µL of chloroform solution at three spots and chromatograph in a system of solvents *glacial acetic acid R*/*water R/dioxane R* (10:25:65). When the solvent front passes 14 cm, dry the plate at 120 °C for 30 min, cool, spray with a solution of 35 g/L *phosphomolybdic acid R* in *2-propanol R*, and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until a white background is obtained. The chromatograms show four clearly separated, well-defined spots.

Silica gel OC for chiral separations.

Silica gel for chromatography, very finely divided, with a particle size of 5 µm, coated with the following derivative:



Silica gel for chromatography, aminopropylmethylsilyl.

Silica gel with a fine particle size, chemically modified by aminopropylmethylsilyl groups on the surface.

Silica gel for chromatography, aminopropylsilyl.

Silica gel with a fine particle size, chemically modified by bonding aminopropylsilyl groups on the surface.

Silica gel, anhydrous. [112926-00-8].

Partly dehydrated polymerised, amorphous silicic acid, absorbing at 20 °C about 30% of its mass of water. Practically insoluble in water, partly soluble in sodium hydroxide solutions. Contains a suitable indicator for the determination of moisture content; on the label, the colour change at the transition of the hydrated form to the anhydrous form is indicated.

Silica gel for chromatography, butylsilyl.

Silica gel, very finely divided, chemically modified at the surface by butylsilyl groups.

Spheroidal silicon dioxide: 30 nm.

Pore volume: 0.6 cm^3/g.

Specific surface area: 80 m^2/g.

Silica gel for chromatography, hexylsilyl.

Silica gel, very finely divided chemically modified at the surface by hexylsilyl groups.

Silica gel for chromatography, hydrophilic.

Silica gel, very finely divided, modified at the surface to impart hydrophilic properties.

Silica gel for chromatography, dimethyloctadecylsilyl.

Silica gel, very finely divided, chemically modified at the surface by dimethyloctadecylsilyl groups.

Specific surface area: 300 m^2/g.

Silica gel for chromatography, diol.

Spherical silica particles to which dihydroxypropyl groups are bonded. Pore size 10 nm.

Silica gel for chromatography.

Silica gel, very finely divided.

Silica gel for chromatography, strong-anion-exchange.

Silica gel, very finely divided, chemically modified at the surface with quaternary ammonium groups.

pH limit for use: 2 to 8.

Silica gel for chromatography, strong-cation-exchange.

Silica gel, very finely divided, chemically modified at the surface by sulfonic acid groups.

Silica gel for chromatography, amylase modified.

Silica gel, very finely divided, with a particle size of 10 μm, chemically modified at the surface by amylase.

Silica gel for chromatography, nitrile.

Silica gel, very finely divided, chemically modified at the surface by cyanopropylsilyl groups.

Silica gel for chromatography, nitrile R1.

Silica gel, very finely divided, consisting of porous spherical particles with chemically bonded nitrile groups.

Silica gel for chromatography, nitrile R2.

Ultrapure silica gel chemically modified at the surface by the introduction of cyanopropylsilyl groups. Contains less than 20 ppm of metals.

Silica gel for chromatography, octadecanoylaminopropylsilyl.

Silica gel, finely divided, chemically modified at the surface by aminopropylsilyl groups that are acylated by octadecanoyl groups.

Silica gel for chromatography, octadecylsilyl.

Silica gel, very finely divided, chemically modified at the surface by octadecylsilyl groups.

Silica gel for chromatography, octadecylsilyl R1.

Silica gel, ultra-pure, very finely divided with a surface chemically modified by the bonding of octadecylsilyl groups.

Contains less than 20 ppm of metals.

Silica gel for chromatography, octadecylsilyl R2.

Silica gel, ultra-pure, very finely divided, with a pore size of 15 nm, chemically modified at the surface by the bonding of octadecylsilyl groups (20% carbon load), optimised for the analysis of polycyclic aromatic hydrocarbons.

Silica gel for chromatography, octadecylsilyl, base-deactivated.

Silica gel, very finely divided; before the introduction of octadecylsilyl groups, pre-treated by thorough washing and hydrolysing of most superficial siloxane bridges to minimise interaction with the basic components.

Silica gel for chromatography, octadecylsilyl, end-capped, base-deactivated.

Silica gel is very finely divided and has a pore size of about 10 nm; contains about 16% of carbon. Before the introduction of octadecylsilyl groups, it is pre-treated by thorough washing and hydrolysing of most superficial siloxane bridges. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica gel for chromatography, octadecylsilyl, end-capped.

Silica gel, very finely divided, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica gel for chromatography, octadecylsilyl, end-capped R1.

Silica gel, ultra-pure, very finely divided with a pore size of 10 nm, chemically modified at the surface by the bonding of octadecylsilyl groups (19% carbon content). To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Contains less than 20 ppm of metals.

Silica gel for chromatography, octylsilyl.

Silica gel, very finely divided, chemically modified at the surface by the bonding of octylsilyl groups.

Silica gel for chromatography, octylsilyl R1.

Silica gel, very finely divided, chemically modified at the surface by octylsilyl and methyl groups (double-bonded phase).

Silica gel for chromatography, octylsilyl R2.

Silica gel is ultra-pure, very finely divided with a pore size of 10 nm, and a surface chemically modified with octylsilyl groups (contains 19% of carbon). Contains less than 20 ppm of metals.

Silica gel for chromatography, octylsilyl, base-deactivated.

Silica gel, very finely divided; pretreated before the bonding of octylsilyl groups by careful washing and hydrolysing most of the superficial siloxane bridges to minimise the interaction with basic components.

Silica gel for chromatography, octylsilyl, end-capped.

Silica gel, very finely divided, with a particle size from 3 µm to 10 µm, chemically modified at the surface by the bonding of octylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica gel for chromatography, trimethylsilyl.

Silica gel, very finely divided, chemically modified at the surface by the bonding of trimethylsilyl groups.

Silica gel for chromatography, phenylsilyl.

Silica gel, very finely divided, chemically modified at the surface by the bonding of phenyl groups.

Silica gel for chromatography, phenylsilyl R1.

Silica gel, finely divided, with a particle size of 5 µm, chemically modified at the surface by phenyl groups.

Silicon dioxide spheroidal: 8 nm.

Specific surface area: 180 m^2/g.

Carbon content: 5.5%.

Silica gel for chromatography, cyanosilyl.

Silica gel, very finely divided, chemically modified at the surface by cyanosilyl groups.

Silica gel for size-exclusion chromatography.

Silica gel, very finely divided, with a particle size of 10 µm and a very hydrophilic surface. The average pore diameter is about 30 nm. It is compatible with aqueous solutions with the pH 2 to 8 and organic solvents. Suitable for the separation of proteins with molecular masses from 1×10^3 to 3×10^5.

Sinensetin. $C_{20}H_{20}O_7$. (M_r 372.4). [2306-27-6]. 3',4',5,6,7-Pentamethoxyflavone.

White or almost white crystalline powder. Practically insoluble in water, soluble in 96% ethanol.

The melting point is about 177 °C.

Absorbance (*2.1.2.24*). The ultraviolet spectrum of the synensetin solution in *methanol R* shows three absorption maxima at 243 nm, 268 nm, and 330 nm.

Quantitation. Determination is carried out by liquid chromatography (*2.1.2.28*) as prescribed in the monograph *Java tea*. The contents of sinensetin calculated by the normalisation procedure must be minimum 95%.

Squalane. $C_{30}H_{62}$. (M_r 422.8). [111-01-3]. 2,6,10,15,19,23-Hexamethyltetracosan.

A colorless oily liquid. Freely soluble in fatty oils, slightly soluble in acetone, 96% ethanol, glacial acetic acid, and methanol.

d_{20}^{20} is from 0.811 to 0.813.

n_D^{20} is from 1.451 to 1.453.

Weak cationic resin.

See *Weak cation-exchange resin R.*

Ion-exchange resin, strongly acidic.

Resin in a protonated form with sulfonic acid groups attached to a lattice consisting of polystyrene cross-linked with 8% of divinylbenzene. Produced in the form of spherical granules; unless otherwise specified, the particle size is between 0.3 mm and 1.2 mm.

Reservoir. Between 4.5 mmol/g and 5 mmol/g with a water content of 50% to 60%.

Column preparation. Unless otherwise specified, use a tube with a disk of porous glass fused inside, 400 mm long, 20 mm in internal diameter, and a filling height of about 200 mm. Pre-mix the resin with *water R*. Inject the resulting suspension into the tube, preventing the formation of air bubbles between the particles. During operation, the liquid must not fall below the surface of the resin. If the resin is in a protonated form, wash with *water R* until no greater than 0.05 mL of *0.1 M sodium hydroxide* is sufficient to neutralise 50 mL, using 0.1 mL of *methyl orange solution R* as indicator. If the resin is in a sodium form or needs to be regenerated, pass slowly about 100 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R* through the column and then wash with *water R* as described above.

Styrene-divinylbenzene copolymer.

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Ethylvinylbenzene-divinylbenzene copolymer.

Porous, rigid, spherical cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Ethylvinylbenzene-divinylbenzene copolymer R1.

Porous, rigid, cross-linked, spherical polymer beads, with a nominal specific surface area of 500 m^2/g to 600 m^2/g and having pores with a mean diameter of 7.5 nm. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Sorbitol. $C_6H_{14}O_6$. (M_r 182.2). [50-70-4]. D-glucite (D-sorbitol).

Contains not less than 97.0% and not greater than 102.0% of $C_6H_{14}O_6$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder.

Very soluble in water, practically insoluble in 96% ethanol.

Has polymorphism.

See *Sorbitol*.

Alcohol. [64-17-5].

See *Ethanol (96%) R*.

Alcohol (*X*%, *V/V*).

See *Ethanol (x% V/V) R*.

Alcohol, aldehyde-free.

Mix 1200 mL of *96% ethanol R* with 5 mL of a 400 g/L solution of *silver nitrate R* and 10 mL of a cooled 500 g/L solution of *potassium hydroxide R*, shake, allow to stand for several days, and filter.

Distill the filtrate immediately before use.

Stearic acid. $C_{18}H_{36}O_2$. (M_r 284.5). [57-11-4]. Octadecanoic acid.

White or almost white powder or flakes. Oily to the touch, practically insoluble in water, soluble in hot 96% ethanol.

The melting point is about 70 °C.

Stearic acid used to quantify the number of fatty acids as prescribed in the monograph Saw Palmette fruit *must additionally meet the following requirement.*

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Saw Palmetto fruit.*

The stearic acid content calculated by the internal normalisation procedure should be minimum 98%.

Streptomycin sulfate. $C_{42}H_{84}X_{14}O_{36}Б_3$.

(M_r 1457). [3810-74-0]. Bis[*N*,*N*′-bis(aminoiminomethyl)-4-*O*-[5-deoxy-2-*O*-[2-deoxy-2 (methylamino)-(-L-glucopyranosyl]-3-*S*-form-α -E-lyxofuranosyl]-D-streptamine] trisulfate.

The antibiotic is produced by certain strains of *Streptomycesgriseus* or produced by any other means. Stabilisers can be added.

The antimicrobial activity should be at least 720 IU/mg calculated on a dry substance basis.

Methods of production of the substance should exclude or minimise the content of substances that lower blood pressure.

The method of production is considered validated if the substance passes the following test:

Abnormal toxicity (*2.1.6.3*). Inject a solution containing 1 mg of the substance in 0.5 mL of *water for injection R* to each mouse.

White or almost white powder, hygroscopic.

Very soluble in water, practically insoluble in ethanol.

Strontium carbonate. $SrCO_3$. (M_r 147.6). [1633-05-2].

White or almost white crystalline powder.

Contains not less than 99.5% of $SrCO_3$.

Sudan red G. $C_{17}H_{14}NO_2$. (M_r 278,3). 1-[(2-Methoxyphenyl)azo]naphthalene-2-ol.

Schultz No. 149.

Colour Index (C.I.) No. 12150.

Solvent Red 1.

Reddish-brown powder. Practically insoluble in water.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel G R* as the coating substance. Apply 10 µL of a 0.1 g/L solution in *methylene chloride R* to the chromatographic plate and develop in the same solvent. The development length of the solvent front is about 10 cm from the start line. The resulting chromatogram must show only one principal spot.

Sudan orange. $C_{16}H_{12}N_2O$. (M_r 248.3). [842-07-9].

Colour Index (C.I.) No. 12055.

1-(Phenylazo)naphthalene-2-ol. Sudan I.

Orange-red powder. Practically insoluble in water, soluble in methylene chloride.

The melting point is about 131 °C.

Sudan III. $C_{22}H_{16}N_4O$. (M_r 352.4).

Brown powder with a green metallic lustre.

Insoluble in water, soluble in chloroform and glacial acetic acid, sparingly soluble in ethanol, fatty and essential oils.

Sudan III solution.

Dissolve 0.01 g of *Sudan III R* in 5 mL of *96% ethanol* and add 5 mL of *glycerol R*.

Sulfamic acid. H_3NO_3S. (M_r 97.1). [5329-14-6].

White or almost white crystalline powder or crystals. Freely soluble in water, sparingly soluble in acetone, 96% ethanol, and methanol.

The melting point is about 205 °C with decomposition.

Sulfanilamide. C6H8N2O2S. (*Mr* 172.2). [63-74-1]. 4-Aminobenzenesulfonamide.

White or almost white powder. Slightly soluble in water, freely soluble in boiling water, acetone, dilute acids, and solutions of alkali metal hydroxides, sparingly soluble in 96% ethanol and petroleum ether.

The melting point is about 165 °C.

Sulfanilic acid. $C_6H_7NO_3S$.

(M_r 173.2). [121-57-3]. 4-Aminobenzenesulfonic acid.

Colourless crystals. Sparingly soluble in water, practically insoluble in 96% ethanol.

Sulfane blue. $C_{27}H_{31}N_2NaO_6S_2$. (M_r 566.6). [129-17-9]. Disulfine blue. Acid Blue 1. Patent Blue VF. Blue VS. Sodium [[[4-(diethylamino)phenyl](2,4-di-sulfonatophenyl)methylene] cyclohexa-2,5-di-en-1-ylidene]diethylammonium.

Schultz No. 769.

Colour Index (C.I.) No. 42045.

Violet powder. Soluble in water. Dilute solutions have a blue colour, which turns yellow with the addition of concentrated hydrochloric acid.

Sulfathiazol. $C_9H_9N_3O_2S_2$. (M_r 255.3). [72-14-0]. 4-Amino-M-(thiazol-2-yl)benzene-sulfonamide.

White or yellowish white powder or crystals. Very slightly soluble in water, soluble in acetone, slightly soluble in 96% ethanol. Soluble in dilute mineral acids, solutions of hydroxides and carbonates of alkali metals.

The melting point is about 200 °C.

Sulfomolybdic reagent R2.

Dissolve about 50 mg of *ammonium molybdate R* in 10 mL of *sulfuric acid R.*

Sulfomolybdic reagent R3.

Dissolve 2.5 g of *ammonium molybdate R* by heating in 20 mL of *water R*. Dilute 28 mL of *sulfuric acid R* to 50 mL with *water R*, then cool. Mix both solutions and dilute to 100 mL with *water R*.

Store in a polyethylene container.

Sulfosalicylic acid. $C_7H_6O_6S{\cdot}2H_2O$.

(M_r 254.2). [5965-83-3].2-Hydroxy-5-sulfobenzoic acid.

White or almost white crystalline powder or crystals. Very soluble in water and 96% ethanol.

The melting point is about 109 °C.

Antimony potassium tartrate. $C_8H_4K_2O_{12}Sb_2{\cdot}3H_2O$.

(M_r 668). [28300-74-5]. Dipotassium di[tartrato(4-)O^1,O^2,O^3,O^4]bis[antimonate(III)]trihydrate.

White or almost white granular powder or clear colorless crystals. Soluble in water and glycerin, freely soluble in boiling water, practically insoluble in 96% ethanol. The aqueous solution has a slightly acidic reaction.

Antimony (III) chloride. $SbCl_3$. (M_r 228.1). [10025-91-9]. Antimony trichloride.

Colourless crystals or clear crystalline mass. Hygroscopic, freely soluble in ethanol, hydrolysed with water.

Store in an airtight container in a dry place.

Antimony(III) chloride solution.

Wash 30 g of *antimony(III) chloride R* quickly with two portions of *ethanol-free chloroform R*, each of 15 mL; discard the washing solutions and dissolve the washed crystals immediately in 100 mL of *ethanol-free chloroform R* under low heating. Store the solution over several grams of *anhydrous sodium sulfate R*.

Antimony chloride solution R1.

Solution A. Dissolve 110 g of *antimony chloride R* in 400 mL of *ethylene chloride R*, add 2 g of *anhydrous aluminum oxide R*, mix, and filter through a glass filter (40) (*2.1.1.2*). Dilute the filtrate to 500.0 mL with *ethylene chloride R* and stir. The absorbance (*2.1.2.24*) of the resulting solution measured at a wavelength of 500 nm in a cell 2 cm thick should not exceed 0.07.

Solution B. In a fume hood, mix 100 mL of freshly distilled *acetyl chloride R* and 400 mL of *ethylene chloride R.*

Mix 90 mL of solution A and 10 mL of solution B.

Store in amber ground-glass-stoppered bottles.

Storage: use within 7 days. The reagent is not suitable if staining appears.

Suspension of rabbit red blood cells.

Prepare a 1.6% (*V/V*) suspension of rabbit erythrocytes as follows: defibrinate 15 mL of freshly drawn rabbit blood by shaking with glass beads, centrifuge at 2000 g for 10 min and wash the erythrocytes with three quantities, each of 30 mL, of a 9 g/L solution of *sodium chloride R*. Dilute 1.6 mL of the suspension of erythrocytes to 100 mL with a mixture of *phosphate buffer solution pH 7.2 R* / 9 g/L solution of *sodium chloride R* (1:9).

Tagatose. $C_6H_{12}O_6$. (M_r 180.16). [87-81-0]. D-*lyxo*-Hexulose.

White or almost white powder.

$[\alpha]_D^{20}$-2.3. Determination is carried out using a 21.9 g/L solution.

The melting point is from 134 °C to 135 °C.

Thallous sulfate. Tl_2SO_4. (M_r 504.8). [7446-18-6]. Dithallium sulfate.

White or almost white, rhomboid prisms. Slightly soluble in water, practically insoluble in 96% ethanol.

Talc. $Mg_3Si_4O_{10}(OH)_2$. (M_r 379.3). [14807-96-6].

The powder of natural hydrated magnesium silicate can have a diverse composition of bound minerals, among which chlorites (hydrated aluminum and magnesium silicates), magnesites (magnesium carbonate), calcites (calcium carbonate) and Dolomites (calcium and magnesium carbonates) predominate.

White or almost white light homogeneous, greasy to the touch (non-abrasive).

Practically insoluble in water, in 96% ethanol, in dilute solutions of acids and alkali metal hydroxides.

Tannic acid. [1401-55-4]. Tannic acid.

Glistening scales or yellowish or light brown amorphous powder. Very soluble in water, freely soluble in 96% ethanol, soluble in acetone.

Store in a place protected from light.

Theophylline. $C_7H_8N_4O_2$. (M_r 180.2). [58-55-9].

1,3-Dimethyl-3,7-dihydro-1H-purine-2,6-dione.

Contains not less than 99.0% and not greater than 101.0% of $C_7H_8N_4O_2$ calculated on a dry substance basis.

White or almost white crystalline powder.

Slightly soluble in water, sparingly soluble in 96% ethanol, soluble in solutions of alkali metal hydroxides, in ammonia, and in mineral acids.

α-Terpinene. $C_{10}H_{16}$. (M_r 136.2). [99-86-5]. 1-Isopropyl-4-methylcyclohexa-1,3-diene.

Clear, almost colorless liquid.

d_4^{20} is about 0.837.

n_D^{20} is from 1.478.

The boiling point is about 174 °C.

α-Terpinene used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Tea tree oil*.

The α-terpinene content calculated by the internal normalisation procedure should be minimum 90%.

γ-Terpinene. $C_{10}H_{16}$. (M_r 136.2). [99-85-4]. 1-Isopropyl-4-methylcyclo-hexa-1,4-diene.

Oily liquid.

γ-Terpinene used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil.*

Test solution. Test substance.

The γ-terpinene content calculated by the internal normalisation procedure should be minimum 93.0%.

Terpinen-4-ol. $C_{10}H_{18}O$. (M_r 154.2). [562-74-3].

4-Methyl-1-(1-methylethyl)cyclohex-3-en-1-ol. *p*-Ment-1-en-4-ol.

A colorless oily liquid.

Terpinene-4-ol used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as specified in the monograph *Lavender oil.*

Test solution. Test substance.

The terpinen-4-ol content calculated by the internal normalisation procedure should be minimum 90.0%.

α-Terpineol. $C_{10}H_{18}O$. (M_r 154.2). [98-55-5]. (*RS*)-2-(4-Methylcyclohex-3-enyl)-2-propanol.

Colourless crystals. Practically insoluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.935.

n_D^{20} is about 1.483.

$[\alpha]_D^{20}$ is about 92.5.

The melting point is about 35 °C.

It can contain from 1% to 3% of β-terpineol.

α-Terpineol used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Anise oil.*

Test solution. 100 g/L in *hexane R.*

The *α*-terpineol content calculated by the internal normalisation procedure should be minimum 97.0%.

Testosterone. $C_{19}H_{28}O_2$. (M_r 288.4). [58-22-0]. 17β-Hydroxyandrost-4-en-3-one.

Contains not less than 97.0% and not greater than 103.0% of $C_{19}H_{28}O_2$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless or yellowish-white crystals.

Practically insoluble in water, freely soluble in alcohol and methylene chloride, practically insoluble in fatty oils.

The melting point is about 155 °C.

Testosterone propanate. $C_{22}H_{32}O_3$. (M_r344.5). [57-85-2]. 3-Oxoandrost-4-en-17β-ilpropanoate.

Contains not less than 97.5% and not greater than 102.0% of $C_{22}H_{32}O_3$ calculated on a dry substance basis.

White or almost white powder or colorless crystals.

Practically insoluble in water, freely soluble in acetone and 96% ethanol, soluble in fatty oils.

Tetrabutylammonium hydroxide. $C_{16}H_{37}NO{\cdot}30H_2O$. ($M_r$ 800). [147741-30-8]. Contains not less than 98,0% of $C_{16}H_{37}NO{\cdot}30H_2O$.

White or almost white crystals. Soluble in water.

Quantitation. Dissolve 1.000 g in 100 mL of *water R* and immediately titrate with *0.1 M hydrochloric acid* potentiometrically (*2.1.2.19*). In parallel, conduct a blank determination.

1 mL of *0.1 M hydrochloric acid* is equivalent to 80.0 mg of $C_{16}H_{37}NO{\cdot}30H_2O$.

Tetrabutylammonium hydroxide solution (104 g/L).

A solution containing 104 g/l of $C_{16}H_{37}NO$ (M_r 259.5) prepared by diluting a reagent of an appropriate grade.

Tetrabutylammonium hydroxide solution (400 g/L).

A solution containing 400 g/l of $C_{16}H_{37}NO$ (M_r 259.5) of the appropriate grade.

Tetrabutylammonium hydrogen sulfate. $C_{16}H_{37}NO_4S$. (M_r 339.5). [32503-27-8].

Crystalline powder or colorless crystals. Freely soluble in water and methanol.

The melting point is from 169 °C to 173 °C.

Absorbance (*2.1.2.24*). NMT 0.05.

Measure the absorbance of a 50 g/L solution in the wavelength range from 240 nm to 300 nm.

Tetrabutylammonium dihydrogen phosphate. $C_{16}H_{38}NO_4P$ (M_r 339.5). [5574-97-0].

White or almost white powder, hygroscopic.

pH (*2.1.2.3*). About 7.5. Measure the pH of a 170 g/L solution.

Absorbance (*2.1.2.24*). About 0.10.

Measure the absorbance of a 170 g/L solution at a wavelength of 210 nm.

Store in an airtight container.

Tetrabutylammonium iodide. $C_{16}H_{36}IN$. (M_r 369.4). [311-28-4].

Contains not less than 98.0% of $C_{16}H_{36}IN$.

White or slightly coloured crystalline powder or crystals. Soluble in 96% ethanol.

Sulfated ash (*2.1.4.14*). NMT 0.02%.

Quantitation. Dissolve 1,200 g in 30 mL of *water R,* add 50.0 mL of *0.1 M silver nitrate solution* and 5 mL of *dilute nitric acid R*. Titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate solution,* using 2 mL of *ferric ammonium sulfate solution R2* as indicator.

1 mL of *0.1 M silver nitrate solution* is equivalent to 36.94 mg of $C_{16}H_{36}IN$.

Tetraheptylammonium bromide. $C_{28}H_{60}BrN$. (M_r 490.7). [4368-51-8].

White or slightly coloured crystalline powder or crystals.

The melting point is from 89 °C to 91 °C.

Tetrahexylammonium hydrogen sulfate.

$C_{24}H_{53}NO4S$. (M_r 451.8). [32503-34-7].

N,*N*,*N*-trihexylhexane-1-aminohydrogen sulfate.

White or almost white crystals.

The melting point from 100 °C to 102 °C.

Tetrahydrofuran. C4H8O. (*Mr* 72,1). [109-99-9]. Tetramethylene oxide.

A clear, colorless, flammable liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.89.

Do not distill if the tetrahydrofuran does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter, fill completely with tetrahydrofuran, shake, and allow to stand protected from light for 30 min. No staining should be observed.

Tetrahydrofuran used in spectrophotometry complies with the following additional test.

Minimum transmittance (*2.1.2.24*). Determination is carried out using *water R* as compensation liquid.

20% at a wavelength of 255 nm,

80% at a wavelength of 270 nm,

98% at a wavelength of 310 nm.

Tetradecane. $C_{14}H_{30}$. (M_r 198.4). [629-59-4]. *n*-Tetradecane.

Contains not less than 99.5% (m/m) of $C_{14}H_{30}$.

A colorless liquid.

d_{20}^{20} is about 0.76.

n_D^{20} is about 1.429.

The boiling point is about 252 °C.

The melting point is about -5 °C.

Tetradecylammonium bromide. $C_{40}H_{84}BrN$. (M_r 659). [14937-42-9]. Tetradecylammonium bromide.

White or slightly coloured crystalline powder or crystals.

The melting point is from 88 °C to 89 °C.

Tetrazolium blue. $C_{40}H_{32}C1_2N_8O_2$.

(M_r 728). [1871-22-3]. 3,3'-(3,3'-Dimethoxy [1,1'-biphenyl]-4,4'-diyl)bis[2,5-diphenyl-2*H*-tetrazolium]dichloride.

Yellow crystals. Slightly soluble in water, freely soluble in 96% ethanol and methanol, practically insoluble in acetone.

The melting point is about 245 °C with decomposition.

Tetramethylammonium hydroxide. $C_4H_{13}NO{\cdot}5H_2O$. (M_r 181.2). [10424-65-4]. Tetramethylammonium hydroxide pentahydrate.

HPLC Grade.

Tetramethylammonium hydroxide solution. [75-59-2].

Contains not less than 10.0% (*m/m*) of $C_4H_{13}NO$ (M_r 91.2).

Clear, colorless or slightly coloured liquid. Miscible with water and 96% ethanol.

Quantitation. Dissolve 1.000 g in 50 mL of water R *and titrate with* 0.05 M sulfuric acid, *using 0.1 mL of* methyl red solution R *as indicator.*

1 mL of *0.05 M sulfuric acid solution* is equivalent to 9.12 mg of $C_4H_{13}XO$.

Diluted tetramethylammonium hydroxide solution.

Dilute 10 mL of a solution of *tetramethylammonium hydroxide R* to 100 mL with *aldehyde-free ethanol R*. Prepare immediately before use.

Tetramethylammonium hydrogen sulfate. $C_4H_{13}NO_4S$. (M_r 171.2). [80526-82-5].

Hygroscopic powder.

The melting point is about 295 °C.

Tetramethylammonium chloride. $C_4H_{12}C1N$ (M_r 109.6). [75-57-0].

Colourless crystals. Soluble in water and 96% ethanol.

The melting point is about 300 °C with decomposition.

Tetramethyldiaminodiphenylmethane.

$C_{17}H_{22}N_2$ (M_r 254.4). [101-61-1]. 4,4'-Methylene-bis-(*N*,*N*-dimethylaniline).

White to bluish-white crystals or leaves. Practically insoluble in water, slightly soluble in 96% ethanol, soluble in mineral acids.

The melting point is about 90 °C.

Tetramethyldiaminodiphenylmethane reagent.

Solution A. Dissolve 2.5 g of *tetramethyldiaminodiphenylmethane R* in 10 mL of *glacial acetic acid R* and add 50 mL of *water R.*

Solution B. Dissolve 5g of *potassium iodide R* in 100 mL of *water R.*

Solution C. Dissolve 0.30 g of *ninhydrin R* in 10 mL of *glacial acetic acid R* and add 90 mL of *water R.*

Mix solutions A and B, and add 1.5 mL of solution C to the resulting solution.

Tetramethylsilane. $C_{14}H_{12}Si$. (M_r 88.2). [75-76-3].

A clear, colorless liquid. Very slightly soluble in water, soluble in acetone, and 96% ethanol.

d_{20}^{20} is about 0.64.

n_D^{20} is about 1.358.

The boiling point is about 26 °C.

Tetramethylsilane used in nuclear magnetic resonance spectroscopy complies with the following additional test.

In the NMR spectrum of about 10% (*V/V*) solution of tetramethylsilane in *deutered chloroform R*, the intensity of any foreign signal, excluding those that correspond to rotation of lateral ties and chloroform, must not exceed the intensity of the C-13 side lines, located at a distance of 59.1 Hz on either side of the main signal of tetramethylsilane.

Tetramethylethylenediamine. $C_6H_{16}N_2$. *(M_r* 116.2). [110-18-9].

N,N,N',N'-Tetramethylethylenediamine.

A colorless liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is about 0.78.

n_D^{20} is about 1.418.

The boiling point is about 121 °C.

Tetrachloroethane. $C_2H_2Cl_4$. (M_r 167.9). [79-34-5]. 1,1,2,2-Tetrachloroethane.

A clear, colorless liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 1.59.

n_D^{20} is about 1.495.

Distillation range (*2.1.2.11*). 145 °C to 147 °C; not less than 95% must be distilled.

Tetraethylammonium hydroxide solution. $C_8H_{21}NO$. (M_r 147.3). [77-98-5].

A 200 g/L solution; colorless liquid, a strong alkali.

d_{20}^{20} is about 1.01.

n_D^{20} is about 1.372.

HPLC Grade.

Tetraethylammonium hydrogen sulfate. $C_8H_{21}NO_4S$. (M_r 227.3). [16873-13-5].

Hygroscopic powder.

The melting point is about 245 °C.

Tetraethylene pentamine. $C_8H_{23}N_5$. (M_r 189.3). [112-57-2]. 3,6,9-Triazoundecane-1,11-diamine.

A colorless liquid. Soluble in acetone.

n_D^{20} is about 1.506.

Store in a cool dry place.

Thiamazole. $C_4H_6N_2S$. (M_r 114.2). [60-56-0]. Methimazole. 1-Methyl-1*H*-imidazole-2-thiol.

White or almost white crystalline powder. Freely soluble in water, soluble in 96% ethanol and methylene chloride.

The melting point is about 145 °C.

2-(2-Thienyl)acetic acid. $C_6H_6O_2S$. (M_r 142.1). [1918-77-0].

Brown powder. The melting point is about 65 °C.

Thymine. $C5H_6N_2O_2$. (M_r 126.1). [65-71-4]. 5-Methylpyrimidine-2,4-(1*H*,3*H*)-dione.

Short needle-like crystals or plates. Slightly soluble in cold water, soluble in hot water, soluble in dilute solutions of alkali metal hydroxides.

Thymol. $C_{10}H_{14}O$. (M_r 150.2). [89-83-8]. 5-Methyl-2-(methylethyl)phenol.

Colourless crystals.

Very slightly soluble in water, very soluble in 96% ethanol, freely soluble in essential and fatty oils, sparingly soluble in glycerol. Soluble in dilute solutions of alkali metal hydroxides.

Thymol used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil.*

Test solution. 0.1 g in 10 mL of *acetone R.*

The thymol content calculated by the method of internal standardisation must be minimum 95.0%.

Thymol blue. $C_{27}H_{30}O_5S$. (M_r 466.6). [76-61-9]. Thymolsulfonphthalein. 4,4'-(3H-2,1-Benz-oxa-thiol-3-or-den)bis[2-isopropyl-5-methyl-phenol]5,5-dioxide.

Brownish green to greenish blue crystalline powder. Slightly soluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Thymol blue solution.

Dissolve 0.1 g of *thymol blue R* in a mixture of 2.15 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R*, dilute to 100 mL with *water R.*

Test for sensitivity.

To 100 mL of *carbon dioxide-free water R*, add 0.1 mL of a solution of thymol blue and 0.2 mL of *0.02 M sodium hydroxide;* blue colour develops, which should turn yellow when not greater than 0.15 mL of *0.02 M hydrochloric acid* is added.

Colour change. Red to yellow in the pH range of 1.2 to 2.8. Olive-green to blue in the pH range of 8.0 to 9.6.

Thymolphthalein. $C_{28}H_{30}O_4$. (M_r 430.5). [125-20-2]. 3,3-Bis(4-hydroxy-5-isopropyl-2-me-thylphenyl)-3H-isobenzofuran-1-one.

White to yellowish white powder. Practically insoluble in water, soluble in 96% ethanol and dilute solutions of alkali metal hydroxides.

Thymolphthalein solution.

A 1 g/l solution of *96% ethanol R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.2 mL of thymolphthalein solution, the solution is colorless; when adding not greater than 0.05 mL of 0.1 M sodium hydroxide, the blue color develops.

Colour change. Colourless to blue in the pH range of 9.3 to 10.5.

Thioacetamide. C_2H_5NS. (M_r75.1). [62-55-5].

Crystalline powder or colorless crystals. Freely soluble in water and 96% ethanol.

The melting point is about 113 °C.

Thioacetamide solution.

A 40 g/L solution.

Thioacetamide reagent.

To 0.2 mL of *thioacetamide solution R*, add 1 mL of a mixture of 5 mL of *water R*, 15 mL of *1 M sodium hydroxide*, and 20 mL of *85% glycerol R*, heat on a water-bath for 20 s. Prepare immediately before use.

Thiobarbituric acid. $C_4H_4N_2O_2S$. (M_r 144.2). [504-17-6].

4,6-Dihydroxy-2-sulfanilamides.

Thioglycolic acid. $C_2H_4O_2S$. (M_r 92.1). [68-11-1]. 2-Mercapto-acetic acid.

A colorless liquid. Miscible with water, soluble in 96% ethanol.

Thiomersal. $C_9H_9HgNaO_2S$. (M_r 404.8). [54-64-8]. Sodium mercurothiolate. Sodium 2-[(ethylmercurio)thio]benzoate.

Light yellowish white crystalline powder. Very soluble in water, freely soluble in 96% ethanol.

Thiourea. CH_4N_2S. (M_r 76.1). [62-56-6].

White or almost white crystalline powder or crystals. Soluble in water and 96% ethanol.

The melting point is about 178 °C.

Tyramine. $C_8H_{11}NO$. (M_r 137.2). [51-67-2]. 4-(2-Aminoethyl)phenol.

Crystals. Slightly soluble in water, soluble in hot anhydrous ethanol.

The melting point is from 164 °C to 165 °C.

Tyrosine. $C_9H_{11}NO_3$. (M_r 181.2). [60-18-4]. 2-Amino-3-(4-hydroxyphenyl)propionic acid.

White crystalline powder, or crystals, or colorless crystals.

Slightly soluble in water, practically insoluble in acetone and anhydrous ethanol, soluble in dilute hydrochloric acid and solutions of alkali metal hydroxides.

Titanium. Ti. (A_r 47.88). [7440-32-6].

Contains not less than 99% of Ti.

Metal powder or thin wire, maximum 0.5 mm in diameter, or sponge.

The melting point is about 1668 °C.

The density is about 4.507 g/cm^3.

Titanium dioxide. TiO_2. (*Mr* 79.9). [13463-67-7].

Contains not less than 98.0% and not greater than 100.5% of TiO_2.

White or almost white powder.

Practically insoluble in water. Non-soluble in dilute mineral acids, slowly dissolves in hot concentrated sulfuric acid.

Titanium chloride. $TiCl_3$. (M_r 154.3). [7705-07-9].

Titanium trichloride.

Reddish-violet crystals, deliquescent. Soluble in water and 96% ethanol.

The melting point is about 440 °C.

Store in an airtight container.

Titanium chloride solution.

A 150 g/L solution in a 100 g/L solution of hydrochloric acid (HCl).

d_{20}^{20} is about 1.19.

Titanium chloride and sulfuric acid reagent.

Mix carefully 20 mL of *titanium(III) chloride solution R* with 13 mL of *sulfuric acid R*, add a sufficient amount of *concentrated hydrogen peroxide solution R* to obtain a yellow colour, heat until white vapours are liberated, and cool. Dilute with *water R* and repeat the evaporation and addition of *water R* to obtain a colorless solution. Dilute to 100 mL with *water R*.

Titan yellow. $C_{28}H_{19}N_5Na_2O_6S_4$. ($M_r$ 696). [1829-00-1]. Thiazole yellow. Disodium 2,2'-[(1-triazen-1,3-diyl)di-4,1-phenylene] bis-[6-methyl-benzothiazole-7-sulfonate].

Schultz No. 280.

Colour Index (C. I.) No. 19540.

Yellowish-brown powder. Freely soluble in water and 96% ethanol.

Titan yellow paper.

Immerse filter paper strips in *titan yellow solution R*, allow to stand for several minutes, and dry at room temperature.

Titan yellow solution.

A 0.5 g/L solution.

Test for sensitivity. To 10 mL of *water R*, add 0.1 mL of titan yellow solution, 0.2 mL of *magnesium standard solution (10 ppm Mg^{2+}) R*, and 1.0 mL of *1 M sodium hydroxide*. A distinct pink colour is visible by comparison of the resulting solution with a reference solution prepared in a similar manner omitting the magnesium.

Tosylarginine methyl ester hydrochloride.

$C_{14}H_{23}ClN_4O_4S$. (M_r 378.9). [1784-03-8]. *N*-Tosyl-L-arginine methyl ester hydrochloride. Methyl(S)-5-guanidino-2-(4-methylbenzolsulfonamide)valerate hydrochloride.

$[\alpha]_D^{20}$ is between -12 and -16. Determination is carried out using a 40 g/L solution.

The melting point is about 145 °C.

Tosylarginine methyl ether hydrochloride solution.

Add 5 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* to *98.5 mg of tosylarginine methyl ester hydrochloride R, shake until dissolved. Add 2.5 mL of* methyl red mixed solution *methyl red R*, and dilute to 25.0 mL with *water R*.

Tosyl-lysyl-chloromethane hydrochloride.

$C_{14}H_{22}Cl_2N_2O_3S$. (M_r 369.3). [4238-41-9]. *N*-Tosyl-L-lysyl chloromethane hydrochloride. (*3S*)-7-Amino-1-chloro-3-(4-methylbenzenesulfanylamido)heptan-2-one hydrochloride.

$[\alpha]_D^{20}$ is between -7 and 9. Determination is carried out using a 20 g/L solution.

The melting point is about 155 °C with decomposition.

$E_{1cm}^{1\%}$ is from 310 to 340. Determination is carried out at 230 nm, using *water R* as compensation liquid.

Tosylphenylalanylchloromethane.

$C_{17}H_{18}ClNO_3S$. (M_r 351.9). [402-71-1]. *N*-Tosyl-L-phenylalanyl chloromethane.

$[\alpha]_D^{20}$ is between 85 and -89. Determination is carried out using a 10 g/L solution in *96% ethanol R*.

The melting point is about 105 °C.

$E_{1cm}^{1\%}$ is from 290 to 320. Determination is carried out at 228.5 nm in *96% ethanol R*.

***o*-Tolidine**. $C_{14}H_{16}N_2$. (M_r 212.3). [119-93-7].

3,3'-Dimethylbenzidine.

Contains not less than 97.0% of $C_{14}H_{16}N_2$.

Light brown crystalline powder.

The melting point is about 130 °C.

o-Tolidine solution.

Dissolve 0.16 g of *o-tolidine R* in 30.0 mL of *glacial acetic acid R*, add 1.0 g of *potassium iodide R* and dilute to 500.0 mL with *water R*.

o-Toluidine. C_7H_9N. (M_r 107.2). [95-53-4]. 2-Methylaniline.

Pale-yellow liquid that turns reddish-brown on exposure to air and light. Slightly soluble in water, soluble in 96% ethanol and dilute acids.

d_{20}^{20} is about 1.01.

n_D^{20} is about 1.569.

The boiling point is about 200 °C.

Store in an airtight container, protected from light.

***p*-Toluidine**. C_7H_9N. (M_r 107.2). [106-49-0]. 4-Methylaniline.

Lustrous plates or flakes. Slightly soluble in water, freely soluble in acetone, and 96% ethanol.

The melting point is about 44 °C.

***o*-Toluidine hydrochloride**. $C_7H_{10}ClN$. (M_r 143.6). [636-21-5]. 2-Methylaniline hydrochloride. 2-Methylbenzenamine hydrochloride.

Contains not less than 98,0% of $C_7H_{10}ClN$.

The melting point is from 215 °C to 217 °C.

Toluidine blue. $C_{15}H_{16}ClN_3S$. (M_r 305.8). [92-31-9]. Toluidine blue O. 3-Amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride.

Schultz No. 1041.

Colour Index (C. I.) No. 52040.

Dark green powder. Soluble in water, slightly soluble in 96% ethanol.

Toluene. C_7H_8. (M_r92.1). [108-88-3]. Methylbenzene.

A clear, colorless, flammable liquid. Very slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20} is from 0.865 to 0.870.

The boiling point is about 110 °C.

Toluene, sulfur-free.

Complies with the requirements prescribed for *toluene R* with the following additional requirements.

Sulfur compounds. To 10 mL of toluene, add 1 mL of *anhydrous ethanol R,* 3 mL of *potassium plumbite solution R,* and boil under reflux condenser for 15 min. Allow to stand for 5 min. No darkening is produced in the aqueous layer.

Thiophen-related substances. Shake 2 mL of toluene with 5 mL of *isatin reagent R* for 5 min and allow to stand for 15 min; no blue staining is produced in the lower layer.

***o*-Toluenesulfonamide**. $C_7H_9NO_2S$. (M_r171.2). [88-19-7].2-Methylbenzenesulfonamide.

White or almost white crystalline powder. Slightly soluble in water, soluble in 96% ethanol, and solutions of alkali metal hydroxides.

The melting point is about 156 °C.

***p*-Toluenesulfonamide.** [70-55-3]

See *Toluenesulfonamide R.*

Toluenesulfonamide. $C_7H_9NO_2S$. (M_r 171.2). [70-55-3]. 4-Methylbenzenesulfonamide. *p*-Toluenesulfonamide.

Contains not less than 99.0% of $C_7H_9NO_2S$.

White or almost white crystalline powder. Slightly soluble in water, soluble in 96% ethanol, and solutions of alkali metal hydroxides.

The melting point is about 136 °C.

Toluenesulfonic acid. $C_7H_8O_3S{\cdot}H_2O$. (M_r 190.2). [6192-52-5]. 4-Methyl-benzenesulfonic acid.

Contains not less than 87.0% of $C_7H_8O_3S$.

White or almost white crystalline powder or crystals. Freely soluble in water, soluble in 96% ethanol.

Tragacanth. [9000-65-1].

An air-solidifying adhesive mass extracted from natural or specially made incisions in the trunk and branches of *Astragalus gummifer Labil.* and some other *Astragalus* species from Western Asia.

Threonine. C4H9NO3. (*Mr* 119.1). [72-19-5]. (2S,3R)-2-Amino-3-hydroxybutanoic acid.

The product of fermentation or hydrolysis of protein.

Contains not less than 99.0% and not greater than 101.0% of $C_4H_9NO_3$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Soluble in water, practically insoluble in 96% ethanol.

Triamcinolone. $C_{21}H_{27}FO_6$. (M_r 394.4). [124-94-7]. 9-Fluoro-11β,16α,17,21-tetrahydroxy-pregna-1,4-diene-3,20-dione.

Crystalline powder.

The melting point is from 262 °C to 263 °C.

Triacetin. $C_9H_{14}O_6$. (*Mr* 218.2). [102-76-1]. Propane-1,2,3-triyl triacetate. Glycerol triacetate.

Almost clear colorless or yellowish liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 1.16.

n_D^{20} is about 1.43.

The boiling point is about 260 °C.

Tricosane. $C_{23}H_{48}$. (M_r 324.6). [638-67-5].

White or almost white crystals. Practically insoluble in water, soluble in hexane.

The melting point is about 48 °C.

Trimethylpentane. C_8H_{18}. (M_r 114.2). [540-84-1]. Isooctane. 2,2,4-Trimethylpentane.

Colourless flammable liquid. Practically insoluble in water, soluble in anhydrous ethanol.

d_{20}^{20} is from 0.691 to 0.696.

n_D^{20} is from 1.391 to 1.393.

Distillation range (*2.1.2.11*). Between 98 °C and 100 °C; not less than 95% must be distilled.

Trimethylpentane used in spectrophotometry complies with the following additional test.

Absorbance (*2.1.2.24*): NMT 0.01.

Determination is carried out at 250-420 nm, using *water R* as compensation liquid.

Trimethylpentane R1.

Complies with the requirements prescribed for *trimethylpentane R* with the following modification.

Absorbance (*2.1.2.24*). NMT 0.07.

Determination is carried out at 220-360 nm, using *water R* as the compensation liquid.

Tris(hydroxymethyl)aminomethane.

$C_4H_{11}NO_3$. (M_r 121.1). [77-86-1]. Aminomethyl of dimetri(methanol). Tromethamine.

Contains not less than 99.0% and not greater than 100.5% of $C_4H_{11}NO_3$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals. Freely soluble in water, sparingly soluble in alcohol, very slightly soluble in ethyl acetate.

Tris(hydroxymethyl)aminomethane solution.

A solution of *Tris(hydroxymethyl)aminomethane R* contains 24.22 g of $C_4H_{11}NO_3$ per 1000.0 mL.

Triscyanoethoxypropane. $C_{12}H_{17}N_3O_3$. (M_r 251.3). 1,2,3-Tris (2-cyanoethoxy)propane.

Brown-yellow viscous liquid. Soluble in methanol.

Used as a stationary phase in gas chromatography.

d220 around 1.11.

Viscosity (*2.1.2.9*). About 172 MPa· s.

Triphenylmethanol. $C_{19}H_{16}O$. (M_r 260.3). [76-84-6]. Triphenylcarbinol.

Colourless crystals. Practically insoluble in water, freely soluble in 96% ethanol.

Triphenyltetrazolium chloride. $C_{19}H_{15}ClN_4$. (M_r 334.8). [298-96-4]. 2,3,5-Triphenyl-2*H*-tetrazolium chloride.

Contains not less than 98.0% of $C_{19}H_{15}ClN_4$.

Pale yellow or dull yellow powder. Soluble in water, in acetone, and 96% ethanol.

The melting point is about 240 °C with decomposition.

Quantitation. Dissolve 1.000 g in a mixture of 5 mL of *dilute nitric acid R* and 45 mL of *water R*, add 50.0 mL of *0.1 M silver nitrate*, and heat to boiling. Cool, add 3 mL of *dibutyl phthalate R*, shake vigorously, and titrate with *0.1 M ammonium thiocyanate solution*, using 2 mL of *ferric ammonium sulfate R2* solution as indicator.

1 mL of *0.1 M silver nitrate solution* is equivalent to 33.48 mg of $C_{19}H_{15}ClN_4$.

Store in a place protected from light.

Triphenyltetrazolium chloride solution.

A 5 g/L solution in *aldehyde-free alcohol (96%) R.*

Store in a place protected from light.

Trifluoroacetic acid. QHF^^

(*Mr* 114.0). [76-05-1].

Contains not less than 99% of C2HFA.

Liquid miscible with acetone and 96% ethanol.

d220 about 1.53.

The boiling point is about 72 °C.

Use a grade suitable for protein sequencing.

Store in an airtight container.

Trifluoroacetic anhydride. $C_4F_6O_3$. (M_r 210.0). [407-25-0].

A colorless liquid.

d_{20}^{20} is about 1.5.

Trichlorotrifluoroethane. $C_2Cl_3F_3$. (M_r 187.4). [76-13-1]. 1,1,2-Trichloro-1,2,2-trifluoroethane.

Colourless, volatile liquid. Practically insoluble in water, miscible with acetone.

d20 is about 1.58.

Distillation range (*2.1.2.11*). From 47 °C to 48 °C; not less than 95% must be distilled.

Trichloroacetic acid. $C_2HCl_3O_2$. (M_r 163.4). [76-03-9].

Colourless crystals or crystalline mass. Very deliquescent in air, very soluble in water and 96% ethanol.

Store in an airtight container.

Trichloroacetic acid solution.

Dissolve 40.0 g of *trichloroacetic acid R* in *water R* and dilute to 1000.0 mL with the same solvent . Determine the concentration by titration with *0.1 M sodium hydroxide* and, if necessary, adjust to a concentration of 40 ± 1 g/L.

1,1,1-Trichloroethane. $C_2H_3Cl_3$. (M_r 133. 4). [71-55-6]. Methyl chloroform.

Non-flammable liquid. Practically insoluble in water, soluble in acetone and methanol.

d_{20}^{20} is about 1.34.

n_D^{20} is about 1.438.

The boiling point is about 74 °C.

Trichloroethylene. C_2HCl_3. (M_r 131.4). [79-01-6].

A colorless liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 1.46.

n_D^{20} is about 1.477.

Triethanolamine. $C_6H_{15}NO_3$**.** (M_r 149.2). [102-71-6]. 2,2',2"-Nitrilotriethanol.

Contains not less than 99.0% (*m/m*) and not greater than 103.0% (*m/m*) of $C_6H_{15}XO_3$ from the total number of bases calculated with reference to the anhydrous substance.

Clear, viscous, colorless or slightly yellow liquid, very hygroscopic.

Miscible with water and 96% ethanol, soluble in methylene chloride.

Triethylamine. $C_6H_{15}N$. (M_r 101.2). [121-44-8]. *N*,*N*-Diethylethanamine.

A colorless liquid. Slightly soluble in water at temperatures below 18.7 °C, miscible with 96% ethanol.

d_{20}^{20} is about 0.727.

n_D^{20} is about 1.401.

The boiling point is about 90 °C.

Triethylenediamine. $C_6H_{12}N_2$. (M_r 112.2). 1,4-Diazabicyclo[2.2.2]octane.

Crystals, very hygroscopic. Easily sublimated at room temperature.

Freely soluble in water, acetone, and anhydrous ethanol.

The boiling point is about 174 °C.

The melting point is about 158 °C.

Store in an airtight container.

Thrombin, bovine. [9002-04-4].

A preparation of the enzyme, obtained from bovine plasma, that converts fibrinogen into fibrin. A yellowish-white powder.

Store at a temperature below 0 °C.

Thrombin, human. [9002-04-4].

Dried human thrombin. A preparation of the enzyme which converts human fibrinogen into fibrin. Obtained from liquid human plasma by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength and temperature.

A yellowish-white powder. Freely soluble in a 9 g/L solution of sodium chloride forming a cloudy, pale yellow solution.

Store in a glass container, sealed under nitrogen, protected from light, at a temperature below 25 °C.

Human thrombin solution.

Reconstitute human *thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Thromboplastin.

A drug preparation containing the membrane glycoprotein tissue factor and phospholipid, either purified from animal brain (usually rabbit) or human placenta or manufactured using recombinant DNA technology with added phospholipids. The preparation is formulated for routine use in the prothrombin time test and may contain calcium.

TLC silica gel plate.

Support of glass, metal or plastic, coated with a layer of silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the packing in the tests where it is used.

The sorbent may contain an organic binder.

Chromatographic separation capacity. Apply to the plate an appropriate volume (10 µL for a normal TLC plate and 1 µL to 2 µL for a fine particle size plate) of *TLC performance test solution R*. Develop in a solvent system using a mixture of *methanol R - toluene R* (20:80). When the solvent front passes two-thirds of the length of the plate, it is considered suitable if four clearly separated spots are visible on it:

- a spot of bromocresol green with R_F of NMT 0.15,
- a spot of methyl orange with an R_F ranging from 0.1 to 0.25,
- a spot of methyl red with an R_F ranging from 0.35 to 0.55,
- spot of Sudan red G in the R_F range from 0.75 to 0.98.

Solution for the suitability test of TLC plates.

Mix 1.0 mL of a 0.5 g/L solution of *sudan red G R* in *toluene R*, a freshly prepared 0.5 g/L solution of *methyl orange R* in *ethanol R*, a 0.5 g/L solution of *bromocresol green R* in *acetone R*, a 0.25 g/L solution of *methyl red R* in *acetone R* and dilute the resulting solution to 10.0 mL with *acetone R*.

TLC silica gel F_{254} plate.

Complies with the requirements prescribed for *TLC plates with a layer of silica gel R* with the following modifications.

Contains a fluorescent indicator with maximum absorption at a wavelength of 254 nm.

Fluorescence suppression. The plate is applied in five points successively increasing volumes from 1 µl to 10 µl for a conventional TLC plate and from 0.2 µl to 2 µl for an HPTLC plate of a 1 g/L solution of *benzoic acid R* in a mixture of *ethanol R-cyclohexane R* (15:85). Chromatograph in the same mixture of solvents. When the solvent front passes half the length of the plate, remove the plate from the chamber and dry until the solvents evaporate. The plate is viewed in UV light at a wavelength of 254 nm. For normal TLC plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 2 µg and greater. For HPTLC plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 0.2 µg and greater.

TLC silica gel G plate.

Complies with the requirements prescribed for *TLC plates with a layer of silica gel R* with the following modification.

Contains calcium sulfate hemihydrate as binder.

TLC silica gel GF_{254} plate.

Complies with the requirements prescribed for *TLC plates with a layer of silica gel R* with the following modifications.

Contains calcium sulfate hemihydrate as binder and a fluorescent indicator having a maximum absorption at 254 nm.

Fluorescence suppression. Complies with the requirements subscribed for *TLC plates with a layer of silica gel F_{254} R.*

TLC silica gel, silanised plate.

Support of glass, metal or plastic, coated with a layer of silanised silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the packing in the tests where it is used.

The sorbent may contain an organic binder.

Chromatographic separation capacity. Introduce 0.1 g each of *methyl laurate R, methyl myristate R, methyl palmitate R and methyl stearate R* into a 250 mL conical flask. Add 40 mL of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Cool down, transfer the solution to the separation funnel with 100 mL of *water R*, acidify with *dilute hydrochloric acid R* to a pH value between 2 and 3, and shake with three quantities of *methylene chloride R*, each of 10 mL. Dry the combined methylene chloride extracts over *anhydrous sodium sulfate R*, filter, and evaporate to dryness on a water-bath.

Dissolve the dry residue in 50 mL of *methylene chloride R* (test solution). Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *TLC silanised silica gel plate R*. The required volume of the test solution is applied to the plate at three points (about 10 µL for a normal TLC plate and 1-2 µL for a HPTLC plate with a small particle size). Develop in the solvent system of *glacial acetic acid R*/*water R*/*dioxane R* (10:25:65). When the solvent front passes two-thirds of the length of the plate, remove the plate from the chamber and dry at 120 °C for 30 min. Cool the plate, spray with a 35 g/L solution of *phosphomolybdic acid R* in *2-propanol R*, and heat at a temperature of 150 °C until the spots become visible. Then treat the plate with ammonia vapour until a white background is obtained. A plate is considered suitable if four clearly separated spots are visible on it.

TLC silica gel F_{254}, silanised plate.

Complies with the requirements prescribed for *TLC plates with a layer of silanised silica gel R* with the following modification.

Contains a fluorescent indicator with maximum absorption at a wavelength of 254 nm.

Thujone. $C_{10}H_{16}O$. (M_r 152.2). [76231-76-0]. 4-Methyl-1-(1-methylethyl)bicyclo[3.1.0]hexane-3-one.

Colourless or almost colorless liquid. Practically insoluble in water, soluble in 96% ethanol and many other organic solvents.

Black ink solution.

Dilute liquid black ink with *water R* in a ratio of 1:10.

Low-pressure hydrocarbons (type L).

Oily mass. Soluble in benzene and toluene.

Carbon dioxide. CO_2. (M_r 44.01). [124-38-9].

Contains not less than 99.5% (*V/V*) of CO_2 in the gaseous state.

Colorless gas. 1 volume of the gas is dissolved in approximately 1 volume of water at 20 °C and a pressure of 101 kPa.

Carbon dioxide R1. CO_2. (M_r 44.01).

Contains not less than 99.995% (*V/V*) of CO_2.

Carbon monoxide. Less than 5 ppm.

Oxygen. Less than 25 ppm.

Nitric acid. Less than 1 ppm.

Carbon dioxide R2. CO_2. (M_r 44.01). [124-38-9].

Contains not less than 99% (*V/V*) of CO_2.

Carbon disulfide. CS_2 (M_r 76.1). [75-15-0].

Colourless or yellowish flammable liquid. Practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20} is about 1.26.

The boiling point is from 46 °C to 47 °C.

Carbon monoxide. CO. (M_r 28.01). [630-08-0].

Contains not less than 99.97% (*V/V*) of CO.

Carbon monoxide R1. CO. (M_r 28.01). [630-08-0].

Contains not less than 99% (*V/V*) of CO.

Carbon tetrachloride. CCl_4. (M_r 153.8). [56-23-5]. Tetrachloromethane.

A colorless liquid. Practically insoluble in water, miscible with 96% ethanol.

d_{20}^{20} is from 1.595 to 1.598.

The boiling point is from 76 °C to 77 °C.

Activated carbon. [64365-11-3].

It is obtained from plant material by appropriate carbonation processes that provide high adsorption capacity.

Black light powder, free from sandiness.

Practically insoluble in all common solvents.

Carbon for chromatography, graphitised.

Carbon chains with a chain length greater than C_9.

The *particle size* is from 400 µm to 850 µm.

The relative density is 0.72.

Surface area 10 m^2/g.

Do not use at temperatures above 400 °C.

Carbon for chromatography, graphitised R1.

Porous spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms.

The *particle size* is from 5 µm to 7 µm.

The porosity of 0.7 cm^3/g.

Acetic anhydride. $C_4H_6O_3$. (M_r 102.1). [108-24-7].

Contains not less than 97.0% (*m/m*) of $C_4H_6O_3$.

A clear, colorless liquid.

The boiling point is from 136 °C to 142 °C.

Quantitation. Place 2.00 g in a flask fitted with a ground-glass stopper, dissolve in 50.0 mL of *1 M sodium hydroxide*, boil under reflux condenser for 1 h, and titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* used for titration of 1 g (*V*1). Place 2.00 g in a flask fitted with a ground-glass stopper, dissolve in 20 mL of *cyclohexane R*, cool in ice, then add the cooled mixture of 10 mL of *aniline R* and 20 mL of *cyclohexane R*, boil with reflux condenser for 1 h, add 50.0 mL of a *1 M sodium hydroxide*, vigorously mix, and titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* used for titration of 1 g (*V*2).

The percentage of $C_4H_6O_3$ is calculated using the formula: 10,2 ($V_1 - V_2$).

Acetic anhydride solution R1.

Dissolve 25.0 mL of *acetic anhydride R* in *anhydrous pyridine R* and adjust to 100.0 mL with the same solvent.

Store protected from light and air.

Acetic anhydride of sulfuric acid solution.

Carefully mix 5 mL of *acetic anhydride R* and 5 mL of *sulfuric acid R*. Add the resulting mixture dropwise to 50 mL of *anhydrous ethanol R*.

Prepare immediately before use.

Acetic acid, anhydrous. $C_2H_4O_2$. (*Mr* 60.1). [64-19-7].

Contains not less than 99.6% (*m/m*) of $C_2H_4O_2$.

Colourless liquid or white or almost white shiny fern-like crystals. Easily miscible or easily soluble in water, 96% ethanol, 85% glycerol, and most fatty and essential oils.

d_{20}^{20} is from 1.052 to 1.053.

The boiling point is from 117 °C to 119 °C.

A 100 g/L solution is a strong acid (*2.1.2.4*).

A 5 g/l acetic acid solution, neutralised with a *dilute ammonia solution R2*, gives the reaction (b) to acetates (*2.1.3.1*).

Freezing point (*2.1.2.17*). Not less than 15.8 °C.

Water (*2.1.5.12*). NMT 0.4%.

If the water content exceeds 0.4%, add the calculated amount of *acetic anhydride R.*

Store in a place protected from light.

Glacial acetic acid. $C_2H_4O_2$. (*Mr* 60.1). [64-19-7].

Contains not less than 99.0% (*m/m*) and not greater than 100.5% (*m/m*) of $C_2H_4O_2$.

A crystalline mass or clear, colourless, volatile liquid.

Miscible with water, 96% ethanol, and methylene chloride.

Acetic acid.

Contains not less than 290 g/L and not greater than 310 g/L of $C_2H_4O_2$ (M_r 60.1).

Dilute 30 g of *glacial acetic acid R* to 100 mL with *water R*.

Dilute acetic acid.

Contains not less than 115 g/L and not greater than 125 g/L of C2H4O2. (*Mr* 60.1).

Dilute 12 g of *glacial acetic acid R* to 100 mL with *water R*.

Dilute acetic acid R1.

Contains not less than 57.5 g/L and not greater than 62.5 g/L of C2H4O2. (*Mr* 60.1).

Dilute 6 g of *glacial acetic acid R* to 100 mL with *water R*.

Uridine. $C_9H_{12}N_2O_6$. (M_r 244.2). [58-96-8]. 1-β-D-Ribofuranosylpurine.

White or almost white crystalline powder. Soluble in water.

The melting point is about 165 °C.

Coagulation factor V solution.

Coagulation factor V solution may be prepared by the following method or by any other method which excludes factor VIII.

Prepare the factor V reagent from freshly oxalated bovine plasma by fractionation at 4 °C with a saturated solution of *ammonium sulfate R* prepared at 4 °C. Separate the fraction that is deposited in the saturation range between 38% and 50% and contains factor V without significant contamination with factor VIII. Remove the ammonium sulfate by dialysis and dilute with a 9 g/L *sodium chloride R* solution to obtain a solution containing 10% to 20% of the amount of factor V present in normal fresh human blood plasma.

Quantitation of factor V. Prepare two dilutions of the factor V preparation in an *imidazole buffer solution R pH 7.3*, containing one volume of the preparation in 10 and 20 volumes of the buffer solution, respectively. Test each dilution as follows: mix 0.1 mL of *plasma substrate deficient in factor V R*, 0.1 mL of the test solution, 0.1 mL of the *thromboplastin reagent R*, and 0.1 mL of a 3.5 g/L solution of *calcium chloride R*, and measure the blood clotting time, i.e. the range between the moment of adding the calcium chloride solution and the first sign of fibrin formation, which can be observed visually or using an appropriate apparatus. In the same way, the blood clotting time is determined (two parallel definitions) of four solutions of ordinary human blood plasma in *imidazole buffer solution pH 7.3 R*, containing, respectively, 1 volume in 10 (corresponds to 100% of factor V), 1 volume in 50 (20%), 1 volume in 100 (10%), and 1 volume in 1000 (1%). Using two-way logarithmic paper plot the average coagulation times for each dilution of human plasma against the equivalent percentage of factor V and read the percentage of factor V for the two dilutions of the factor V solution by interpolation. The mean of the two results gives the percentage of factor V in the test solution. Store the solution frozen at a temperature not higher than -20 °C.

Factor Xa, bovine, coagulation. [9002-05-5].

An enzyme which converts prothrombin to thrombin. The semi-purified preparation is obtained from liquid bovine plasma and it may be prepared by activation of the zymogen factor X with a suitable activator such as Russell's viper venom.

Store the lyophilised preparation at a temperature of -20 °C, store the frozen solution at a temperature below -20 °C.

Factor Xa solution, bovine.

Reconstitute as directed by the manufacturer and dilute with tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R.

The change in absorbance should not exceed 0.20 per min. Measure at 405 nm (*2.1.2.24*) against *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* as compensation liquid.

Factor Xa solution, bovine R1.

Reconstitute as directed by the manufacturer and dilute to 1.4 nkat/mL with *tris(hydroxymethyl)EDTA buffer solution pH 8.4 R.*

Phenazone. $C_{11}H_{12}N_2O$. (M_r 188.2). [60-80-0]. 1,5-Dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one.

Contains not less than 99.0% and not greater than 101.0% of $C_{11}H_{12}N_2O$ calculated on a dry substance basis.

White or almost white crystalline powder or colorless crystals.

Very soluble in water, 96% ethanol, and methylene chloride.

Phenanthrene. $C_{14}H_{10}$. (M_r 178.2). [85-01-8].

White or almost white crystals. Practically insoluble in water, sparingly soluble in 96% ethanol.

The melting point is about 100 °C.

Phenanthroline hydrochloride. $C_{12}H_9C1N_2 \cdot H_2O$. ($M_r$ 234.7). [3829-86-5]. 1,10-Phenanthroline hydrochloride monohydrate.

White or almost white powder. Freely soluble in water, soluble in 96% ethanol.

The melting point is about 215 °C with decomposition.

Phenylalanine. $C_9H_{11}NO_2$. (M_r 165.2). [63-91-2]. (2S)-2-Amino-3-phenylpropanoic acid.

The product of fermentation or hydrolysis of protein.

Contains not less than 98.5% and not greater than 101.0% of $C_9H_{11}NO_2$ calculated on a dry substance basis.

White or almost white crystalline powder or shiny white flakes.

Sparingly soluble in water, very slightly soluble in 96% ethanol, soluble in dilute mineral acids and in dilute solutions of alkali metal hydroxides.

Phenylhydrazine hydrochloride. $C_6H_9ClN_2$. (M_r 144.6). [59-88-1].

White or almost white crystalline powder that turns brown under exposure to air. Soluble in water and 96% ethanol.

The melting point is about 245 °C with decomposition.

Store in a place protected from light.

Phenylhydrazine hydrochloride solution.

Dissolve 0.9 g of *phenylhydrazine hydrochloride R* in 50 mL of *water R*, decolourise with *activated carbon R*, and filter. Add 30 mL of *hydrochloric acid R* to the filtrate and dilute to 250 mL with *water R*.

Phenylhydrazine solution in sulfuric acid.

Dissolve 65 mg of *phenylhydrazine hydrochloride R*, previously recrystallised from *85% (V/V) ethanol R*, in a mixture of *water R*/*sulfuric acid R* (80:170) and dilute to 100 mL with the same mixture of solvents.

Prepare immediately before use.

α-Phenylglycine. $C_8H_9NO_2$. (M_r 151.2). [2835-06-5].

(*RS*)-2-Amino-2-phenylacetic acid.

D-Phenylglycine. $C_8H_9NO_2$. (M_r 151.2). [875-74-1]. (2*R*)-2-Amino-2-phenylacetic acid.

Contains not less than 99% of $C_8H_9NO_2$.

White or almost white crystalline powder.

***p*-Phenylenediamine dihydrochloride**. $C_6H_{10}Cl_2N_2$. (M_r 181.1). [615-28-1]. 1,4-Diaminobenzene dihydrochloride.

White or slightly coloured crystalline powder or crystals. Turns red in the air. Freely soluble in water, slightly soluble in 96% ethanol.

Phenyl isothiocyanate. C_7H_5NS. (M_r 135.2). [103-72-0].

Liquid. Insoluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 1.13.

n_D^{20} is about 1.65.

The boiling point is about 221 °C.

The melting point is about -21 °C.

Phenoxybenzamine hydrochloride. $C_{18}H_{23}Cl_2NO$. (M_r 340.3).

N-(2-Chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)-benzylamine hydrochloride.

Contains not less than 97.0% and not greater than 103.0% of $C_{18}H_{23}C1_2NO$ calculated on the dried basis.

White or almost white crystalline powder. Sparingly soluble in water, freely soluble in 96% ethanol.

The melting point is about 138 °C.

Loss on drying (*2.1.2.31*). NMT 0.5%.

Dry over *phosphorus pentoxide R* at a pressure not exceeding 670 Pa for 24 h.

Quantitation. Dissolve 0.500 g in 50.0 mL of *chloroform-free ethanol R* and extract in three portions with *0.01 M hydrochloric acid*, each of 20 mL. Discard the acid layer and filter the chloroform layer through cotton wool. Dilute 5.0 mL of the resulting filtrate to 500.0 mL with *ethanol-free chloroform R*. Measure the absorbance of the resulting solution in a closed cell at a maximum at a wavelength of 272 nm. Calculate the content of $C_{18}H_{23}Cl_2NO$ taking the specific absorption index equal to 56.3.

Store in a place protected from light.

Phenoxyacetic acid. $C_8H_8O_3$. (M_r 152.1). [122-59-8]. 2-Phenoxyethanoic acid.

Almost white crystals. Sparingly soluble in water, freely soluble in 96% ethanol and glacial acetic acid.

The melting point is about 98 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Phenoxymethylpenicillin*; the resulting chromatogram shows only one principal spot.

Phenoxyethanol. $C_8H_{10}O_2$. (M_r 138.2). [122-99-6]. 2-Phenoxyethanol.

A clear, colourless, oily liquid. Slightly soluble in water, freely soluble in 96% ethanol.

d_{20}^{20} is about 1.11.

n_D^{20} is about 1.537.

Freezing point (*2.1.2.17*). Not less than 12 °C.

Phenol. C_6H_6O. (M_r 94.1). [108-95-2].

Contains not less than 99.0% and not greater than 100.5% of C_6H_6O.

Colourless or faintly pink, or pale yellowish crystals or crystalline mass. Deliquescent.

Soluble in water, very soluble in 96% ethanol, glycerol, methylene chloride.

Phenol red. [143-74-8].

Bright red to dark red crystal powder. Very slightly soluble in water, slightly soluble in 96% ethanol.

Phenol red solution.

Dissolve 0.1 g of *phenol red R* in a mixture of 2.82 mL of *0.1 M sodium hydroxide* and 20 mL of *96% ethanol R*, dilute to 100 mL with *water R*.

Test for sensitivity. Add 0.1 mL of the phenol red solution to 100 mL of *carbon dioxide-free water R*. The solution is yellow. Not greater than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to reddish-violet.

Colour change. Yellow to reddish-violet in the pH range of 6.8 to 8.4.

Phenol red solution R2.

Solution A. Dissolve 33 mg of *phenol red R* in 1.5 mL of a *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*.

Solution B. Dissolve 25 mg of *ammonium sulfate R* in 235 mL of *water R*, add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Mix solution B with 25 mL of solution A. If necessary, adjust the pH of the solution to 4.7.

Phenol red solution R3.

Solution A. Dissolve 33 mg of *phenolic red R* in 1.5 mL of *dilute sodium hydroxide solution R* and dilute to 50 mL with *water R*.

Solution B. Dissolve 50 mg of *ammonium sulfate R* in 235 mL of *water R*, add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Mix solution B with 25 mL of solution A. If necessary, adjust the pH of the solution to 4.7.

Phenolphthalein. $C_{20}H_{14}O_4$. (M_r 318.3). [77-09-8]. 3,3-Bis(4-hydroxy-phenyl)-3*H*-iso-benzofuran-1-one.

White to yellowish white powder. Practically insoluble in water, soluble in 96% ethanol.

Phenolphthalein solution.

Dissolve 0.1 g of *phenolphthalein R* in 80 mL of *96% ethanol R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 100 mL of *carbon dioxide-free water R*, add 0.1 mL of phenolphthalein solution; when adding no greater than 0.2 mL of *0.02 M sodium hydroxide*, the colour of the solution should change from colorless to pink.

Colour change. colorless to bright pink in the pH range of 8.2 to 10.0.

Phenolphthalein solution R1.

A 10 g/l solution of *96% ethanol R*.

Phenolphthalein paper.

Immerse filter paper strips for a few minutes in *phenolphthalein solution R* and allow to dry.

Fenchone. $C_{10}H_{16}O$. (M_r 152.2). [7787-20-4]. (1*R*)-1,3,3-Trimethylbicyclo[2.2.1]heptane-2-one.

Oily liquid. Miscible with 96% ethanol, practically insoluble in water.

n_D^{20} is about 1.46.

The boiling point is from 192 °C to 194 °C.

Fenchone used in gas chromatography complies with the following test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) under the conditions specified in the monograph *Bitter fennel*, using fenchone as the test solution.

The fenchone content calculated by the internal normalisation procedure should be minimum 98.0%.

Ferroin. [14634-91-4].

Dissolve 0.7 g of *ferrous sulfate R* and 1.76 g of *phenanthroline hydrochloride R* in 70 mL of *water R* and dilute to 100 mL with the same solvent.

Test for sensitivity. To 50 mL of *dilute sulfuric acid R*, add 0.1 mL of *ferroin R*. After adding 0.1 mL of *0.1 M ammonium cerium nitrate*, the colour of the solution should change from red to bright blue.

Ferrocyphene. $C_{26}H_{16}FeN_6$. (M_r 468.3). [14768-11-7]. Dicyanobis(1,10-phenanthroline)iron(II).

Violet-bronze crystal powder. Practically insoluble in water and 96% ethanol.

Store in a dark, dry place.

Fibrin congo red.

Allow 1.5 g of fibrin to stand overnight in 50 mL of a 20 g/L solution of *congo red R* in *96% (V/V) ethanol R* and filter. Wash fibrin with *water R* and store under *ether R*.

Cut the washed fibrin into small pieces and allow to stand overnight in a 20 g/L solution of *congo red R* in *alcohol (90%, V/V) R*, and filter; wash the fibrin with *water R* and store under *ether R*.

Fibrin blue.

Mix 1.5 g of fibrin with 30 mL of a 5 g/L *indigo carmine R* solution in a 1% (*V/V*) *dilute hydrochloric acid R solution*, heat the mixture to 80 °C, and allow to stand at this temperature for about 30 min with stirring, then cool and filter. Wash the precipitate thoroughly, resuspending in a *1% (V/V) dilute hydrochloric acid solution R* and stirring for about 30 minutes, then filter. Wash the precipitate three times, dry at 50 °C, and grind.

Fibrinogen. [9001-32-5]. Human fibrinogen, freeze-dried.

Sterile lyophilised preparation of the plasma protein fraction containing a soluble component of human plasma that is converted to fibrin when thrombin is added. Obtained from human plasma as prescribed in the monograph *Human plasma for fractionation*. The preparation may contain excipients such as salts, buffers, and stabilisers.

The reconstituted solution must contain not greater than 10 g/L of fibrinogen.

Hygroscopic white or pale yellow powder or free-flowing dry substance.

The drug solution is prepared directly before use.

Fixing solution.

To 250 mL of *methanol R*, add 0.27 mL of *formaldehyde R* and dilute to 500.0 mL with *water R*.

Fixing solution for isoelectric focusing in polyacrylamide gel.

A solution containing 35 g of *sulfosalicylic acid R* and 100 g of *trichloroacetic acid R* per 1 L of *water R*.

Phloroglucinol. $C_6H_6O_3 \cdot 2H_2O$. (M_r 162.1). [6099-90-7]. Benzene-1,3,5-triol.

White or yellowish crystals. Slightly soluble in water, soluble in 96% ethanol.

The melting point is about 223 °C (instantaneous melting method).

Phloroglucinol solution.

To 1 mL of a 100 g/L solution of *phloroglucinol R* in *96% ethanol R*, add 9 mL of *hydrochloric acid R*.

Store in a place protected from light.

Fluorescein. $C_{20}H_{12}O_5$. (M_r 332.3). [2321-07-5]. 3',6'-Dihydroxispiro[isobenzofuran-1(3*H*), 9'-[9*H*]xanthene]-3-one.

Orange-red powder. Practically insoluble in water, soluble in warm 96% ethanol, solutions of alkali metal hydroxides. In solution, fluorescein shows green fluorescence.

The melting point is about 315 °C.

Fluorescein-conjugated rabies antiserum.

Immunoglobulin fraction with a high rabies antibody titer prepared from the sera of suitable animals that have been immunised with inactivated rabies virus; the immunoglobulin is conjugated with fluorescein isothiocyanate.

Flufenamic acid. $C_{14}H_{10}F_3NO_2$. (M_r 281.2). [530-78-9]. 2-[[3-(Trifluoromethyl)phenyl]amino]benzoic acid.

Pale yellow crystalline powder or needle-like crystals. Practically insoluble in water, freely soluble in 96% ethanol.

The melting point is from 132 °C to 135 °C.

Folic acid. $C_{19}H_{19}N_7O_6$. (M_r 441.4). [75708-92-8]. (2*S*)-2-[[4-[[(2-amino-4-oxo-1,4-di-hydropteridine-6-yl)methyl]amino]benzoyl]amino] pentandionic acid.

Contains not less than 96.0% and not greater than 102.0% of $C_{19}H_{19}N_7O_6$ calculated with reference to the anhydrous substance.

Yellowish or orange crystalline powder.

Practically insoluble in water and in most organic solvents. Soluble in dilute acids and alkali solutions.

Formaldehyde. [50-00-0].

See *Formaldehyde solution R.*

Formaldehyde solution.

Formaldehyde solution (35%) contains not less than 34.5% (*m/m*) and not greater than 38.0% (*m/m*) of formaldehyde (CH_2O; M_r 30.03).

Contains methanol as a stabiliser.

A clear, colorless liquid.

Miscible with water and 96% ethanol. It may become cloudy during storage.

Formaldehyde solution in sulfuric acid.

Mix 2 mL of a solution of *formaldehyde R* with 100 mL of *sulfuric acid R*.

Formamide. CH_3NO. (M_r 45.0). [75-12-7].

Clear, colourless, oily, hygroscopic liquid. Miscible with water and 96% ethanol. Hydrolysed with water.

d_{20}^{20} is about 1.134.

The boiling point is about 210 °C.

Contains not less than 99.5% of CH_3NO.

Store in an airtight container.

Formamide, treated.

Disperse 1.0 g of *sulfamic acid R* in 20.0 mL of *formamide R* containing 5% (*V/V*) of *water R.*

Formamide R1.

Complies with the requirements prescribed for *formamide R* with the following additional test.

Water (*2.1.5.12*). NMT 0.1%. Determination is carried out with an equal volume of *anhydrous methanol R.*

Phosphorus (V) oxide. P_2O_5. (M_r 141.9). [1314-56-3]. Diphosphorus pentoxide. Phosphoric anhydride.

White or almost white amorphous powder, deliquescent. Forms hydrates with water with the release of heat.

Store in an airtight container.

Phosphoric acid. H_3PO_4. (M_r 98.0). [7664-38-2].

Contains not less than 84.0% (*m/m*) and no greater than 90.0% (*m/m*) H_3PO_4.

Clear, colorless syrupy liquid that causes corrosion. When stored at low temperatures, it can solidify into a colorless crystalline mass that does not melt at temperatures below 28 °C.

Miscible with water and 96% ethanol.

d_{20}^{20} is about 1.7.

Phosphoric acid, dilute.

Contains not less than 9.5% (*m/m*) and not greater than 10.5% (*m/m*) of H_3PO_4 (*Mr* 98.0).

Preparation: to 885 g of *water R* add 115 g of *phosphoric acid R* and mix.

Dilute phosphoric acid R1.

Dilute 93 mL of *dilute phosphoric acid R* to 1000 mL with *water R.*

Phosphotungstic acid solution.

To 10 g of *sodium tungstate R,* add 8 mL of *phosphoric acid R* and 75 mL of *water R,* heat with a reverse condenser for 3 h, allow to cool, and dilute with to 100 mL *water R.*

Phosphomolybdic acid.

$12MoO_3 \cdot H_3PO_4 \cdot xH_2O$. [51429-74-4].

Small orange-yellow crystals. Freely soluble in water, soluble in 96% ethanol.

Phosphomolybdic acid solution.

Dissolve 4 g of *phosphomolybdic acid R* in *water R*, dilute to 40 mL with the same solvent. Cautiously with cooling, add 60 mL of *sulfuric acid R*. Prepare immediately before use.

Phosphomolybdotungstic reagent.

Dissolve 100 g of *sodium tungstate R* and 25 g of *sodium molybdate R* in 700 mL of *water R*, add 100 mL of *hydrochloric acid R* and 50 mL of *phosphoric acid R*. Heat the mixture in a glass flask under a reflux condenser for 10 h, add 150 g of *lithium sulfate R*, 50 mL of *water R*, and a few drops of *bromine R*. Boil to remove the excess of bromine (15 min), cool, dilute to 1000 mL with *water R*, and filter. The reagent must have a yellow colour. The reagent is not suitable for use if it becomes green but can be regenerated by boiling with a few drops of *bromine R*. Excess bromine must be removed by boiling.

Store at a temperature between 2 °C and 8 °C.

Diluted phosphomolybdotungstic reagent..

Mix *phosphomolybdotungstic reagent R* with *water R* (1:2).

Fructose. $C_6H_{12}O_6$. (M_r 180.2). [57-48-7].

White or almost white crystalline powder.

Very soluble in water, soluble in 96% ethanol.

Phthalazine. $C_8H_6N_2$. (M_r 130.1). [253-52-1].

Pale yellow crystals. Freely soluble in water, soluble in ethanol, ethyl acetate, and methanol.

The melting point is from 89 °C to 92 °C.

Phthalic acid. $C_8H_6O_4$. (M_r 166.1). [88-99-3]. Benzene-1,2-dicarboxylic acid.

White or almost white crystalline powder. Soluble in hot water and 96% ethanol.

Pthtalic aldehyde. $C_8H_6O_2$. (M_r 134.1). [643-79-8]. Benzene-1,2-dicarboxaldehyde.

Yellow crystalline powder. The melting point is about 55 °C.

Store protected from light and air.

Phthalaldehyde reagent.

Dissolve 2.47 g of *boric acid R* in 75 mL of *water R*, adjust the pH to 10.4 with a solution of 450 g/L of *potassium hydroxide R*, and adjust to 100 mL with *water R*. Dissolve 1.0 g of *phthalic aldehyde R* in 5 mL of *methanol R*, add 95 mL of the prepared solution of boric acid and 2 mL of *thioglycolic acid R*, and adjust the pH to 10.4 with a solution of 450 g/L of *potassium hydroxide R*.

Store in a place protected from light.

Use within 3 days.

Phthalic anhydride. $C_8H_4O_3$. (M_r 148.1). [85-44-9]. Isobenzofuran-1,3-dione.

Contains not less than 99.0% of $C_8H_4O_3$. White or almost white flakes. The melting point is between 130 °C and 132 °C. *Quantitation.* Dissolve 2.000 g in 100 mL of *water R*, boil under a reflux condenser for 30 min, allow to cool, and titrate with *1 M sodium hydroxide solution*, using a *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide solution* is equivalent to 74.05 mg of $C_8H_4O_3$.

Phthalic anhydride solution.

Dissolve 42 g of *phthalic anhydride R* in 300 mL of *anhydrous pyridine R* and allow to stand for 16 h.

Store in a place protected from light.

Storage: use within 7 days.

Phthalein purple. $C_{32}H_{32}B_2O_{12} \cdot xH_2O$. ($M_r$ 637 for anhydrous substance). [2411-89-4]. Metalphthalein. 2,2′,2″,2‴-[o-Cresolphthalein-3′,3″-bis(methylenenitrilo)]tetra-acetic acid. (1,3-Dihydro-3-oxo-isobenzofuran-1-ylidene)bis[(6-hydroxy-5-methyl-3,1- phenylene)bis(methyleneimino)diacetic acid].

Yellowish white to brownish powder. Practically insoluble in water, soluble in 96% ethanol. The reagent is available in the form of sodium salt: yellowish white to brownish powder; soluble in water, practically insoluble in 96% ethanol.

Test for sensitivity. Dissolve 10 mg in 1 mL of a solution of *concentrated ammonia R* and dilute to 100 mL with *water R*. To 5 mL of the solution add 95 mL of *water R*, 4 mL of *concentrated ammonia R*, 50 mL of *ethanol (96%) R* and 0.1 mL of *0.1 M barium chloride*. The solution is blue-violet. Add 0.15 mL of *0.1 M sodium edetate*. The solution becomes colourless.

2-Fluoro-2-deoxy-B-glucose. $C_6H_{11}FO_5$. (M_r 182.2). [86783-82-6].

White or almost white crystalline powder.

The melting point is from 174 °C to 176 °C.

2-Fluoro-2-deoxy-D-mannose. $C_6H_{11}FO_5$. (M_r 182.1). [38440-79-8].

Colourless, soft substance.

Fluorodinitrobenzene. $C_6H_3FN_2O_4$. (M_r 186.1). [70-34-8].

1-Fluoro-2,4-dinitrobenzene.

Pale-yellow crystals or liquid. Soluble in propylene glycol.

The melting point is about 29 °C.

Contains not less than 99,0% of $C_6H_3FN_2O_4$. Carry out determination by gas chromatography.

1-Fluoro-2-nitro-4-(trifluoromethyl)-benzene.

C7H3F4NO2. (*Mr* 209.1). [367-86-2].

The melting point is about 197 °C.

Hydrofluoric acid. HF. (M_r 20.01). [7664-39-3].

Contains not less than 40.0% (*m/m*) HF.

A clear, colorless liquid.

Loss on ignition. NMT 0.05% (w/w).

evaporate hydrofluoric acid in a platinum crucible, ignite the residue carefully to a constant mass.

Quantitation. In an accurately weighed ground-glass-stoppered flask containing 50.0 mL of *1 M sodium hydroxide*, add 2 g of hydrofluoric acid, and weigh. Titrate with *0.5 M sulfuric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide solution* is equivalent to 20.01 mg of HF.

Store in a polyethylene container.

Fucose. $C_6H_{12}O_5$. (M_r 164.2). [6696-41-9]. 6-Deoxy-1-L-galactose.

White or almost white powder. Soluble in water and 96% ethanol.

$[\alpha]_D^{20}$ is about -76. Determination is carried out in a 90 g/L solution of 24 h after dissolution.

The melting point is about 140 °C.

Fuchsin, basic. [632-99-5].

Rosaniline hydrochloride mixture ($C_{20}H_{20}ClN_3$; M_r 337.9; Colour Index (C. I.) No. 42510; Schultz No. 780) and *para*-rosaniline hydrochloride (($C_{19}H_{18}ClN_3$; M_r 323.8; Colour Index No. 42500; Schultz No. 779).

If necessary, carry out the purification as follows: Dissolve 1 g of basic fuchsin in 250 mL of *dilute hydrochloric acid R*, alow to stand for 2 h at room temperature, filter; neutralise the resulting filtrate with a *dilute sodium hydroxide solution R* and add 1 mL to 2 mL of its excess. Filter through a glass filter (40) (*2.1.1.2)*, wash the precipitate with *water R*, dissolve in 70 mL of *methanol R*, previously heated to boiling, and add 300 mL of *water R* at 80 °C. Cool and filter; dry the crystals in vacuo.

Crystals with a greenish-bronze lustre. Soluble in water and 96% ethanol.

Store in a place protected from light.

Fuchsin, decolourised solution.

Dissolve 0.1 g of *basic fuchsin R* in 60 mL of *water R*, add a solution containing 1 g of *anhydrous sodium sulfite R* or 2 g of *sodium sulfite R* in 10 mL of *water R*. Slowly, with constant stirring, add 2 mL of *hydrochloric acid R*, dilute to 100 mL with *water R*.

Keep in a dark place for at least 12 hours, discolour with *activated carbon R*, and filter. If the solution becomes cloudy, it is filtered before use. If a violet staining appears when the solution is standing, discolour it again with *activated carbon R*.

Test for sensitivity. To 1.0 mL, add 1.0 mL of *water R* and 0.1 mL of *aldehyde-free alcohol R*. Add 0.2 mL of a solution containing 0.1 g/L of formaldehyde (CH_2O, M_r 30.0).

Within 5 minutes, a light pink colour of the solution should appear.

Store in a dark place.

Fuchsin solution, decolourised R1.

To 1 g of *basic fuchsin R*, add 100 mL of *water R*, heat to 50 °C and cool, stirring periodically. Allow to stand for 48 h, stir and filter. To 4 mL of filtrate, add 6 mL of *hydrochloric acid R*, mix, and dilute to 100 mL with *water R*. The solution is used 1 h after preparation.

Furfural. $C_5H_4O_2$. (*Mr* 96.1). [98-01-1]. 2-Furaldehyde. 2-Furancarboxaldehyde.

Colourless or brownish-yellow, clear oily liquid. Miscible with 11 volumes of water, miscible with 96% ethanol.

d_{20}^{20} is from 1.155 to 1.161.

Distillation range (*2.1.2.11*). From 159 °C and 163 °C; not less than 95% must be distilled.

Store in a dark place.

Calconecarboxylic acid. $C_{21}H_{14}N_2O_78$. (M_r 438.4). [3737-95-9]. 2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)naphthalene-3-carboxylic acid.

Brownish black powder. Slightly soluble in water, very slightly soluble in acetone and 96% ethanol, sparingly soluble in dilute solutions of sodium hydroxide.

Calconecarboxylic acid indicator.

Mix one volume of *calconecarboxylic acid R* with 99 volumes of *sodium chloride R*.

Test for sensitivity. Dissolve 50 mg of the calconecarboxylic acid indicator in a mixture of 2 mL of *strong sodium hydroxide solution R* and 100 mL of *water R*; blue colour develops, which should turn violet when adding 1 mL of a 10 g/L solution of *magnesium sulfate R* and 0.1 mL of a 1.5 g/L solution of *calcium chloride R*; when adding 0.15 mL of *0.01 M sodium edetate*, blue colour appears again.

Quinaldine red. $C_{21}H_{23}IN_2$. (M_r 430.3). [117-92-0]. 2-[2-[4-(Dimethyl-but) phenyl] ethenyl]-1-ethylquinoline iodide.

Dark bluish black powder. Sparingly soluble in water, freely soluble in 96% ethanol.

Quinaldine red solution.

Dissolve 0.1 g of *quinaldine red R* in *methanol R* and dilute to 100 mL with the same solvent.

Colour change. Colourless to red in the pH range of 1.4 to 3.2.

Quinhydrone. $C_{12}H_{10}O_4$. (M_r 218.2). [106-34-3]. Equimolecular compound of 1,4-benzoquinone and hydroquinone.

Lustrous crystals or dark green crystalline powder. Slightly soluble in water, sparingly soluble in hot water, soluble in 96% ethanol and concentrated ammonia solution.

The melting point is about 170 °C.

Quinidine. $C_{20}H_{24}N_2O_2$. (M_r 324.4). [56-54-24]. (*S*)-(6-Methoxyquinol-4-yl)[(2*R*,4*S*,5*R*) 5-vinyl-quinuclidine-2-yl]methanol.

White or almost white crystalline powder. Very slightly soluble in water, sparingly soluble in 96% ethanol, slightly soluble in methanol.

$[\alpha]_D^{20}$ is about +260. Determination is carried out using a 10 g/L solution in *anhydrous ethanol R*.

The melting point is about 172 °C.

Store in a place protected from light.

Quinidine sulfate. $C_{40}H_{50}N_4O_8S{\cdot}2H_2O$. ($M_r$ 783). [6591-63-5]. Bis[(8)-[(2R, 4S, 5R)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinoline-4-yl)methanol]sulfate dihydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_{40}H_{50}N_4O_8S$ calculated on a dry substance basis.

White or almost white crystalline powder or silky colorless needles.

Slightly soluble in water, soluble in boiling water, and 96% ethanol, practically insoluble in acetone.

Quinine. $C_{20}H_{24}N_2O_2$. (M_r 324.4). [130-95-0]. (*R*)-(6-Methoxyquinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinyl-quinuclidine-2-yl]methanol.

White or almost white microcrystalline powder. Very slightly soluble in water, slightly soluble in boiling water, very soluble in anhydrous ethanol.

$[\alpha]_D^{20}$ is about -167. Determination is carried out using a 10 g/L solution in *anhydrous ethanol R*.

The melting point is about 175 °C.

Store in a place protected from light.

Quinine hydrochloride. $C_{20}H_{25}ClN_2O_2 \cdot 2H_2O$. ($M_r$ 396,9). [6119-47-7]. (*R*)-[(2S,4S,5R)-5-Ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxychi-nolin-4-yl)methanol hydrochloride dihydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_{20}H_{25}ClN_2O_2$ calculated on a dry substance basis.

White or almost white or colorless fine silky needles, often in clusters.

Soluble in water, freely soluble in 96% ethanol.

Quinine sulfate. $C_{40}H_{50}N_4O_8S \cdot 2H_2O$. ($M_r$ 783). [6119-70-6].

Alkaloid monosulfates expressed as bis [(*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinoline-4-yl)methanol] sulfate dihydrate.

Contains not less than 99.0% and not greater than 101.0% of $C_{40}H_{50}N_4O_8S$ calculated on a dry substance basis.

White or almost white crystalline powder or thin colorless needles.

Slightly soluble in water, sparingly soluble in boiling water, and 96% ethanol.

Chloral. $C_2H_3Cl_3O_2$. (M_r 165.4). [302-17-0]. 2,2,2-Trichloroethane-1,1-diol.

Contains not less than 98.5% and not greater than 101.0% of C2H3CI3O2.

Colourless clear crystals.

Very soluble in water, freely soluble in 96% ethanol.

Chloral hydrate solution.

A 80 g per 20 mL solution of *water R.*

Chloramine. $C_7H_7ClNNaO_2S \cdot 3H_2O$. ($M_r$ 281.7). [7080-50-4]. Sodium N-chloro-4-methylbenzene-sulfonimidate trihydrate.

Contains not less than 98.0% and not greater than 103.0% of $C_2H_7ClNNaO_2S \cdot 3H2O$.

White or slightly yellowish crystalline powder.

Freely soluble in water, soluble in 96% ethanol.

Chloramine solution.

A 20 g/L solution.

Prepare immediately before use.

Chloramine solution R1.

0.1 g/l *chloramine R* solution. Prepare immediately before use.

Chloramine solution R2.

A 0.2 g/L solution of *chloramine R.*

Prepare immediately before use.

Chloraniline. $C6H_6CIN$. (M_r 127.6). [106-47-8]. 4-Chloraniline.

Crystals. Soluble in hot water, freely soluble in 96% ethanol.

The melting point is about 71 °C.

Chloroacetanilide. C_8H_8CINO. (M_r 169.6). [539-03-7]. 4'-Chloroacetanilide.

Crystalline powder. Practically insoluble in water, soluble in 96% ethanol.

The melting point is about 178 °C.

4-Chlorobenzenesulfonamide. $C_6H_6CINO_2S$. (M_r 191.6). [98-64-6].

White or almost white powder.

The melting point is about 145 °C.

Chlorobutanol. $C_4H_7C1_3O$. (M_r 177.5). [57-15-8]. 1,1,1-Trichloro-2-methylpropane-2-ol-1.

Contains not less than 98.0% and not greater than 101.0% of $C_4H_7C1_3O$ calculated with reference to the anhydrous substance.

White or almost white crystalline powder or colorless crystals. Easily sublimated.

Slightly soluble in water, very soluble in 96% ethanol, soluble in 85% glycerol.

The melting point is about 95 °C (without pre-drying).

Perchloric acid. $HClO_4$. (M_r 100.5). [7601-90-3].

Contains not less than 70.0% (*m/m*) and not greater than 73.0% (*m/m*) of $HClO_4$.

A clear, colorless liquid. Freely miscible with water.

d_{20}^{20} is about 1.7.

Quantitation. To 2.50 g of perchloric acid, add 50 mL of *water R* and titrate with *1M sodium hydroxide*, using 0.1 mL of *methyl red solution R*.

1 mL of *1 M sodium hydroxide solution* is equivalent to 100.5 mg of HCIO4.

Perchloric acid solution.

Dilute 8.5 mL of *perchloric acid R* to 100 mL with *water R.*

Hydrochloric acid. HCl. (M_r 36.46). [7647-01-0].

Contains not less than 35.0% (*m/m*) and no greater than 39.0% (*m/m*) of HCl.

Clear, colorless fuming liquid. Water-miscible.

d_{20}^{20} is about 1.18.

2 M hydrochloric acid.

Dilute 206.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

3 M hydrochloric acid.

Dilute 309.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

6 M hydrochloric acid.

Dilute 618.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

Hydrochloric acid R1.

Contains 250 g/l of HCI.

Dilute 70 g of *hydrochloric acid R* to 100 mL with *water R*.

Hydrochloric acid, brominated.

To 100 mL of *hydrochloric acid R*, add 1 mL of *bromine solution R.*

Hydrochloric acid in methanol.

Dilute 4.0 mL of *hydrochloric acid R* to 1000.0 mL with *methanol R2*.

Hydrochloric acid in ethanol.

Dilute 5.0 mL of *1 M hydrochloric acid* to 500.0 mL with *96% ethanol R*.

Dilute hydrochloric acid.

Contains 73 g/l of HCI.

Dilute 20 g of *hydrochloric acid R* to 100 mL with *water R*.

Dilute hydrochloric acid R1.

Contains 0.37 g/l of HCl.

Dilute 1.0 mL of *dilute hydrochloric acid R* to 200.0 mL with *water R*.

Dilute hydrochloric acid R2.

Dilute 30 mL of *1 M hydrochloric acid* to 1 L with *water R*; the pH of the solution is 1.6 ± 0.1.

Hydrochloric acid, lead-free.

Complies with the requirements prescribed for *hydrochloric acid R* with the following additional test.

Lead (*2.1.2.21, Method I).* NMT 20 ppm. Determination is carried out by atomic emission spectrometry.

Test solution. Place 200 g of hydrochloric acid in a quartz crucible, evaporate almost to dryness, add 5 mL of nitric acid to the resulting residue, prepared by distillation of *nitric acid R* at a temperature below the boiling point, and evaporate to dryness. To the residue obtained, add 5 mL of nitric acid prepared by distillation of *nitric acid R* at a temperature below the boiling point.

Reference solutions. Prepare reference solutions using a *standard solution of lead ions (0.1 ppm Pb^{2+}) R*, diluted with nitric acid prepared by distillation of *nitric acid R* at a temperature below the boiling point.

The emission intensity is measured at a wavelength of 220.35 nm.

Hydrochloric acid, heavy metal-free.

Complies with the requirements prescribed for *hydrochloric acid R* with the following maximum concentration of heavy metals:

As NMT 0.005 ppm.
Cd – NMT 0.003 ppm.
Cu – NMT 0.003 ppm.
Fe – NMT 0.05 ppm.
Hg – NMT 0.005 ppm.
Ni – NMT 0.004 ppm.
Pb NMT 0.001 ppm.
Zn – NMT 0.005 ppm.

Hydrochloric acid, dilute, heavy metal-free.

Complies with the requirements prescribed for *dilute hydrochloric acid R* with the following maximum concentration of heavy metals:

As NMT 0.005 ppm.
Cd – NMT 0.003 ppm.
Cu – NMT 0.003 ppm.
Fe – NMT 0.05 ppm.
Hg – NMT 0.005 ppm.
Ni – NMT 0.004 ppm.

Pb NMT 0.001 ppm.

Zn – NMT 0.005 ppm.

2-Chloro-2-deoxy-B-glucose. $C_6H_{11}ClO_5$. (M_r 198.6). [14685-79-1].

White or almost white crystals. Very hygroscopic powder. Soluble in water and dimethylsulfoxide, practically insoluble in 96% ethanol.

2-Chloro-*N*-(2,6-dimethylphenyl)acetamide. $C_{10}H_{12}ClNO$. (M_r 197.7). [1131-01-7].

3-Chloro-2-methylaniline. C_7H_8ClN. (M_r 141.6). [87-60-5]. 6-Chloro-2-toluidine.

Immiscible with water, slightly soluble in anhydrous ethanol.

d_{20}^{20} is about 1,171.

n_D^{20} is about 1.587.

The boiling point is about 115 °C.

The melting point is about 2 °C.

2-Chloronicotinic acid. $C_6H_4ClNO_2$. (M_r 157.6). [2942-59-8]. 2-Chlorpyridine-3-carboxylic acid.

White or almost white powder.

The melting point is about 177 °C.

Contains not less than 95% of $C_6H_4ClNO_2$.

2-Chloro-4-nitroaniline. $C_6H_5ClN_2O_2$. (M_r 172.6). [121-87-9].

Yellow crystalline powder. Freely soluble in methanol.

The melting point is about 107 °C.

Store in a place protected from light.

2-Chloro-5-nitrobenzoic acid. $C_7H_4ClNO_4$. (M_r 201.6). [2516-96-3].

The melting point is from 165 °C to 168 °C.

Chlorogenic acid. $C_{16}H_{18}O_9$. (M_r 354.3). [327-97-9]. (1*S*,3*R*,4*R*,5*R*)-3-[(3,4-Dihydroxycinnamoyl)oxy]-1,4,5- trihydroxycyclohexanecarboxylic acid.

White or almost white crystalline powder. Freely soluble in boiling water, acetone, and 96% ethanol.

d_D^{25} is about -35.2.

The melting point is about 208 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Belladonna leaf dry extract, standardised* under the conditions described in *Identification A*; the chromatogram shows only one principal spot.

Chlorogenic acid used in liquid chromatography complies with the following additional test.

Quantitation. Determination is carried out by liquid chromatography (*2.1.2.28*) as prescribed in the monograph *Artichoke Leaf.*

The content must be minimum 97.0%

Chloroform. $CHCl_3$. (M_r 119.4). [67-66-3]. Trichloromethane.

A clear, colorless liquid. Slightly soluble in water, miscible with 96% ethanol.

d_{20}^{20}*0* is from 1.475 to 1.481.

The boiling point is about 60 °C.

Ethanol. From 0.4% (*m/m*) to 1.0% (*m/m*).

Chloroform, acidified.

To 100 mL of *chloroform R*, add 10 mL of *hydrochloric acid R*, shake, allow to stand, and separate 2 layers.

Chloroform, ethanol-free.

Wash 200 mL of *chloroform R* with *water R*, shaking with four portions, each of 100 mL. Dry over 20 g of *anhydrous sodium sulfate R* for 24 h. Distill the filtrate over 10g of *anhydrous sodium sulfate R*, discarding the first 20 mL of the distillate.

Prepare immediately before use.

Chloroform stabilised with amylene. $CHCl_3$. (*Mr* 119.4).

A clear, colorless liquid. Slightly soluble in water, miscible with 96% ethanol.

Water. NMT 0.05%.

Residue on evaporation. NMT 0.001%.

Minimum transmittance (*2.1.2.24*). Determined using *water R* as compensation liquid:

not less than 50% at a wavelength of 255 nm, not less than 80% at a wavelength of 260 nm, not less than 98% at a wavelength of 300 nm.

Quantitation. NLT 99.8% of $CHCl_3$.

Carry out determination by gas chromatography.

Chloroplatinic acid. $H_2Cl_6Pt{\cdot}6H_2O$. (M_r 517.9). [18497-13-7]. Hexachloroplatinic(IV) acid hexahydrate.

Contains not less than 37.0% (*m/m*) of platinum (A_r 195.1).

Brownish-red crystals or crystalline mass. Very soluble in water, soluble in 96% ethanol.

Quantitation. Ignite 0.200 g of chloroplatinic acid at a temperature of 900 ± 50 °C to a constant mass and weigh the residue (platinum).

Store in a place protected from light.

3-Chloropropane-1,2-diol. $C_3H_7ClO_2$. (M_r 110.5). [96-24-2].

A colorless liquid. Soluble in water and 96% ethanol.

d_{20}^{20} is about 1.322.

n_D^{20} is about 1.480.

The boiling point is about 213 °C.

5-Chlorosalicylic acid. $C_7H_5ClO_3$. (*Mr* 172.6). [321-14-2].

White or almost white crystalline powder. Soluble in methanol.

The melting point is about 173 °C.

Chlorthiazide. $C_7H_6ClN_3O_4S_2$. (M_r 295.7). [58-94-6]. 6-Chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide.

Contains not less than 98.0% of $C_7H_6ClN_3O_4S_2$.

White or almost white crystalline powder. Very slightly soluble in water, sparingly soluble in acetone, slightly soluble in 96% ethanol, soluble in dilute solutions of alkali metal hydroxides.

Chlorotrimethylsilane. C_3H_9ClSi. (M_r 108.6). [75-77-4].

Clear, colorless liquid, fuming in the air.

d_{20}^{20} is about 0.86.

n_D^{20} is about 1.388.

The boiling point is about 57 °C.

Chloroacetic acid. $C_2H_3ClO_2$. (M_r 94.5). [79-11-8].

Colourless or white or almost white crystals, deliquescent. Very soluble in water, soluble in 96% ethanol.

Store in an airtight container.

Chlorophenol. C_6H_5ClO. (M_r 128.6). [106-48-9]. 4-Chlorophenol.

Colourless or almost colorless crystals. Slightly soluble in water, very soluble in 96% ethanol, and solutions of alkali metal hydroxides.

The melting point is about 42 °C.

2-Chloroethanol. C_2H_5ClO. (M_r 80.5). [107-07-3.]

A colorless liquid. Soluble in 96% ethanol.

d_{20}^{20} is about 1.197.

n_D^{20} is about 1.442.

The boiling point is about 130 °C.

The melting point is about -89 °C.

2-Chloroethanol solution.

Dissolve 125 mg of *2-chloroethanol R* in *2-propanol R* and dilute to 50 mL with the same solvent. Dilute 5 mL of the resulting solution to 50 mL with *2-propanolol R*.

Chloroethylamine hydrochloride. $C_2H_7Cl_2N$. (M_r 116.0). [870-24-6]. 2-Chloroethanamine hydrochloride.

The melting point is about 145 °C.

(2-Chloroethyl)diethylamine hydrochloride. $C_6H_{15}Cl_2N$. (M_r172.1). [869-24-9].

White or almost white crystalline powder. Very soluble in water and methanol, freely soluble in methylene chloride, practically insoluble in hexane.

The melting point is about 211 °C.

Cholesterol. $C_{27}H_{46}O$. (M_r 386.7). [57-88-5].

Contains NLT 95.0% of cholest-5-en-3β-ol and NLT 97.0% and NMT 103.0% of sterols equivalent to the dried substance.

White or almost white crystalline powder.

Practically insoluble in water, sparingly soluble in acetone and 96% ethanol. Sensitive to light.

Choline chloride. $C_5H_{14}ClNO$. (M_r 139.6). [67-48-1].

(2-Hydroxyethyl)trimethylammonium chloride.

Crystals, deliquescent. Very soluble in water and 96% ethanol.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Suxamethonium chloride*, using 5 µl of a 0.2 g/L solution in *methanol R*; the chromatogram shows only one principal spot.

Store in an airtight container.

Chromatical hexahydrate.

$[Cr(H_2O)_4Cl_2]Cl{\cdot}2H_2O$. ($M_r$ 266.5). [10060-12-5].

Dark green and hygroscopic crystalline powder.

Store in a dry place, protected from oxidising agents.

Chromium(VI) oxide. CrO_3. (M_r100.0). [1333-82-0]. Chromium oxide(VI).

Needle-like dark brownish-red crystals or granules, deliquescent. Very soluble in water.

Store in an airtight container.

Chromazurol S. $C_{23}H_{13}Cl_2Na_3O_9S$. (M_r 605). [1667-99-8]. Trisodium 5-[(3-carboxylato--5-methyl-4-oxocyclohexa-2,5-diene-1-ylidene) (2,6-dichloro-3-sulfonatophenyl)methyl]-2-hydroxy-3-methylbenzoate.

Schultz No. 841.

Colour Index (C.I.) No. 43825.

Brownish black powder. Soluble in water, slightly soluble in 96% ethanol.

Chromic potassium sulfate. $CrK(SO_4)_2 \cdot 12H_2O$. (M_r 499.4). [7788-99-0]. Chrome alum.

Large, violet-red or black crystals. Freely soluble in water, practically insoluble in 96% ethanol.

Chrome mixture.

Saturated solution of *chromium(VI) oxide R* in *sulfuric acid R.*

Chrome dark blue. $C_{16}H_9C1N_2Na_2O_9S_2$. (M_r 518.8). [1058-92-0]. Disodium 2-[(5-chloro-2-oxyphenyl)azo]-1,8-dioxynaphthalene-3,6-di-sulfonate. Acid chrome dark blue. Acid blue 13.

Dark brown or black powder. Freely soluble in water.

Chrome dark blue indicator.

Grind 0.25 g of *dark blue chrome R* and 25 g of *sodium chloride R* in a mortar and mix.

Chrome dark blue solution.

Dissolve 0.5 g of *chrome dark blue R* in 10 mL of *ammonia buffer solution pH 10.0 R* and adjust to 100 mL with *95% (V/V) ethanol*.

Use within 1 month.

Chromotrope II B. $C_{16}H_9N_3\ Na_2O_{10}S_2$. ($M_r$ 513.4). [548-80-1]. Disodium 4,5-dihydroxy-3-(4-nitrophenylazo)naphthalene-2,7-disulfonate.

Schultz No. 67.

Colour Index (C.I.) No. 16575.

Reddish-brown powder. Soluble in water to form a yellowish red solution, practically insoluble in 96% ethanol.

Chromotrope II B solution.

A 0.05 g/L solution in *sulfuric acid R.*

Chromotropic acid, sodium salt. $C_{10}H_6Na_2O_8S_2 \cdot 2H_2O$. ($M_r$ 400.3). [5808-22-0]. Disodium 4,5-dihydroxynaphthalene-2,7-disulfonate dihydrate. Disodium 1,8-dihydroxy-naphthalene-3,6-disulfonate dihydrate.

Schultz No. 1136.

A yellowish-white powder. Soluble in water, practically insoluble in 96% ethanol.

Chromogenic substrate R1.

Dissolve N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-T-arginine-4-nitroanilide dihydrochloride in *water R* to obtain 0.003 M solution. Before use, dilute with *tris(hydroxymethyl)aminomethane edetate buffer solution pH 8.4 R* to obtain a 0.0005 M solution.

Chromogenic substrate R2.

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to obtain 0.003 M solution. Before use, dilute with *tris(hydroxymethyl)aminomethane edetate buffer solution pH 8.4 R* to obtain a 0.0005 M solution.

Cesium chloride. CsCl. (M_r 168.4). [7647-17-8]. Cesium chloride.

White or almost white powder. Very soluble in water, freely soluble in methanol, practically insoluble in acetone.

Cellulose for chromatography. [9004-34-6].

White or almost white fine homogeneous powder with an average particle size of fewer than 30 µm.

Preparation of a thin layer. Suspend 15 g in 100 mL of *water R* and homogenise in an electric mixer for 60 s. Coat thoroughly cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose for chromatography R1.

Microcrystalline cellulose.

Preparation of a thin layer. Suspend 25 g of cellulose in 90 mL of *water R* and homogenise in an electric mixer for 60 s. Coat thoroughly cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose for chromatography F_{254}.

Microcrystalline cellulose F_{254}. White or almost white fine homogeneous powder with an average particle size of fewer than 30 µm, containing a fluorescent indicator with a maximum intensity at a wavelength of 254 nm.

Preparation of a thin layer. Suspend 25 g in 100 mL of *water R* and homogenise in an electric mixer for 60 s. Coat thoroughly cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cerium nitrate. $Ce(NO_3)_3 \cdot 6H_2O$. (M_r 434.3). [10294-41-4]. Cerium trinitrate hexahydrate.

colorless to slightly yellow crystalline powder. Freely soluble in water and 96% ethanol.

Cerium sulfate. $Ce(SO_4)_2 \cdot 4H_2O$. (M_r 404.3). [10294-42-5]. Cerium(IV) sulfate tetrahydrate.

Yellow or orange-yellow crystalline powder or crystals. Very slightly soluble in water, slowly soluble in dilute acids.

Cetyltrimethylammonium bromide. $C_{19}H4_2BrN$. (M_r 364.5). [57-09-0]. Cetrimonium bromide. *N*-Hexadecyl-*N*,*N*,*N*-trimethylammonium bromide.

White or almost white crystalline powder. Soluble in water, freely soluble in 96% ethanol.

The melting point is about 240 °C.

Cetrimide. [8044-71-1].

It consists of trimethyltetradecylammonium bromide and may contain minor amounts of dodecyl and hexadecyltrimethylammonium bromides.

Contains not less than 96.0% and not greater than 101.0% of alkyltrimethylammonium bromides calculated as $C_{17}H_{38}BrN$ (M_r 336.4) with reference to the dried substance.

White or off-white, loose, free-flowing powder.

Freely soluble in water and alcohol.

Cetyl alcohol. $C_{16}H_{34}O$. (M_r 242.4). [36653-82-4]. Hexadecane-1-ol.

Contains not less than 95.0% of $C_{16}H_{34}O$.

The melting point is about 48 °C.

Cianopramine solution. [506-68-3].

To bromine *water R*, add dropwise with cooling *0.1 M ammonium thiocyanate* until the yellow colour is discharged.

Prepare immediately before use.

Cyanoguanidine. C_2H4N_4. (M_r 84.1). [461-58-5]. Dicyandiamide.

1-Cyanoguanidine.

White or almost white crystalline powder. Sparingly soluble in water and 96% ethanol, practically insoluble in methylene chloride.

The melting point is about 210 °C.

Cyanocobalamin. $C_{63}H_{88}CoN_{14}O_{14}P$. ($M_r$ 1355). [68-19-9]. (-(5,6-Dimethylbenzimidazole-1-yl)cobamide cyanide.

Contains not less than 96.0% and not greater than 102.0% of $C6_3H_{88}CoN_{14}O_{14}P$ calculated on a dry substance basis. Obtained by fermentation.

Dark red crystalline powder or dark red crystals.

Sparingly soluble in water and 96% ethanol, practically insoluble in acetone. Anhydrous substance is very hygroscopic.

Cyanoacetic acid. $C_3H_3NO_2$. (M_r 85.1). [372-09-8].

White or yellowish white hygroscopic crystals. Freely soluble in water.

Store in an airtight container.

Cyclohexane. C_6H_{12}. (M_r 84.2). [110-82-7].

A clear, colorless, flammable liquid. Practically insoluble in water, miscible with organic solvents.

d_{20}^{20} is about 0.78.

The boiling point is about 80.5 °C.

Cyclohexane used in spectrophotometry complies with the following additional test.

The *absorbance* (*2.1.2.24*) is determined using *water R* as the compensation liquid.

NMT 0.35 at a wavelength of 220 nm,
NMT 0.16 at a wavelength of 235 nm,
NMT 0.05 at a wavelength of 240 nm,
NMT 0.01 at a wavelength of 250 nm.

Cyclohexane R1.

Complies with the requirements prescribed for *cyclohexane R* with the following additional requirement.

The fluorescence, measured at 460 nm (under illumination with an excitant light beam at 365 nm) is not more intense than that of a solution containing 0.002 ppm of *quinine R* in *0.05 M sulfuric acid*.

Cyclohexylamine. $C_6H_{13}N$. (M_r 99.2). [108-91-8].

A colorless liquid. Soluble in water, miscible with the most common solvents.

n_D^{20} is about 1,460.

The boiling point is from 134 °C to 135 °C.

Cyclohexylenedinitrilotetraacetic acid. $C_{14}H_{22}N_2O_8,H_2O$. (M_r 364.4). *trans*-Cyclohexylene-1,2-dinitrile-*N*,*N*,*N'*,*N'*-tetra-acetic acid.

White or almost white crystalline powder.

The melting point is about 204 °C.

3-Cyclohexylpropanoic acid. $C_9H_{16}O_2$. (M_r 156.2). [701-97-3].

A colorless liquid.

d_{20}^{20} is about 0.998.

n_D^{20} is about 1.4648.

The boiling point is about 130 °C.

α-Cyclodextrin. $C_{36}H_{60}O_{30}$. (M_r 972). [10016-20-3]. Cyclohexanes(14)-(α-D-glucopyranosil). Cyclomaltohexaose. Alphadex.

β-Cyclodextrin. $[C_6H_{10}O_5]_7$. (M_r 1135). [7585-39-9].

Cycloheptakis-(14)-(α-D-glucopyranosyl) (cyclomaltoheptaose or β-cyclodextrin).

Contains not less than 98.0% and not greater than 101.0% $[C_6H_{10}O_5]_7$ calculated on a dry substance basis.

White or almost white amorphous or crystalline powder.

Sparingly soluble in water and propylene glycol, practically insoluble in anhydrous ethanol and methylene chloride.

β-Cyclodextrin for chiral chromatography, modified.

A 30% solution of 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethyl-silyl-β-cyclodextrin in *poly(dimethyl)(85)(diphenyl)(15)siloxane R*.

β-Cyclodextrin for chiral chromatography, modified R1.

A 30% solution of 2,3-di-*O*-acetyl-6-*O*-*tert*-butylsilyl-β-cyclodextrin in *poly(dimethyl)(85)(diphenyl)(15)siloxane R*.

***p*-Cymene.** $C_{10}H_{14}$. (M_r 134.2). [99-87-6]. 1-Isopropyl-4-methylbenzene.

A colorless liquid. Practically insoluble in water, soluble in 96% ethanol.

d_{20}^{20} is about 0.858.

n_D^{20} is about 1.4895.

The boiling point is from 175 °C to 178 °C.

p-Cymene used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) under the conditions specified in the monograph *Peppermint oil*, using *p*-cymene as the test solution.

p-Cymene content calculated by the internal normalisation procedure should be minimum 96.0%.

Cynarin. $C_{25}H_{24}O_{12}$. (M_r 516.4). [30964-13-7]. (1α, 3α, 4α, 5β)-1,3-Bis[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]-oxy]4,5-dihydroxy-cyclohexanecarboxylic acid.

White or almost white odorless amorphous mass.

Cineole. $C_{10}H_{18}O$. (M_r 154.3). [470-82-6]. 1,8-Cineole. Eucalyptol. 1,8-Epoxy-*p*-mentane.

A colorless liquid. Practically insoluble in water. Miscible with anhydrous ethanol.

d_{20}^{20} is from 0.922 to 0.927.

n_D^{20} is from 1.456 to 1.459.

Freezing point (*2.1.2.17*). Between 0 °C and 1 °C.

Distillation range (*2.1.2.11*). From 174 °C to 177 °C.

Phenol. Shake 1 g with 20 mL of *water R*. Allow to separate and add to 10 mL of the aqueous layer 0.1 mL of *ferric chloride solution R1*. No violet colour develops.

Turpentine oil. Dissolve 1 g of cineole in 5 mL of *90% (V/V) ethanol R*, add freshly prepared *bromine water* dropwise. Not greater than 0.5 mL is required to give a yellow colour lasting for 30 min.

Residue on evaporation. NMT 0.05%.

To 10.0 mL, add 25 mL of *water R*, evaporate in a water bath, dry the residue to a constant mass at 100-105 °C.

Cineole used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Peppermint oil*, using cineole as the test solution.

The cineole content calculated by the internal normalisation procedure should be minimum 98.0%.

1,4-Cineole. $C_{10}H_{18}O$. (M_r 154.3). [470-67-7]. 1-Methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1] heptane. 1-Isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptane.

A colorless liquid.

d_4^{20} is about 0.900.

n_D^{20} is about 1.445.

The boiling point is about 173 °C.

Zinc. Zn. (A_r 65.4). [7440-66-6].

Contains not less than 99.5% of Zn.

Silver-white cylinders, granules, pellets or filings with a blue sheen.

Arsenic (*2.1.4.2, Method A*). Maximum 0.2 ppm. Dissolve 5.0 g of zinc in a mixture of 15 mL of *hydrochloric acid R* and 25 mL of *water R*.

Zinc, activated.

Place the zinc cylinders or pellets in a conical flask, add a sufficient amount of 50 ppm solution of *chloroplatinic acid R* to completely cover the metal, wash the metal with water after 10 min, remove the water, and immediately dry.

Arsenic. To 5 g of the activated zinc, add 15 mL of *hydrochloric acid R*, 25 mL of *water R*, 0.1 mL of a solution of *stannous chloride R*, and 5 mL of a solution of *potassium iodide R*. Then proceed as prescribed in the test for arsenic (*2.1.4.2, Method A*). No stain should be observed on the *mercuric bromide paper R*.

Activity. Repeat the test for arsenic, using the same reagents, add a solution containing 1 µg of arsenic. A noticeable staining appears on *mercuric bromide paper R*.

Zinc acetate. $(C_2H_3O_2)Zn{\cdot}2H_2O$. ($M_r$ 219.5). [5970-45-6]. Zinc acetate dihydrate.

White or almost white shiny crystals, slightly efflorescent in the air. Freely soluble in water, soluble in 96% ethanol. At a temperature of 100 °C, it loses its crystallisation water.

d20 about 1.735.

The melting point is about 237 °C.

Zinc acetate solution.

Dissolve 5.9 g of *zinc acetate R* by stirring in a mixture of 600 mL of *water R* and 150 mL of *glacial acetic acid R*. When mixing, add 150 mL of *concentrated ammonia solution R*, cool to room temperature, adjust the pH of the *ammonia solution R* to 6.4, dilute to 1000 mL with *water R*.

Zinc oxide. ZnO. (M_r 81.4). [1314-13-2]. Zinc oxide.

Contains not less than 99.0% and not greater than 100.5% of ZnO calculated with reference to the ignited substance.

White or slightly yellowish white soft amorphous powder, free of solid particles.

Practically insoluble in water and 96% ethanol, soluble in dilute mineral acids.

Zinc powder. Zn. (A_r 65.4). [7440-66-6]. Contains not less than 90.0% of Zn (A_r 65.4).

Very fine grey powder. Soluble in *dilute hydrochloric acid R*.

Zinc sulfate. $ZnSO_4{\cdot}7H_2O$. (M_r 287.5). [7446-20-0]. Zinc sulfate heptahydrate.

Contains not less than 99.0% and not greater than 104.0% of $ZnSO_4{\cdot}7H_2O$.

White or almost white crystalline powder or colorless clear crystals. Efflorescent in air.

Very soluble in water, practically insoluble in 96% ethanol.

Zinc chloride. $ZnCl_2$. (M_r 136.3). [7646-85-7]. Zinc chloride.

Contains not less than 95.0% and not greater than 100.5% of $ZnCl_2$.

White or almost white crystalline powder or white sticks. Deliquescent.

Very soluble in water, freely soluble in 96% ethanol and glycerol.

Zinc chloride-formic acid solution.

Dissolve 20 g of zinc chloride R *in 80 g of a 850 g/L solution of* anhydrous formic acid R.

Zinc chloride solution, iodinated.

Dissolve 20 g of *zinc chloride R* and 6.5 g of *potassium iodide R* in 10.5 mL of *water R*, add 0.5 g of *iodine R*, and shake for 15 min. Filter, if necessary.

Store in a place protected from light.

Zinc iodide and starch solution.

To a solution of 2 g of *zinc chloride R* in 10 mL of *water R*, add 0.4 g of *soluble starch R*, and heat until the starch dissolves. After cooling to room temperature, add 1.0 mL of a colorless solution containing 0.10 g of *zinc R* in the form of sawdust and 0.2 g of *iodine R* in *water R*, dilute to 100 mL with *water R*, filter.

Store in a place protected from light.

Test for sensitivity. Dilute 0.05 mL of *sodium nitrite solution R* to 50 mL with *water R*. To 5 mL of the resulting solution, add 0.1 mL of *dilute sulfuric acid R* and 0.05 mL of the prepared zinc iodide and starch solution and stir; the solution turns blue.

Cinchonidine. $C_{19}H_{22}N_2O$. (M_r 294.4). [485-71-2]. (*R*)-(Quinol-4-yl) [(2S,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

White or almost white crystalline powder. Very slightly soluble in water and petroleum ether, sparingly soluble in 96% ethanol.

$[\alpha]_D^{20}$ is between -105 and -110. Carry out determination using a 50 g/l solution of *96% ethanol R*.

The melting point is about 208 °C with decomposition.

Store in a place protected from light.

Cinchonine. $C_{19}H_{22}N2_O$. (M_r 294.4). [118-10-5]. (*S*)-(Quinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidine-2-yl]methanol.

White or almost white crystalline powder. Very slightly soluble in water, sparingly soluble in 96% ethanol and methanol.

$[\alpha]_D^{20}$ is between + 225 and + 230. Carry out determination using a 50 g/l solution of *96% ethanol R*.

The melting point is about 263 °C.

Store in a place protected from light.

Zirconyl nitrate. A basic salt corresponding approximately to the formula $ZrO(NO_3)_2 \cdot 2H_2O$. [14985-18-3].

White or almost white crystalline powder or crystals. Hygroscopic, water-soluble. Clear or slightly opalescent aqueous solution.

Store in an airtight container.

Zirconyl nitrate solution.

1 g/L solution in a mixture of *water R*/*hydrochloric acid R* (40: 60).

Zirconyl chloride. A basic salt corresponding approximately to the formula $ZrCl_2O \cdot 8H_2O$. [15461-27-5].

Contains not less than 96.0% of $ZrCl_2O \cdot 8H_2O$.

White or almost white crystalline powder or crystals. Freely soluble in water and 96% ethanol.

Quantitation. Dissolve 0,600 g in a mixture of 5 mL of *nitric acid R* and 50 mL of *water R*, add 50.0 mL of *0.1 M silver nitrate solution*, 3 mL of *dibutyl phthalate R* solution, shake and titrate with *0.1 M ammonium thiocyanate solution* to reddish-yellow colouration, using 2 mL of *ferric ammonium sulfate solution R2* as indicator.

1 mL of *0.1 M silver nitrate solution* is equivalent to 16.11 mg of $ZrCl_2O \cdot 8H_2O$.

L-Cysteine. $C_3H_7NO_2S$. (M_r 121.1). [52-90-4].

Powder. Freely soluble in water, 96% ethanol, and acetic acid, practically insoluble in acetone.

Cysteine hydrochloride. $C_3H_8ClNO_2S \cdot H_2O$. (M_r 175.6). [7048-04-6]. (2*R*)-2-Amino-3-sulfanylpropanoic acid hydrochloride monohydrate.

Contains not less than 98.5% and not greater than 101.0% of $C_3H_8ClNO_2S \cdot H_2O$ calculated on a dry substance basis.

The product of fermentation or hydrolysis of proteins.

White or almost white crystalline powder or colorless crystals.

Freely soluble in water, slightly soluble in 96% ethanol.

L-Cystine. $C_6H_{12}N_2O_4S_2$. (M_r 240.3). [56-89-3].

White or almost white crystalline powder. Practically insoluble in water and 96% ethanol, soluble in dilute solutions of alkali metal hydroxides.

[a] 20 from-218 to-224. Determination is carried out in *1 M hydrochloric acid*.

The melting point is 250 °C with decomposition.

Citral. $C_{10}H_{16}O$. (M_r 152.2). [5392-40-5].

A mixture of (2*E*) and (2*Z*)-3,7-Dimethylocta-2,6-dienal.

Light yellow liquid. Practically insoluble in water, miscible with 96% ethanol and glycerin.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel* GF_{254} *R* as the coating substance. On the chromatographic plate, apply 10 µL of a 1 g/L solution in *toluene R*. Develop using a mixture of *ethyl acetate R/toluene R* solvents (15:85). When the solvent front passes 15 cm, remove the plate from the chamber and dry in air. View in UV light at a wavelength of 254 nm. The resulting chromatogram must show only one principal spot.

Citral used in gas chromatography complies with the following additional test.

Quantitation. Carry out determination by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Citronella oil*.

The citral (neral + geranial) content calculated by internal normalisation procedure should be minimum 95,0%.

Citrated rabbit plasma.

Collect blood by intracardiac puncture from a rabbit kept fasting for 12 h, using a plastic syringe with a No. 1 needle containing a suitable volume of 38 g/L solution of *sodium citrate R* so that the final volume ratio of citrate solution to blood is 1:9. Separate the plasma by centrifugation at acceleration from 1500 *g* to 1800 *g* and temperature from 15 °C to 20 °C for 30 min.

Store at a temperature between 0 °C and 6 °C.

Use within 4 h of collection.

Citronellal. $C_{10}H_{18}O$. (M_r 154.3). [106-23-0]. 3,7-Dimethyl-6-octanal.

Very slightly soluble in water, soluble in 96% ethanol.

d_4^{20} from 0.848 to 0.856.

n_D^{20} is about 1.446.

Citronellal used in gas chromatography complies with the following additional test.

Quantitation. Carry out determination by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Citronella oil.*

The citronellal content calculated by the internal normalisation procedure should be at least 95.0%.

Citronellyl acetate. $C_{12}H_{22}O_2$. (M_r 198.3). [150-84-5]. 3,7-Dimethyl-6-octen-1-ilacetate.

d_4^{20} 0.890.

n_D^{20} is about 1.443.

The boiling point is 229 °C.

Citronellyl acetate used in gas chromatography complies with the following additional test.

Quantitation. Carry out determination by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Citronella oil.*

The citronellyl acetate content calculated by internal normalisation procedure should be minimum 95.0%.

Store in an airtight container, protected from light.

Citronellol. $C_{10}H_{20}O$. (M_r 156.3). [106-22-9]. 3,7-Dimethyloct-6-en-1-ol.

Colourless clear liquid. Practically insoluble in water, miscible with 96% ethanol.

d_4^{20} 0.857.

n_D^{20} is about 1.456.

The boiling point is from 220 °C to 222 °C.

Citronellol used in gas chromatography complies with the following additional test.

Quantitation. Carry out determination by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Citronella oil.*

The citronellol content calculated by the internal normalisation procedure should be minimum 95.0%.

Store in an airtight container, protected from light.

Citropten. $C_{11}H_{10}O_4$. (M_r 206.2). [487-06-9]. Limettin. 5,7-Dimethoxy-2*H*-1-benzopyran-2-one.

Acicular crystals. Practically insoluble in water and petroleum ether, freely soluble in acetone and 96% ethanol.

The melting point is about 145 °C.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*), using *silica gel* GF_{254} *R* as the coating substance. On the chromatographic plate, apply 10 µL of a 1 g/L solution in *toluene R*. Develop using a mixture of *ethyl acetate R/toluene R* solvents (15:85). When the solvent front passes 15 cm, remove the plate from the chamber and dry in air. View in UV light at a wavelength of 254 nm. The resulting chromatogram must show only one principal spot.

Oxalic acid. $C_2H_2O_4{\cdot}2H_2O$. (M_r 126.1). [6153-56-6]. Ethanedicarboxylic acid dihydrate.

White or almost white crystals. Soluble in water, freely soluble in 96% ethanol.

Oxalic acid and sulfuric acid solution.

A 50 g/L solution of *oxalic acid R* in a cooled mixture of equal volumes of *sulfuric acid R* and *water R*.

Eugenol. $C_{10}H_{12}O_2$. (M_r 164.2). [97-53-0]. 4-Allyl-2-methoxyphenol.

Colourless or pale yellow oily liquid that darkens and becomes more viscous on exposure to air and light. Practically insoluble in water, miscible with 96% ethanol, fatty and essential oils.

d_{20}^{20} is about 1.07.

The boiling point is about 250 °C.

Eugenol used in gas chromatography complies with the following additional test.

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Clove oil*, using eugenol as the test solution.

The eugenol content calculated by the internal normalisation procedure should be minimum 98.0%.

Store in a place protected from light.

Emetine dihydrochloride. $C_{29}H_{42}C_{12}N_2O_4{\cdot}5H_2O$. ($M_r$ 644). [316-42-7]. (2*S*,3*R*,11b*S*)-2-[[(1*R*)- 6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinoline-1-yl]methyl]-3-ethyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2*H*-benzo[a]quinolysine dihydrochloride.

Emetine hydrochloride pentahydrate contains NLT 98.0% and NMT 102.0% of $C_{29}H_{42}Cl_2N_2O_4$calculated on a dry substance basis.

White or slightly yellowish crystalline powder. Freely soluble in water and alcohol.

Emodin. $C_{15}H_{10}O_5$. (M_r 270.2). [518-82-1]. 1,3,8-Trihydroxy-6-methylanthraquinone.

Needle-like orange-red crystals. Practically insoluble in water, soluble in 96% ethanol and solutions of alkali metal hydroxides.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Rhubarb*. The chromatogram must show only one principal spot.

Erythritol. $C_4H_{10}O_4$. (M_r 122.1). [149-32-6]. (2*R*,3*S*)-butane-1,2,3,4-tetrol(mesoerythritol).

Contains not less than 96.0% and not greater than 102.0% of $C_4H_{10}O_4$ calculated on a dry substance basis.

White or almost white crystalline powder or free-flowing granules.

Freely soluble in water, very slightly soluble in 96% ethanol.

Erucamide. $C_{22}H_{43}O$. (M_r 337.6). [112-84-5]. (Z)-Docos-13-enoamide.

Yellowish or white powder or granules. Practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

The melting point is about 70 °C.

Esculin. $C_{15}H_{16}O_9{\cdot}1S\ H_2O$. ($M_r$ 367.3). [531-75-9]. 6-(β-D-Glucopyranosyloxy)-7-hydroxy-2*H*-chromene-2-one.

White or almost white powder or colorless crystals. Sparingly soluble in water and 96% ethanol, freely soluble in hot water and hot 96% ethanol.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Eleutherococcus*; the chromatogram shows only one principal spot.

Estragole. $C_{10}H_{12}O$. (M_r 148.2). [140-67-0]. 1-Methoxy-4-prop-2-enylbenzene.

Liquid. Miscible with 96% ethanol.

n_D^{20} is about 1.52.

The boiling point is about 216 °C.

Estragole *used in gas chromatography complies with the following test.*

Quantitation. Determination is carried out by gas chromatography (*2.1.2.27*) as prescribed in the monograph *Anise oil*, using estragole as the test solution.

The estragole content calculated by internal normalisation procedure should be minimum 98,0%.

Estradiol. $C_{18}H_{24}O_2$. (M_r 272.4). [50-28-2]. Estra-1,3,5(10)-triene-3,17 β-diol. β-Estradiol.

Prismatic crystals, stable in air. Practically insoluble in water, freely soluble in 96% ethanol, soluble in acetone and dioxane, sparingly soluble in vegetable oils.

The melting point is from 173 °C to 179 °C.

17α-Estradiol. $C_{18}H_{24}O_2$. (M_r 272.4). [57-91-0].

White or almost white crystalline powder or colorless crystals.

The melting point is from 220 °C to 223 °C.

Escin. [6805-41-0].

A mixture of related saponins derived from the seeds of *Aesculus hippocastanum L.*

Almost white or slightly reddish or yellowish very fine amorphous powder.

Chromatography. Determination is carried out by thin-layer chromatography (*2.1.2.26*) as prescribed in the monograph *Senega root*, using 20 µl of the solution. After spraying the chromatogram with a solution of *anisaldehyde R* and heating, a principal spot with *RF* about 0.4 should be detected on the chromatogram of the test solution.

Ethanol. [64-17-5].

See *Ethanol, anhyndrous R.*

Ethanol, anhydrous. C_2H_6O. (M_r 46.07). [64-17-5].

At a temperature of 20 °C, contains not less than 99.5% *(V/V)* of C_2H_6O (99.2% *m/m*), calculated from the relative density using alcohol-metric tables.

Colourless, clear, volatile, flammable liquid. Hygroscopic.

Miscible with water and methylene chloride. Burns with a blue, smokeless flame.

The boiling point is about 78 (C.

Ethanol R1.

Complies with the requirements specified in the monograph *Ethanol, anhydrous R* with the following additional requirement.

Methanol. The determination is carried out by gas chromatography *(2.1.2.27)*.

Test solution. Ethanol to be examined.

Reference solution. Dilute 0.50 mL of *anhydrous methanol R* to 100.0 mL with the substance to be examined. Dilute 1.0 mL of the resulting solution to 100.0 mL with the substance to be examined. The chromatographic procedure may be carried out using a flame-ionisation detector under the following conditions:

- a glass column 2 m × 2 mm filled with a *ethylvinylbenzene-divinylbenzene copolymer R*, particle size from 75-100 µm;
- *nitrogen for chromatography R as* carrier gas;
- flow rate of 30 mL/min;
- column temperature 130 °C;
- injection port - 150 °C;
- detector temperature 200 °C.

Inject 1 mL of the test solution and the reference solution alternately, three times. After each chromatography, heat the column to 230 °C for 8 minutes. Integrate the methanol peak.

Calculate the percentage methanol content using the formula:

$$\frac{a \cdot b}{c - b},$$

where *a* is the percentage of methanol in the reference solution;

b is the area of the methanol peak on the chromatogram of the test solution;

c is the area of the methanol peak in the chromatogram of the reference solution.

Ethanol (96%). [64-17-5].

At a temperature of 20 °C, contains not less than 95.1% (*v/v*) (92.6% *m/m*) and not greater than 96.9% (*v/v*) (95.2% *m/m*) C_2H_6O (M_r 46.07), calculated from the relative density using alcohol-metric tables, and water.

Colourless, clear, volatile, flammable liquid. Hygroscopic.

Miscible with water and methylene chloride. Burns with a blue, smokeless flame.

The boiling point is about 78 °C.

Ethanol (*x*% *V/V*).

To obtain a solution in which the ethanol content corresponds to the value *x*, mix appropriate volumes of *water R* and *96% alcohol R*, taking into account the effects of warming and volume contraction inherent to the preparation of such a mixture.

Ethanolamine. C_2H_7NO. (M_r 61.1). [141-43-5]. 2-Aminoethanol.

Clear, colourless, viscous, hygroscopic liquid. Miscible with water and methanol.

d_{20}^{20} is about 1.04.

n_D^{20} is about 1.454.

The melting point is about 11 °C.

Store in an airtight container.

Ethyl acrylate. $C_5H_8O_2$. (M_r 100.1). [140-88-5]. Ethylprop-2-enoate.

A colorless liquid.

d_{20}^{20} is about 0.924.

n_D^{20} is about 1.406.

The boiling point is about 99 °C.

The melting point is about -71 °C.

Ethyl acetate. $C_4H_8O_2$. (M_r 88.1). [141-78-6].

A clear, colorless liquid. Soluble in water, miscible with 96% ethanol.

d_{20}^{20} is from 0.901 to 0.904.

The boiling point is from 76 °C to 78 °C.

Ethyl acetate, treated

Disperse 200 g of *sulfamic acid R* in *ethyl acetate R* and dilute to 1000 mL with the same solvent. Stir the resulting suspension for 3 days and filter through a paper filter. Use within 1 month.

Ethylbenzene. C_8H_{10}. (M_r 106.2). [100-41-4].

Contains not less than 99.5% (*m/m*) of C_8H_{10}, determined by gas chromatography.

A clear, colorless liquid. Practically insoluble in water, soluble in acetone and 96% ethanol.

d_{20}^{20} is about 0.87.

n_D^{20} is about 1.496.

The boiling point is about 135 °C.

Ethyl benzoate. $C_9H_{10}O_2$. (M_r 150.2). [93-89-0].

Clear, colourless, light-refractive liquid. Practically insoluble in water, miscible with 96% ethanol and petroleum ether.

d_{20}^{20} is about 1.050.

n_D^{20} is about 1.506.

The boiling point of 211 °C to 213 °C.

Ethyl-5-bromovalerate. $C_7H_{13}BrO_2$. (M_r 209.1). [14660-52-7]. Ethyl-5-bromopentanoate.

A clear, colorless liquid.

d_{20}^{20} is about 1.321.

The boiling point is from 104 °C to 109 °C.

2-Ethylhexane-1,3-diol. $C_8H_{18}O_2$. (M_r 146.2). [94-96-2].

Slightly oily liquid. Soluble in anhydrous ethanol, 2-propanol, propylene glycol, and castor oil.

*d*20 is about 0.942.

ρ*20* is about 1.451.

The boiling point is about 244 °C.

2-Ethylhexanoic acid. $C_8H_{16}O_2$. (M_r 144.2). [149-57-5].

A colorless liquid.

d_{20}^{20} is about 0.91.

n_D^{20} is about 1.425.

Related substances. The determination is carried out by gas chromatography *(2.1.2.27)*. Chromatograph 1 µL of the solution prepared as follows: suspend 0.2 g of 2-ethylhexane acid in 5 mL of *water R*, add 3 mL of a *dilute hydrochloric acid R* and 5 mL of *hexane R*, shake for 1 min, after separating the layers, use the upper layer. Carry out the chromatographic procedure in accordance with the test for 2-ethyl-hexanoic acid specified in the monograph on *Amoxicillin sodium*.

The sum of the area of any peaks, apart from the principal peak and the peak due to the solvent, is not greater than 2.5 per cent of the area of the principal peak.

Ethylene bis[3,3-di(3-tert-butyl-4-hydroxiphenyl)butyrate]. [32509-66-3].

See *Ethylenebis[3,3-di(3-(1,1-dimethyl-ethyl)-4-hydroxyphenyl)butyrate] R.*

Ethylene bis[3,3-di(3-(1,1-dimethylethyl)- 4-hydroxyphenyl)butyrate]. $C_{50}H_{66}O_8$. (M_r 795). [32509-66-3]. Ethylene bis [3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate].

Crystalline powder. Practically insoluble in water and petroleum ether, very soluble in acetone and methanol.

The melting point is about 165 °C.

Ethylene glycol. $C_2H_6O_2$. (M_r 62.1). [107-21-1].

Ethane-1,2-diol.

Contains not less than 99.0% of $C_2H_6O_2$.

Colourless, slightly viscous, hygroscopic liquid. Miscible with water and 96% ethanol.

d_{20}^{20} is from 1.113 to 1.115.

n_D^{20} is about 1.432.

The melting point is about -12 °C.

The boiling point is about 198 °C.

Acidity. To 10 mL, add 20 mL of *water R* and 1 mL of a *phenolphthalein solution R*; the colour of the solution should turn pink with the addition of not greater than 0.15 mL of *0.02* M *sodium hydroxide.*

Water (2.1.5.12). NMT 0.2%.

Ethylene glycol monomethyl ether.

$C_3H_8O_2$. (M_r 76.1). [109-86-4]. 2-Methoxyethanol.

Contains not less than 99.0% of $C_3H_8O_2$.

A clear, colorless liquid. Miscible with water, acetone and 96% ethanol.

d_{20}^{20} is about 0.97.

n_D^{20} is about 1.403.

The boiling point is about 125 °C.

Ethylene glycol monoethyl ether. $C_4H_{10}O_2$. (M_r 90.1). [110-80-5]. 2-Ethoxyethanol.

Contains not less than 99.0% of $C_4H_{10}O_2$.

A clear, colorless liquid. Miscible with water, acetone and 96% ethanol.

d_{20}^{20} is about 0.93.

n_D^{20} is about 1.406.

The boiling point is about 135 °C.

Ethylenediamine. $C_2H_8N_2$. (M_r 60.1). [107-15-3]. Ethane-1,2-diamine.

Clear, colourless, fuming liquid with a strongly alkaline reaction.

Miscible with water and 96% ethanol. The boiling point is about 116 °C.

(Ethylenedinitrilo)tetraacetic acid. $C_{10}H_{16}N_2O_8$. (M_r 292.2). [60-00-4]. *N*,*N*-1,2-ethanediylbis[*N*-(carboxymethyl)glycine]. Edetic acid.

White or almost white crystalline powder. Very slightly soluble in water.

The melting point is about 250 °C with decomposition.

Ethylene oxide. C_2H_4O. (M_r 44.05). [75-21-8]. Oxirane.

Colorless flammable gas. Very soluble in water and anhydrous ethanol.

The condensation temperature is about 12 °C.

Ethylene oxide solution.

Weigh a quantity of cool *ethylene oxide stock solution R*, equivalent to 2.5 mg of ethylene oxide into a cool flask and dilute to 50.0 g with *macrogol 200 R1*, mix thoroughly. Dilute 2.5 g of the resulting solution to 25.0 mL with *macrogol 200 R1* (5 µg of ethylene oxide per 1 g of solution).

Prepare immediately before use.

The solution can be prepared using a suitable commercial reagent instead of *ethylene oxide stock solution R* by dilution.

Ethylene oxide solution R1.

Dilute accurately weighed 1.0 mL of the cooled *ethylene oxide stock solution R* to 50.0 mL with *macrogol 200 R1* and mix thoroughly. Dilute 2.5 g of the resulting solution to 25.0 mL with *macrogol 200 R1*. Calculate the exact amount of ethylene oxide in ppm from the volume determined by weighing and taking the relative density of *macrogol 200 R1* as 1.127.

Prepare immediately before use.

The solution can be prepared using a suitable commercial reagent instead of *ethylene oxide stock solution R* by dilution.

Ethylene oxide solution R2.

Weigh 1.00 g of the cooled *ethylene oxide stock solution R*, (equivalent to 2.5 mg of ethylene oxide) into a previously weighed cold flask containing 40.0 g of cooled *macrogol 200 R1*, and mix. Determine the exact mass and dilute to a calculated mass so as to obtain a solution containing 50 µg of ethylene oxide per 1 g of the solution. Weigh 10.00 g, place in a flask containing about 30 mL of *water R*, mix and dilute to 50.0 mL with *water R* (10 µg/mL of ethylene oxide).

Prepare immediately before use.

The solution can be prepared using a suitable commercial reagent instead of *ethylene oxide stock solution R* by dilution.

Ethylene oxide solution R3.

Dilute 10.0 mL of *ethylene oxide solution R2* to 50.0 mL with *water R* (2 µg/mL of ethylene oxide).

Prepare immediately before use.

Ethylene oxide solution R4.

Dilute 1.0 mL of *ethylene oxide stock solution R1* to 100.0 mL with *water R*. Dilute 1.0 mL of the resulting solution to 25.0 mL with *water R*.

Ethylene oxide stock solution.

All operations for the preparation of these solutions must be conducted in a fume cupboard. The operator must protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store solutions in an airtight container in a refrigerator at 4 °C to 8 °C. Conduct all tests three times.

Into a dry, clean test-tube, cooled in a mixture of 1 part of *sodium chloride R* and 3 parts of crushed ice, introduce a flow current of *ethylene oxide R gas*, allowing condensation onto the inner wall of the test-tube. Using a glass syringe, previously cooled to -10 °C, inject about 300 µL (corresponding to about 0.25 g) of liquid *ethylene oxide R* into 50 mL of *macrogol 200 R1*.

Determine the absorbed amount of ethylene oxide by weighing before and after absorption (M_{eo}). Dilute to 100.0 mL with *macrogol 200 R1*. Mix well before use.

Quantitation. To 10 mL of a 500 g/L suspension of *magnesium chloride R* in *anhydrous ethanol R*, add 20.0 mL of *0.1 M alcoholic hydrochloric acid.* Stopper the flask, shake to obtain a saturated solution and allow to stand overnight to equilibrate. Weigh 5.00 g of a 2.5 g/L basic solution of *ethylene oxide R* into the flask and allow to stand for 30 min. Titrate with *0.1 M alcoholic potassium hydroxide solution* potentiometrically (*2.1.2.19*).

Carry out a blank titration using the same quantity of *macrogol 200 R1* instead of the ethylene oxide stock solution.

The content of ethylene oxide in milligrams per gram is calculated using the formula:

$$\frac{(V_0 - V_1) \cdot f \cdot 4{,}404}{m},$$

where V_0, V_1 are volumes of *0.1 M alcoholic potassium hydroxide solution* used for the blank titration and the assay, respectively;

f is the correction factor to the molarity of *0.1 M alcoholic potassium hydroxide solution;*

m is the weight of the test sample in grams.

Ethylene oxide stock solution R1.

A 50 g/L solution of ethylene oxide R *in* methanol R.

Use either a suitable commercial reagent or prepare a solution corresponding to the aforementioned composition.

Ethylene oxide stock solution R2.

A 50 g/L solution of ethylene oxide R *in* methylene chloride R.

Use either a suitable commercial reagent or prepare a solution corresponding to the aforementioned composition.

Ethylene chloride. $C_2H_4Cl_2$. (M_r 99.0). [107-06-2]. 1,2-Dichloroethane.

A clear, colorless liquid. Soluble in about 120 parts of water and 2 parts of 96% ethanol.

d_{20}^{20} is about 1.25.

Distillation range (*2.1.2.11*). From 82 °C to 84 °C; not less than 95% must be distilled.

N-Ethylmaleimide. $C_6H_7NO_2$. (M_r 125.1). [128-53-0]. 1-Ethyl-1*H*-pyrrole-2,5-dione.

Colourless crystals. Sparingly soluble in water, freely soluble in 96% ethanol.

The melting point is from 41 °C to 45 °C.

Store at 2 °C to 8 °C.

Ethyl methanesulfonate. $C_3H_8O_3S$. (M_r 124.2). [62-50-0].

A clear, colorless liquid.

Contains not less than 99,0% of $C_3H_8O_3S$.

The *density* is about 1.206 g/cm^3 (20 °C).

n_D^{20} is about 1.418.

The boiling point is about 213 °C.

Ethyl methyl ketone. [78-93-3].

See *Methyl ethyl ketone R.*

2-Ethyl-2-methylsuccinic acid. $C_7H_{12}O_4$. (M_r 160.2). [631-31-2]. 2-Ethyl-2-methylbutanediacarboxylic acid.

The melting point is from 104 °C to 107 °C.

Ethyl parahydroxybenzoate. $C_9H_{10}O_3$. (M_r 166.2). [120-47-8]. Ethyl-4-hydroxybenzoate.

Contains not less than 98.0% and not greater than 102.0% of $C_9H_{10}O_3$.

White or almost white crystalline powder or colorless crystals.

Very slightly soluble in water, freely soluble in 96% ethanol and methanol.

2-Ethylpyridine. C_7H_9N. (M_r 107.2). [100-71-0].

Colourless or brownish liquid.

d_{20}^{20} is about 0.939.

n_D^{20} is about 1.496.

The boiling point is about 149 °C.

Ethyl formate. $C_3H_6O_2$. (M_r 74.1). [109-94-4]. Ethyl methanoate.

A clear, colorless, flammable liquid. Freely soluble in water, miscible with 96% ethanol.

d_{20}^{20} is about 0.919.

n_D^{20} is about 1.36.

The boiling point is about 54 °C.

Ethyl cyanoacetate. $C_5H_7NO_2$. (M_r 113.1). [105-56-6].

A colorless or light yellow liquid. Slightly soluble in water, miscible with 96% ethanol.

The boiling point is from 205 °C to 209 °C with decomposition.

Ethion. $C_9H_{22}O_4P_2S_4$. (M_r 384.5). [563-12-2].

The melting point is from -24 °C to -25 °C.

A suitable standard sample solution (10 ng/µl in cyclohexane) can be used.

Ethoxychrysoidine hydrochloride.

$C_{14}H_{17}ClN_4O$. (M_r 292.8). [2313-87-3]. 4-[(4-Ethoxyphenyl)-diazenyl]phenylene-1,3-diamine hydrochloride.

Reddish powder. Soluble in 96% ethanol.

Ethoxychrysoidine solution.

A 1 g/l solution of *96% ethanol R.*

Test for sensitivity.

To a mixture of 5 mL of *dilute hydrochloric acid R* and 0.05 mL of an ethoxychrysoidine solution, add 0.05 mL of a *0.0167 M bromide-bromate solution.* The colour of the solution should change from red to light yellow within 2 min.

Bovine euglobulins.

Use fresh bovine blood collected in an anticoagulant solution (for example, sodium citrate solution). Discard any haemolysed blood. Centrifuge at acceleration of 1500 *g* to 1800 *g* at a temperature of 15 °C to 20 °C to obtain a plasma supernatant with a low platelet content.

To 1 L of bovine plasma, add 75 g of *barium sulfate R*, shake for 30 min, then centrifuge at 1500-1800 *g* at 15-20 °C, and separate a clear supernatant. Add 10 mL of 0.2 mg/mL solution of *aprotinin R* and shake until mixed. In a container with a minimum capacity of 30 L in a chamber at 4 °C, introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide, and immediately add, while stirring, the supernatant obtained from the plasma. A white precipitate is formed. For precipitation, allow to stand at 4 °C for 10-15 h. Separate the clear supernatant using a siphon. The precipitate is collected by centrifugation at a temperature of 4 °C.

Suspend the precipitate by mechanical dispersion in 500 mL of *distilled water R* at 4 °C, shake for 5 min, and separate by centrifugation at 4 °C. The precipitate is mechanically dispersed in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, adjust the pH to 7.2 - 7.4 with a 10 g/L solution of *sodium hydroxide R* and filter through a glass filter (*2.1.1.2*). Crush the resulting precipitate in a mortar, wash the filter and mortar with 40 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, dilute to 100 mL with the same solution, and lyophilise. Typically, the recovery is 6 g to 8 g of euglobulins from 1000 mL of bovine plasma.

Suitability test. Prepare a solution using a *phosphate buffer solution pH 7.4 R* containing 30 g/l of *bovine albumin R*.

In a test tube with a diameter of 8 mm, placed in a water bath at 37 °C, add 0.2 mL of a 100 IU/mL urokinase reference solution and a 20 IU/mL solution of 0.1 mL of *human thrombin R*. Quickly enter 0.5 mL of a solution containing 10 mg of bovine euglobulins per millilitre. A dense clot should form in less than 10 s. Note the time elapsed between the addition of a solution of bovine euglobulins and the destruction of the clot. The lysis time should not exceed 15 min.

Store in a dry place at a temperature of 4 °C. Shelf life is 1 year.

Euglobulins, human.

For the preparation, use fresh human blood collected into an anticoagulant solution (for example, sodium citrate solution) or human blood for transfusion that has been collected in plastic blood bags and which has just reached its expiry date. Discard any haemolysed blood. Centrifuge at acceleration from 1500 *g* to 1800 *g* at a temperature of 15 °C to obtain a plasma supernatant with a low platelet content. Iso-group plasmas may be mixed.

To 1 L of plasma, add 75 g of *barium sulfate R*, shake for 30 min, then centrifuge at 15 °C with an acceleration of at least 15000 *g*, and draw off the clear supernatant.

Add 10 mL of 0.2 mg/mL solution of *aprotinin R* and shake until mixed. In a container with a minimum capacity of 30 L in a chamber at 4 °C, introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide, and immediately add, while stirring, the supernatant obtained from the plasma; a white precipitate is formed. For precipitation, allow to settle at 4 °C for 10-15 h. Remove the clear supernatant by siphoning. Collect a precipitate by centrifugation at 4 °C. Suspend the precipitate by mechanical dispersion in 500 mL of *distilled water R* at 4 °C, shake for 5 min, and separate by centrifugation at 4 °C. Disperse the precipitate mechanically in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, and adjust the pH to 7.2-7.4 with a solution of *sodium hydroxide R*. Filter through a glass filter (*2.1.1.2)*. Crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of human plasma.

Suitability test. For the test, prepare a solution using *phosphate buffer solution pH 7.2 R* containing 30 g/L of *bovine albumin*. In a test tube with a diameter of 8 mm, placed in a water bath at 37 °C, introduce 0.1 mL of a solution of a reference preparation of streptokinase containing 10 IU/mL of streptokinase activity and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL. Quickly add 1 mL of a solution containing 10 mg/mL of human euglobulins. A dense clot should form in less than 10 seconds. Note the time that elapses between the addition of the solution of human euglobulins and breaking of the clot. The lysis time should not exceed 15 min.

Store in an airtight container at a temperature of 4 °C.

Shelf life is 1 year.

Ether. $C_4H_{10}O$. (M_r 74.1). [60-29-7].

Clear, colourless, volatile, highly mobile, highly flammable liquid. Hygroscopic, water-soluble, miscible with 96% ethanol.

d_{20}^{20} is from 0.713 to 0.715.

The boiling point is from 34 °C to 35 °C.

Do not distill if the ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill the cylinder completely with the test ether, stir vigorously, and allow to stand for 30 min, protected from light. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Store in an airtight container in a dark place at a temperature not exceeding 15 °C.

Ether, peroxide-free. $C_4H_{10}O$. (M_r 74.1). Anaesthetic ether.

Diethyl ether, which allows the presence of a suitable non-volatile antioxidant of the appropriate concentration.

Clear, colourless, volatile, highly mobile liquid.

Soluble in 15 parts of water, miscible with 96% ethanol and fatty oils.

Succinic acid. $C_4H_6O_4$. (M_r 118.1). [110-15-6]. Butanedicarboxylic acid.

White or almost white crystalline powder or colorless crystals. Soluble in water and 96% ethanol.

The melting point is from 184 °C to 187 °C.

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2.2.1.2. Standard solutions for limit tests

In standard solutions, the quantitative content of ions/elements is indicated in parentheses. For ions, indicate the numerical value of the charge, and then the charge sign ("+"or" -"), for example, palladium ion standard solution (500 ppm Pd^{2+}) — palladium chloride solution.

For elements that are part of complex ions/compounds, indicate their degree of oxidation, that is, the sign of the conditional charge ("+"or" -"), and then a numeric value. For example, palladium standard solution (20 ppm Pd^{+2}) is a solution of the $H_2[PdCl_4]$ complex.

Aluminium standard solution (200 ppm Al^{3+}).

Dissolve a quantity of *aluminium potassium sulfate R*, equivalent to 0.352 g of $AlK(SO_4)_2 \cdot 12H_2O$, in *water R*. Add 10 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*.

Aluminium standard solution (100 ppm Al^{3+}).

Dissolve 8.947 g of *aluminium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Aluminium standard solution (10 ppm Al^{3+}).

Dissolve a quantity of *aluminium nitrate R* equivalent to 1.39 g of $Al(NO_3)_3 \cdot 9H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Aluminium standard solution (2 ppm $Al3^{+}$).

Dissolve a quantity of *aluminium potassium sulfate R* equivalent to 0.352 g of $AlK(SO_4)_2 \cdot 12H_2O$ in 10 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Ammonium standard solution (200 ppm NH_4^+).

Place 0.593 g of *ammonium chloride R,* dried in a desiccator over sulfuric acid to a constant mass, in a 1000 mL volumetric flask, dissolve in *water R*, and dilute to 1000.0 mL with the same solvent, and mix.

Ammonium standard solution (100 ppm NH_4^+).

Dissolve a quantity of *ammonium chloride R* equivalent to 0.741 g of NH4Cl in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute 10.0 mL of the resulting solution to 25.0 mL with *water R* immediately before use.

Ammonium standard solution (3 ppm NH_4^+).

Dissolve a quantity of *ammonium chloride R* equivalent to 0.889 g NH^4Cl in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Ammonium standard solution (2 ppm NH_4^+).

Dilute 1 mL of the *ammonium standard solution* (200 ppm NH_4^+) *R* to 100.0 mL with *water R* and stir. Prepare immediately before use.

Ammonium standard solution (2.5 ppm $_N^H$4+).

Dissolve a quantity of *ammonium chloride R* equivalent to 0.741 g of NH_4Cl in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Ammonium standard solution (1 ppm NH_4^+).

Dilute *ammonium standard solution (2.5 ppm NH4) R* to 2.5 times its volume with *water R* immediately before use.

Acetaldehyde standard solution (100 ppm C_2H_4O).

Dissolve 1.0 g of *acetaldehyde R* in *2-propanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the resulting solution to 500.0 mL with *2-propanol R*.

Prepare immediately before use.

Acetaldehyde standard solution (100 ppm C2H4O) R1.

Dissolve 1.0 g of *acetaldehyde R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the resulting solution to 500.0 mL with *water R*.

Prepare immediately before use.

Barium standard solution (0.1% Ba^{2+}).

Dissolve a quantity of *barium chloride R* equivalent to 0.178 g of $BaCl_2{\cdot}2H2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

Barium standard solution (50 ppm Ba^{2+}).

Dissolve a quantity of *barium chloride R* equivalent to 0.178 g of $BaCl_2{\cdot}2H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution with *distilled water R* to 20 times its volume immediately before use.

Barium standard solution (2 ppm Ba^{2+}).

Dilute $^{barium\ standard\ solution\ (50\ ppm\ Ba)\ R}$ *to 25 times its volume with* distilled water R *immediately before use.*

Vanadium standard solution (1 g/L V^{+5}).

Dissolve a quantity of *ammonium vanadate R* equivalent to 0.230 g of NH_4VO_3 in *water R* and dilute to 100.0 mL with the same solvent.

Bismuth standard solution (100 ppm Bi^{3+}).

Dissolve a quantity of *bismuth R* equivalent to 0.500 g of Bi in 50 mL of *nitric acid R* and dilute to 500.0 mL with *water R*.

Dilute the solution to 10 times its volume with *dilute nitric acid R* immediately before use.

Hydrogen peroxide standard solution (10 ppm H_2O_2).

Dilute 10.0 mL of *dilute hydrogen peroxide solution R* to 300.0 mL with *water R*. Dilute 10.0 mL of the resulting solution to 1000.0 mL with *water R*.

Prepare immediately before use.

Hydrogen peroxide standard solution (2 ppm H_2O_2).

Dilute 10.0 mL of *dilute hydrogen peroxide solution R* to 300.0 mL with *water R*. Dilute 2.0 mL of the resulting solution to 1000.0 mL with *water R*.

Prepare immediately before use.

Germanium standard solution (100 ppm Ge^{+4}).

Dissolve a quantity of *ammonium hexafluorohermanate (IV) R* equivalent to 0.307 g of $(NH_4)_2GeF_6$ in 0.01% (*V/V*) *hydrofluoric acid R* and dilute to 1000 mL with *water R*.

Glyoxal standard solution (20 ppm $C_2H_2O_2$).

Weigh a quantity of *glyoxal solution R* equivalent to 0.200 g of $C_2H_2O_2$ into a volumetric flask and dilute to 100.0 mL with *anhydrous ethanol R*.

Dilute the solution to 100 times its volume with *anhydrous ethanol R* immediately before use.

Glyoxal standard solution (2 ppm C2H2O2).

Immediately before use, dilute glyoxal standard solution (20 ppm $C_2H_2O_2$) R *to 10 times its volume with* anhydrous ethanol R.

Iron standard solution (0.1% Fe^{2+}).

Dissolve 0.100 g of Fe in the smallest amount necessary of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 mL with *water R*.

Iron standard solution (250 ppm Fe^{3+}).

Dissolve 4.840 g of *ferric chloride R* in 150 g/L of *hydrochloric acid R* and dilute to 100.0 mL with the same acid.

Dilute the solution with *water R* to 40 times its volume immediately before use.

Iron standard solution (20 ppm Fe^{3+}).

Dissolve a quantity of *ferric ammonium sulfate R* equivalent to 0.863 g of $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 25 mL of the *dilute sulfuric acid R* and dilute to 500.0 mL with *water R*.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Iron standard solution (10 ppm Fe^{2+}).

Dissolve a quantity of *ferric ammonium sulfate R*, equivalent to 7.022 g of $FeNH_4(SO_4) \cdot 6H_2O$, in 25 mL of *dilute sulfuric acid R* and dilute to 1000.0 mL with *water R* .

Dilute the solution to 100 times its volume with *water R* immediately before use.

Iron standard solution (8 ppm Fe^{2+}).

Dissolve 80 mg of *iron R* in 50 mL of 220 g/L *hydrochloric acid R* and dilute to 1000,0 mL with *water R*.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Iron standard solution (2 ppm Fe^{3+}).

Immediately before use, dilute *iron standard solution (20 ppm Fe^{3+}) R* to 10 times its volume with *water R*.

Iodide standard solution (10 ppm I^-).

Dissolve a quantity of *potassium iodide R* equivalent to 0.131 g of KI in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Cadmium standard solution (0.1% Cd^{2+}).

Dissolve a quantity of *cadmium R* equivalent to 0.100 g of Cd in the minimum required equal volumes of *hydrochloric acid R* and *water R*, dilute to 100.0 mL with *1% (V/V) hydrochloric acid R*.

Cadmium standard solution (10 ppm Cd^{2+}).

Dilute cadmium standard solution (0.1% Cd^{2+}) R *to 100 times its volume with a 1%* (V/V) solution of hydrochloric acid R *immediately before use.*

Potassium standard solution (0.2% K^+).

Dissolve a quantity of *potassium sulfate R* equivalent to 0.446 g of K_2SO_4 in *distilled water R* and dilute to 100.0 mL with the same solvent.

Potassium standard solution (600 ppm K^+).

Dissolve a quantity of *potassium sulfate R* equivalent to 2.676 g of K_2SO_4in 100.0 mL of *water R.*

Dilute the solution with *water R* to 20 times its volume immediately before use.

Potassium standard solution (100 ppm K^+).

Dissolve a quantity of *potassium sulfate R* equivalent to 0.446 g of K_2SO_4in 100.0 mL of *water R.*

Dilute the solution with *water R* to 20 times its volume immediately before use.

Potassium standard solution (20 ppm K^+).

Dilute *potassium standard solution (100 ppm K+) R* to 5 times its volume with *water R* immediately before use.

Calcium standard solution (400 ppm Ca^{2+}).

Dissolve a quantity of *calcium carbonate R* equivalent to 1.000 g of $CaCO_3$ in 23 mL of *1M hydrochloric acid* and dilute to 100.0 mL with *distilled water R.*

Dilute the solution with *distilled water R* to 10 times its volume immediately before use.

Calcium standard solution (100 ppm Ca^{2+}).

Dissolve a quantity of *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ in 3 mL of *acetic acid R* and dilute to 250.0 mL with *distilled water R.*

Dilute the solution with *distilled water R* to 10 times its volume immediately before use.

Calcium standard solution (100 ppm Ca^{2+}) R1.

Dissolve a quantity of *anhydrous calcium chloride R* equivalent to 2.769 g of $CaCl_2$ in *dilute sulfuric acid R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Calcium standard solution alcoholic (100 ppm Ca^{2+}).

Dissolve a quantity of *calcium carbonate R* equivalent to 2.50 g of $CaCO_3$ in 12 mL of *acetic acid R* and dilute to 1000.0 mL with *distilled water R.*

Dilute the solution to 10 times its volume with *96% ethanol R* immediately before use.

Calcium standard solution (10 ppm Ca^{2+}).

Dissolve a quantity of *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ in 3 mL of *acetic acid R* and dilute to 250.0 mL with *distilled water R.*

Dilute the solution to 100 times its volume with *distilled water R* immediately before use.

Cobalt standard solution (100 ppm Co^{2+}).

Dissolve a quantity of *cobalt nitrate R* equivalent to 0.494 g of $Co(NO_3)_2 \cdot 6H_2O$ in 500 mL of *1M nitric acid* and dilute the clear solution to 1000 mL with *water R.*

Magnesium standard solution (0.1% Mg^{2+}).

Dissolve a quantity of *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4 \cdot 7H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

Magnesium standard solution (1000 ppm Mg^{2+}).

Dissolve 5.275 g of *magnesium nitrate R* in 16 mL of *dilute nitric acid R* and dilute to 500.0 mL with *water R.*

Standardisation. Carry out the determination of magnesium by complexometry (*2.5.11*).

Magnesium standard solution (100 ppm Mg^{2+}).

Dissolve a quantity of *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4 \cdot 7H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Magnesium standard solution (10 ppm Mg^{2+}).

Dilute *magnesium standard solution (100 ppm Mg^{2+}) R* to 10 times its volume with *water R* immediately before use.

Magnesium standard solution (10 ppm Mg^{2+}) R1.

Dissolve 8.365 g of *magnesium chloride R* in *dilute hydrochloric acid R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Manganese standard solution (1000 ppm Mn^{+2}).

Dissolve a quantity of *manganese sulfate* equivalent to 3.08 g of $MnSO_4 \cdot H_2O$ in 500 mL of *1 M nitric acid* and dilute the solution to 1000 mL with *water R.*

Manganese standard solution (100 ppm Mn^{+2}).

Dissolve a quantity of *manganese sulfate* equivalent to 3.08 g of $MnSO_4 \cdot H_2O$ in 500 mL of *1 M nitric acid solution* and dilute the clear solution to 1000 mL with *water R.*

Copper liposoluble standard solution (1000 ppm Cu).

A copper (metal) organic compound in an oil.

Copper standard solution (0.1% Cu^{+2}).

Dissolve a quantity of *copper sulfate pentahydrate R* equivalent to 0.393 g of $CuSO_4 \cdot 5H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

Copper standard solution (10 ppm Cu^{+2}).

Dilute *copper standard solution (0.1% Cu^{2+}) R* 100 times its volume with *water R* immediately before use.

Copper standard solution (0.1 ppm Cu^{+2}).

Dilute *copper standard solution (10 ppm Cu^{2+}) R* to 100 times its volume with *water R* immediately before use.

Arsenic standard solution (10 ppm As^{+3}).

Dissolve a quantity of *arsenic(III) oxide R*, equivalent to 0.330 g of As_2O_3, in 5 mL of a *dilute sodium hydroxide solution R* and dilute to 250.0 mL with *water R*.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Arsenic standard solution (1 ppm As^{+3}).

Dilute *arsenic standard solution (10 ppm As^{+3}) R* to 10 times its volume with *water R* immediately before use.

Arsenic standard solution (0.1 ppm As^{+3}).

Dilute *arsenic standard solution (1 ppm As^{+3}) R* to 10 times its volume with *water R* immediately before use.

Sodium standard solution (1000 ppm Na^{+}).

Dissolve a quantity of *anhydrous sodium carbonate R* equivalent to 2.305 g of Na_2CO_3 in a mixture of 25 mL of *water R* and 25 mL of *nitric acid R* and dilute to 1000.0 mL with *water R.*

Sodium standard solution (200 ppm Na^{+}).

Dissolve a quantity of *sodium chloride R* equivalent to 0.509 g of NaCl in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Sodium standard solution (50 ppm Na^{+}).

Dilute the sodium standard solution (200 ppm Na^{+}) R *to 4 times its volume with* water R.

Nickel liposoluble standard solution (1000 ppm Ni).

A nickel (metal) organic compound in an oil.

Nickel standard solution (10 ppm Ni^{2+}).

Dissolve a quantity of *nickel sulfate R* equivalent to 4.78 g of $NiSO_4 \cdot 7H_2O$ in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Nickel standard solution (5 ppm Ni^{2+}).

Dilute nickel standard solution (10 ppm Ni^{2+}) R*to twice its volume with* water for chromatography R.

Nickel standard solution (0.2 ppm Ni^{2+}).

Immediately before use, dilute *nickel standard solution (10 ppm Ni^{2+}) R* to 50 times its volume with *water R.*

Nickel standard solution (0.1 ppm Ni^{2+}).

Immediately before use, dilute *nickel standard solution (10 ppm Ni^{2+}) R* to 100 times its volume with *water R.*

Nitrate standard solution (100 ppm NO_3^{-}).

Dissolve a quantity of *potassium nitrate R* equivalent to 0.815 g of KNO_3 in *water R* and dilute to 500.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Nitrate standard solution (10 ppm NO_3^-).

Dilute *nitrate standard solution (100 ppm NO_3^-) R* to 10 times its volume with *water R* immediately before use.

Nitrate standard solution (2 ppm NO_3^-).

Dilute *nitrate standard solution (10 ppm NO_3^-) R* to 5 times its volume with *water R* immediately before use.

Tin liposoluble standard solution (1000 ppm Sn).

A tin (metal) organic compound in an oil

Tin standard solution (5 ppm Sn^{2+}).

Dissolve a quantity of *tin R* equivalent to 0.500 g of Sn in a mixture of 5 mL of *water R* and 25 mL of *hydrochloric acid R*, dilute to 1000.0 mL with *water R.*

Dilute the solution to 100 times its volume with *2.5% (V/V) hydrochloric acid R* immediately before use.

Tin standard solution (0.1 ppm Sn^{2+}).

Dilute *tin standard solution (5 ppm Sn^{2+}) R* to 50 times its volume with *water R* immediately before use.

Palladium standard solution (500 ppm Pd^{2+}).

Dissolve 50.0 mg of *palladium R* in 9 mL of *hydrochloric acid R* and dilute to 100.0 mL with *water R.*

Palladium standard solution (20 ppm Pd^{+2}).

Dissolve 0.333 mg of *palladium chloride R* in 2 mL of warm *hydrochloric acid R* and dilute to 1000.0 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Palladium standard solution (0.5 ppm Pd^{2+}).

Dilute 1 mL of palladium standard solution (500 ppm Pd^{2+}) R *with a mixture of 0.3 volume of* nitric acid R *and 99.7 volume of* water R.

Platinum standard solution (30 ppm Pt^{+4}).

Dissolve 80 mg of *chloroplatinic acid R* in *1 M hydrochloric acid* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *1 M hydrochloric acid* immediately before use.

Mercury standard solution (1000 ppm Hg^{2+}).

Dissolve a quantity of *mercuric chloride R* equivalent to 1.354 g of $HgCl_2$ in 50 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R.*

Mercury standard solution (10 ppm Hg^{2+}).

Dissolve a quantity of *mercuric chloride R* equivalent to 0.338 g of $HgCl_2$ in 250.0 mL of *water R.*

Dilute the solution to 100 times its volume with *water R* immediately before use.

Lead liposoluble standard solution (1000 ppm of Pb).

A lead (metal) organic compound in an oil.

Lead standard solution (0.1% of Pb^{2+}).

Dissolve a quantity of *lead(II) nitrate R*, equivalent to 0.400 g of $Pb(NO_3)_2$ in *water R* and adjust to 250.0 mL with the same solvent.

Lead standard solution (0.1% of Pb^{2+}) R1.

Dissolve a quantity of *lead(II) nitrate R* equivalent to 0.400 g of $Pb(NO_3)_2$ in *dilute lead-free nitric acid R* and dilute to 250.0 mL with the same solvent.

Lead standard solution (100 ppm of Pb^{2+}).

Dilute *lead standard solution (0.1% of Pb^{2+}) R* to 10 times of its volume with *water R* immediately before use.

Lead standard solution (10 ppm of Pb^{2+}).

Dilute *lead standard solution (100 ppm of Pb^{2+}) R* to 10 times its volume with *water R* immediately before use.

Lead standard solution (10 ppm of Pb^{2+}) R1.

Dissolve 0.160 g of *lead(II) nitrate R* in 100 mL of *water R*, add 1 mL of *lead-free nitric acid R*, and dilute to 1000.0 mL with *water R*.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Lead standard solution (10 ppm of Pb^{2+}) R2.

Dilute lead standard solution (0.1% of Pb^{2+}) R1 *to 100 times its volume with* dilute lead-free nitric acid R. Use within 1 week.

Lead standard solution (2 ppm of Pb^{2+}).

Dilute *lead standard solution (10 ppm of Pb^{2+}) R* to 5 times its volume with *water R* immediately before use.

Lead standard solution (1 ppm of Pb^{2+}).

Dilute *lead standard solution (10 ppm of Pb^{2+}) R* to 10 times its volume with *water R* immediately before use.

Lead standard solution (0.5 ppm of Pb^{2+}).

Dilute lead standard solution (10 ppm of Pb^{2+}) R *to 20 times its volume with* dilute lead-free nitric acid R. Use within 1 day.

Lead standard solution (0.25 ppm of Pb^{2+}).

Dilute *lead standard solution (1 ppm of Pb^{2+}) R* to 4 times its volume with *water R* immediately before use.

Lead standard solution (0.1 ppm of Pb^{2+}).

Dilute *lead standard solution (1 ppm of Pb^{2+}) R* to 10 times its volume with *water R* immediately before use.

Selenium standard solution (100 ppm Se^{+4}).

Dissolve 0.100 g of *selenium R* in 2 mL of *nitric acid R*, evaporate to dryness. Dissolve the residue with 2 mL of *water R* and evaporate to dryness; repeat this operation three times. Dissolve the residue in 50 mL of *dilute hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

Selenium standard solution (1 ppm Se^{+4}).

Dissolve a quantity of *selenious acid R* equivalent to 6.54 mg of H_2SeO_3 in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution with *water R* to 40 times its volume immediately before use.

Silver standard solution (5 ppm Ag^{+}).

Dissolve a quantity of *silver nitrate R* equivalent to 0.790 g of $AgNO_3$ in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Strontium standard solution (1.0% Sr^{2+}).

Cover with *water R*, *strontium carbonate R* equivalent to 1.6849 g of $SrCO_3$. Cautiously add *hydrochloric acid R* until all the solid has dissolved and there is no sign of further effervescence, and dilute to 100.0 mL with *water R*.

Sulfate standard solution (100 ppm SO_4^{2-}).

Dissolve a quantity of *potassium sulfate R* equivalent to 0.181 g of K_2SO_4 in *distilled water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution with *distilled water R* to 10 times its volume immediately before use.

Sulfate standard solution (10 ppm SO_4^{2-}).

Dissolve a quantity of *potassium sulfate R* equivalent to 0.181 g of K_2SO_4 in *distilled water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *distilled water R* immediately before use.

Sulfate standard solution (10 ppm SO_4^{2-}) R1.

Dissolve a quantity of *potassium sulfate R* equivalent to 0.181 g of K_2SO_4 in *30% (V/V) ethanol R* and dilute to 100.0 mL with the same solvent.

Dilute the solution with *30% (V/V) ethanol R* to 100 times its volume immediately before use.

Sulfite standard solution (80 ppm SO_3^{2-}).

Dissolve 3.150 g of *anhydrous sodium sulfite R* in freshly prepared *distilled water R* and dilute to 100.0 mL with the same solvent. Dilute 0.5 mL of the resulting solution to 100.0 mL with freshly prepared *distilled water R*.

Sulfite standard solution (1.5 ppm SO_3^{2-}).

Dissolve a quantity of *sodium metabisulphite R* equivalent to 0.152 g of $Na_2S_2O_5$ in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. To 3.0 mL of the resulting solution, add 4.0 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

Antimony standard solution (100 ppm Sb^{+5}).

Dissolve a quantity of *antimony potassium tartrate R* equivalent to 0.274 g of $C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$ in 500 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

Antimony standard solution (1 ppm Sb^{+5}).

Dissolve *antimony potassium tartrate R* equivalent to 0.274 g $C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$ in 20 mL of *hydrochloric acid R1* and dilute the clear solution to 100.0 mL with *water R*. To 10.0 mL of the resulting solution, add 200 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*. To 100.0 mL of this solution, add 300 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*.

Prepare the dilute solutions immediately before use.

Thallium standard solution (10 ppm Tl^{+}).

Dissolve *thallium sulfate R* equivalent to 0.125 g of Tl_2SO_4 in a 9 g/L solution of *sodium chloride R*, and dilute to 1000.0 mL with the same solvent. Dilute 10.0 mL of the resulting solution to 100.0 mL with a 9 g/L solution of *sodium chloride R*.

Titanium standard solution (100 ppm Ti^{3+}).

Dissolve 100.0 mg of *titanium R*, if necessary, by heating in 100 mL of *hydrochloric acid R*, dilute to 150 mL with *water R*; allow to cool and dilute to 1000.0 mL with *water R*.

Ferrocyanide standard solution (100 ppm of $[Fe(CN)_6]^{4-}$).

Dissolve *potassium ferrocyanide R* equivalent to 0.20 g $K_4[Fe(CN)_6] \cdot 3H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Ferrocyanide standard solution (50 ppm of $[Fe(CN)_6]^{3-}$).

Dissolve *potassium ferricyanide R* equivalent to 0.78 g of $K_3[Fe(CN)_6]$ in *water R*, and dilute to 100.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Formaldehyde standard solution (5 ppm of CH_2O).

Dilute a quantity of *formaldehyde solution R* equivalent to 1.0 g of CH_2O to 1000.0 mL with *water R*.

Dilute the solution to 200 times its volume with *water R* immediately before use.

Phosphate standard solution (200 ppm of PO_4^{3-}).

Dissolve *potassium dihydrogen phosphate R* equivalent to 0.286 g of KH_2PO_4 in *water R* and dilute to 1000.0 mL with the same solvent.

Phosphate standard solution (5 ppm of PO_4^{3-}).

Dissolve *potassium dihydrogen phosphate R* equivalent to 0.716 g of KH_2PO_4 in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Fluoride standard solution (10 ppm of F^-).

Dissolve in *water R sodium fluoride R* previously dried at 300 °C for 12 h, equivalent to 0.442 g of NaF, and dilute to 1000.0 mL with the same solvent (1 mL = 0.2 mg F).

Store in a polyethylene container.

Dilute the solution with *water R* to 20 times its volume immediately before use.

Fluoride standard solution (1 ppm of F^-).

Dilute *fluoride standard solution (10 ppm F^-) R* to 10 times its volume with *water R* immediately before use.

Chloride standard solution (50 ppm of Cl^-).

Dissolve *sodium chloride R* equivalent to 0.824 g of NaCl in *water R*, and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 10 times its volume with *water R* immediately before use.

Chloride standard solution (8 ppm of Cl^-).

Dissolve *sodium chloride R* equivalent to 1.32 g of NaCl in *water R* and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Chloride standard solution (5 ppm of Cl^-).

Dissolve *sodium chloride R* equivalent to 0.824 g of NaCl in *water R*, and dilute to 1000.0 mL with the same solvent.

Dilute the solution to 100 times its volume with *water R* immediately before use.

Chromium liposoluble standard solution (1000 ppm Cr).

A chromium (metal) organic compound in an oil

Chromium standard solution (0.1% of Cr^{+6}).

Dissolve *potassium dichromate R* equivalent to 2.83 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

Chromium standard solution (100 ppm of Cr^{+6}).

Dissolve *potassium dichromate R* equivalent to 0.283 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

Chromium standard solution (0.1 ppm of Cr^{+6}).

Dilute chromium standard solution (100 ppm of Cr^{+6}) *R* to 1000 times its volume with *water R* immediately before use.

Zinc standard solution (5 mg/mL Zn^{2+}).

Dissolve 3.15 g of *zinc oxide R* in 15 mL of *hydrochloric acid R* and dilute to 500.0 mL with *water R.*

Zinc standard solution (100 ppm Zn^{2+}).

Dissolve a quantity of *zinc sulfate R* equivalent to 0.440 g of $ZnSO_4 \cdot 7H_2O$ in 1 mL of *acetic acid R* and dilute to 100.0 mL with *water R.*

Dilute the solution to 10 times its volume with *water R* immediately before use.

Zinc standard solution (10 ppm Zn^{2+}).

Immediately before use, dilute *zinc standard solution (100 ppm Zn^{2+}) R* to 10 times its volume with *water R.*

Zinc standard solution (5 ppm of Zn^{2+}).

Dilute *zinc standard solution (100 ppm of Zn^{2+}) R* to 20 times its volume with *water R* immediately before use.

Zirconium standard solution (1 g/l of Zr^{+4}).

Dissolve *zirconyl nitrate R* equivalent to 0.293 g of $ZrO(NO_3)_2 \cdot 2H_2O$ in a mixture of *hydrochloric acid R/water R* (2:8, *V/V*) and dilute to 100.0 mL with the same mixture of solvents.

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2.2.1.3 Buffer solutions

Buffered acetone solution.

Dissolve 8.15 g of *sodium acetate R* and 42 g of *sodium chloride R* in *water R*, add 68 mL of *0.1 M hydrochloric acid*, 150 mL of *acetone R* and dilute to 500 mL with *water R.*

Buffer solution pH 2.0.

Dissolve 6.57 g of *potassium chloride R* in *water R*, add 119.0 mL of *0.1 M hydrochloric acid* and dilute to 1000.0 mL with *water R.*

Phosphate buffer solution pH 2.0.

Dissolve 8.95 g of *sodium hydrogen phosphate R* and 3.40 g of *potassium dihydrogen phosphate R* in *water R*; dilute to 1000.0 mL with the same solvent. If necessary, adjust the pH of the solution with *phosphoric acid R.*

0.125 M Phosphate buffer solution pH 2.0.

Dissolve 17.0 g of *potassium dihydrogen phosphate R* and 17.8 g of *anhydrous disodium hydrogen phosphate R* in *water R* and dilute to 1000.0 mL with *water R.* If necessary, adjust the pH of the solution with *phosphoric acid R.*

Sulfate buffer solution pH 2.0.

Solution A. Dissolve 132.1 g of *ammonium sulfate R* in *water R* and dilute to 500.0 mL with the same solvent.

Solution B. With continuous cooling and stirring, carefully add 14 mL of *sulfuric acid R* to approximately 400 mL of *water R*; cool and dilute to 500.0 mL with *water R.*

Mix equal volumes of solutions A and B. If necessary, adjust the pH.

Buffer solution pH 2.2.

Mix 6.7 mL of *phosphoric acid R* with 55.0 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R.*

Buffer solution pH 2.5.

Dissolve 100 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust the pH with *hydrochloric acid R* to 2.5, and dilute to 1000.0 mL with *water R.*

Buffer solution pH 2.5 R1.

To 4.9 g of dilute phosphoric acid R, *add 250 mL of* water R, *adjust the pH with a* dilute sodium hydroxide solution R *and dilute to 500.0 mL with* water R.

0.2 M Phosphate buffer solution pH 2.5.

Dissolve 27.2 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust the pH to 2.5 with *phosphoric acid R*, and dilute to 1000.0 mL with *water R.*

Phosphate buffer solution pH 2.8.

Dissolve 7.8 g of *sodium dihydrogen phosphate R* in 900 mL of *water R* adjust the pH to 2.8 of the solution with *phosphoric acid R* and dilute to 1000 mL with the same solvent.

Buffer solution pH 3.0.

Dissolve 21.0 g of *citric acid monohydrate R* in 200 mL of *1 M sodium hydroxide* and dilute to 1000 mL with *water R.* Dilute 40.3 mL of the resulting solution to 100.0 mL with *0.1 M hydrochloric acid.*

0.1 M Phosphate buffer solution pH 3.0.

Dissolve 12.0 g of *anhydrous sodium dihydrogen phosphate R* in *water R*, adjust the pH of the solution with *dilute phosphoric acid R1* and dilute to 1000 mL with *water R.*

Phosphate buffer solution pH 3.0.

Mix 0.7 mL of *phosphoric acid R* with 100 mL of *water R* and dilute to 900 mL with the same solvent. Adjust the pH to 3.0 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water R.*

0.25 M Citrate buffer solution pH 3.0.

Dissolve 5.3 g of *citric acid monohydrate R* in 80 mL of *water R*, adjust the pH with *1 M sodium hydroxide* and dilute the solution to 100.0 mL with *water R.*

Phosphate buffer solution pH 3.0 R1.

Dissolve 3.40 g of *potassium dihydrogen phosphate R* in 900 mL of *water R.* Adjust the pH of the solution to 3.0 with *phosphoric acid R* and dilute to 1000.0 mL with *water R.*

Phosphate buffer solution pH 3.2.

To 900 mL of a 4 g/L solution of *sodium dihydrogen phosphate R*, add 100 mL of a 2.5 g/L solution of *phosphoric acid R*. If necessary, adjust the pH.

Phosphate buffer solution pH 3.2 R1.

Adjust the pH of a 35.8 g/L solution of *disodium hydrogen phosphate R* to pH 3.2 with *dilute phosphoric acid R*. Dilute 100.0 mL of the resulting solution to 2000.0 mL with *water R.*

Phosphate buffer solution pH 3.25.

Dissolve about 1.36 g of *potassium dihydrogen phosphate R* in 1000 mL of *water R* and adjust the pH of the solution to 3.25 ± 0.05 with *dilute phosphoric acid R*, filter through a 0.45 µm (or less) membrane filter.

Phosphate buffer solution pH 3.4.

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH with *phosphoric acid R.*

Buffer solution pH 3.5.

Dissolve 25.0 g of *ammonium acetate R* in 25 mL of *water R*, and add 38.0 mL of *hydrochloric acid R1*. If necessary, adjust the pH with *dilute hydrochloric acid R* or *dilute ammonia solution R1* and dilute to 100.0 mL with *water R*.

Phosphate buffer solution pH 3.5.

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH of the solution with *phosphoric acid R*.

Buffer solution pH 3.6.

To 250.0 mL of *0.2 M potassium hydrophthalate R*, add 11.94 mL of *0.2 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

Buffer solution pH 3.7.

To 15.0 mL of *acetic acid R*, add 60 mL of *96% ethanol R* and 20 mL of *water R*; adjust the pH to 3.7 with *ammonia solution R* and dilute to 100.0 mL with *water R*.

Buffered copper sulfate solution pH 4.0.

Dissolve 0.25 g of *copper (II) sulfate R* and 4.5 g of *ammonium acetate R* in *dilute acetic acid R* and dilute to 100.0 mL with the same solvent.

0.1 M Sodium acetate buffer solution pH 4.0.

Dissolve 822 mg of *sodium acetate R* in 100 mL of *water R* (solution A). Dilute 1.44 mL of *glacial acetic acid R* with 250 mL of *water R* (solution B). Titrate 100 mL of solution B with 20 mL of solution A.

Acetate buffer solution pH 4.4.

Dissolve 136 g of *sodium acetate R* and 77 g of *ammonium acetate R* in *water R* and dilute with the same solvent to 1000.0 mL, then add 250.0 mL of *glacial acetic acid R* , and mix.

Phthalate buffer solution pH 4.4.

Dissolve 2.042 g of *potassium hydrophthalate R* in 50 mL of *water R*, add 7.5 mL of *0.2 M sodium hydroxide*, and dilute to 200.0 mL with *water R*.

Acetate buffer solution pH 4.5.

Dissolve 77.1 g of *ammonium acetate R* in *water R*, add 70 mL of *glacial acetic acid R*, and dilute to 1000.0 mL with *water R*.

0.5 M Ammonium acetate buffer solution pH 4.5.

Mix 14.3 mL of *glacial acetic acid R* and 470 mL of *water R*, adjust the pH to 4.5 with *concentrated ammonia solution R* and dilute to 500.0 mL with *water R*.

0.05 M Phosphate solution pH 4.5.

Dissolve 6.80 g of *potassium dihydrogen phosphate R* in 1000.0 mL of *water R*. The pH value of the solution should be 4.5.

Sodium acetate buffer solution pH 4.5.

Dissolve 63 g of *anhydrous sodium acetate R* in *water R*, add 90 mL of *acetic acid R*, adjust the pH to 4.5 and dilute to 1000 mL with *water R*.

Acetate buffer solution pH 4.6.

Dissolve 5.4 g of *sodium acetate R* in 50 mL of *water R*, add 2.4 g of *glacial acetic acid R* and dilute to 100.0 mL with *water R*. If necessary, adjust the pH.

Succinate buffer solution pH 4.6.

Dissolve 11.8 g of *succinic acid R* in a mixture of 600 mL of *water R* and 82 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Acetate buffer solution pH 4.7.

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of this solution with 250 mL of *dilute acetic acid R*. Shake twice with a freshly prepared, filtered, 0.1 g/L solution of *dithizone R* in *chloroform R*. Shake with *carbon tetrachloride R* until the extract is colourless. Filter the aqueous layer to remove traces of carbon tetrachloride.

Acetate buffer solution pH 4.7 R1.

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of the resulting solution with 250 mL of *dilute acetic acid R*.

Acetate buffer solution pH 5.0.

To 120 mL of a 6 g/L solution of *glacial acetic acid R*, add 100 mL of *0.1 M potassium hydroxide* and about 250 mL of *water R*, mix, adjust the pH to 5.0 with a 6 g/L solution of *acetic acid R* or *0.1 M solution of potassium hydroxide* and dilute the resulting solution to 1000.0 mL with *water R*.

0.2 M Deuterated sodium phosphate buffer solution pH 5.0.

Dissolve 2.76 g of *sodium dihydrogen phosphate monohydrate R* in 90 mL of *deuterium oxide R*, adjust the pH with a deuterated solution of *phosphoric acid R* or *1 M sodium hydroxide R*, dilute to 100 mL with *deuterium oxide R*, and mix.

Phosphate buffer solution pH 5.0.

Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust the pH with *1 M potassium hydroxide* and dilute to 1000 mL with *water R*.

Citrate buffer solution pH 5.0.

Prepare a solution containing 20.1 g/L of citric acid monohydrate R *and 8.0 g/L of* sodium hydroxide R*solution. Adjust the pH with* dilute hydrochloric acid R.

Buffer solution pH 5.2.

Dissolve 1.02 g of *potassium hydrophthalate R* in 30.0 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

0.067 M Phosphate buffer solution pH 5.4.

Mix the corresponding volumes of a 23.99 g/L solution of *disodium hydrophosphate R* and a 9.12 g/L solution of *sodium dihydrophosphate monohydrate R* to obtain a solutionwith pH 5.4.

Acetate-edetate buffer solution pH 5.5.

Dissolve 250 g of *ammonium acetate R* and 15 g of *sodium edetate R* in 400 mL of *water R* and add 125 mL of *glacial acetic acid R*.

Buffer solution pH 5.5.

Dissolve 54.4 g of *sodium acetate R* in 50 mL of *water R*, if necessary, heat to a temperature of 35 °C. After cooling, slowly add 10 mL of *anhydrous acetic acid R*, shake and dilute to 100.0 mL with *water R*.

Phosphate buffer solution pH 5.5.

Solution A. Dissolve 13.61 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Solution B. Dissolve 35.81 g of *disodium hydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Mix 96.4 mL of solution A and 3.6 mL of solution B.

Phosphate-citrate buffer solution pH 5.5.

Mix 56.85 mL of a 28.4 g/L solution of *anhydrous disodium hydrogen phosphate R* and 43.15 mL of a 21 g/L solution of *citric acid monohydrate R*.

Phosphate buffer solution pH 5.6.

Solution A. Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent.

Solution B. Dissolve 1.161 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent.

Mix 94.4 mL of solution A and 5.6 mL of solution B. If necessary, adjust the pH to 5.6 with solution A or solution B.

Phosphate buffer solution pH 5.8.

Dissolve 1.19 g of *disodium dihydrogen phosphate dihydrate R* and 8.25 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Acetate buffer solution pH 6.0.

Dissolve 100 g of *ammonium acetate R* in 300 mL of *water R*, add 4.1 mL of *glacial acetic acid R*. If necessary, adjust the pH with *ammonia solution R* or *acetic acid R* and dilute to 500.0 mL with *water R*.

Diethylammonium phosphate buffer solution pH 6.0.

Dilute 68 mL of *phosphoric acid R* to 500 mL with *water R*. To 25 mL of the resulting solution, add 450 mL of *water R* and 6 mL of *diethylamine R*. If necessary, adjust the pH to 6 ± 0.05 with *diethylamine R* or *phosphoric acid R*, and dilute to 500.0 mL with *water R*.

1 M Morpholinoethanesulfonate buffer solution pH 6.0.

Dissolve 48.8 g of *2-[N-morpholino]ethanesulfonic acid R* in 160 mL of *water R* and add 25 mL of *2 M sodium hydroxide R*. Adjust the pH to 6.0 with *2 M sodium hydroxide R*and dilute to almost 250 mL. If necessary, adjust the pH with *2 M sodium hydroxide R* and dilute to 250.0 mL with *water R*.

Phosphate buffer solution pH 6.0.

Mix 63.2 mL of a 71.5 g/L solution of disodium hydrogen phosphate dodecahydrate R *and 36.8 mL of a 21 g/L solution of* citric acid monohydrate R.

Phosphate buffer solution pH 6.0 R1.

Dissolve 6.8 g of sodium dihydrogen phosphate R *in* water R *and dilute to 1000.0 mL with* water R. Adjust the pH with *strong sodium hydroxide solution R.*

Phosphate buffer solution pH 6.0 R2.

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R,* add 28.5 mL of *0.2 M solution of sodium hydroxide* and dilute to 1000.0 mL with *water R.*

Phosphate buffer solution pH 6.4.

Dissolve 2.5 g of *disodium hydrogen phosphate dodecahydrate R,* 2.5 g of *sodium dihydrogen phosphate R,* and 8.2 g of *sodium chloride R* in 950 mL of *water R.* If necessary, adjust the pH with *1 M sodium hydroxide* or *1 M hydrochloric acid* to 6.4 and dilute to 1000.0 mL with *water R.*

0.5 M Phthalate buffer solution pH 6.4.

Dissolve 100 g of *potassium hydrophthalate R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary, adjust the pH with a *strong sodium hydroxide solution R.*

Buffer solution pH 6.5.

Dissolve 60.5 g of *disodium hydrogen phosphate R* and 46 g of *potassium dihydrogen phosphate R* in *water R,* add 100 mL of *0.02 M sodium edetate,* 20 mg of *mercuric chloride R,* and dilute to 1000.0 mL with *water R.*

Imidazole buffer solution pH 6.5.

Dissolve 6.81 g of *imidazole R,* 1.23 g of *magnesium sulfate R,* and 0.73 g of *calcium sulfate R* in 752 mL of *0.1 M hydrochloric acid.* If necessary, adjust the pH and dilute to 1000.0 mL with *water R.*

0.1 M Phosphate buffer solution pH 6.5.

Dissolve 13.80 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of *distilled water R,* adjust the pH with a 400 g/L solution of *sodium hydroxide R,* and dilute with *distilled water R* to 1000.0 mL.

Phosphate buffer solution pH 6.5.

Dissolve 2.75 g of sodium dihydrogen phosphate R *and 4.5 g of* sodium chloride R *in 500 mL of* water R. *Adjust the pH with* phosphate buffer solution pH 8.5 R.

Buffer solution pH 6.6.

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R,* add 89.0 mL of *0.2 M sodium hydroxide* and dilute to 1000.0 mL with *water R.*

0.1 M Phosphate buffer solution pH 6.7.

Dissolve 15.6 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Dissolve 17.8 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1000.0 mL with the same solvent. Mix the solutions, if necessary, adjust the pH to 6.7.

Phosphate buffered saline pH 6.8.

Dissolve 1.0 g of *potassium dihydrogen phosphate R,* 2.0 g of *dipotassium hydrogen phosphate R,* and 8.5 g of *sodium chloride R* in 900 mL of *water R.* If necessary, adjust the pH and dilute to 1000.0 mL with the same solvent.

Phosphate buffer solution pH 6.8.

Mix 77.3 mL of a 71.5 g/L solution of disodium hydrogen phosphate dodecahydrate R *and 22.7 mL of a 21 g/L solution of* citric acid monohydrate R.

Phosphate buffer solution pH 6.8 R1.

To 51.0 mL of a 27.2 g/L solution of *potassium dihydrogen phosphate R,* add 49.0 mL of a 71.6 g/L solution of *disodium hydrogen phosphate dodecahydrate R,* if necessary, adjust the pH.

Store at a temperature between 2 °C and 8 °C.

1M Tris-hydrochloride buffer solution pH 6.8.

Dissolve 60.6 g of *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R,* adjust the pH with *hydrochloric acid R,* and dilute to 500.0 mL with *water R.*

Buffer solution pH 7.0.

To 1000 mL of a solution containing 18 g/L of *disodium hydrogen phosphate dodecahydrate R* and 23 g/L of *sodium chloride R,* add a sufficient amount (about 280 mL) of a solution containing 7.8 g/L of *sodium dihydrogen phosphate R* and 23 g/L of *sodium chloride R* to adjust the pH.

Dissolve in the ressulting solution sufficient *sodium azide R* to obtain a 0.2 g/L solution.

Maleate buffer solution pH 7.0.

Dissolve 10.0 g of *sodium chloride R,* 6.06 g of *tris(hydro-xymethyl)aminomethane R,* and 4.90 g of *maleic anhydride R* in 900 mL of *water R*, adjust the pH with a 170 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R.*

Store at 2 °C to 8 °C. Use within 3 days.

0.025 M Phosphate buffer solution pH 7.0.

Mix 1 volume of 0.063 M phosphate buffer solution pH 7.0 R *with 1.5 volumes of* water R.

0.03 M Phosphate buffer solution pH 7.0.

Dissolve 5.2 g of *dipotassium hydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust the pH of the solution to 7.0 ± 0.1 with *phosphoric acid R,* and dilute to 1000 mL with *water for chromatography R*.

0.05 M Phosphate buffer solution pH 7.0.

Mix 34 mL of water R *and 100 mL of* 0.067 M phosphate buffer solution pH 7.0 R.

0.063 M Phosphate buffer solution pH 7.0.

Dissolve 5.18 g of *anhydrous disodium hydrogen phosphate R* and 3.65 g of *sodium dihydrogen phosphate monohydrate R* in 950 mL of *water R,* adjust the pH with *phosphoric acid R* and dilute to 1000.0 mL with *water R.*

0.067 M Phosphate buffer solution pH 7.0.

Solution A. Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent.

Solution B. Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 100.0 mL with the same solvent.

Mix 38.9 mL of solution A and 61.1 mL of solution B, if necessary, adjust the pH.

0.1 M Phosphate buffer solution pH 7.0.

Dissolve 1.361 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent. Adjust the pH with a 35 g/L solution of *disodium hydrogen phosphate dodecahydrate R.*

Phosphate buffer solution pH 7.0.

Mix 82.4 mL of a 71.5 g/L solution of disodium hydrogen phosphate dodecahydrate R *and 17.6 mL of a 21 g/L solution of* citric acid monohydrate R.

Phosphate buffer solution pH 7.0 R1.

Mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and 148.2 mL of a 8 g/L solution of *sodium hydroxide R,* if necessary, adjust the pH and dilute to 1000.0 mL with *water R.*

Phosphate buffer solution pH 7.0 R2.

Mix 50.0 mL of a solution of 136 g/L *potassium dihydrogen phosphate R* and 29, 5 mL of *1 M sodium hydroxide solution R* and dilute to 100.0 mL with *water R*, if necessary, adjust the pH to 7.0 ±0.1.

Phosphate buffer solution pH 7.0 R3.

Dissolve 5 g of *potassium dihydrogen phosphate R* and 11 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R,* adjust the pH with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R* to a value of 7.0 and dilute to 1000 mL with *water R,* then mix.

Phosphate buffer solution pH 7.0 R4.

Dissolve 28.4 g of *anhydrous sodium dihydrogen phosphate R* and 18.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 500 mL with the same solvent.

Phosphate buffer solution pH 7.0 R5.

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* in 800 mL of *water R,* adjust the pH with a 30% (*m/m*) solution of *phosphoric acid R,* and dilute to 1000 mL with *water R.*

Phosphate buffer solution pH 7.0 R6.

Dissolve 3.56 of *disodium hydrogen phosphate dihydrate R* in 950 mL of *water for chromatography R,* adjust the pH *with phosphoric acid R* and dilute to 1000 mL with *water for chromatography R.*

Phosphate buffer solution pH 7.0 R7.

Dissolve 35g of *dipotassium hydrogen phosphate R* in 900 mL of *water R,* adjust the pH with *dilute phosphoric acid R* to a value of 7.0, and dilute to 1000.0 mL with *water R.*

Potassium phosphate buffer solution pH 7.0.

Dissolve 10 mg of *bovine albumin R* and 68 mg of *potassium dihydrogen phosphate R* in 30 mL of *water R.* If necessary, adjust the pH of *potassium hydroxide R* to 7.0, dilute to 50 mL with *water R*, and filter.

Sodium/calcium acetate buffer solution pH 7.0.

Dissolve 10 mg of *bovine albumin R* and 32 mg of *calcium acetate R* in 60 mL of *water R*. Add 580 µL of *glacial acetic acid*, adjust the pH with *2 M sodium hydroxide* to 7.0, dilute to 100 mL with *water R*, and filter.

Tetrabutylammonium buffer solution pH 7.0.

Dissolve 6.16 g of *ammonium acetate R* in a mixture of 15 mL of a 400 g/L solution of *tetrabutylammonium hydroxide R* and 185 mL of *water R*, adjust the pH with *nitric acid R.*

Buffered salt solution pH 7.2.

Dissolve 8.0 g of *sodium chloride R,* 0.2 g of *potassium chloride R,* 0.1 g of *anhydrous calcium chloride R,* 0.1 g of *magnesium chloride R,* 3.18 g of *disodium hydrogen phosphate dodecahydrate R,* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with *water R.*

Buffer solution pH 7.2.

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R,* add 175.0 mL of *0.2 M sodium hydroxide.* Dilute to 1000.0 mL with *water R* and, if necessary, adjust the pH.

Phosphate-albumin buffered saline pH 7.2.

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R,* 7.6 g of *sodium chloride R,* and 10 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use, adjust the pH using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R.*

Phosphate-albumin buffered saline pH 7.2 R1.

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R,* 7.6 g of *sodium chloride R,* and 1 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent.

Immediately before use, adjust the pH using dilute sodium hydroxide solution R *or* dilute phosphoric acid R.

Phosphate buffer solution pH 7.2.

Mix 87.0 mL of a 71.5 g/L solution of disodium hydrogen phosphate dodecahydrate R *and 13.0 mL of a 21 g/L solution of* citric acid monohydrate R.

Imidazole buffer solution pH 7.3.

Dissolve 3.4 g of *imidazole R* and 5.8 g of *sodium chloride R* in *water R*, add 18.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R.* If necessary, adjust the pH.

Barbital buffer solution pH 7.4.

Mix 50 mL of a solution containing 19.44 g/L of *sodium acetate R* and 29.46 g/L of *sodium barbital R* in *water R* with 50.5 mL of *0.1 M hydrochloric acid*, add 20 mL of a 85 g/L solution of *sodium chloride R* and dilute to 250 mL with *water R.*

Buffer solution pH 7.4.

Dissolve 0.6 g of *potassium dihydrogen phosphate R,* 6.4 g of *disodium hydrogen phosphate dodecahydrate R,* and 5.85 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary, adjust the pH.

Phosphate buffered saline pH 7.4.

Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R,* 0.19 g of *potassium dihydrogen phosphate R,* 8.0 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary, adjust the pH.

Phosphate buffer solution pH 7.4.

To 393.4 mL of 0.1 M sodium hydroxide, *add 250.0 mL of* 0.2 M potassium dihydrogen phosphate R.

Tris(hydroxymethyl)aminomethane buffer solution pH 7.4.

Dissolve 30.3 g of *tris(hydroxymethyl)aminomethane R* in approximately 200 mL of *water R*, add 183 mL of *1 M hydrochloric acid*, and dilute to 500.0 mL with *water R.*

Note: *the pH of the solution is 7.7-7.8 at room temperature and 7.4 at 37 °C. This solution is stable for several months at 4 °C.*

Tris(hydroxymethyl)aminomethane-sodium chloride buffer solution pH 7.4.

Dissolve 6.08 g of *tris(hydroxymethyl)aminomethane R* and 8.77 g of *sodium chloride R* in 500 mL of *distilled water R*, add 10.0 g of *bovine albumin R*. Adjust the pH with *hydrochloric acid R* and dilute to 1000.0 mL with *distilled water R*.

Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1.

Dissolve 0.1 g of *bovine albumin R* in a mixture of 2 mL of *tris (hydroxymethyl)aminomethane buffer solution pH of 7.4 R* and 50 mL of a 5.84 mg/mL solution of *sodium chloride R*, and dilute to 100.0 mL with *water R*.

Tris-sodium acetate buffer solution pH 7.4.

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*, adjust the pH with *sulfuric acid R* to 7.4, and dilute to 1000.0 mL with *water R*.

Tris-sodium acetate-sodium chloride buffer solution pH 7.4.

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R* and 14.6 g of *sodium chloride R* in 900 mL of *water R*, add 0.50 g of *bovine albumin R*, adjust the pH with *sulfuric acid R* to 7.4, and dilute to 1000.0 mL with *water R*.

Borate buffer solution with pH 7.5.

Dissolve 2.5 g of *sodium chloride R*, 2.85 g of *disodium tetraborate R*, and 10.5 g of *boric acid R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary, adjust the pH.

Store at 2 °C to 8 °C.

Buffer (HEPES) solution pH 7.5.

Dissolve 2.38 g of *2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid R* in approximately 90 mL of *water R*, adjust the pH with *sodium hydroxide solution R* to 7.5, and dilute to 100 mL with *water R*.

0.05 M Phosphate buffer solution pH 7.5.

Dissolve 0.89 g of *disodium hydrogen phosphate dihydrate R* in approximately 80 mL of *water R*, adjust the pH to 7.5 with a 8.5% (*V/V*) solution of *phosphoric acid R* and dilute to 100.0 mL with *water R*.

0.2 M Phosphate buffer solution pH 7.5.

Dissolve 27.22 g of *potassium dihydrogen phosphate R* in 930 mL of *water R*, adjust the pH with a 300 g/L solution of *potassium hydroxide R* to 7.5, and dilute to 1000.0 mL with *water R*.

0.33 M Phosphate buffer solution pH 7.5.

Solution A. Dissolve 119.31 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Solution B. Dissolve 45.36 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Mix 85 mL of solution A and 15 mL of solution B, if necessary, adjust the pH.

0.05 M Tris-hydrochloride buffer solution pH 7.5.

Dissolve 6.057 g of *tris(hydroxymethyl)aminomethane R* in *water R*, if necessary, adjust the pH with *hydrochloric acid R*, and dilute to 1000.0 mL with *water R*.

1 M Tris-hydrochloride buffer solution pH 7.5.

Dissolve 12.11 g of *tris(hydroxymethyl)aminomethane R* in 90 mL of *water R*, adjust the pH with *hydrochloric acid R* to 7.5, and dilute to 100.0 mL with *water R*.

Tris(hydroxymethyl)aminomethane buffer solution pH 7.5.

Dissolve 7.27 g of *tris(hydroxymethyl)aminomethane R* and 5.27 g of *sodium chloride R* in *water R*, if necessary, adjust the pH, and dilute to 1000.0 mL with *water R*.

Sodium citrate buffer solution pH 7.8 (0.034 M sodium citrate and 0.101 M sodium chloride).

Dissolve 10.0 g of *sodium citrate R* and 5.90 g of *sodium chloride R* in 900 mL of *water R*, adjust the pH with *hydrochloric acid R*, and dilute to 1000.0 mL with *water R*.

0.0015 M Borate buffer solution pH 8.0.

Dissolve 0.572 g of *disodium tetraborate R* and 2.94 g of *calcium chloride R* in 800 mL of *water R*, adjust the pH with *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

Buffer solution pH 8.0.

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R*, add 46.8 mL of *0.2 M sodium hydroxide* and dilute to 200.0 mL with *water R*.

Buffer solution pH 8.0 R1.

Dissolve 20 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust the pH with *phosphoric acid R*, and dilute to 1000 mL with *water R*.

0.02 M Phosphate buffer solution pH 8.0.

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R*, add 46.8 mL of *0.2 M sodium hydroxide* and dilute to 500.0 mL with *water R*.

0.02 M Sodium phosphate buffer solution pH 8.0.

Dissolve 0.31 g of *sodium dihydrogen phosphate R* in 70 mL of *water R*, adjust the pH with *1 M sodium hydroxide solution* to 8.0 and dilute the solution to 100 mL with *water R*.

0.1 M Phosphate buffer solution pH 8.0.

Dissolve 0.523 g of *potassium dihydrogen phosphate R* and 16.73 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

1 M Phosphate buffer solution pH 8.0.

Dissolve 136.1 g of *potassium dihydrogen phosphate R* in *water R*, adjust the pH with *1 M sodium hydroxide*, and dilute to 1000.0 mL with *water R*.

1 M Tris-hydrochloride buffer solution pH 8.0.

Dissolve 121.1 g of *tris(hydroxymethyl)aminomethane R* and 1.47 g of *calcium chloride R* in 900 mL of *water R*, adjust the pH with *hydrochloric acid R*, and dilute to 1000.0 mL with *water R*.

Tris-hydrochloride buffer solution pH 8.0.

Dissolve 1.21 g of tris(hydroxymethyl)aminomethane R *and 29.4 mg of* calcium chloride R *in* water R, *adjust the pH with* 1 M hydrochloric acid, *and dilute to 100.0 mL with* water R.

Tris-sodium acetate buffer solution pH 8.0.

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*, adjust the pH with *sulfuric acid R* to 8.0, and dilute to 1000 mL with *water R*.

Tris-sodium acetate-sodium chloride buffer solution pH 8.0.

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R*, and 14.6 g of *sodium chloride R* in 900 mL of *water R*, add 0.50 g of *bovine albumin R* adjust the pH with *sulfuric acid R* to 8.0 and dilute the solution to 1000 mL with *water R*.

Tris(hydroxymethyl)aminomethane buffer solution pH 8.1.

Dissolve 0.294 g of calcium chloride R *in 40 mL of a solution of* tris(hydroxymethyl)aminomethane R, *adjust to the pH with* 1 M hydrochloric acid, *and dilute to 100.0 mL with* water R.

Tris-glycine buffer solution pH 8.3.

Dissolve 6.0 g of *tris(hydroxymethyl)aminomethane R* and 28.8 g of *glycine R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use, add 10 volumes of *water R* to 1 volume of the solution.

Tris-hydrochloride buffer solution pH 8.3.

Dissolve 9.0 g of *tris(hydroxymethyl)aminomethane R* in 2900 mL of *water R*, adjust the pH with *1 M hydrochloric acid*, and dilute to 3000.0 mL with *water R*.

Barbital buffer solution pH 8.4.

Dissolve 8.25 g of *sodium barbital R* in *water R* and dilute to 1000.0 mL with the same solvent.

Tris-EDTA BSA buffer solution pH 8.4.

Dissolve 6.1 g of*tris(hydroxymethyl)aminomethane R*, 2.8 g of *sodium edetate R*, 10.2 g of *sodium chloride R*, and 10 g of *bovine albumin R* in *water R*, adjust the pH with *1 M hydrochloric acid* to 8.4, and dilute to 1000.0 mL with *water R*.

Tris-(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4.

Dissolve 5.12 g of *sodium chloride R*, 3.03 g of *tris(hydroxymethyl)aminomethane R*, and 1.40 g of *sodium edetate R* in 250 mL of *distilled water R*, adjust the pH with *hydrochloric acid R* to 8.4, and dilute the solution to 500.0 mL with *distilled water R*.

Guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.5.

Dissolve 1.0 g of *sodium edetate R*, 12.1 g of *tris(hydroxymethyl)aminomethane R*, and 57.0 g of *guanidine hydrochloride R* in 35 mL of *water R*, adjust the pH with *hydrochloric acid R* to 8.5 and dilute the solution to 100 mL with *water R*.

Phosphate buffer solution pH 8.5.

Dissolve 3.5 g of *dipotassium hydrogen phosphate R* and 4.5 g of *sodium chloride R* in 500 mL of *water R*, adjust the pH with a mixture of equal volumes of *dilute phosphoric acid R* and *water R*.

Tris acetate buffer solution pH 8.5.

Dissolve 0.294 g of *calcium chloride R* and 12.11 g of *tris-(hydroxymethyl)aminomethane R* in *water R*, adjust the pH with *acetic acid R*, and dilute to 1000.0 mL with *water R*.

Barbital buffer solution pH 8.6 R1.

Dissolve 1.38 g of *barbital R*, 8.76 g of *sodium barbitatal R*, and 0.38 g of *calcium lactate pentahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

1.5 M Tris-hydrochloride buffer solution pH 8.8.

Dissolve 90.8 g *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R*, adjust the pH with *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

3 M Tris-hydrochloride buffer solution pH 8.8.

Dissolve 363.3 g of *tris(hydroxymethyl)aminomethane R* in 500 mL of *water R*, adjust the pH with *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

Phosphate buffer solution pH 9.0.

Dissolve 1.74 g of *potassium dihydrogen phosphate R* in 80 mL of *water R* if necessary, adjust the pH with *1 M potassium hydroxide*, and dilute to 100.0 mL with *water R*.

Buffer solution pH 9.0.

Dissolve 6.18 g of *boric acid R* in *0.1 M potassium chloride R* and dilute to 1000.0 mL with the same solvent, add 420.0 mL of *0.1 M sodium hydroxide*.

Buffer solution pH 9.0 R1.

Dissolve 6.20 g of *boric acid R* in 500 mL of *water R*, adjust the pH with *1 M sodium hydroxide* (about 41.5 mL) and dilute to 1000.0 mL with *water R*.

Tris(hydroxymethyl)aminomethane buffer solution pH 9.0.

Dissolve 1.21 g of tris(hydroxymethyl)aminomethane R *in 950 mL of* water for chromatography R, *adjust the pH with* acetic acid R *to a value of 9.0 and dilute to 1000.0 mL with* water for chromatography R.

0.05 M Tris-hydrochloride buffer solution pH 9.0.

Dissolve 0.605 g of *tris(hydroxymethyl)aminomethane R* in *water R*, adjust the pH with *1 M hydrochloric acid R* and dilute to 100.0 mL with *water R*.

Ammonium chloride buffer solution pH 9.5.

Dissolve 33.5 g of *ammonium chloride R* in 150 mL of *water R*, add 42.0 mL of *concentrated ammonia solution R* and dilute to 250.0 mL with *water R*.

Store in a polyethylene container.

Ammonium buffer solution pH 10.0.

Dissolve 5.4 g of *ammonium chloride R* in 20 mL of *water R*, add 35.0 mL of *ammonia solution R* and dilute to 100.0 mL with *water R*.

Borate buffer solution pH 10.0.

Place 12.4 g of *boric acid R* in a 500.0 mL volumetric flask, add 300 mL of *water R* to suspend boric acid. Add 100 mL of a 56 g/L solution of *potassium hydroxide R* and mix until boric acid is dissolved. Adjust the pH to 10.0, slowly adding a 56 g/L solution of *potassium hydroxide R* (usually about 60 mL is required), mix, and dilute to almost 500 mL with *water R*. If necessary, adjust the pH with *boric acid R* or a 56 g/L solution of *potassium hydroxide R* and dilute to 500.0 mL with *water R*.

Diethanolamine buffer solution pH 10.0.

Dissolve 96.4 g of *diethanolamine R* in *water R*, dilute to 400 mL with the same solvent, add 0,5 mL of a 186 g/L solution of *magnesium chloride R*, adjust the pH with *1 M hydrochloric acid*, and dilute to 500,0 mL with *water R*.

0.1 M Ammoniim carbonate buffer solution pH 10.3.

Dissolve 7.91 g of *ammonium carbonate R* in 800 mL of *water R*, adjust the pH with *dilute sodium hydroxide solution R*, and dilute to 1000.0 mL with *water R*.

Ammonium chloride buffer solution pH 10.4.

Dissolve 70 g of *ammonium chloride R* in 200 mL of *water R*, add 330 mL of *concentrated ammonia solution R* and dilute to 1000.0 mL with *water R*. If necessary, adjust the pH to 10.4 with *ammonia R*.

Borate buffer solution pH 10.4.

Dissolve 24.64 g of *boric acid R* in 900 mL of *distilled water R*, adjust the pH with a 400 g/L solution of *sodium hydroxide R*, and dilute to 1000 mL with *distilled water R*.

Ammonium chloride buffer solution pH 10.7.

Dissolve 67.5 g of *ammonium chloride R* in *water R*, add 570 mL of *concentrated ammonia solution R* and dilute to 1000.0 mL with *water R*.

Buffer solution pH 10.9.

Dissolve 6.75 g of *ammonium chloride R* in *ammonia solution R* and dilute to 100.0 mL with the same solvent.

Total-ionic-strength-adjustment buffer.

Dissolve 58.5 g of *sodium chloride R*, 57.0 mL of *glacial acetic acid R*, 61.5 g of *sodium acetate R*, and 5.0 g of *cyclohexylenedinitrilotetra-acetic acid R* in *water R* and dilute to 500.0 mL with the same solvent. Adjust the pH with a 335 g/L solution of *sodium hydroxide R* to 5.0-5.5 and dilute the solution to 1000.0 mL with *distilled water R*.

Total-ionic-strength-adjustment buffer R1.

Solution A. Dissolve 210 g of citric acid monohydrate R *in 400 mL of* distilled water R, *adjust the pH with* concentrated ammonia solution R *to 7.0, and dilute to 1000.0 mL with* distilled water R.

Solution B. Dissolve 132 g of *ammonium phosphate R* in *distilled water R* and dilute to 1000.0 mL with the same solvent.

Solution C. To a suspension of 292 g of *(ethylenedinitrilo)tetra-acetic acid R* in approximately 500 mL of *distilled water R*, add about 200 mL of *concentrated ammonia solution R* until dissolved, adjust the pH with *concentrated ammonia solution R* to 6-7, and dilute to 1000,0 mL with *distilled water R*.

Mix equal volumes of solutions A, B, and C and adjust to pH 7.5 with *concentrated ammonia R*.

Buffer solution pH 11.

Dissolve 6.21 g of *boric acid R*, 4.00 g of *sodium hydroxide R*, 3.70 g of *potassium chloride R* in 500 mL of *water R* and dilute to 1000 mL with the same solvent.

0.1 M Phosphate buffer solution pH 11.3.

Dissolve 17.4 g of *dipotassium hydrogen phosphate R* in approximately 950 mL of *water R*, adjust to pH 11.3 with a 100 g/L solution of *potassium hydroxide R*, and dilute to 1000.0 mL with *water R*. Filter through a 0.45 µm membrane filter.

2.2.2. REAGENTS, PRIMARY STANDARDS FOR VOLUMETRIC SOLUTIONS

202020001-2019

2.2.2.1. Original reference substances for volumetric solutions

Primary standard substances for volumetric solutions are indicated by the suffix RV. (main reagent.) Primary reference standards of suitable quality may be obtained from commercial sources or prepared by the following methods.

Benzoic acid. $C_7H_6O_2$. (M_r 122.1). [65-85-0].

Sublime *benzoic acid R* in a suitable apparatus.

Potassium bromate. $KBrO_3$. (M_r 167.0). [7758-01-2].

Recrystallise *potassium bromate R* from boiling *water R.* Collect the crystals and dry to a constant mass at 180 °C.

Potassium hydrogen phthalate. $C_8H_5KO_4$. (M_r 204.2). [877-24-7].

Recrystallise potassium hydrophthalate R from boiling *water R*. Collect the crystals at a temperature above 35 °C and dry to a constant mass at 110 °C.

Arsenic trioxide. AS_2O_3. (M_r 197.8). [1327-53-3].

Sublimate *arsenic oxide R* in a suitable apparatus.

Store over *anhydrous silica gel R.*

Sodium carbonate. Na_2CO_3. (M_r 106.0). [497-19-8].

Filter a saturated solution of *sodium carbonate R* at room temperature. Slowly pass a flow of *carbon dioxide R* through the filtrate under constant cooling and stirring. After 2 h, collect the precipitate on a glass filter (*2.1.1.2*), wash the filter with ice *water R*, saturated with carbon dioxide. Dry at 100-105 °C and ignite to a constant mass at 270-300 °C, stirring periodically.

Sodium chloride NaCl. (M_r 58.44). [7647-14-5].

To 1 volume of *saturated sodium chloride solution R,* add 2 volumes of *hydrochloric acid R*. Collect the resulting crystals and wash with *hydrochloric acid R1,* which is removed by heating in a water bath. Ignite to a constant mass at 300 °C.

Sulfanilic acid. $C_6H_7NO_3S$. (M_r 173.2). [121-57-3].

Recrystallise *sulfanilic acid R* from boiling *water R*, filter, and dry to constant mass at 100-105 °C.

Zinc. Zn. (A_r 65.4). [7440-66-6].

Use zinc with a content of minimum 99.9% of Zn.

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2.2.2.2. Volumetric solutions

Volumetric solutions are solutions with accurately known concentrations intended for titrimetric (volumetric) analysis.

Volumetric solutions are prepared according to the usual chemical analytical methods. The accuracy of the apparatus used is verified to ensure that it is appropriate for the intended use. The concentration of volumetric solutions is expressed in terms of molarity, i.e. the number of moles, the amount of substance dissolved in 1 L of solution. A solution containing *x* moles in 1 L is referred to as *x* M solution.

Volumetric solutions do not differ from the prescribed strength by greater than 10%. The molarity of the volumetric solutions is determined by an appropriate number of titrations. The relative standard deviation does not exceed 0.2%. Volumetric solutions are standardised by the methods described below.

If a volumetric solution is to be used in a quantitative analysis in which the end-point is determined by an electrochemical process (for example, amperometry or potentiometry), the solution is standardised by the same method. The composition of the media in which a volumetric solution is standardised should be the same as that in which it is to be used.

Solutions more dilute than those described below are obtained by dilution with *carbon dioxide-free water R* in accordance with the instructions for standardisation. The correction factors of these solutions are the same as those from which the dilutions were prepared.

1 M nitric acid solution.

Dilute 96.6 g of *nitric acid R* to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*, titrate with the prepared nitric acid solution potentiometrically (2.1.2.19), or using 0.1 mL of a *methyl orange R solution* as indicator, until a reddish-yellow colour is obtained.

1 mL of *1 M nitric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

0.1 M Ammonium thiocyanate.

Dissolve 7.612 g of *ammonium thiocyanate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of *0.1 M silver nitrate solution*, add 25 mL of *water R*, 2 mL of *dilute nitric acid R*, 2 mL of *ferric ammonium sulfate R2*, and titrate with the ammonium thiocyanate solution until a reddish-yellow colour is obtained.

0.01 M Ammonium thiocyanate.

Dilute 100.0 mL of 0.1 M ammonium thiocyanate to 1000.0 mL with water.

Standardisation. To 20.0 mL of *0.01 M silver nitrate*, add 25 mL of *water R*, 2 mL of *dilute nitric acid R*, 2 mL of *ferric ammonium sulfate R2*, and then proceed as indicated in the case of 0.1 M ammonium thiocyanate standardisation.

1 mL of *0.01 M silver nitrate solution* is equivalent to 0.7612 mg of NH4SCN.

0.1 M Ammonium and cerium nitrate.

Shake for 2 min a solution containing 56 mL of *sulfuric acid R* and 54.82 g of *ammonium and cerium nitrate R*, add five successive quantities, each of 100 mL, of *water R*, shaking after each addition. Dilute the clear solution to 1000.0 mL with *water R*. Standardise the solution after 10 days.

Standardisation. Titrate 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a 49 g/L solution of *sulfuric acid R* with the prepared ammonium and cerium nitrate solution potentiometrically (2.1.2.19), or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2 \cdot 4H2O$.

Store in a place protected from light.

0.01 M Ammonium and cerium nitrate.

To 100.0 mL of *0.1 M ammonium and cerium nitrate*, add 30 mL of *sulfuric acid R* and dilute to 1000.0 mL with *water R*.

0.1 M Ammonium and cerium sulfate.

Dissolve 65.0 g of *ammonium and cerium sulfate R* in a mixture of 500 mL of *water R* and 30 mL of *sulfuric acid R*; allow to cool and dilute to 1000.0 mL with *water R*.

Standardisation. Titrate 0.300 g of *ferrous ethylendiammonium sulfate RV* in 50 mL of a 49 g/L solution of *sulfuric acid R* with a prepared ammonium and cerium sulfate solution potentiometrically (2.1.2.19), or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium sulfate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2 \cdot 4H2O$.

0.01 M Ammonium and cerium sulfate.

To 100.0 mL of *0.1 M ammonium and cerium sulfate*, add 30 mL of *sulfuric acid R* and dilute the solution to 1000.0 mL with *water R*.

0.05 M Barium perchlorate.

Dissolve 15.8 g of *barium hydroxide R* in a mixture of 7.5 mL of *perchloric acid R* and 75 mL of *water R*, adjust the solution to pH 3 by adding *perchloric acid R* and filter if necessary. Add 150 mL of *96% ethanol R* and dilute to 250 mL with *water R*, then dilute to 1000.0 mL with *buffer solution pH 3.7 R*.

Standardisation. To 5.0 mL of *0.05 M sulfuric acid* add 5 mL of *water R*, 50 mL of *buffer solution pH 3.7 R*, and 0.5 mL of *alizarin S solution R* and titrate with the barium perchlorate solution until an orange-red colour appears. Standardise immediately before use.

0.025 M Barium perchlorate.

Dilute 500.0 mL of *0.05 M barium perchlorate* to 1000.0 mL with *buffer solution pH 3.7 R*.

0.005 M Barium perchlorate.

Dilute 10.0 mL of *0.05 M barium perchlorate* to 100.0 mL with the *buffer solution*. Preparation of the buffer solution: To 15.0 mL of *acetic acid R*, add 60.0 mL of *2-propanol R*, adjust to pH 3.7 with a solution of *ammonia R*, and dilute to 100.0 mL with *water R*.

0.1 M Barium chloride.

Dissolve 24.4 g of *barium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of the barium chloride solution add 60 mL of *water R*, 3 mL of *concentrated ammonia R*, and 0.5-1 mg of *phthalein purple R* and titrate with *0.1 M sodium edetate*. When the solution begins to decolourise, add 50 mL of *96% ethanol R* and continue the titration until the blue-violet colour disappears.

0.004 M Benzethonium chloride.

Dissolve in *water R* 1.792 g of *benzethonium chloride R*, previously dried to constant mass at 100-105 °C, and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.350 g of the dried benzethonium chloride in 35 mL of a mixture consisting of 30 volumes of *anhydrous acetic acid R* and add 70 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, using 0.05 mL of *crystal violet solution R* as indicator. At the same time, carry out a blank titration.

1 mL of *0.1 M perchloric acid solution* is equivalent to 44.81 mg of $C_{27}H_{42}ClNO_2$.

0.0167 M Bromide-bromate.

Dissolve 2.7835 g of *potassium bromate RV* and 13 g of *potassium bromide R* in *water R* and dilute to 1000.0 mL with the same solvent.

0.01 M Bismuth nitrate.

Dissolve 4.86 g of *bismuth nitrate pentahydrate R* in 60 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R* .

Standardisation. To 25.0 mL of the bismuth nitrate solution, add 50 mL of *water R* and titrate with *0.01 M sodium edetate* using 0.05 mL of a 1 g/L solution of *xylenol orange R* as indicator.

0.1 M Ferric ammonium sulfate.

Dissolve 50.0 g of *ferric ammonium sulfate R* in a mixture of 6 mL of *sulfuric acid R* and 300 mL of *water R* and dilute to 1000.0 mL with *water R.*

Standardisation. To 10.0 mL of the prepared ferric ammonium sulfate solution, add 35 mL of *water R*, 3 mL of *hydrochloric acid R* and 1 g of *potassium iodide R* and after 10 min titrate with *0.1 M sodium thiosulfate* potentiometrically (2.1.2.19), or using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 48.22 mg of $FeNH_4(SO_4)_2 \cdot 12H_2O$.

0.1 M Ferrous sulfate.

Dissolve 27.80 g of *ferrous sulfate R* in 500 mL of *dilute sulfuric acid R* and dilute to 1000.0 mL with *water R.*

Standardisation. To 25.0 mL of the ferrous sulfate solution, add 3 mL of *phosphoric acid R* and titrate immediately with *0.02 M potassium permanganate*.

Standardise immediately before use.

0.1 M Iodine.

Dissolve 25.5 g of *iodine R* and 40 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of the resulting iodine solution, add 1 mL of *dilute acetic acid R*, 40 mL of *water R* and titrate with a *0.1 M sodium thiosulfate solution,* using *starch solution R as indicator.*

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 12.69 mg of I_2.

Store in a place protected from light.

0.5 M Iodine.

Dissolve 127 g of *iodine R* and 200 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.*To 2.0 mL of the iodine solution add 1 mL of *dilute acetic acid R and 50 mL of water R* and titrate with *0.1 M sodium thiosulfate,* using *starch solution R* as indicator.

Store in a place protected from light.

0.05 M Iodine.

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of the iodine solution, add 1 mL of *dilute acetic acid R,* 40 mL of *water R,* and titrate with a *0.1 M sodium thiosulfate* potentiometrically (2.1.2.19), or using *starch solution R* as indicator.

Store in a place protected from light.

0.01 M Iodine.

Add 0.3 g of *potassium iodide R* to 20.0 mL of *0.05 M iodine* and dilute to 100.0 mL with *water R.*

Standardisation. To 25 mL of the resulting iodine solution, add 1 mL of *dilute acetic acid R*, 25 mL of *water R* and titrate with a *0.01 M sodium thiosulfate* using *starch solution R* as indicator.

1 mL of 0.01 M sodium thiosulfate solution is equivalent to 1.269 mg of I_2.

0.033 M Potassium bromate.

Dissolve 5.5670 g of *potassium bromate RV* in *water R* and dilute to 1000.0 mL with the same solvent.

0.02 M Potassium bromate.

Dissolve 3.340 g of *potassium bromate RV* in *water R* and dilute to 1000.0 mL with the same solvent.

0.0167 M Potassium bromate.

Prepare by diluting *0.033 M Potassium bromate.*

0.0083 M Potassium bromate.

Prepare by diluting *0.033 M Potassium bromate.*

1 M Potassium hydroxide.

Dissolve 60 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Titrate 20.0 mL of the potassium hydroxide solution with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator.

0.1 M Potassium hydroxide.

Dissolve 6 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Titrate 0.150 g of potassium hydrogen phthalate RV in 50 mL of *water R* with a prepared *potassium hydroxide solution* potentiometrically (2.1.2.19), or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M potassium hydroxide* is equivalent to 20.42 mg of C8H5KO4.

0.5 M Potassium hydroxide in alcohol (60%, *V/V*).

Dissolve 3 g of *potassium hydroxide R* in *aldehyde-free alcohol R (60%, V/V)* and dilute to 100.0 mL with the same solvent.

Standardisation. Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *96% ethanol R*, titrate with a prepared potassium hydroxide solution potentiometrically (2.1.2.19), or using 0.1 mL of a *phenolphthalein solution R* as indicator.

1 mL of *0.5 M potassium hydroxide in alcohol (60‰, V/V)* is equivalent to 61.06 mg of $C_7H_6O_2$.

0.5 M Potassium hydroxide, alcoholic.

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100.0 mL with *aldehyde-free alcohol R.*

Standardisation. Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *96% ethanol R*, titrate with a prepared potassium hydroxide solution potentiometrically (2.1.2.19), or using *0.1 mL of a phenolphthalein solution R* as indicator.

1 *mL of 0.5 M of potassium hydroxide alcohol solution* is equivalent to 61.06 mg of $C_7H_6O_2$.

For dilution, use the *aldehyde-free alcohol R.*

0.1 M Potassium hydroxide, alcoholic.

Dilute 20.0 mL of *0.5 M alcoholic potassium hydroxide solution* to 100.0 mL with *aldehyde-free alcohol R.*

0.01 M Potassium hydroxide, alcoholic.

Dilute 2.0 mL of *0.5 M alcoholic potassium hydroxide solution* to 100.0 mL with *aldehyde-free alcohol R.*

0.1 M Potassium hydrogen phthalate.

Place about 800 mL of *anhydrous acetic acid R* in a conical flask, add 20.42 g of *potassium hydrogen phthalate RV*, and heat on a water-bath until dissolved, protected from humidity.

Cool to 20 °C and dilute to 1000.0 mL with *anhydrous acetic acid R.*

0.0167 M Potassium dichromate.

Dissolve 4.90 g of *potassium dichromate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the potassium dichromate solution add 1 g of *potassium iodide R*, 7 mL of *dilute hydrochloric acid R*, 250 mL of *water R* and titrate with *0.1 M sodium thiosulfate*, using 3 mL of *starch solution R* as indicator, until the colour changes from blue to light green.

0.05 M Potassium iodate.

Dissolve 10.70 g of *potassium iodate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dilute 15.0 mL of the prepared potassium iodate solution to 40.0 mL with *water R*, add 1 g of *potassium iodide R* and 5 mL of *dilute sulfuric acid R*, and titrate with *0.1 M sodium thiosulfate* potentiometrically (2.1.2.19) or using 1 mL of *starch solution R* added at the end of the titration as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 3.567 mg of KIO_3.

0.0167 M Potassium iodate.

Dissolve 3.567 g of *potassium iodate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the prepared potassium iodate solution, add 2 g of *potassium iodide R*, 25 mL of *dilute sulfuric acid R*, and titrate with *0.1 M sodium thiosulfate*, using1 mL of *starch solution R* added at the end of the titration as indicator.

1 mL of *0.1 M sodium thiosulfate solution* is equivalent to 3.567 mg of KIO_3.

0.001 M Potassium iodide.

Dilute 10.0 mL of *potassium iodide solution R* to 100.0 mL with *water R.* Dilute 5.0 mL of the resulting solution to 500.0 mL with *water R.*

0.02 M Potassium permanganate.

Dissolve 3.2 g of *potassium permanganate R* in *water R* and dilute to 1000.0 mL with the same solvent. Heat the solution for 1 h on a water bath, allow to cool and filter through a sintered glass filter (*2.1.1.2).*

Standardisation. Dissolve 0.300 g of *ferrous ethylendiammonium sulfate RV* in 50 mL of a 49 g/L solution of *sulfuric acid R* and titrate with a prepared potassium permanganate solution potentiometrically (2.1.2.19), or using 0.1 mL of *ferroin R* as indicator. Standardise immediately before use.

1 mL of *0.02 M potassium permanganate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)2{\cdot}4H_2O$.

Store in a place protected from light.

0.1 M Lanthanum nitrate.

Dissolve 43.30 g of *lanthanum nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20 mL of the lanthanum nitrate solution, add 15 mL of *water R* and 25 mL of *0.1 M sodium edetate.* Add about 50 mg of *xylenol orange triturate R* and about 2 g of *hexamethylenetetramine R* and titrate with *0.1 M zinc sulfate* until the colour changes from yellow to violet-pink.

1 mL of *0.1 M sodium edetate solution* is equivalent to 43.30 mg of $La(NO_3)_3{\cdot}6H_2O$.

0.1 M Lithium methoxide.

Dissolve 0.694 g of *lithium R* in 150 mL of *anhydrous methanol R* and dilute to 1000.0 mL with *toluene R.*

Standardisation. To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the lithium methoxide solution until a blue colour is obtained. Immediately add 0.200 g of *benzoic acid RV*, stir to effect solution, and titrate with the lithium methoxide solution until the blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. The volume of titrant used in the second titration ascertains the exact strength of the lithium methoxide solution. Standardise immediately before use.

1 mL of *0.1 M lithium methylate solution* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Magnesium chloride.

Dissolve 20.33 g of *magnesium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Carry out the determination of magnesium by complexometry (*2.1.5.11).*

0.02 M Copper sulfate.

Dissolve 5.0 g of *copper sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the copper sulfate solution add 2 g of *sodium acetate R* and 0.1 mL of *pyridylazonaphthol solution R* and titrate with *0.02 M sodium edetate* until the colour changes from violet-blue to bright green. Titrate slowly towards the end of the titration.

0.1 M Sodium arsenite.

Dissolve *arsenious trioxide RV* equivalent to 4.946 g of As_2O_3 in a mixture of 20 mL of *strong sodium hydroxide solution R* and 20 mL of *water R*, dilute to 400 mL with *water R* and add *dilute hydrochloric acid R* until the solution is neutral to *litmus paper R.* Dissolve 2 g of *sodium hydrogen carbonate R* in the solution and dilute to 500.0 mL with *water R.*

1 M Sodium hydroxide.

Dissolve 42 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 1.50 g of *potassium hydrogen phthalate RV* in 50 mL of *water R* and titrate with a prepared sodium hydroxide solution potentiometrically (2.1.2.19), or using 0.1 mL of a *phenolphthalein solution R* as indicator.

1 mL of *1 M of sodium hydroxide* is equivalent to 204.2 mg of $C_8H_5KO_4$.

If sodium hydroxide free from carbonate is prescribed, prepare it as follows. Dissolve *sodium hydroxide R* in *water R* to give a concentration of 400-600 g/L and allow to stand. Decant the clear supernatant, taking precautions to avoid the introduction of carbon dioxide, and dilute with *carbon dioxide-free water R* to the required molarity. The solution complies with the following test. Titrate 20.0 mL of hydrochloric acid of the same molarity with the solution of sodium hydroxide, using 0.1 mL of *phenolphthalein solution R* as indicator. At the end-point add just sufficient of the acid to discharge the pink colour; concentrate the solution to 20 mL by boiling; during boiling add just sufficient acid to discharge the pink colour, which should not reappear after prolonged boiling. The volume of acid used does not exceed 0.1 mL.

0.1 M Sodium hydroxide.

Dilute 100.0 mL of *1 M sodium hydroxide* to 1000.0 mL with *carbon dioxide-free water R*.

*Standardisation.*Carry out the titration as described for *1 M sodium hydroxide solution* using 0.150 g of *potassium hydrogen phthalate RV* in 50 mL of *water R.*

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.42 mg of C8H5KO4.

Standardisation (for use in the quantitation of halide salts of organic bases). Dissolve 0.100 g of *benzoic acid RV* in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *96% ethanol R*. Carry out the titration (*2.1.2.19),* using the sodium hydroxide solution. Note the volume of the sodium hydroxide solution added between the two points of inflexion.

1 mL of *0.1 M sodium hydroxide solution* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Sodium hydroxide, ethanolic.

To 250 mL of *anhydrous ethanol R* add 3.3 g of *strong sodium hydroxide solution R.*

Standardisation. Dissolve 0.100 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *96% ethanol R* and titrate with the ethanolic sodium hydroxide solution potentiometrically *(*2.1.2.19*),* using 0.2 mL of *thymolphthalein solution R* as indicator. Standardise immediately before use.

1 mL of *0.1 M ethanolic sodium hydroxide solution* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Sodium methylate.

Cool 175 mL of *anhydrous methanol R* in ice water and add, in small portions, about 2.5 g of freshly cut *sodium R*. When the metal is dissolved, dilute to 1000.0 mL with *toluene R.*

Standardisation. To 10 mL of *dimethylformamide R*, add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the prepared sodium methylate solution until the blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*, mix until dissolved, and titrate with the prepared sodium methylate solution until the blue colour is obtained again. Protect the solution from atmospheric carbon dioxide throughout the titration. The sodium methylate solution is standardised by the volume of the titrant used in the second titration. Standardise immediately before use.

1 mL of *0.1 M sodium methylate solution* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Sodium nitrite.

Dissolve 7.5 g of *sodium nitrite R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.150 g of *sulfanilic acid RV* in 50 mL of *dilute hydrochloric acid R* and determine the primary aromatic amino group *(2.1.5.8)* electrometrically using a prepared sodium nitrite solution. Standardise immediately before use.

1 mL of *0.1 M sodium nitrite solution* is equivalent to 17.32 mg of C_6H_7XO3S.

0.1 M Sodium periodate.

Dissolve 21.4 g of *sodium periodate R* in about 500 mL of *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. In a stoppered flask, introduce 5.0 mL of the prepared sodium periodate solution and add 100 mL of *water R*, 10 mL of *potassium iodide R*, and 5 mL of *hydrochloric acid R1*, close the flask, mix, allow to stand for 2 min, and titrate with *0.1 M sodium thiosulfate* until a slightly yellow colour is obtained. Carry out the titration potentiometrically *(*2.1.2.19*)* or add 2 mL of *starch solution R* and titrate slowly until the colour is completely discharged.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.674 mg $NaIO_4$ or 0.125 mL of *0.1 M sodium periodate*.

0.1 M Sodium thiosulfate.

Dissolve 25 g of *sodium thiosulfate R* and 0.2 g of *sodium carbonate R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of *0.033 M potassium bromate*, add 40 mL of *water R*, 10 mL of *potassium iodide solution R* and 5 mL of *hydrochloric acid R1* and titrate with the sodium thiosulfate solution, using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.783 mg $KBrO_3$ or 0.5 mL of *0.033 M potassium bromate.*

0.1 M Sodium edetate.

Dissolve 37.5 g of *sodium edetate R* in 500 mL of *water R*, add 100 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.120 g of *zinc RV* in 4 mL of *hydrochloric acid R1*, add *diluted sodium hydroxide solution R* until the solution is weakly acid and carry out the quantitation of zinc by complexometry (*2.1.5.11*).

1 mL of 0.1 M sodium edetate solution is equivalent to 6.538 mg of Zn.

Store in a polyethylene container.

0.02 M Sodium edetate.

Dissolve 7.444 g of *sodium edetate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.100 g of *zinc RV* in 4 mL of *hydrochloric acid R1* and add 0.1 mL of *bromine water R*; drive off the excess of bromine by boiling. Transfer the solution to a volumetric flask and dilute to 100.0 mL with *water R*, transfer 25.0 mL of the solution to a 500 mL conical flask and dilute to 200 mL with *water R.* Add about 50 mg of *xylenol orange indicator R* and *hexamethylenetetramine R* until the solution becomes violet-pink. Add 2 g of *hexamethylenetetramine R* in excess. Titrate with the sodium edetate solution until the violet-pink colour changes to yellow.

1 mL of *0.02 M sodium edetate solution* is equivalent to 1.308 mg of Zn.

0.1 M Silver nitrate.

Dissolve 17.0 g of *silver nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 50 mg of *sodium chloride RV* in *water R*, add 5 mL of *dilute nitric acid R*, dilute to 50 mL with *water R*, and titrate with the prepared silver nitrate solution potentiometrically (2.1.2.19).

1 mL of *0.1 M silver nitrate solution* is equivalent to 5.844 mg of NaCl.

Store in a place protected from light.

0.001 M Silver nitrate.

Dilute 5.0 mL of *0.1 M silver nitrate* to 500.0 mL with *water R*.

0.5 M Sulfuric acid.

Mix 28 mL of *sulfuric acid R* with *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R* and titrate with a prepared sulfuric acid solution potentiometrically (2.1.2.19), or using 0.1 mL of a solution of *methyl orange R* as indicator until a reddish-yellow colour appears.

1 mL of *0.5 M sulfuric acid* is equivalent to 121.1 mg of C4H11NO3.

0.05 M Sulfuric acid.

Dilute 100.0 mL of *0.5 M sulfuric acid* to 1000.0 mL with *water R*.

Standardisation. Carry out the titration described for *0.5 M sulfuric acid*, using 0.100 g of *sodium carbonate RV*, dissolved in 20 mL of *water R.*

1 mL of *0.05 M sulfuric acid solution* is equivalent to 5.30 mg of Na_2CO_3.

0.1 M Lead nitrate.

Dissolve 33 g of *lead nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Take 20.0 mL of the prepared lead nitrate solution and carry out the determination of lead by complexometry (*2.1.5.11*).

0.05 M Lead nitrate.

Dilute 50.0 mL of *0.1 M lead nitrate* to 100.0 mL with *water R.*

0.1 M Tetrabutylammonium hydroxide.

Dissolve 40 g of *tetrabutylammonium iodide R* in 90 mL of *anhydrous methanol R*, add 20 g of finely powdered *silver oxide R* and shake vigorously for 1 h. Centrifuge a few millilitres of the mixture and test the supernatant for iodides.

If a positive reaction is obtained, add an additional 2 g of *silver oxide R* and shake for a further 30 min. Repeat this procedure until the liquid is free from iodides, filter the mixture through a fine sintered-glass filter (*2.1.1.2*) and rinse the reaction vessel and filter with three quantities, each of 50 mL, of *toluene R.* Add the washings to the filtrate and dilute to 1000.0 mL with *toluene R*. Pass dry carbon dioxide-free nitrogen through the solution for 5 min.

Standardisation. To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the tetrabutylammonium hydroxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*, stir to effect solution, and titrate with the tetrabutylammonium hydroxide solution until the pure blue colour is again obtained. From the volume of titrant used in the second titration ascertain the exact strength of the tetrabutylammonium hydroxide solution. Protect the solution from atmospheric carbon dioxide throughout the titration. Standardise immediately before use.

1 mL of *0.1 M tetrabutylammonium hydroxide solution* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M tetrabutylammonium hydroxide solution in 2-propanol.

Prepare according to the instructions described for *0.1 M tetrabutylammonium hydroxide solution*, using *2-propanol R* instead of *toluene R* as the solvent; standardise as described given for *0.1 M tetrabutylammonium hydroxide solution*.

1 M Hydrochloric acid.

Dilute 103.0 g of *hydrochloric acid R* to 1000.0 mL with *water R.*

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R* and titrate with prepared hydrochloric acid potentiometrically (2.1.2.19*)* or using 0.1 mL of *methyl orange solution R* as indicator until the solution becomes yellowish-red.

1 mL of *1 M hydrochloric acid* is equivalent to 121.1 mg of C4H11NO3.

0.1 M Hydrochloric acid.

Dilute 100.0 mL of *1 M hydrochloric acid* to 1000.0 mL with *carbon dioxide-free water R*.

Standardisation. Carry out the titration in accordance with the instructions given for *1 M hydrochloric acid* using 95 mg of *trometamol RV* dissolved in 20 mL of *water R*.

1 mL of *0.1 M hydrochloric acid* is equivalent to 12.11 mg of $C_4H_{11}NO_3$.

0.1 M Hydrochloric acid, alcoholic.

Dilute 9.0 mL of *hydrochloric acid R* to 1000.0 mL with *aldehyde-free alcohol R*.

0.1 M Perchloric acid.

Place 8.5 mL of *perchloric acid R* in a volumetric flask containing about 900 mL of *glacial acetic acid R* and mix. Add 30 mL of *acetic anhydride R*, dilute to 1000.0 mL with *glacial acetic acid R*, mix and allow to stand for 24 h. Determine the water content (2.1.5.12) without the addition of methanol and, if necessary, adjust the water content to 0.1-0.2% by adding either *acetic anhydride R* or *water R*. Allow to stand for 24 h

Standardisation. Dissolve 0.350 g of *potassium hydrogen phthalate RV* in 50 mL of *anhydrous acetic acid R*, warming gently if necessary. Allow to cool protected from the air, and titrate with the prepared perchloric acid solution potentiometrically (2.1.2.19), using 0.05 mL of *crystal violet solution R* as indicator. Note the temperature of the perchloric acid solution at the time of the titration. If the temperature at which a quantitation is carried out is different from that at which the *0.1 M perchloric acid* has been standardised, the volume *(V_c)* used in the assay is calculated as follows:

$$V_c = V\,[1 + (t_1 - t_2) \cdot 0.0011],$$

where t_1 is the temperature during standardisation;

t_2 is the temperature during the quantitation;

V is the volume actually used for titration, in millilitres.

1 mL of *0.1 M perchloric acid solution* is equivalent to 20.42 mg of $C_8H_5KO_4$.

0.05 M Perchloric acid.

Dilute 50.0 mL of *0.1 M perchloric acid* to 100.0 mL with *anhydrous acetic acid R*.

0.02 M Perchloric acid.

Dilute 20.0 mL of *0.1 M perchloric acid* to 100.0 mL with *anhydrous acetic acid R*.

0.1 M Acetic acid.

Dilute 6.0 g of *glacial acetic acid R* to 1000.0 mL with *water R*.

Standardisation. To 25.0 mL of the prepared acetic acid solution, add 0.5 mL of *phenolphthalein solution R* and titrate with *0.1 M sodium hydroxide*.

0.1 M Cerium sulfate.

Dissolve 40.4 g of *cerium sulfate R* in a mixture of 500 mL of *water R* and 50 mL of *sulfuric acid R*; allow to cool and dilute to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a 49 g/L solution of *sulfuric acid R*, titrate with the prepared cerium sulfate solution potentiometrically (2.1.2.19), or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M cerium sulfate* is equivalent to 38.21 mg of Fe(C2H10N2)(SO4)2·4H2O.

0.05 M Zinc chloride.

Dissolve 6.82 g of *zinc chloride R*, weighed with appropriate precautions, in *water R*. If necessary, add dropwise *dilute hydrochloric acid R* until the opalescence disappears and dilute to 1000.0 mL with *water R*.

Standardisation. To 20.0 mL of the zinc chloride solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.1.5.11*).

0.1 M Zinc sulfate.

Dissolve 29 g of *zinc sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the zinc sulfate solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.1.5.11*).

2.3. GENERAL TEXTS

2.3.1. GENERAL TEXTS ON MICROBIOLOGY

203010001-2019

2.3.1.1. Efficacy of antimicrobial preservation

This chapter establishes general requirements for evaluating the efficacy of antimicrobial preservatives in drug preparations.

Antimicrobial preservatives are intended to prevent the proliferation of microorganisms or limit microbial contamination of the drug product during storage and use, especially in the case of multi-dose containers. Antimicrobial preservatives must not be used as a substitute for good manufacturing practice (GMP) requirements. The effective concentration of a preservative in the finished drug product should be lower than a dose toxic to humans.

The efficacy of antimicrobial preservatives is the ability of a substance to inhibit the growth of microorganisms throughout the shelf life of a drug product.

Test for efficacy of preservatives is a procedure that consists in artificial contamination of a drug preparation with suspensions of certain test microorganisms, incubation of contaminated samples at a certain temperature, sampling at specified time intervals, and counting viable microbial cells in 1 g (mL) of the preparation during the test period, calculating and evaluating the results obtained.

It is unacceptable to add preservatives to a preparation for intracavitary, intracardiac, and intraocular injections in contact with the cerebrospinal fluid, as well as for a single dose exceeding 15 mL.

1. TEST STRAINS OF MICROORGANISMS: USING AND HANDLING

The efficacy of preservatives for preparations is determined against certain types of bacteria, yeast and moulds. The following test strains are used for the test (Table 2.3.1.1.-1).

Table 2.3.1.1.-1. – *Test strains of microorganisms*

Microorganism	Strain number
Escherichia coli	GCPM 240533; ATCC 25922, ATCC 8739; NCTC 12923; NCTC 12241; DSM 1103; CIP 53.126, NCIMB 8545
Pseudomonas aeruginosa	GCPM 190155; ATCC 9027; NCTC 12924; CIP 82.118, NCIMB 8626
Staphylococcus aureus	GCPM 201108; ATCC 6538; NCTC 10788, NCIMB 9518, CIP 4.83
Candida albicans	GKPM 303903; GKPM 303901; PKNRY401/NCTC 885-653; ATSS 10231; NCPF3179, IP 48.72
Aspergillus brasiliensis	RKPGB 106; ATSS 9642, ATSS 16404, VCM F-1119; VCM F-3882; NCPF 2275, IMI 149007, IP1431.83

Notes.

1. In addition to the indicated test strains, other microorganisms can be used, which should be typical in terms of cultural, morphological, tinctorial, and biochemical properties.

2. The set of test strains of microorganisms can be reduced or increased depending on the method of application, composition, or possible microbial contaminants of the test drug preparation. For example, *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92) can be used to test oral preparations containing high concentrations of sugar; for preparations containing benzalkonium chloride, it is advisable to include *Burkholderia cepacia* (ATCC 25416, ATCC 177759), etc.

All test strains of microorganisms obtained from official collections with a manufacturer's certificate in ampoules, on discs or in any other form should be restored by the methods described in the instructions attached to the test strains or in accordance with monograph 2.1.6.6.

Cultures of bacteria and fungi are re-inoculated making no greater than 5 passages. The conditions for cultivating test strains for inoculate preparation are presented in Table 2.3.1.1.-2. The culture media used are described in monograph 2.1.6.6.

Note. The use of alternative liquid and agar culture media is allowed.

Control of growth properties of the employed culture media is carried out as specified in monograph 2.1.6.6.

When preparing the inoculate, daily cultures of test strains of bacteria and *C. albicans* are washed off the surface of the mown agar with a sterile 0.9% sodium chloride solution. Adjust the concentration of bacterial cells to 10^9 CFU/mL, and *C. albicans* to 10^7 CFU/mL, using a reference standard of opalescence or a variety of instrumental techniques, including turbidimetric method.

In the case of using fluid culture media for the cultivation of test strains, separate bacteria and *C. albicans* cells by centrifugation, wash and re-suspend with a sterile 0.9% sodium chloride solution to a concentration of 1 x 10^7— 1 x 10^8 CFU/mL.

Use a sterile 0.9% sodium chloride solution containing 0.05% polysorbate 80 to wash off *A. brasiliensis* conidia. Determine the number of *A. brasiliensis* conidia in 1 mL of the washings using a Goryaev chamber or a dish agar method. Dilute the resulting suspension to a concentration of 10^7 conidia in 1 mL.

Dilute standardised suspensions of all test microorganisms to a concentration of 10^7 - 10^8 CFU/mL.

Select a suitable sample from each suspension and count the number of colony-forming units in 1 mL of each suspension by inoculating on dishes or membrane filtration (2.1.6.6). The obtained value is used to determine the number of viable microorganisms in the inoculum and the baseline to use in the test. Use the inoculate immediately after preparation.

2. TEST PROCEDURE

To determine the efficacy of preservatives, use final drug products in intact containers (primary packaging).

In each sterile vial with the product to be examined, introduce a suspension containing one of the test strains of microorganisms, providing a concentration of 10^5-10^6 cells per millilitre or gram of the preparation, and mix. The volume of the inoculate should be 0.5-1% of the sample volume. Mix thoroughly to ensure a uniform distribution of microorganisms in the sample.

Heat the samples of a solid, ointment-based preparation to a temperature of 42.5 ± 2.5 °C. Mix the inoculate of each standardised microbial suspension with the drug preparation for not less than 1 min until a homogeneous emulsion is achieved. To improve mixing, a certain (validated) amount of a sterile surfactant, such as polysorbate 80, may be added unless it affects the viability of microorganisms or the preservative efficacy.

Keep contaminated samples of the preparation at a temperature of 22.5 ± 2.5 °C in a dark place for a certain time.

Table 2.3.1.1.-2. – *Conditions of cultivation for test microorganisms for inoculum preparation*

Test strain	Culture medium	Conditions of incubation	
		temperature	time
Escherichia coli	Soya-bean casein agar or medium No. 1	32.5 ± 2.5 °C	18-24 h
Pseudomonas aeruginosa *Staphylococcus aureus*	Soya-bean casein broth or medium No. 8		
Candida albicans	Sabouraud glucose agar or medium No. 2 Sabouraud liquid medium or soya-bean casein broth	22.5 ± 2.5 °C	48 - 72 h
Aspergillus brasiliensis	Sabouraud glucose agar or medium No. 2	22.5 ± 2.5 °C	6-10 days

Samples are taken from each test specimen (usually 1 mL or 1 g) immediately after inoculation and at specified time intervals (Tables 2.3.1.1.-3 – 2.3.1.1.-5), the number of viable microorganisms is determined by inoculating on dishes or by membrane filtration (2.1.6.6).

Table 2.3.1.1.-3. – *Sterile drug preparations (parenteral preparations, eye preparations, intrauterine preparations, and intramammary preparations)*

		Log reduction				
		6 h	24 h	7 days	14 days	28 days
Bacteria	A	2	3	—	—	NR
	B	—	1	3	—	NI
	C	—	—	1	3	NI
Fungi	A	—	—	2	—	NI
	B	—		—	1	NI
	C	—	—	NI	NI	NI

Symbols: NR – no recovery; NI – no increase in the number of microorganisms compared to the previous result.

Table 2.3.1.1.-4 – *Non-sterile drug preparations used topically, as well as preparations for cutaneous application and preparations for inhalation*

		Log reduction			
		2 days	7 days	14 days	28 days
Bacteria	A	2	3	—	NI
	B	—	—	3	NI
	C	—	—	2	NI
Fungi	A	—	—	2	NI
	B	—	—	1	NI
	C	—	—	NI	NI

Table 2.3.1.1.-5. – *Oral drug preparations, including antacids, water-soluble or water-based preparations, as well as rectal preparations*

		Log reduction	
		14 days	28 days
Bacteria	A	3	NI
	B	1	NI
Fungi	A	1	NI
	B	—	NI

The antimicrobial activity of the product must be eliminated using one of the following methods: by dilution, by membrane filtration, or by the use of a specific inactivator, which is added to the dishes with the culture medium or to the corresponding dilution of the drug before the inoculation. The inactivators used should not affect the viability of microorganisms. Some of the inactivators are described in monograph 2.1.6.6.

3. ACCEPTANCE CRITERIA

The quantity of CFU/mL for each test strain is determined by the dish agar method after the above-mentioned incubation periods of the contaminated sample of the drug product. The change in the number of microbial cells compared to the initial concentration in 1 mL is expressed in decimal logarithms (log). When evaluating the efficacy of antimicrobial activity of preservatives, an increase in CFU/mL is not recorded if the subsequent measurement exceeds the previous one by less than 0.5 IgCFU.

4. QUALITY REQUIREMENTS

Tables 2.3.1.1.-3–2.3.1.1.-5 present criteria for evaluation of antimicrobial activity of preservative(s) in terms of the log reduction in the number of viable microorganisms against the value obtained for the inoculum. The A criteria correspond to the recommended efficiency of the preservative. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B or C criteria must be satisfied.

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2.3.1.2. Requirements for microbiological purity of drug products, pharmaceutical substances, and excipients for their production

This chapter defines the acceptance criteria for the microbiological purity of drug products, pharmaceutical substances, and excipients for their production.

Table 2.3.1.2.-1. – *Acceptance criteria for microbiological quality of drug preparations*

Category	Route of administration	Acceptance criteria
1	Drug preparations that are subject to the "Sterility" requirement	Preparations must be sterile
2	- Topical (on the oral mucosa, nasal, introduction to the external auditory canal, vaginally, etc.) - For external use (on undamaged and (or) damaged skin) - For respiratory use (for inhalation) - Transdermal patches Except for those preparations that must be sterile	- Total aerobic microbial count (TAMC) – maximum 10^2 CFU/g of CFU/mL of the preparation or 1 patch (including the adhesive side and base) - Total yeast and mould count (TYMC) – maximum 10^1 CFU/g of CFU/mL or on 1 patch (including the adhesive side and base) - Absence of *Pseudomonas aeruginosa* in 1 g (mL) of the preparation or on 1 patch (including the adhesive side and base) - Absence of *Staphylococcus aureus* in 1 g (mL) of the drug or on 1 patch (including the adhesive side and base) - Absence of bile-tolerant enterobacteria in 1 g (mL) of aerosol preparations used for respiratory purposes - Absence of *Candida albicans* in 1 g (mL) of vaginal preparations
3A	Oral or rectal preparations	
	Solid (non-aqueous) oral preparations	- TAMC – not greater than 10^3CFU in 1 g - TYMC – not greater than 10^2 CFU in 1 g - Absence of *Escherichia coli* in 1 g
	Rectal preparations	- TAMC – not greater than 10^3 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL)
	Liquid oral preparations	- TAMC – not greater than 10^2 CFU in 1 g (mL) - TYMC – not greater than 10^1 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL)
3B	For oral administration – from raw materials of natural origin, the level of microbial contamination of which cannot be reduced during pre-treatment. Required acceptance criteria for microbiological quality of herbal medicinal products are given in general chapter 5.1.8	- TAMC – not greater than 10**4** CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Bile-tolerant bacteria – NMT 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of bacteria of the genus *Salmonella spp.* in 10 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL)
3C	Provision for premixes for medicated feeding stuffs for veterinary use using excipients of plant origin for which antimicrobial treatment is not feasible	- TAMC – not greater than 10^5 CFU in 1 g (mL) - TYMC – not greater than 10**4** CFU in 1 g (mL) - Bile-tolerant bacteria – NMT 10**4** CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of *Salmonella spp.* bacteria in 25 g (mL)

Table 2.3.1.2.-2. – *Acceptance criteria for the quality of pharmaceutical substances and excipients for the manufacture of drug products in terms of microbiological purity*

Category	Pharmaceutical substances, excipients	Acceptance criteria
1.2.A.	Pharmaceutical substances for the manufacture of non-sterile drug preparations	Substances must be sterile
1.2.B.	Pharmaceutical substances for the production of: - sterile drug products that are sterilised in their containers; - sterile drug products, in the production/manufacture of which sterilising filtration is used to ensure sterility; - non-sterile drug products belonging to category 2	- TAMC – not greater than 10^2 CFU in 1 g (mL) - TYMC – not greater than 10^1 CFU in 1 g (mL) - Absence of bile-tolerant enterobacteria, in 1 g (mL) - Absence of *Pseudomonas aeruginosa* in 1 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL)
2.2	Pharmaceutical substances of synthetic origin for the production of non-sterile drug products	- TAMC – not greater than 10^3 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL)
3.2	Pharmaceutical substances of natural origin for the production of non-sterile drug products. Recommended acceptance criteria for microbiological quality of herbal medicinal products/pharmaceutical substances of plant origin are given in general chapter 5.1.8.	- TAMC – not greater than 10^4 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of *Salmonella spp.* in 25 g (mL) - Absence of *Pseudomonas aeruginosa* in 1 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL) - Bile-tolerant bacteria – NMT 10^2 CFU in 1 g (mL)
4.2	Excipients of natural origin (wheat flour, starch, talc, etc.)	- TAMC – not greater than 10^3 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of *Salmonella spp.* in 25 g (mL) - Absence of *Pseudomonas aeruginosa* in 1 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL) - Bile-tolerant bacteria – NMT 10^2 CFU in 1 g (mL)

Notes to Tables 2.3.1.2.-1.–2.3.1.2.-2.

1. If any pathogenic bacteria are detected during the test, other than those indicated above, it is considered that the quality of drug preparations, pharmaceutical substances, and excipients does not meet the requirements for "Microbiological purity".

2. Other standards may be specified as an exception in the normative quality document, depending on the composition of drugs and the features of the manufacturing process. The implementation of other rules requires justification.

3. Much stricter requirements may be provided for drug products used in children, for example:

- in 1 g (mL) of preparations for children (0 to1 year) – not greater than 50 aerobic bacteria and yeast and mould (in total) in the absence of bile-tolerant enterobacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

- in 1 g (mL) of preparations for children (older than 1 year) – no greater than 500 aerobic microorganisms and 50 yeast and mould (in total) in the absence of bile-tolerant enterobacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

4. Category 1.2.B includes thermolabile pharmaceutical substances that are used in the production of sterile drug products that are not sterilised in the final packaging. Such pharmaceutical substances cannot be subjected to thermal sterilisation due to their structure and physical and chemical properties. In this case, during the production of a drug product, it is allowed to use a sterilising filtration of a solution of a pharmaceutical substance or a solution of a pharmaceutical substance with excipients.

Non-sterile drug products may be contaminated with microorganisms. A limited number of bacteria and fungi are allowed in the absence of certain species that pose a danger to human health. The presence of microorganisms in non-sterile drug products may have an adverse effect on the patient's health and lead to a decrease in the therapeutic effectiveness of the drug. Therefore, manufacturers must ensure an appropriate level of microbiological purity of drugs by following the current guidelines for good manufacturing practice, storage and distribution of drug preparations (GxP).

Microbiological examination of non-sterile drug preparations is carried out in accordance with the methods given in chapters 2.1.6.6, 2.1.6.7.

Tables 2.3.1.2.-1 – 2.3.1.2. -2 show the quality requirements for drug preparations and pharmaceutical substances, as well as excipients.

For non-sterile drug preparations, the acceptance criteria based on the calculation of the total aerobic microbial count (TAMC) and the total yeast and mould count (TYMC) are given in Tables 2.3.1.2.-1 and 2.3.1.2.-2. Acceptance criteria are set based on individual results or on the average of all counts when using replication (for example, direct inoculation). The results are interpreted as follows:

- at 10^1 CFU – NMT 20 CFU/g (mL)
- at 10^2 CFU – NMT 200 CFU/g (mL)
- at 10^3 CFU – NMT 2000 CFU/g (mL), etc.

When establishing regulatory requirements for the quality of individual drugs and drafting a quality regulatory document, both digital category designations and indications of quality requirements in accordance with the method of application can be used.

For pharmaceutical substances used in the production of drug products (with the exception of herbal preparations), the total aerobic microbial count should not exceed 10^3 CFU in 1 g (mL), and the total yeast and mould count – NMT 10^2 CFU in 1 g (mL).

Based on the risk analysis, other quality acceptance criteria for pharmaceutical substances and excipients may be specified in the regulatory documentation (Table 2.3.1.2.-2). This takes into account the specifics of the manufacturing process and the purpose of the drug, which uses a pharmaceutical substance, as well as the availability of excipients of the required quality.

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2.3.1.3. Viral safety

This chapter contains general requirements concerning the viral safety of medicinal products whose manufacture has involved the use of materials of human or animal origin.

Medicinal products may be subjected to viral contamination during their manufacture. The risk of viral contamination exists for all medicinal products, the production of which involves raw materials and source materials of animal or human origin.

The main reasons for contamination include the use of infected materials (raw materials, cell cultures) and accidental introduction of a virus during the manufacturing process.

The risk of viral contamination exists for medicinal products manufactured:

- from the blood, urine, and other biological liquids of human or animal origin;
- from human or animal organs and tissues;
- using the *in vivo* culture method;
- by *in vitro* cultivation of human or animal cell lines.

The chapter does not apply to non-traditional transmissible agents, such as causative agents of transmissible spongiform encephalopathy in cattle and scrapie (prurigo) in sheep and goats.

The requirements for ensuring viral safety of medicinal products obtained from materials of human or animal origin are established by the competent authority in accordance with the requirements of the current regulatory legal acts of the Member States of the Union.

RISKS OF VIRAL CONTAMINATION

The main causes of viral contamination of medicinal products include:

- the use of a source material obtained from an infected person or animal;
- foreign viruses introduced during the manufacturing process;
- the use of contaminated reagents or products of animal origin in the manufacture of medicinal products;
- infected donor cells and cell lines contaminated with viruses prior to their use as MCB and WCB;
- a contaminating virus introduced when creating a production cell line under inappropriate conditions.

To ensure the viral safety of medicinal products during the manufacturing process, the following measures should be taken:

1. Selection of raw materials and source materials and testing for viral contaminants that are pathogenic for humans;
2. Assessing and testing the capacity of the production process to remove and/or inactivate viruses;
3. Testing for viral contamination at appropriate and critical stages of production.

However, it should be borne in mind that none of these measures can fully guarantee the absence of viruses. Therefore, it is necessary to use a more comprehensive approach. Measures taken to manage the risk of viral contamination of medicinal products manufactured using raw materials of animal or human origin should be aimed at minimising the risk rather than eliminating it. Any residual risk should be assessed in relation to the possible benefits of using a particular material or raw material in the production of medicinal products.

REQUIREMENTS FOR RAW MATERIALS

To minimise the risk of viral contamination, the following conditions must be met when selecting raw materials:

1. Raw materials of human origin (blood, urine, or other human biological liquids) are collected from healthy donors. Donors of blood and blood plasma, urine, or other biological liquids must be examined in accordance with the legal documents in force in the territory of the Member States of the Union.
2. Raw materials of animal origin should be selected only from animals from farms that are safe in terms of infectious diseases. Raw materials must be subject to mandatory veterinary and sanitary examination in accordance with the requirements of regulatory legal acts in force in the territories of the Member States of the Union and be accompanied by appropriate supporting documents.
3. The genus and source of origin of animals intended for the production of biotechnological medicinal products should be determined, including the genotype and age. Animals must be taken from closed-type farms free from infectious diseases. The status of the farm must be confirmed by appropriate documents.

4. Materials and reagents of biological origin (such as sheep red blood cells, fetal calf serum, bovine serum albumin, human transferrin, insulin, trypsin, etc.), culture media used in the manufacture of medicinal products must be free from viral contamination and have the required quality.

DETECTION OF VIRAL CONTAMINATION

Testing of raw materials of human or animal origin is a mandatory requirement for minimising the risk of viral contamination. For example, human blood plasma is subject to mandatory testing for the absence of surface antigen of the hepatitis B virus (HBsAg), antibodies to the hepatitis C virus, antigen p24HIV-1, antibodies to HIV-1, HIV-2. If there is a high probability of viral contamination of a raw material or cell substrate, specific tests and/or techniques may be required to detect viruses. The testing procedure and the scope of tests for viral contamination and inactivation of viruses at the "critical" stages in the production of drug products depend on various factors that need to be taken into account individually. If the raw materials used (organs, tissues, biological liquids) or cell lines are obtained from anthropoid apes or monkeys, they must be additionally checked for the presence of human viruses, primarily for viruses that cause immunodeficiency and hepatitis, unless a different method is justified in the quality regulatory document. Molecular genetics methods are also used to detect viral contamination.

Special attention should be paid to viruses that often contaminate the animal species from which the cell line is derived. It has to be considered certain cell lines contain endogenous viruses, such as retroviruses, which are difficult or even impossible to neutralise. Moreover, potential viral contamination can lead to the formation of both complete viral genomes and sub-genomic viral fragments, leading to the reproduction of infectious viral particles. The possible mutation of endogenous viruses during prolonged cultivation must also be considered.

The presence of nucleotide sequences of viral genomes does not preclude the use of the cells in which they are found, but any identified viral nucleic acid must be identified. When creating a cell line that secretes monoclonal antibodies, the cell bank should be monitored for the presence of not only human viruses but also viruses from mice and other rodents.

A cell line that produces any viruses capable of infecting human cells can only be used in exceptional circumstances. All products obtained by using such cell lines should be considered individually in each particular case. If any cell line secretes infectious viruses, appropriate precautions should be taken to protect the operating personnel from infection.

Special attention should be paid to the use of cell lines transformed by the Epstein-Barr virus in the production of monoclonal antibodies. Human B-lymphocytes transformed by this virus do not secrete viral particles but contain a complex of copies of the viral genome and its nucleotide sequences, which can be determined by polymerase chain reaction (PCR) or by co-cultivation with the corresponding indicator cell line.

Appropriate control tests should be performed to detect viruses in the materials and reagents used (for example, trypsin obtained from pigs is tested for the presence of swine parvovirus). It is also necessary to monitor the blood serum of cattle. It should not contain potentially dangerous viruses for humans — such as bovine diarrhea virus, infectious bovine rhinotracheitis, and parainfluenza 3).

It should not be overlooked that all types of tests have limited sensitivity; for example, the ability to detect low virus concentrations in the test depends on the size of the test sample. Therefore, none of these approaches can accurately determine the viral safety of a drug preparation.

VIRUS INACTIVATION OR ELIMINATION PROCESSES

If necessary, the elimination and/or inactivation of viruses present in a medicinal product is carried out by subjecting raw materials or excipients to the following treatment methods:

- physical (sterilisation, steam treatment, dry heating, radiation, filtration); (sterilisation with saturated water vapour under pressure, hot air, filtration, ionising radiation);

- chemical (using detergents to destruct supercapsids of viruses containing lipids);

- combined methods (neutralisation with specific antibodies, virus elimination by chromatographic methods, heating in the form of a suspension with chemical agents, etc.).

Any of the treatment methods used should be validated and should provide a significant reduction in the risk of viral contamination of medicinal products during their production.

VALIDATION OF VIRAL INACTIVATION OR ELIMINATION PROCESSES

Testing can detect one or more types of viruses, but no single test can confirm the presence of all known viruses. Moreover, in order to obtain a positive result, any analytical system requires certain minimal viral contamination. Tests are also limited by statistical errors in sampling and examination of samples. In this regard, confirmation of the absence of viruses in a biological medicinal product in many cases occurs not only through direct testing for their presence but also by confirming that the production process is able to eliminate or inactivate them.

Validation of virus elimination processes is one of the most important factors that ensure the safety of products that are based on a potentially infected material, such as blood plasma.

Due to the fact that there are known cases of drug contamination with viral agents that were not known at the time of production, it is of particular importance to assess the effectiveness of elimination processes.

If the stock material or raw material is insufficiently characterised, such as human or animal blood, tissues, and organs, or if the cells were cultivated *in vivo*, the possibility of viral contamination increases. Therefore, the manufacturing process must generally include one or more effective steps for the inactivation and/or elimination of viruses. In many cases, the absence of viruses in the final product is confirmed not only by their direct detection using various validated procedures but also by the ability of the applied cleaning procedure to eliminate and/or inactivate viruses. The type and scope of virus tests and the determination of virus elimination required at different stages of the drug production process depend on various factors and should be considered for each specific case and consistently. The following factors must be taken into account: the nature of viruses; the properties of the Bank of cells and their characteristics; the components of the culture medium; cultivation techniques; layout of production premises and specification of equipment; test results on the presence/absence of viruses after cell culture process; the manufacturing process of the product and its capacity to remove and/or inactivate viruses; product type and its estimated clinical use.

Virus purification methods and techniques for controlling the degree of purification during production process, including requirements for controlled indicators, should be described in detail, justified, and validated. Make sure that the purification methods used do not adversely affect the properties of the medicinal product. When employing purification methods that include affinity chromatography using monoclonal antibodies, measures must be taken to ensure that these and other materials used in production that are potential contaminants do not impair the quality and safety of the final product.

Criteria for recycling any intermediate or final semi-finished product must be accurately established, validated, and justified.

To prevent viruses from entering finished dosage forms, a provision is made to introduce into the production processes several stages of inactivation and/or removal of viruses, for which a decrease in the concentration of model viruses has been proven. The inclusion of methods for inactivation/removal of potential viral contaminants should not reduce the biological activity of the medicinal product.

TESTS FOR THE ABSENCE OF CONTAMINATION BY INFECTIOUS VIRUSES AT THE PRODUCTION STAGES

Tests should be carried out using methods characterised by specificity and analytical sensitivity.

The absence of markers of hepatitis B and C viruses, HIV-1 and HIV-2 should be confirmed using validated methods for medicinal products obtained from blood, blood plasma, urine, organs, and tissues.

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2.3.1.4. Requirements for microbiological purity of pharmaceutical substances of plant origin, herbal medicinal products and extracts used in their preparation

This general chapter presents recommended acceptance criteria for the microbiological purity of herbal preparations/pharmaceutical substances and herbal medicinal products.

During the microbiological analysis of herbal preparations/pharmaceutical substances and herbal medicinal products, the quantitation of aerobic microorganisms, yeast and mould, as well as the isolation of certain types of pathogenic bacteria is carried out. The analysis is conducted in accordance with chapters 2.1.6.6, 2.1.6.7 and 2.1.6.9. Acceptance criteria for microbiological purity of herbal preparations/pharmaceutical substances and herbal medicinal products are presented in Table 2.3.1.4.-1.

When establishing regulatory requirements for the quality of individual drugs and drafting a quality regulatory document, both digital category designations and indications of quality requirements in accordance with the method of application can be used.

If necessary, other acceptance criteria may be established for microbiological purity of herbal preparations, which must be justified and proven during the validation.

Since herbal preparations/pharmaceutical substances and herbal medicinal products are heterogeneous in terms of the number of aerobic bacteria and fungi, the results of the microbiological examination are interpreted as follows:

- at 10^5 CFU – NMT $5 \cdot 10^5$
- at 10^7 CFU – $5 \cdot 10^7$, etc.

Table 2.3.1.4.-1. – *Acceptance criteria for herbal preparations/pharmaceutical substances and herbal medicinal products based thereon*

Category	Pharmaceutical substances of vegetal origin and herbal drug preparations present in the dosage form	Acceptance criteria
4.A	Pharmaceutical substances and herbal medicinal products containing herbal drugs intended for the preparation of infusions and decoctions using boiling water	- TAMC – not greater than 10^7 CFU per 1 g - TYMC – not less than 10^5 CFU in 1 g - *Escherichia coli* – NMT 10^3 CFU in 1 g - Absence of *Salmonella spp.* in 25 g
4.B	Herbal drug preparations intended for obtaining dosage forms without using boiling water	- TAMC – not greater than 10^5 CFU in 1 g - TYMC – not greater than 10^4 CFU in 1 g - Bile-tolerant bacteria – NMT 10^3 CFU in 1 g - Absence of *Escherichia coli* in 1 g - Absence of *Salmonella spp.* in 25 g
3.2	Pharmaceutical substances are substances (products) obtained after processing herbal raw materials using methods such as extraction, distillation, extraction, fractionation, purification, concentration, fermentation, etc.	- TAMC – not greater than 10^4 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of *Salmonella spp.* in 25 g (mL) - Absence of *Pseudomonas aeruginosa* in 1 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL) - Bile-tolerant bacteria – NMT 10^2 CFU in 1 g (mL)
3.B	Herbal medicinal products for oral use made from herbal raw materials, the level of microbial contamination of which cannot be reduced during pre-treatment	- TAMC – not greater than 10^4 CFU in 1 g (mL) - TYMC – not greater than 10^2 CFU in 1 g (mL) - Bile-tolerant bacteria – NMT 10^2 CFU in 1 g (mL) - Absence of *Escherichia coli* in 1 g (mL) - Absence of *Salmonella spp* in 10 g (mL) - Absence of *Staphylococcus aureus* in 1 g (mL)

2.3.2.0. Residual organic solvents

RESIDUAL SOLVENTS, LIMITING RESIDUAL SOLVENT LEVELS IN ACTIVE SUBSTANCES, EXCIPIENTS AND MEDICINAL PRODUCTS

This general monograph specifies permitted limits for the content of solvents that may remain in active pharmaceutical substances, excipients, and medicinal products after processing. These requirements apply to all active pharmaceutical substances, excipients, and drug products, regardless of whether or not they are the subject of the monograph of the Pharmacopoeia. All substances and medicinal products should be checked for possible content of residual organic solvents.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs since the solvents employed may vary from one manufacturer to another, and the requirements of this general chapter are applied via the general monograph on *Substances for Pharmaceutical Use*. Information about the solvents used in the production process must be submitted as part of the registration dossier to the competent authority.

Where only Class 3 solvents are used, a test for *loss on drying* may be applied or a specific determination of the particular solvent may be made. If for a Class 3 solvent a justified and authorised limit higher than 0.5 per cent is applied, a specific determination of the solvent is a mandatory requirement.

When Class 1 residual solvents or Class 2 residual solvents (or Class 3 residual solvents which exceed the 0.5%) are used, the procedure described in the general chapter (2.1.4.19). Identification and control of residual solvents. Otherwise, a suitable validated procedure should be used.

When a quantitation of a residual solvent is carried out, the result is taken into account for the calculation of the content of the substance except where a test for drying is carried out.

1. INTRODUCTION

The objective of this chapter is to recommend acceptable amounts of residual organic solvents in drug products for the safety of the patient. The chapter recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual organic solvents.

Residual organic solvents in drug products are defined in accordance with this chapter as volatile organic substances used or formed in the production of active pharmaceutical substances, excipients, or in the preparation of medicinal products. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of an active substance may enhance the yield or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. This chapter does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

Since there is no therapeutic benefit, all residual organic solvents should be removed to the extent possible to meet product specifications, good manufacturing practices (*GMP*), or other quality-based requirements. Drug products should contain no higher levels of residual solvents than can be supported by safety data.

Some solvents that are known to cause unacceptable toxicities (Class 1, Table 2.3.2.0.-3) should be avoided in the production of active substances, excipients, or drug products unless their use can be strongly justified in a "risk-benefit" assessment. Some solvents associated with less severe toxicity (Class 2, Table 2.3.2.0.-4) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, Table 2.3.2.0.-5) should be used where practical. The complete list of solvents included in this chapter is given in Appendix 1.

This list is not exhaustive and may be supplemented by other solvents used. The recommended acceptable standards for Class 1 and 2 residual organic solvents, as well as the classification of solvents, may change as new data on their safety become available. The rationale for safety data in a marketing application for a new medicinal product containing a new solvent not included in the list of solvents (Appendix 1) may be based on concepts of this chapter.

2. SCOPE

Residual organic solvents in active substances, excipients, and in medicinal products are within the scope of this chapter. Therefore, testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. It is only necessary to test for solvents that are used or produced in the manufacture or purification of active substances, excipients, or medicinal products. Although manufacturers may choose to test the medicinal product, a cumulative method may be used to calculate the residual solvent levels in the medicinal product from the levels in the ingredients used to produce the medicinal product. If the calculation results in a level equal to or below that recommended in this chapter, no testing of the medicinal product for residual organic solvents needs to be considered. If, however, the calculated level is above the recommended level, the medicinal product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. Medicinal product should also be tested if a solvent is used during its manufacture.

This general monograph does not apply to potentially new active substances, excipients, or medicinal products used during the clinical research stages of development, nor does it apply to existing marketed drug products.

The chapter applies to all dosage forms and routes of administration. Higher levels of residual organic solvents may be acceptable in certain cases such as short term (30 days or less) or topical application. Justification for these levels should be made on a case by case basis.

See Appendix 2 for additional background information related to residual organic solvents.

3. GENERAL PRINCIPLES

3.1. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

The term "tolerable daily intake" (TDI) is used by the *International Program on Chemical Safety* (IPCS) to describe exposure limits of toxic chemicals, and "acceptable daily intake" (ADI) is used by the World Health Organization (WHO) and other national and international health authorities and institutes.

The new term "permitted daily exposure" (PDE) is defined in the present monograph as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADI's of the same substance.

Residual organic solvents assessed in this chapter are listed in Appendix 1 by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

Class 1 solvents: Solvents to be avoided (highly toxic solvents)

These include known human carcinogens, strongly suspected human carcinogens, and environmental hazards.

Class 2 solvents: Solvents to be limited (non-genotoxic solvents)

These include non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity.

This class also includes solvents suspected of other significant but reversible toxicities.

Class 3 solvents: Solvents with low toxic potential (low toxic solvents)

These include solvents with low toxic potential to man; no health-based exposure limit is needed. Class 3 solvents have PDEs of 50 mg or more per day.

3.2. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The methods for establishing permitted daily exposures for residual organic solvents is presented in Appendix 3.

3.3. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS

Two options are available when setting limits for Class 2 solvents.

Option 1: The concentration limits in ppm stated in Table 2.3.2.0.-4 can be used, calculated using equation (1) below by assuming a product mass of 10 g administered daily.

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{dose}}, \qquad (1)$$

where *PDE* is given in terms of mg/day and *dose* is given in g/day

These limits of residual organic solvents are considered acceptable for all substances, excipients, or products. Therefore this option may be applied if the daily dose is not known or fixed. If the content of residual organic solvents in all excipients and active pharmaceutical substances in a formulation meet the limits given in Table 2.3.2.0.-4, then these components may be used in any proportion. No further calculation is necessary, provided the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day should be considered under Option 2.

Method 2. It is not considered necessary for each component of the medicinal product to comply with the limits given in Option 1. The PDE in terms of mg/day, as stated in Table 2.3.2.0.-4, can be used with the known maximum daily dose and the equation (1) to determine the concentration of residual solvent allowed in a medicinal product. Such limits are considered acceptable, provided that it has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, a reasonable variation in the manufacturing process, and the limits should meet contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the medicinal product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the use of Option 1 and Option 2 applied to acetonitrile in a medicinal product. PDE to acetonitrile is 4.1 mg per day.

Thus, the Option 1 limit is 410 ppm. The maximum administered daily mass of a medicinal product is 5.0 g. The drug contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in Table 2.3.2.0.-1.

Excipient 1 meets the Option 1 limit, but the drug substance, excipient 2, and medicinal product do not meet the Option 1 limit. Nevertheless, the content of acetonitrile in the medicinal product, established using Option 2, does not exceed the allowable rate of 4.1 mg per day and, therefore, complies with the recommendations of this monograph.

Consider another example for acetonitrile as a residual organic solvent. The maximum administered daily mass of a drug product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile is given in Table 2.3.2.0.-2.

In this case, the concentration of acetonitrile in the medicinal product does not meet the acceptable standard either using Option 1 or using Option 2.

The manufacturer could test the medicinal product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the product should take other steps to reduce the amount of acetonitrile in the medicinal product. If all of these steps fail to reduce the level of residual solvent, in exceptional cases the manufacturer could provide a summary of efforts made to reduce the solvent level to meet the guideline value, and provide a risk-benefit analysis to support allowing the product to be utilised containing residual solvent at a higher level.

3.4. ANALYTICAL PROCEDURES

Residual organic solvents are generally determined using chromatographic techniques, in particular, gas chromatography. Any suitable procedures described in the Pharmacopoeia can be used to determine the content of residual organic solvents. In other words, manufacturers should be free to choose the most appropriate validated analytical procedure for a particular application.

Table 2.3.2.0.-1 – *Composition of the medicinal product and the calculated maximum content of acetonitrile (Example 1)*

Component	Amount in formulation, g	Acetonitrile content, ppm	Daily exposure, mg
Active pharmaceutical ingredient	0.3	800	0.24
Excipient 1	0.9	400	0.36
Excipient 2	3.8	800	3.04
Medicinal product	5.0	728	3.64

Table 2.3.2.0.-2 – *Composition of the medicinal product and the estimated limit of acetonitrile (Example 2)*

Component	Amount in formulation, g	Acetonitrile content, ppm	Daily exposure, mg
Active pharmaceutical ingredient	0.3	800	0.24
Excipient 1	0.9	2000	1.80
Excipient 2	3.8	800	3.04
Medicinal product	5.0	1016	5.08

If only *Class 3* solvents are present, non-specific control methods may be used, such as, for example, loss on drying.

Validation of procedures for controlling residual organic solvents must meet the EAEU document "Guidelines for validation of analytical procedures".

3.5. INFORMATION ON THE CONTENT OF RESIDUAL ORGANIC SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual organic solvents in excipients or active pharmaceutical substances in order to meet the criteria of this chapter. Information about the content of residual organic solvents, which can be transmitted to pharmaceutical manufacturers by suppliers of active substances or excipients, can be presented in the following ways:

- only Class 3 residual organic solvents may be present. Loss on drying is less than 0.5%;

- only Class 2 residual organic solvents may be present; the content of each of them does not exceed the limit calculated by Option 1; the supplier then indicates the name of each of the residual organic solvents;

- Class 2 and 3 residual organic solvents may be present; the content of each of the Class 2 solvents does not exceed the permissible standards in accordance with Option 1, and the content of Class 3 solvents is less than 0.5%.

If Class 1 solvents may be present, each of them must be identified and quantified.

"May be present" refers to the solvent used in the final manufacturing step and to solvents that are used in earlier manufacturing steps and not removed consistently by an approved (validated) process.

If Class 2 residual organic solvents are present in quantities higher than the permissible standards in accordance with Option 1, and the content of Class 3 residual organic solvents exceeds 0.5%, each of them must be identified and quantified.

4. LIMITS OF RESIDUAL ORGANIC SOLVENTS

4.1. SOLVENTS TO BE AVOIDED

Class 1 solvents should not be used in the production of active pharmaceutical ingredients, excipients and medicinal products due to their high toxicity and harmful effects on the environment. However, if their use is unavoidable for the production of a drug that has a strong therapeutic effect, their quantities should be limited in accordance with Table 2.3.2.0.-3 unless otherwise justified. 1,1,1-Trichloroethane is included in Table 2.3.2.0.-3 because it is dangerous to the environment. The established acceptable limit of 1500 ppm is based on a review of the safety data.

4.2. SOLVENTS TO BE LIMITED

The content of solvents listed in Table 2.3.2.0.-4 should be limited in pharmaceutical products due to their toxicity.

Table 2.3.2.0.-3. – *Class 1 solvents in drug preparations and substances for pharmaceutical use (solvents to be avoided)*

Solvent	Concentration limit, ppm	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

Table 2.3.2.0.-4. – *Class 2 solvents in drug preparations and substances for pharmaceutical use*

Solvent	PDE, mg/day	Concentration limit, ppm
Acetonitrile	4.1	410
Hexane	2.9	290
N,*N*-Dimethylacetamide	10.9	1090
N,*N*-Dimethylformamide	8.8	880
1,2-Dimethoxyethane	1.0	100
1,4-Dioxane	3.8	380
Dichloromethane	6.0	600
1,2-Dichloroethene	18.7	1870
Xylene*	21.7	2170
Cumene	0.7	70
Methanol	30.0	3000
Methylbutyl ketone	0.5	50
Methyl isobutyl ketone	45.0	4500
N-Methylpyrrolidone	5.3	530
Methylcyclohexane	11.8	1180
2-Methoxyethanol	0.5	50
Nitromethane	0.5	50
Pyridine	2.0	200
Sulpholane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloroethene	0.8	80
Formamide	2.2	220
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
Ethyleneglycol	6.2	620
2-Ethoxyethanol	1.6	160

* Usually 60% of *m*-xylene, 14% of *p*-xylene, 9% of *o*-xylene, and 17% of ethylbenzene.

PDE data are given with an accuracy of 0.1 mg/day, and concentrations - up to 10 ppm. The set values do not reflect the required analytical accuracy of the determination. The accuracy must be established during the procedure validation process.

4.3. LOW TOXIC SOLVENTS

Class 3 solvents (shown in Table 2.3.2.0.-5) can be classified as less toxic and have a lower risk to human health.

Table 2.3.2.0.-5. – *Class 3 solvents that should be limited by GMP or other quality requirements*

Anisole	2-Methyl-1-propanol
Acetone	Formic acid
1-Butanol	Pentane
2-Butanol	1-Pentanol
Butyl acetate	1-Propanol
tert-Butylmethyl ether	2-Propanol
Heptane	Propyl acetate
Dimethylsulfoxide	Triethylamine
Isobutyl acetate	Acetic acid
Isopropyl acetate	Ethanol
Methyl acetate	Ethyl acetate
3-Methyl-1-butanol	Ethyl ether
Methylethylketone	Ethyl formate

Table 2.3.2.0.-6. – *Solvents for which no valid toxicity data are available*

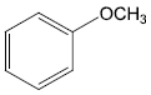
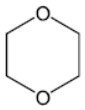
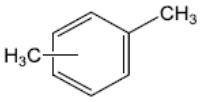
1,1-Dimethoxymethane	Methylisopropyl ketone
2,2-Dimethoxypropane	Methyltetrahydrofuran
1,1-Diethoxypropane	Petroleum ether
Isooctane	Trifluoroacetic acid
Isopropyl ether	Trichloroacetic acid

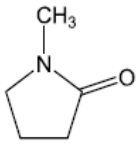
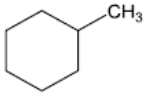
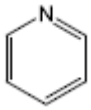
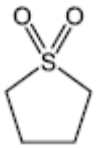
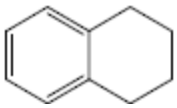
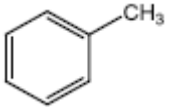
Class 3 does not include solvents known to be hazardous to human health in concentrations that are normally allowed in drug products. However, for many Class 3 solvents, no long-term toxicity or carcinogenicity studies have been conducted. Available data indicate that they are less toxic in acute or short-term trials and give a negative result in genotoxicity tests (do not show genotoxicity). It is considered that the content of these residual organic solvents of 50 mg/day or less (corresponding to 5000 ppm or 0.5% according to Option 1) is acceptable without justification. Higher values may also be acceptable, provided that they are determined by production capabilities that meet the requirements of good manufacturing practice (*GMP*).

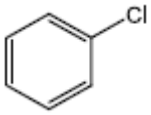
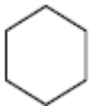
4.4. SOLVENTS FOR WHICH NO ADEQUATE TOXICOLOGICAL DATA ON WHICH TO BASE A PDE WAS FOUND

The solvents listed in Table 2.3.2.0.-6 may also be of interest to manufacturers of excipients, active pharmaceutical ingredients, or medicinal products. However, no valid toxicity data was found for PDE calculation. Manufacturers should supply justification for residual levels of these solvents in pharmaceutical products.

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GENERAL CHAPTER

Solvent	Other names	Structure	Class
Anisole	Methoxybenzene		Class 3
Acetone	2-Propanone, propane-2-one	CH_3COCH_3	Class 3
Acetonitrile		CH_3CN	Class 2
Benzene			Class 1
1-Butanol	*n*-Butyl alcohol, butane-1-ol	$CH_3[CH_2]_3OH$	Class 3
2-Butanol	*sec*-Butyl alcohol, butane-2-ol	$CH_3CH_2CH(OH)CH_3$	Class 3
Butyl acetate	Acetic acid butyl ether	CH3COO[CH2]3CH3	Class 3
tert-Butylmethyl ether	2-Methoxy-2-methylpropane	$(CH_3)_3COCH_3$	Class 3
Hexane	*n*-Hexane	$CH_3[CH_2]_4CH_3$	Class 2
Heptane	*n*-Heptane	$CH_3[CH_2]_5CH_3$	Class 3
N,*N*-Dimethylacetamide	DMA	$CH_3CON(CH_3)_2$	Class 2
Dimethylsulfoxide	Methylsulfinylmethane, methylsulfoxide, DMSO	$(CH_3)_2SO$	Class 3
N,*N*-Dimethylformamide	DMFA	$HCON(CH_3)_2$	Class 2
1,2-Dimethoxyethane	Ethylene glycol dimethyl ether, monoglyme, dimethyl cellosolve	$H_3COCH_2CH_2OCH_3$	Class 2
1,4-Dioxane	*p*-Dioxane, [1,4] dioxane		Class 2
Dichloromethane	Methylene chloride	CH_2Cl_2	Class 2
1,2-Dichloroethane	*sim*-Dichloroethane, ethylene dichloride, ethylene chloride	CH_2ClCH_2Cl	Class 1
1,1-Dichloroethene	1,1-Dichloroethylene, vinylidene chloride	$H_2C=CCl_2$	Class 1
1,2-Dichloroethene	1,2-Dichlorethylene, acetylindole	ClHC=CHCl	Class 2
Isobutyl acetate	Isobutyl acetic ether	$CH_3COOCH_2CH(CH_3)_2$	Class 3
Isopropyl acetate	Isopropyl acetic acid ether	$CH_3COOCH(CH_3)_2$	Class 3
Xylene*	Dimethylbenzene		Class 2

Solvent	Other names	Structure	Class
Cumene	Isopropylbenzene, (1-methylethyl)benzene		Class 2
Methanol	Methyl alcohol	CH_3OH	Class 2
Methyl acetate	Acetic acid methyl ether	CH_3COOCH_3	Class 3
3-Methyl-1-butanol	Isoamyl alcohol, isopentyl alcohol, 3-methylbutane-1-ol	$(CH_3)_2CHCH_2CH_2OH$	Class 3
Methylbutyl ketone	2-Hexanone, hexane-2-one	$CH_3[CH_2]_3COCH_3$	Class 2
Methyl isobutyl ketone	4-Methylpentan-2-one, 4-methyl-2-pentanon, MIBK	$CH_3COCH_2CH(CH_3)_2$	Class 3
N-Methylpyrrolidone	1-Methylpyrrolidine-2-one, 1-methyl-2-pyrrolidinone		Class 2
2-Methyl-1-propanol	Isobutyl alcohol, 2-methylpropan-1-ol	$(CH_3)_2CHCH_2OH$	Class 3
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylethylketone	2-Butanone, MEK, butane-2-one	$CH_3CH_2COCH_3$	Class 3
2-Methoxyethanol	Methyl cellosolve	$CH_3OCH_2CH_2OH$	Class 2
Formic acid		$HCOOH$	Class 3
Nitromethane		CH_3NO_2	Class 2
Pentane	*n*-Pentane	$CH_3[CH_2]_3CH_3$	Class 3
1-Pentanol	Amyl alcohol, pentane-1-ol, pentyl alcohol	$CH_3[CH_2]_3CH_2OH$	Class 3
Pyridine			Class 2
1-Propanol	Propane-1-ol, Propyl alcohol	$CH_3CH_2CH_2OH$	Class 3
2-Propanol	Propane-2-ol, Isopropyl alcohol	$(CH_3)CHOH$	Class 3
Propyl acetate	Acetic acid propyl ether	$CH_3COOCH_2CH_2CH_3$	Class 3
Sulpholane	Tetrahydrothiophene-1,1-dioxide		Class 2
Tetrahydrofuran	Tetramethylene Oxide, Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2

Solvent	Other names	Structure	Class
1,1,1-Trichloroethane	Methyl chloroform	CH_3CCl_3	Class 1
1,1,2-Trichloroethene	Trichloroethene	$HClC=CCl_2$	Class 2
Carbon tetrachloride	Tetrachloromethane	CCl_4	Class 1
Acetic acid	Ethanoic acid	CH_3COOH	Class 3
Formamide	Methanamide	$HCONH_2$	Class 2
Chlorobenzene			Class 2
Chloroform	Trichloromethane	$CHCl_3$	Class 2
Cyclohexane	Hexamethylene		Class 2
Ethanol	Ethyl alcohol	CH_3CH_2OH	Class 3
Ethyl acetate	Acetic acid ethyl ether	$CH_3COOCH_2CH_3$	Class 3
Ethyleneglycol	1,2-Dihydroxyethane, 1,2-ethanediol	$HOCH_2CH_2OH$	Class 2
Ethyl ether	Diethyl ether, ethoxyethane, 1,1'-oxybisethane	$CH_3CH_2OCH_2CH_3$	Class 3
Ethyl formate	Formic acid ethyl ether	$HCOOCH_2CH_3$	Class 3
2-Ethoxyethanol	Cellosolve	$CH_3CH_2OCH_2CH_2OH$	Class 2

* Usually 60% of *m*-xylene, 14% of *p*-xylene, 9% of *o*-xylene, and 17% of ethylbenzene.

APPENDIX 2. ADDITIONAL BACKGROUND

A2.1. Environmental regulation of organic volatile solvents

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in Environmental Health Criteria (*EHC*) monographs and the Integrated Risk Information System (*IRIS*). The objectives of such groups as the International Programme on Chemical Safety (*IPCS*), the United States Environmental Protection Agency (*USEPA*), and the United States Food and Drug Administration (*USFDA*) include the determination of acceptable exposure levels of chemicals. The goal is the protection of human health and maintenance of environmental integrity against the possible deleterious effects of chemicals resulting from long-term environmental exposure.

The methods involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter-term study data can be used with modification of the approach such as the use of larger safety factors. The approach described therein relates primarily to long-term or life-time exposure of the general population in the ambient environment, i.e. ambient air, food, drinking water, etc.

A2.2. Residual organic solvents in pharmaceutical products

Exposure limits in this chapter are established by referring to methodologies and toxicity data described in *EHC* and *IRIS* monographs. However, some specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits. They are:

1) Patients (not the general population) use pharmaceutical products to treat their diseases or for prophylaxis to prevent infection or disease;

2) The assumption of life-time patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health;

3) Residual organic solvents are unavoidable components in pharmaceutical production and will often be a part of drug products;

4) Residual organic solvents should not exceed recommended levels except in exceptional circumstances;

5) Data from toxicological studies that are used to determine acceptable levels for residual organic solvents should have been generated using appropriate protocols such as those described for example, by OECD and the *USFDA* Red Book.

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 solvents could be determined with the use of a large safety factor (i.e., 10,000 to 100,000) with respect to the no-observed-effect level (NOEL). Detection and quantitation of these solvents should be by state-of-the-art analytical techniques.

Acceptable exposure levels in this guideline for Class 2 solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceutical products (*Pharmacopeial Forum*, Nov-Dec 1989), and the method adopted by *IPCS* for Assessing Human Health Risk of Chemicals (*Environmental Health Criteria* 170, WHO, 1994). These methods are similar to those used by the *USEPA* (*IRIS) and the USFDA* (*Red Book*), etc. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values tabulated in Section 4 of this document.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE}=\frac{\text{NOEL x Weight Adjustment}}{F1 \times F2 \times F3 \times F4 \times F5}.$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of "uncertainty factors" used in Environmental Health Criteria (*Environmental Health Criteria* 170, World Health Organization, Geneva, 1994), and "modifying factors" or "safety factors" in *Pharmacopoeial Forum*. The assumption of 100% systemic exposure is used in all calculations regardless of a route of administration.

The modifying factors are as follows:

*F*1 is a factor to account for extrapolation between species;

*F*1 = 5 for extrapolation from rats to humans;

*F*1 = 12 for extrapolation from mice to humans;

*F*1 = 2 for extrapolation from dogs to humans;

*F*1 = 2.5 for extrapolation from rabbits to humans;

*F*1 = 3 for extrapolation from monkeys to humans;

*F*1 = 10 for extrapolation from other animals to humans.

*F*1 takes into account the comparative surface area: body weight ratios for the species concerned and for man. Surface area is calculated as:

$$S = km^{0.67},$$

where: *m* is the body mass;

k is the constant assumed to be 10.

The bodyweight values used in the equation are shown below in Table 2.3.2.0.-7.

*F*2 is a factor of 10 to account for variability between individuals. A factor, equal to 10 is generally given for all organic solvents, and 10 is used consistently in this chapter.

*F*3 is a variable factor to accounts for toxicity studies of short-term exposure.

*F*3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys).

*F*3 = 1 for reproductive studies in which the whole period of organogenesis is covered.

*F*3 = 2 for a 6-month study in rodents, or a 3.5-year study in non-rodents.

*F*3 = 5 for a 3-month study in rodents, or a 2-year study in non-rodents.

*F*3 = 10 for studies of a shorter duration.

In all cases, the higher factor has been used for study durations between the time points (e.g. a factor of 2 for a 9-month rodent study).

Table 2.3.2.0.-7. – *Values used in calculations in this document*

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Hound body weight (Beagle)	11.5 kg
Rat respiratory volume	290 l/day
Mouse respiratory volume	43 l/day
Rabbit respiratory volume	1440 l/day
Guinea pig respiratory volume	430 l/day
Human respiratory volume	28800 l/day
Hound respiratory volume	9000 l/day
Monkey respiratory volume	1150 l/day
Mouse water consumption	5 mL/day
Rat water consumption	30 mL/day
Rat food consumption	30 g/day

*F*4 is a factor that may be applied in cases of severe toxicity, e.g. non-genotoxic carcinogenicity, neurotoxicity or teratogenicity. In studies of reproductive toxicity, the following factors are used:

*F*4 = 1 for fetal toxicity associated with maternal toxicity;

*F*4 = 5 for fetal toxicity without maternal toxicity;

*F*4 = 5 for a teratogenic effect with maternal toxicity;

*F*4 = 10 for a teratogenic effect without maternal toxicity.

*F*5 is a variable factor that may be applied if the NOEL (no-effect level) was not established. When only a LOEL (the lowest-observed effect level) is available, a factor of up to 10 can be used depending on the severity of the toxicity.

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognised that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider the toxicity study of acetonitrile in mice. The NOEL is calculated to be 50.7 mg/(kg· day). The PDE for acetonitrile in this study is calculated as follows:

$$\text{PDE}= \frac{50.7\ \text{mg}\cdot\ \text{kg}^{-1}\cdot\ \text{day}^{-1}\cdot\ 50\ \text{kg}}{12\text{-}10\cdot 5\text{-}1\text{-}1} = 4.22\ \text{mg*day}^{-1}.$$

In this example:

*F*1 = 12 to account for the extrapolation from mice to humans;

*F*2 = 10 to account for differences between individual humans;

*F*3 = 5 because the duration of the study was only 13 weeks;

*F*4 = 1 because no severe toxicity was encountered;

*F*5 = 1 because the no-effect level was determined.

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or mg/m^3.

Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (*Mr* 153.84) summarised in Pharmeuropa, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\frac{n}{V} = \frac{R}{RT} - \frac{300 \cdot 10^{-6}\ \text{atm} \cdot 153840\ \text{mg}\cdot\text{mol}^{-1}}{0{,}082\ \text{L}\cdot\text{atm}\cdot K^{-1} \cdot \text{mol}^{-1}\cdot 298\text{K}} =$$

$$= \frac{46.15\ \text{mg}}{24.45\ \text{l}} = 1.89\ \text{mg/l}.$$

The relationship 1000 L = 1 m^3 is used to convert to mg/m^3.

2.3.3.1. The use of alcoholometric tables.

When determining the concentration of ethanol in ethyl alcohol, use Alcoholometric table 4.1.-1. *"The ratio between the density of the water-alcohol solution and the ethanol content in the solution"*.

If it is necessary to obtain ethyl alcohol of a certain concentration from the available ethyl alcohol of various concentrations and purified water, follow the alcoholometric tables:

Table 4.1.-2. *"The amount (in grams at 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 kg of alcohol with a concentration of 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 92%"*;

Table 4.1.-3. *"The amount (in millilitres at 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 L of alcohol with a concentration of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%"*;

Table 4.1.-4. *"The amount (in millilitres at 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 L of alcohol with a concentration of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%"*;

Table 4.1.-5. *"Table for obtaining alcohol of various concentrations at 20 °C";*

Table 4.1.-6. *The amount (in millilitres at 20 °C) of water and alcohol with a concentration from 96.6% to 97.0%, which must be mixed to get 1 L (at 20 °C) of alcohol with a concentration of 40%, 70%, 80%, 90%, 95%*.

Alcoholmetric tables 4.1.-1.-5 are given in Appendix 4.1 to the Pharmacopoeia.

2.3.4.0. Polymorphism

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a substance in the solid state to exist in different crystalline forms having the same chemical composition. Solids that are in non-crystalline form are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulfur), the term "allotropy" is used instead of "polymorphism".

The term "pseudopolymorphism" is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include substances where the solvent is trapped in the matrix in variable proportions. However, the term "pseudopolymorphism" is ambiguous because of its use in different circumstances, it is, therefore, preferable to use only the terms "solvates" and "hydrates" when describing such substances.

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, the occurrence of solvates, allotropy, or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given substance have the same chemical behaviour in solution or as a melt; in contrast, their physicochemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At normal temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. Such change is a univariate equilibrium so that at a given pressure this state is characterised by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

If the transformation of one polymorphic form into another is accompanied by a slight change in enthalpy, then *in vivo* these forms easily transform into each other. Therefore, replacing one polymorphic modification with another does not lead to a significant change in the absorption rate, and the bioavailability of the active substance does not undergo significant changes. Significant differences in the free energy of polymorphic modifications cause noticeable changes in bioavailability.

When a substance is crystallised from a solution or melt, the least stable phase is formed first, which is closest to the solution in terms of free energy. For this reason, metastable forms have a less internal coupling of molecules, which is reflected in their physical properties, in particular, in increased solubility. Since dissolution is the limiting stage in the absorption of the active substance from the gastrointestinal tract, metastable forms have greater bioavailability than stable forms and are more appropriate for obtaining active pharmaceutical ingredients. One of the effective agents that inhibit the transition of metastable forms into stable ones and thereby increase their stability is the use of a number of auxiliary substances (methylcellulose, polyvinylpyrrolidone, sodium alginate, propylene glycol alginate, etc.).

Different crystalline forms or solvates may be produced by varying the crystallisation conditions (temperature, pressure, solvent, concentration, rate of crystallisation, seeding of the crystallisation medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism:

- X-ray powder diffraction;
- X-ray diffraction of single crystals;
- thermal analysis (differential scanning calorimetry, thermogravimetry, thermomicroscopy);
- microcalorimetry;
- moisture absorption analysis;
- optical and electron microscopy;
- solid-state nuclear magnetic resonance;
- infrared absorption spectrophotometry;
- Raman spectrometry;
- measurement of solubility and intrinsic dissolution rate;
- density measurement.

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise, solvates are less soluble in their solvent than unsolvated forms.

Establishing the relationship between the crystalline form of a substance and the conditions for its production is a necessary stage in the development of a technology for the production of an active pharmaceutical substance. Minor changes in conditions may result in a substance with a different ratio of polymorphic forms or new polymorphic forms.

Targeted preparation of polymorphic forms of active substances is carried out using methods of equilibrium and non-equilibrium crystallisation.

The method of equilibrium crystallisation is based on isothermal and isoconcentration evaporation of a solvent from a solution in equilibrium with crystals of this polymorphic form. If the target product is a high-temperature modification of the substance, then the requirement for its production is to maintain the crystallisation temperature above the transition temperature of the high-temperature modification to the low-temperature one. When obtaining a low-temperature form, the crystallisation temperature is maintained below the temperature of this transformation. The type of solvent is essential in the equilibrium crystallisation method.

In the non-equilibrium crystallisation method, the process is carried out at a high crystallisation temperature, i.e. with significant supersaturation in the system. For this purpose, the following methods are usually used:

- polythermal crystallisation;
- replacement of solvent;
- spray drying;
- freeze-drying.

According to the method of polythermal crystallisation, a saturated solution of the active pharmaceutical substance is obtained first at a fixed temperature of the solvent (usually elevated), and then it is sharply cooled to a certain temperature, holding it for some time.

According to the solvent replacement method, a saturated solution of the active pharmaceutical substance is obtained in an organic solvent or water, and then water or an organic solvent is added, respectively. As a result of a sharp decrease in the solubility of the substance in the water-organic mixture, crystals of the target form drop out of the solution. The method for introducing the solvent is determined by the solubility of the polymorphic form released. An important condition is also the choice of the crystallisation temperature depending on the nature of the polymorphic modification (high- or low-temperature modification).

The spray drying method consists of dispersing the initial solution into the gas-coolant flow. Depending on the temperature of the coolant, the rate of its delivery, and the type of solvent, a certain rate of evaporation of the solvent is set, which provides a given degree of supersaturation of the solution. Under these conditions, metastable modifications in monotropic systems are easily formed. However, to prevent the establishment of thermodynamic equilibrium, the process is carried out quickly enough, using small amounts of the initial solution.

The freeze-drying method is based on the sublimation of a solvent from a pre-frozen solution. The type of solvent, the freezing rate, the concentration of the initial solution, and the lyophilisation conditions are crucial.

Thus, when obtaining polymorphic forms with optimal therapeutic parameters, preference is given to those methods that provide better solubility and ability to absorb, transform and interact in the body, give them specific adsorption in certain organs and tissues, as well as a certain rate and degree of elimination from the body.

2.3.5.0. Control of Impurities in Substances for Pharmaceutical Use

INTRODUCTION

The monographs of the Pharmacopoeia on substances for pharmaceutical use are designed to ensure acceptable quality for users. The role of the Pharmacopoeia in public health protection requires that adequate control of impurities be provided by monographs. The quality required is based on scientific, technical and regulatory considerations.

Requirements concerning impurities are given in individual monographs and in the general monograph *Substances for pharmaceutical use* which are complementary: individual monographs prescribe acceptance criteria for impurities whereas the general monograph deals with the need for qualification, identification, and reporting of any organic impurities that occur in active (pharmaceutical) substances.

The thresholds for reporting, identification and qualification contained in the general monograph *Substances for pharmaceutical use* apply to all related substances. However, if a monograph does not contain a related substances test based on a quantitative method, any new impurities occurring above a threshold may be overlooked since the test is not capable to detect those impurities.

The requirements of the "Related substances" section of the general monograph *Substances for pharmaceutical use*, notably those concerning thresholds, do not apply to excipients; also excluded from the provisions of this section are: biological and biotechnological products; oligonucleotides; radiopharmaceuticals; fermentation products and semisynthetic products derived therefrom; herbal products and crude products of animal and plant origin. Although the thresholds stated in the general monograph do not apply, the general concepts of reporting, identification (wherever possible) and qualification of impurities are equally valid for all classes of these substances.

BASIS FOR THE ELABORATION OF MONOGRAPHS OF THE PHARMACOPOEIA OF THE UNION

Pharmacopoeia monographs are elaborated on substances that are present in drug products that have been authorised by the competent authority, consequently, these monographs do not necessarily cover all sources of substances for pharmaceutical use in the world market.

Organic and inorganic impurities present in those substances that have been evaluated by the competent authorities are qualified with respect to safety at the maximum allowable content (at the maximum daily dose) unless new safety data which become available after confirmation of the results of the assessment of the lower limits.

The main part of Pharmacopoeia monographs on substances for pharmaceutical use are harmonised with the requirements of European Pharmacopoeia monographs that, in their turn, are elaborated by groups of experts and working parties collaborating with national pharmacopoeia authorities, the competent authorities for marketing authorisation, national control laboratories, and the European Pharmacopoeia laboratory; they are also assisted by the producers of the substances and/or the pharmaceutical manufacturers that use these substances.

CONTROL OF IMPURITIES IN SUBSTANCES FOR PHARMACEUTICAL USE

The quality with respect to impurities is controlled by a set of tests within a monograph. These tests are intended to cover organic and inorganic impurities that are relevant in view of the sources of active substances in authorised medicinal products.

Control of residual solvents is provided by the general monograph *Substances for pharmaceutical use* and general chapter *2.3.2.0. Residual organic solvents*. The certificate of suitability of a monograph for a given source of a substance indicates the residual solvents that are controlled together with the specified acceptance criteria and the validated control procedures (where this differs from those described in general chapter *2.1.4.19. Identification and control of residual solvents*).

As a rule, monographs on organic chemicals usually have a test entitled "Related substances" that covers relevant organic impurities. This test may be supplemented by specific tests where the general test does not control a given impurity or where there are particular reasons (for example, safety reasons) for requiring special control.

Where a monograph has no "Related substances" (or equivalent) test but only specific tests, the user of a substance must nevertheless ensure that there is suitable control of organic impurities. Related substances occurring above the identification threshold are to be identified (wherever possible) and, unless justified, those occurring above the qualification threshold are to be qualified (see also under "Recommendations to users of monographs on active substances").

Where the monograph covers substances with different impurity profiles, it may have a single related substances test to cover all impurities mentioned in the "Impurities" section or several tests may be necessary to give control of all known profiles. Compliance may be established by carrying out only the tests relevant to the known impurity profile for the source of the substance.

Instructions for control of impurities may be included in the "Production" section of a monograph, for example where the only analytical method appropriate for the control of a given impurity is to be performed by the manufacturer since the method is too technically complex for general use or cannot be applied to the final drug substance and/or where validation of the production process (including the purification step) will give sufficient control.

IMPURITIES SECTION IN MONOGRAPHS ON ACTIVE SUBSTANCES

The "Impurities" section in a monograph includes impurities (chemical structure and name wherever possible), which are usually organic, which are known to be detected by the tests prescribed in the monograph. It is based on information available at the time of elaboration or revision of the monograph and is not necessarily exhaustive. The section includes specified impurities and, where so indicated, other detectable impurities.

Specified impurities have an acceptance criterion not greater than that authorised by the competent authorities.

Other detectable impurities are potential impurities with a defined structure but not known to be normally present above the identification threshold in substances used in the production of drug products. They are given in the "Impurities" section for information.

Where an impurity other than a specified impurity is found in an active substance it is the responsibility of the user of the substance to check whether it has to be identified/qualified, depending on its content, nature, maximum daily dose, and relevant identification/qualification threshold, in accordance with the general monograph on *Substances for pharmaceutical use*, "Related substances" section.

INTERPRETATION OF THE TEST FOR RELATED SUBSTANCES IN THE MONOGRAPHS ON ACTIVE SUBSTANCES

A specific monograph on a substance for pharmaceutical use is to be read and interpreted in conjunction with the general monograph on *Substances for pharmaceutical use*.

Where a general acceptance criterion for impurities ("any other impurity", "other impurities", "any impurity") equivalent to a nominal content greater than the applicable identification threshold (see general monograph *Substances for pharmaceutical use*) is prescribed, this is valid only for specified impurities mentioned in the "Impurities" section. The need for identification (wherever possible), reporting, specification, and qualification of other impurities that occur must be considered according to the requirements of the general monograph. It is the responsibility of the user of the substance to determine the validity of the acceptance criteria for impurities not mentioned in the "Impurities" section and for those indicated as "other detectable impurities".

Acceptance criteria for the related substances test are presented in different ways in existing monographs. The decision tree (Figure 2.3.5.0.-1) may be used as an aid in the interpretation of general acceptance criteria and their relation with the "Impurities" section of the monograph.

General acceptance criteria for "other" impurities are expressed in various ways in the monographs: "any other impurity", "other impurities", "any impurity", "any spot", "any band", etc. The general acceptance criteria may apply to certain specified impurities only or to unspecified impurities and certain specified impurities, depending on the nature of the active substance and the applicable identification.

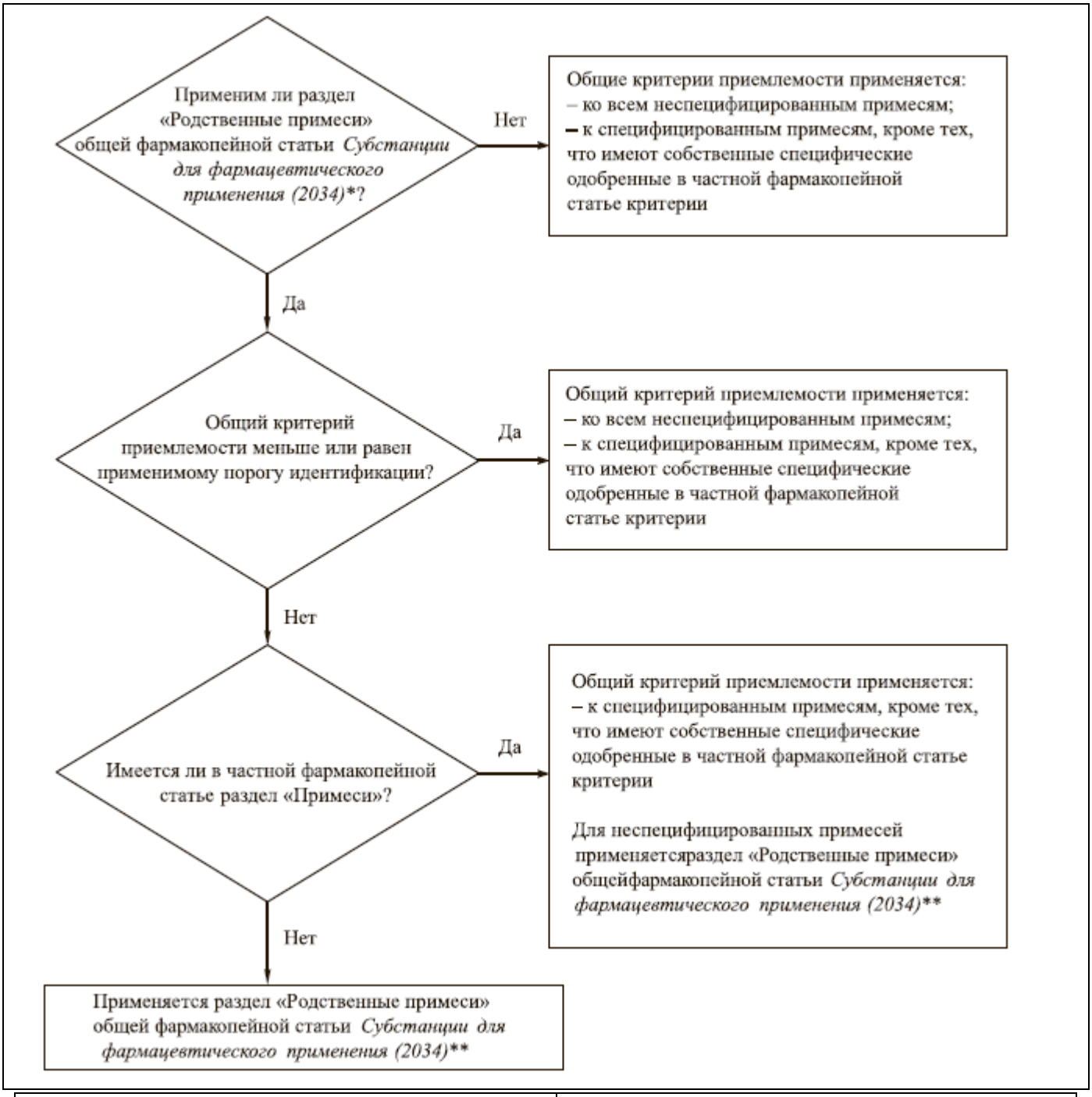
RECOMMENDATIONS TO USERS OF MONOGRAPHS ON ACTIVE SUBSTANCES

Monographs give a specification for suitable quality of substances with impurity profiles corresponding to those taken into account during elaboration and/or revision of the monograph. It is the responsibility of the user of the substance to check that the monograph provides adequate control of impurities for a substance for pharmaceutical use from a given source.

A monograph with the "Related substances" test based on a quantitative method (such as liquid chromatography, gas chromatography and capillary electrophoresis) provides adequate control of impurities for a substance from a given source if impurities present in amounts above the applicable identification threshold are specified impurities mentioned in the "Impurities" section.

If the substance contains impurities other than those mentioned in the "Impurities" section, it has to be verified that these impurities are detectable by the method described in the monograph; otherwise a new method must be developed and revision of the monograph must be requested. Depending on the contents found and the limits proposed, the identification and/or the qualification of these impurities must be considered.

Where a single related substances test procedure covers different impurity profiles, only impurities for the known profile from a single source need to be reported in the certificate of analysis unless the marketing authorisation holder uses active substances with different impurity profiles.



	Does the "Related substances" section of the general monograph on *Substances for pharmaceutical use** apply?
	No
	The general acceptance criteria apply to: - all unspecified impurities; - specified impurities except those that have their own specific criteria in the monograph
	Yes
	Is the general acceptance criterion less than or equal to the applicable identification threshold?
	Does the monograph have an "Impurities" section?
	The general acceptance criterion applies to: - specified impurities except those that have their own specific criteria in the monograph For unspecified impurities, the section "Related impurities" of the general monograph *Substances for pharmaceutical use***
	The "Related substances" section of the general monograph on *Substances for pharmaceutical use*** applies

Figure 2.3.5.0.-1. – *Decision tree for interpretation of general acceptance criteria for "other" impurities in monographs*

* The requirements of this section apply to active substances, with the exception of biological and biotechnological products; oligonucleotides; radiopharmaceuticals; products of fermentation and semi-synthetic products derived therefrom; crude products of animal or plant origin; herbal drug preparations.

IDENTIFICATION OF IMPURITIES (PEAK ASSIGNMENT)

Where a monograph has an individual limit for an impurity, it is often necessary to define means of identification, for example using a reference substance, a representative chromatogram or relative retention.

The user of the substance may find it necessary to identify impurities other than those for which the monograph provides a means of identification, for example to check the suitability of the specification for a given impurity profile by comparison with the "Impurities" section.

The Pharmacopoeia does not provide reference substances, representative chromatograms or information on relative retentions for this purpose, unless prescribed in the monograph, users will therefore have to apply the available scientific techniques/methods for identification.

NEW IMPURITIES/SPECIFIED IMPURITIES ABOVE THE SPECIFIED LIMIT

Where a new manufacturing process or change in an established process leads to the occurrence of a new impurity, it is necessary to apply the conditions of the general monograph on *Substances for pharmaceutical use* regarding identification and qualification and to verify the suitability of the monograph for control of the impurity. A certificate of suitability is a means for confirming for a substance from a given source that the new impurity is adequately controlled or the certificate contains a procedure for control with defined acceptance criteria. In the latter case revision of the monograph will be initiated.

Where a new manufacturing process or change in an established process leads to the occurrence of a specified impurity above the specified limit, it is necessary to apply the requirements of the general monograph on *Substances for pharmaceutical use* regarding qualification.

EXPRESSION OF ACCEPTANCE CRITERIA

The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values.

CHROMATOGRAPHIC METHODS

Various aspects of impurity control are described in chapter *2.1.2.36 Chromatographic separation techniques.*

2.3.6.0. "Characters" Section in Monographs

The statements included in the "Characters" section are not to be interpreted in a strict sense and are not requirements, although they can indirectly contribute to the preliminary assessment of the quality of a medicinal product. For information of users, the methods recommended to authors of monographs as the basis for statements concerning hygroscopicity, crystallinity and solubility are given below.

When describing the properties of drugs, the following characteristics are indicated: appearance, odor, hygroscopicity, crystallinity, solubility, and, if necessary, other indicators.

When describing the appearance, evaluate visually the shape, colour, gloss, clarity, and other properties of the test samples, while non-liquid samples are placed on a sheet of white paper, and liquid samples are examined in clear colorless test tubes and viewed in diffused daylight.

The odor or its absence, if necessary, is determined as specified in chapter *2.1.3.2. Odour*.

Procedures for determining hygroscopicity, crystallinity, and solubility are given below.

HYGROSCOPICITY

Hygroscopicity is the character of substances to absorb water vapour from the air. Wetted hydrophilic substances with a capillary-porous structure and highly water-soluble substances, especially compounds that form crystal hydrates *with* water, are hygroscopic.

This method is to be carried out on a substance that complies with the "Loss on drying" or "Water content" tests specified in the monograph. The procedure gives an indication of the degree of hygroscopicity rather than a true determination.

In a pre-weighed glass vessel (50 mm in external diameter and 15 mm high) with appropriate stopper, place the amount of substance prescribed in the "Loss on drying" or "Water" test in the vessel and weigh. Place the unstoppered vessel in a desiccator at 25 °C containing a saturated solution of ammonium chloride or ammonium sulfate or place it in a climatic cabinet set at 25 ± 1 °C and 80 ± 2% relative humidity. Allow to stand for 24 h. Stopper the weighing vessel and weigh.

Calculate the percentage increase in mass using the expression:

$$\frac{m_3 - m_2}{m_2 - m_1} \cdot 100,$$

where m_1 is the mass of the empty glass vessel, in grams;

m_2 is the mass of the glass vessel with the test sample before exposure to a humid environment, in grams;

m_3 is the mass of the glass vessel with the test sample after exposure to a humid environment, in grams.

The results are interpreted as follows:

- *deliquescent*: sufficient water is absorbed to form a liquid;
- *very hygroscopic*: increase in mass is equal to or greater than 15%;
- *hygroscopic*: increase in mass is less than 15% and equal to or greater than 2%;
- *slightly hygroscopic*: increase in mass is less than 2% and equal to or greater than 0.2%.

CRYSTALLINITY

This procedure is employed to establish the crystalline or amorphous nature of a substance.

Mount a few particles of the substance to be examined in mineral oil on a clean glass slide and examine under a polarising microscope. Crystalline particles exhibit birefringence and extinction positions when the microscope stage is revolved.

SOLUBILITY

To indicate the degree of solubility, use the terms specified in Table 2.3.6.0.-1.

For this test a maximum of 111 mg of substance (for each solvent) and a maximum of 30 mL of each solvent are necessary.

Dissolving procedure

Shake vigorously for 1 min and place in a constant temperature device, maintained at a temperature of 25.0 ± 0.5 °C for 15 min. If the test sample is not completely dissolved, repeat the shaking for 1 min and place the tube and allow to stand at 25 ± 0.5 °C for 15 min.

Determination procedure

Weigh 100 mg of finely powdered test sample (90) in a stoppered tube (16 mm in internal diameter and 160 mm long), add 0.1 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is *very soluble.*

If the test sample is not completely dissolved, add 0.9 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is *freely soluble*.

If the test sample is not completely dissolved, add 2.0 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is considered *soluble*.

If the test sample is not completely dissolved, add 7.0 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is considered *sparingly soluble*.

If the test sample is not completely dissolved, weigh 10 mg of finely powdered test sample (90) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is considered *slightly soluble*.

If the substance is not completely dissolved, weigh 1 mg of finely powdered test sample (90) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under dissolving procedure. If the test sample is completely dissolved, it is considered *very slightly soluble*.

In the case of test samples with a known solubility, it is allowed to perform the test not to the full extent described above, but only for the extreme values of the specified degree of solubility. For example, if the substance is *soluble*, then 100 mg of the powdered substance should not be dissolved in 1.0 mL of the solvent but should be completely dissolved in 3.0 mL of the solvent.

If other characters are specified in the monograph, then tests are performed to determine them, as prescribed in the corresponding general or individual monographs.

Table 2.3.6.0.-1. – *Meanings of terms describing the solubility of substances at temperatures between 15 °C and 25 °C.*

Term		Approximate volume of solvent (mL) required to dissolve 1 g of the substance
in Russian	in English	
Очень легко растворим	Very soluble	less than 1
Легко растворим	Freely soluble	1 to 10
Растворим	Soluble	10 to 30
Умеренно растворим	Sparingly soluble	30 to 100
Мало растворим	Slightly soluble	100 to 1000
Очень мало растворим	Very slightly soluble	1000 to 10,000
Практически нерастворим	Practically insoluble	greater than 10,000

The term "partly soluble" is used to describe a mixture containing both soluble and insoluble components.
The term "miscible" is used to describe a liquid that mixes with the specified solvent in all ratios.

2.3.7.0. Functionality-related characteristics of excipients

This chapter and the sections of monographs "Functionality-related characteristics" are not mandatory and are published for information and recommendations.

INTRODUCTION

Excipients whose safety was evaluated earlier are used in the composition of drug preparations to give them certain functions. The main purpose of the auxiliary substance is to provide the required physicochemical and biopharmaceutical properties of the product.

The functionality-related characteristics of excipients are determined by their physical and chemical properties and, in some cases, by the content of by-products or additives used to improve the desired functionality-related characteristics. In addition, these characteristics may depend on complex interactions between the components of the drug and on external influences during the manufacturing process. Therefore, the functionality-related characteristics of excipients can only be determined in the context of a specific composition and production process, often using several analytical techniques.

Some properties of excipients (such as the particle size of the excipient when used in production or the molecular weight of the polymer material used as a component that increases the viscosity of the drug) may relate to functional characteristics in a broader sense. Such functionality-related characteristics (FRCs) can be controlled and may be subject to a product-specific quality specification when the pharmaceutical development work has demonstrated their critical role in the manufacturing process and quality attributes of the drug product. Such critical FRCs may be described as critical quality attributes (CQAs) for the drug product.

Knowledge of these characteristics can facilitate the application of process analytical technology (PAT).

FRCs are included in monographs for excipients to help drug manufacturers establish quality specification requirements based on standard analytical techniques. These characteristics allow finding mutual understanding between manufacturers and consumers of excipients in order to supply excipients with specified properties. FRCs can be specified (for example, in the certificate) by the manufacturer of excipients with reference to the monograph, which contains a description of the method used to control a certain parameter. The section of monographs "Functionality-related characteristics" includes parameters for which the effect on the functionality of the excipient for the intended use is established. Due to the multi-purpose use of many excipients and the development of new ways to use them, the listed FRCs and methods of their use are not exhaustive.

PHYSICAL GRADES OF SUBSTANCES

Excipients that are particulate solids can be available in a variety of physical grades, for example with regard to particle-size distribution, which is usually controlled by the excipient supplier. However, FRCs for these excipients may concern a wide range of properties, resulting from solid-state properties and properties of the particulate solid, which may not be controlled by the excipient supplier.

Properties of particulate solids include for example particle-size distribution, specific surface area, bulk density, flowability, wettability, and water sorption. Depending on the size range, the particle-size distribution can be determined by a sieve analysis (see general chapter 2.9.38. Particle-size distribution estimation by analytical sieving) or instrumental methods, for example, Particle size analysis by laser light diffraction.

The method described in chapter Specific surface area by gas adsorption is based on the Brunauer-Emmett-Teller (BET) procedure. Methods to characterise flowability and bulk density of powders are described in general chapters Powder flow and Bulk density and tapped density. Solid-state properties may have an impact on the wettability (see general chapter Wettability of porous solids including powders) and water-solid interactions (see general chapter Water-solid interactions: determination of sorption-desorption isotherms and of water activity) of particulate solids.

Examples of solid-state properties to be considered in the development of solid dosage forms include polymorphism, pseudopolymorphism, crystallinity, and density. Procedures of their evaluation them are given in general chapters 2.3.4.0. Polymorphism, 2.3.8.0. Crystallinity and Density of solids.

CLASSES OF SUBSTANCES BASED ON CHEMICAL CHARACTERISTICS

The available excipients are presented as classes with different chemical characteristics and are of natural, semi-synthetic or synthetic origin. Monographs usually control the chemical composition of excipients that are a mixture of related compounds, such as the composition of fatty acids in vegetable oils or surfactants. At the same time, the Pharmacopoeia contains monographs describing a class of plastic materials that may differ in composition depending on the structure of homopolymers, polymer blocks and copolymers, the degree of polymerisation, and, consequently, by mass and molecular weight distribution, the degree of substitution, and, in some cases, even by substituents in the polymer chain. These changes can have a significant impact on the functional characteristics of excipients and should be taken into account in pharmaceutical development; it is preferable to set a range of acceptable values for each parameter that is critical for both the production process and the characteristics of the drug.

"FUNCTIONALITY-RELATED CHARACTERISTICS" SECTION IN MONOGRAPHS

Monographs on excipients may have a section entitled "Functionality-related characteristics" included for information for the user and is not a mandatory part of the monograph. The section gives a statement of characteristics that are known to be relevant for certain uses of the excipient. The use for which the characteristic is relevant is stated, for other uses, the characteristic may be irrelevant. For this reason, the section should not be seen simply as a supplement to the monograph. It is the responsibility of the manufacturer of the drug product to decide how the information on "Functionality-related characteristics" will be applied in the manufacturing process in light of the use of the excipient and data from pharmaceutical development.

The information on the functionality-related characteristics may be given in different ways:

- name of the FRC;
- name of the FRC and a recommended method for its determination, referring wherever possible to a general chapter of the Pharmacopoeia;
- name of the FRC with a recommended method for its determination and typical values, which may be in the form of tolerances from the nominal value.

A given characteristic may be the subject of a mandatory requirement in the monograph. If it is relevant for certain uses, it is also referred to in the "Functionality-related characteristics" section as a relevant characteristic that the manufacturer of the medicinal product may choose to indicate in the excipient grade specification of a particular drug preparation used.

The section on FRCs is intended to reflect current knowledge related to the major uses of an excipient. In view of the multiple uses of some excipients and the continuous development of new uses, the section may not be complete. In addition, the methods cited for the determination of a particular characteristic are given as recommendations, and the use of other methods is not excluded.

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2.3.8.0. Crystallinity

This chapter provides general information on crystallinity and refers to the various techniques described in the Pharmacopoeia that are used for its determination.

INTRODUCTION – THE CONCEPT OF CRYSTALLINITY

Most organic and inorganic substances of pharmaceutical relevance exist as a solid substances, which can be characterised by a structure located between a perfectly ordered crystal and an amorphous substance.

Real crystals lie somewhere between an ideal crystal state and the amorphous state. The position of a crystal on a scale bounded by these two extremes is termed its crystallinity.

A perfectly ordered crystal is an ideal substance state that is seldom, if ever, achieved. The structural units of a crystal, termed unit cells, are repeated regularly and indefinitely in three dimensions in space. The unit cell has a definite orientation and shape defined by the translational vectors *a*, *b* and *c*, and the angles α, β and γ, and hence has a definite volume, *V*, that contains the atoms and molecules necessary for forming the crystal. A crystalline system is defined by three long-range order symmetry operators (translational, orientational and conformational); the various mesophases (liquid crystals, crystals and plastic crystals) have one or two of the long-range symmetry operators and the ideal amorphous state is defined by the absence of all three operators.

Each crystal can be classified as a member of one of 7 possible crystal systems that are defined by the relationships between the individual dimensions *a*, *b* and *c* and between the individual angles α, β and γ of the unit cell. The structure of a given crystal may be classified according to one of the 7 crystal systems, to one of the 14 Bravais lattices and to one of the 230 space groups. All the 230 possible space groups, their symmetries and the symmetries of their diffraction patterns are compiled in the *International Tables for Crystallography*.

Many substances are capable of crystallising in greater than one type of crystal lattice, which is known as polymorphism. The occurrence of polymorphism is a common phenomenon among organic molecules, giving rise to different physicochemical properties. Crystalline polymorphs have the same chemical composition but different internal crystal structures and, therefore, possess different physicochemical properties. The different crystal structures in polymorphs are due to different atomic packing arrangements and/or different conformations of the molecules (see chapter *2.3.4.0. Polymorphism*).

The other extreme of a crystal state is the ideal or true amorphous state, where all long-range order is lost. For most organic systems certain short-range order remains, but this is not expected to extend over distances much larger than nearest neighbour (NN) or next nearest neighbour (NNN) interactions, which are typically less than 2-2.5 nm for small organic molecules.

Amorphous substance is characterised by the absence of distinct reflections in the X-ray powder diffraction pattern.

The crystallinity of a real powder can be considered by two models of crystallinity. In the 1-state model all particles will be of the same crystallinity whereas in the 2-state model each particle can be either crystalline or amorphous, such that the actual crystallinity of the powder is the weighted average of these two extreme crystallinities. Such a powder is obtained when pure crystalline and amorphous phases are physically mixed. In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with different sizes and shapes.

The extent of disorder in a crystalline solid can affect many physicochemical properties of substances for pharmaceutical use. Because of the great relevance of these properties, it is important to be able to assess the extent of disorder or the crystallinity of a solid by a suitable quantitative method.

METHODS FOR MONITORING AND DETERMINING CRYSTALLINITY

Various methods are available for determining the crystallinity of a solid. Many techniques cannot detect or quantify these properties independently, for this reason, it is useful to combine several of the methods described below. Such methods often do not give accurate results and limits of quantitation are usually much greater than those for chemical impurities. In addition, certain assumptions have to be made about the relationship between standards used for calibration, which are typically mixtures of crystalline and amorphous particles (2-state model), and the samples to be analysed that are likely to have at least a small component of a substance exhibiting 1-state model behavior. Finally, the lack of well-defined standards for 100% crystalline or 100% amorphous substance complicates the validation of such methods. As explained above, it is obvious that different amorphous or non-crystalline phases exist and even co-exist in a solid powder. These different non-crystalline forms of a solid can give different responses depending on the techniques used for determining the degree of crystallinity.

X-ray powder diffraction. This method is the most commonly used method for determining the degree of crystallinity, although this method suffers from some limitations due to peak broadening, amorphous halo and preferred orientation, which make interpretation and quantitation difficult.

X-ray powder diffraction alone is often insufficient to distinguish between the different non-crystalline phases. The X-ray diffraction pattern of a purely amorphous and nanocrystalline phase is characteristic of a broad diffuse halo. In-depth analysis of the X-ray diffraction patterns will show that the diffuse halo in the pattern of nanocrystalline material shows some correlation to the pattern of the parent crystalline phase, while in the case of a pure amorphous phase such a correlation does not exist. Additional techniques may be required to establish the true nature of X-ray amorphous materials.

Thermal analysis. Thermal analysis of crystalline materials exhibits a melting transition that is often accompanied by decomposition or evaporation of solvents. In the case of true amorphous substances, thermal analysis reveals a glass transition, whereas only a melt would be expected for a nanocrystalline material.

Microcalorimetry. Microcalorimetry is a highly sensitive technique which allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure. Amorphous parts of a substance can recrystallise by subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The measurement of the heat of recrystallisation enables the amorphous substance to be determined from the enthalpy of recrystallisation. By relating the output from the microcalorimeter for a sample to that obtained for an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1% can be reached.

Solution calorimetry. The crystallinity of the solid test sample is given by the enthalpy of solution of the solid sample (ΔH_x^s) minus the enthalpy of solution of the chosen reference standard of the same substance (ΔH_r^s) when determined under the same conditions. Because the reference standard is usually chosen for its perceived high crystallinity, its enthalpy of solution is usually algebraically greater (more endothermic or less exothermic) than that of the solid test sample in the same solvent. Consequently, the crystallinity determined is a negative quantity with the SI units kJ/mol or J/g (J/kg is avoided because of its unwieldiness and potential for error). The preference for a negative value with respect to a highly crystalline reference standard recognises the fact that most samples have a lower crystallinity than this reference standard.

Near-infrared spectroscopy (NIR). Near-infrared (NIR) spectroscopy (*2.1.2.34*) is another technique used to measure the degree of crystallinity, and has also been proven to be useful in studies of polymorphism. The NIR spectrum of a sample contains both physical and chemical information. Being non-invasive, non-destructive and operable at room temperature, the method is a valuable tool to assess changes in the amorphous and crystalline state.

Infrared absorption spectrophotometry and Raman spectrometry. Infrared absorption spectrophotometry and Raman spectrometry (*2.1.2.23*) are other techniques used to measure the degree of crystallinity, and have also been proven to be useful in studies of polymorphism.

Solid-state NMR. Solid-state nuclear magnetic resonance spectrometry (ss NMR) can be used to provide information about polymorphism and related relative molecular conformations. However, some caution has to be exercised in the interpretation of results, since it is not always simple to distinguish between samples that comprise a mixture of different physical forms (2-state model) and those that comprise crystals having disorder with an exchange that is slow on the NMR timescale. Similarly, samples that contain defects arising from different molecular conformations or slightly different packing arrangements (1-state model) may show additional signals. Solid-state NMR may be quite sensitive to this, even if lattice parameters are hardly affected and, consequently, little or no change is observed by X-ray powder diffraction. It is evident that the crystallinity of substances for pharmaceutical use can be complex, and both crystalline defects and amorphous material may co-exist.

Optical microscopy. A method to detect whether or not particles are crystalline is to use a polarising microscope (*2.1.9.13*), where particles show birefringence and extinction positions when the microscope stage is revolved.

2.3.9.0. Recommendations on Methods for Dosage Forms Testing

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2.3.9.1. Recommendations on Dissolution Testing

This general chapter is non-mandatory; it provides information on dissolution testing, on recommended dissolution media and on the expression of dissolution specifications for oral dosage forms (see general chapter 2.1.9.3. Dissolution test for solid dosage forms). This information represents generally accepted parameters used in the field of dissolution.

In the determination of the dissolution rate of the active substance(s) of a solid dosage form, the following are to be specified:

- the apparatus to be used, and in cases where the flow-through apparatus is specified, the flow-through cell;
- the composition, the volume and the temperature of the dissolution medium;
- the rotational speed or the flow rate of the dissolution medium;
- the time, the method and the amount for sampling of the test solution or the conditions for continuous monitoring;
- the method of analysis;
- acceptance criteria.

The choice of apparatus to be used depends on the physicochemical characteristics of the dosage form. When a large quantity of dissolution medium is required to ensure sink conditions, or when a change of pH is necessary, the flow-through apparatus may be preferred.

EXPERIMENTAL TESTING CONDITIONS

The use of the basket and the paddle apparatus and the reciprocating cylinder apparatus is generally based on the principle of operating under sink conditions, i.e. in such a manner that the substance already in solution does not exert a significant modifying effect on the dissolution rate of the remainder. Sink conditions normally occur in a volume of dissolution medium that is at least 3-10 times the saturation volume.

In general, an aqueous medium is used. The composition of the medium is chosen on the basis of the physicochemical characteristics of the active substance(s) and excipient(s) within the range of conditions to which the drug preparation is likely to be exposed after its administration. This applies in particular to the pH and the ionic strength of the dissolution medium.

The pH of the dissolution medium is usually set between pH 1 and pH 8. In justified cases, a higher pH may be needed. For the lower pH values in the acidic range, as a rule, use *0.1 M hydrochloric acid.* Recommended dissolution media are listed later in this section.

Water is recommended as a dissolution medium only when it is proven that the pH variations do not have an influence on the dissolution characteristics.

In specific cases, and subject to approval by the competent authority, dissolution media may contain enzymes, surfactants, further inorganic substances and organic substances. For the testing of preparations containing poorly aqueous-soluble active substances, modification of the medium may be necessary. In such circumstances, a low concentration of surfactant is recommended; it is recommended to avoid the use of organic solvents.

Gases dissolved in the dissolution medium can affect the results of the dissolution test. This is true in particular for the flow-through apparatus, where de-aeration of the medium is necessary to avoid the formation of gas bubbles in the flow-through cell. A suitable method of de-aeration is as follows: heat the medium while stirring gently to about 41 °C, immediately filter under vacuum using a filter with a porosity of 0.45 μm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min.

A different validated method for removing dissolved gases can also be used.

Using the paddle or basket apparatus, the volume of dissolution medium is normally 500-1000 mL. A rotational speed of between 50 rpm and 100 rpm is normally chosen; it must not exceed 150 rpm.

For the flow-through apparatus, the dissolution medium flow rate is normally set between 4 mL/min and 50 mL/min.

RECOMMENDED DISSOLUTION MEDIA

The following dissolution media can be used.

The composition and preparation of the media are indicated below.

Hydrochloric acid media

- *0.2 M hydrochloric acid.*

- *0.2 M sodium chloride*. Dissolve 11.69 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

For preparing media with the pH values indicated in Table 2.3.9.1.-2, mix 250.0 mL of 0.2 M sodium chloride and the specified volume of *0.2 M hydrochloric acid*, and dilute to 1000.0 mL with *water R*.

The hydrochloric acid media may also be prepared by replacing sodium chloride with potassium chloride.

Table 2.3.9.1.-1. – *Examples of dissolution media*

pH	Dissolution medium
pH 1.0	HCl
pH 1.2	NaCl, HCl
pH 1.5	NaCl, HCl
pH 4.5	Phosphate or acetate buffer solution
pH 5.5 and pH 5.8	Phosphate or acetate buffer solution
pH 6.8	Phosphate buffer solution
pH 7.2 and pH 7.5	Phosphate buffer solution

Acetate buffer solutions

- *2 M acetic acid*. Dilute 120.0 g of *glacial acetic acid R* with *water R* to 1000.0 mL.

- *Acetate buffer solution pH 4.5*. Dissolve 2.99 g of *sodium acetate R* in *water R*, add 14.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

- *Acetate buffer solution pH 5.5*. Dissolve 5.98 g of *sodium acetate R* in *water R*, add 3.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

- *Acetate buffer solution pH 5.8*. Dissolve 6.23 g of *sodium acetate R* in *water R*, add 2.1 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

Phosphate buffer solutions

For preparing buffers with the pH values indicated in Table 2.3.9.1.-3, mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and the specified volume of *0.2 M sodium hydroxide*, and dilute to 1000.0 mL with *water R*.

Other phosphate buffer solutions

- *Phosphate buffer solution pH 4.5*. Dissolve 13.61 g of *potassium dihydrogen phosphate R* in 750 mL of *water R*.

Table 2.3.9.1.-2. – *Media with hydrochloric acid*

pH	HCl (mL)
1.2	425.0
1.3	336.0
1.4	266.0
1.5	207.0
1.6	162.0
1.7	130.0
1.8	102.0
1.9	81.0
2.0	65.0
2.1	51.0
2.2	39.0

Table 2.3.9.1.-3. – *Phosphate buffer solutions*

pH	5.8	6.0	6.2	6.4	6.6	6.8
NaOH (mL)	18.0	28.0	40.5	58.0	82.0	112.0
pH	7.0	7.2	7.4	7.6	7.8	8.0
NaOH (mL)	145.5	173.5	195.5	212.0	222.5	230.5

Adjust the pH (*2.1.2.3*) if necessary with *0.1 M sodium hydroxide* or with *0.1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

- *Phosphate buffer solution pH 5.5 R.*
- *Phosphate buffer solution pH 6.8 R1.*
- *Buffer solution pH 7.2 R.*
– *0.33 M phosphate buffer solution pH 7.5 R.*

Simulated intestinal fluid pH 6.8

Mix 77.0 mL of *0.2 M sodium hydroxide*, 250.0 mL of a solution containing 6.8 g of *potassium dihydrogen phosphate R*, and 500 mL of *water R*, add 10.0 g of *pancreas powder R*, mix and adjust the pH (*2.1.2.3*) if necessary, and dilute to 1000.0 mL with *water R*.

Simulated gastric fluid

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*, add 80 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*. If required, pepsin powder may be omitted.

Increasing pH

For a test involving increasing pH, one of the following sequences may be used:

Time (h)	0-1	1-2	2-3 3-4	4-5 5-6	6-7 7
pH	1.0				
pH	1.2	6.87			
pH	1.2	2.5	4.5	7.0	7.5
pH	1.5	4.5		7.2	

To achieve this pH variation, it is possible either:

- to substitute one buffer solution for another (whole substitution);

- or to remove only half of the medium each time (half change method) and replace it with a buffer solution of higher pH: the initial pH is 1.2 and the second solution is phosphate buffer solution pH 7.5; or,

- or to an initial buffer solution at pH 1.5, to add a dose of a powder mixture containing *tris(hydroxymethyl)aminomethane R* and *anhydrous sodium acetate R* to obtain pH 4.5 and a second dose to obtain pH 7.2, as described below:

- *hydrochloric acid pH 1.5*: dissolve 2 g of *sodium chloride R* in *water R*, add 31.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*;

- *buffer solution pH 4.5:* mix 2.28 g of *tris(hydroxymethyl)aminomethane R* with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the *hydrochloric acid pH 1.5* described above;

- *buffer solution pH 7.2*: mix 2.28 g of *tris(hydroxymethyl)aminomethane* R with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the buffer solution pH 4.5 described above.

The flow-through cell may be used for the continuous change of pH.

QUALIFICATION AND VALIDATION

Due to the nature of the test method, quality by design is an important qualification aspect for *in vitro* dissolution test equipment. Any irregularities such as vibration or undesired agitation by mechanical imperfections are to be avoided.

Qualification of the dissolution test equipment has to consider the dimensions and tolerances of the apparatus. Critical test parameters, such as temperature and volume of dissolution medium, rotational speed or liquid flow rate, sampling, and procedures should be monitored periodically.

The performance of the dissolution test equipment may be monitored by testing a reference product that is sensitive to hydrodynamic conditions. Such tests may be performed periodically or continuously for comparative reasons with other laboratories.

During testing, critical inspection and observation are required, and this approach is especially important to explain any outlying results.

Validation of automated systems, whether concerning the sampling and analytical part of the dissolution media preparation and test performance, has to consider accuracy, precision, and the avoidance of contamination by any dilutions, transfers, cleaning, and sample or solvent preparation procedures.

EXPRESSION OF DISSOLUTION SPECIFICATIONS FOR ORAL DOSAGE FORMS

The dissolution specification is expressed in terms of the quantity (*Q*) of active substance dissolved in a specified time, expressed as a percentage of the content stated on the product label.

Conventional-release dosage forms

In most cases, when tested under reasonable and justified test conditions, the acceptance criteria at level *S*1 are that at least 80% of the active substance is released within a specified time, typically 45 min or less. This corresponds to a *Q* value of 75%, since, as referred to in Table 2.9.3.-1, for level *S*1 the individual value of each of the 6 units tested is not less than (*Q* + 5) %, i.e. not less than 80%.

Typically, a single-point acceptance criterion is sufficient to demonstrate that the majority of the active substance has been released, although in certain circumstances it may be necessary to test at the additional time point(s), in order to demonstrate adequate dissolution.

Prolonged-release solid dosage forms

The dissolution test acceptance criteria for prolonged-release dosage forms is normally expected to consist of 3 or more points. The 1st specification point is intended to prevent the unintended rapid release of the active substance ("dose dumping"). It is therefore set after a testing period corresponding to a dissolved amount of the active substance typically of 20% to 30%. The second specification point defines the dissolution pattern and so is set at around 50% release of the active substance. The final specification point is intended to ensure almost complete release, which is generally understood as greater than 80% release of the active substance.

Delayed-release solid dosage forms

A delayed-release dosage form may release the active substance(s) fractionally or totally according to the formulation design when tested in different dissolution media, e.g. in increasing pH conditions. Dissolution specifications, therefore, have to be decided on a case-by-case basis.

Gastro-resistant dosage forms require at least 2 specification points in a sequential test and 2 different specifications in a parallel test. In a sequential test, the first specification point represents an upper limit and is set after 1 h or 2 h in an acidic medium, and the second after a pre-set time period of testing in an adequate buffer solution (preferably pH 6.8).

In most cases, the acceptance criteria at level *B*1 are that at least 80% of the active substance is released. This corresponds to a *Q* value of 75%, since, as referred to in Table 2.3.9.1.-4, for level *B*1 the individual value of each of the 6 units tested is not less than (*Q* + 5) %, i.e. not less than 80%.

APPENDIX

401000000-2019

4.1. Alcoholimetric Tables

Table 4.1.-1. – *The ratio between the density of the water-alcohol solution and the ethanol content in the solution*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0.99823	0.00	0.00	0.00	0.00
80	12	16	13	16
0.9978	23	29	23	29
6	34	43	34	43
4	44	56	44	56
2	55	70	55	70
0	66	83	66	83
0.9968	77	97	77	97
6	87	1.10	87	1.10
4	98	24	98	24
2	1.09	38	1.09	38
0	20	51	19	51
0.9958	31	65	32	66
6	42	79	41	80
4	52	92	52	93
2	63	2.06	63	2.07
0	74	20	74	21
0.9948	85	34	85	35
6	96	48	96	50
4	2.07	62	2.07	64
2	19	76	18	78
0	29	90	29	92
0.9938	41	3.04	40	3.06
6	52	18	51	20
4	63	32	62	34
2	75	46	73	48
0	86	60	84	63
0.9928	97	74	95	77
6	3.09	89	3.07	92
4	20	4.03	18	4.06
2	32	17	29	20
0	44	32	41	36
0.9918	55	46	52	50
6	67	61	64	65

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	78	75	75	80
2	90	90	87	95
0	4.02	5.05	99	5.10
0.9908	14	20	4.10	25
6	26	35	22	41
4	38	50	34	56
2	50	65	46	71
0	62	80	58	87
0.9898	75	95	70	6.02
6	87	6.10	81	17
4	99	26	94	34
2	5.11	41	5.06	49
0	24	57	19	65
0.9888	37	73	31	81
6	49	88	43	97
4	62	7.04	56	7.13
2	75	20	68	29
0	87	36	81	46
0.9878	6.00	52	94	62
6	13	67	6.05	77
4	26	83	18	94
2	39	99	31	8.10
0	52	8.15	43	27
0.9868	65	32	57	44
6	78	48	69	61
4	92	64	82	77
2	7.05	80	95	93
0	18	97	7.08	9.11
0.9858	32	9.13	21	27
6	45	30	34	45
4	58	47	47	62
2	72	63	60	78
0	85	80	73	96
0.9848	99	97	87	10.13

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
6	8.12	10.13	8.00	30
4	26	30	13	47
2	39	47	26	65
0	53	63	39	82
0.9838	67	80	52	99
6	80	97	66	11.17
4	94	11.14	79	34
2	9.08	31	93	52
0	22	48	9.06	70
0.9828	35	65	19	87
6	49	82	33	12.04
4	63	99	46	22
2	77	12.16	60	40
0	91	34	74	58
0.9818	10.05	51	87	75
6	19	68	10.01	93
4	34	85	14	13.11
2	48	13.03	28	29
0	62	20	42	47
0.9808	76	38	56	66
6	91	55	69	83
4	11.05	73	84	14.02
2	20	90	97	20
0	34	14.08	11.11	38
0.9798	49	26	25	57
6	64	44	40	76
4	78	62	54	94
2	93	79	67	15.12
0	12.07	97	82	31
0.9788	22	15.15	96	50
6	37	34	12.11	69
4	52	52	25	88
2	67	70	39	16.07
0	81	88	53	26
0.9778	96	16.06	68	44
6	13.11	25	83	66
4	27	43	97	83
2	42	61	13.11	17.01
0	57	80	26	21
0.9768	72	98	40	40
6	87	17.17	55	60
4	14.02	35	69	79
2	18	54	84	99

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0	33	73	99	18.19
0.9758	49	91	14.14	38
6	64	18.10	29	58
4	80	29	44	78
2	96	48	59	97
0	15.11	67	74	19.17
0.9748	27	86	89	37
6	43	19.05	15.04	57
4	58	24	19	77
2	74	43	34	97
0	90	62	49	20.16
0.9738	16.05	81	64	36
6	21	20.00	79	56
4	37	19	94	76
2	52	37	16.08	95
0	68	56	23	21.15
0.9728	84	75	38	35
6	99	93	52	54
4	17.15	21.12	67	74
2	30	31	82	94
0	45	49	96	22.13
0.9718	61	68	17.11	33
6	76	86	25	52
4	92	22.05	40	72
2	18.07	23	55	91
0	22	41	69	23.10
0.9708	37	60	84	31
6	52	78	98	50
4	67	96	18.12	69
2	83	23.14	26	88
0	98	32	41	24.07
0.9698	19.13	50	55	26
6	28	68	69	45
4	43	86	83	64
2	58	24.04	97	83
0	73	22	19.12	25.02
0.9688	88	40	26	21
6	20.03	57	39	40
4	18	75	53	59
2	33	93	68	77
0	47	25.11	82	96
0.9678	62	28	95	26.15
6	77	46	20.09	34

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	92	64	24	53
2	21.07	81	37	72
0	21	99	51	91
0.9668	36	26.16	65	27.09
6	50	34	79	28
4	65	51	92	47
2	80	68	21.06	65
0	94	85	19	83
0.9658	22.09	27.03	33	28.02
6	23	20	47	20
4	37	37	60	38
2	52	54	74	56
0	66	71	87	75
0.9648	81	88	22.00	93
6	95	28.05	14	29.12
4	23.09	22	27	29
2	23	38	40	47
0	38	55	53	65
0.9638	52	72	67	83
6	66	88	79	30.00
4	80	29.05	93	18
2	94	21	23.05	36
0	24.08	38	19	54
0.9628	22	54	32	71
6	36	71	45	90
4	50	87	58	31.07
2	64	30.03	70	24
0	78	19	83	42
0.9618	92	35	95	60
6	25.05	52	24.09	78
4	19	68	21	95
2	32	84	34	32.12
0	46	31.00	47	30
0.9608	59	16	59	48
6	73	31	71	63
4	86	47	84	81
2	26.00	63	96	98
0	13	78	25.08	33.14
0.9598	26	94	21	31
6	39	32.09	33	48
4	52	24	45	64
2	65	39	56	81
0	78	54	68	96

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0.9588	92	69	80	34.13
6	27.04	84	92	29
4	17	99	26.04	46
2	30	33.14	16	62
0	43	29	27	79
0.9578	55	44	39	95
6	68	59	51	35.11
4	81	73	62	26
2	94	88	74	43
0	28.06	34.03	86	59
0.9568	19	17	97	75
6	31	31	27.08	90
4	43	45	19	36.06
2	56	60	31	22
0	68	74	42	37
0.9558	80	88	53	53
6	93	35.02	64	68
4	29.05	16	75	84
2	17	30	86	99
0	29	44	97	37.15
0.9548	41	58	28.07	30
6	53	72	19	46
4	65	85	30	51
2	77	99	41	76
0	89	36.13	52	92
0.9538	30.01	26	62	38.06
6	13	40	73	21
4	25	53	83	36
2	36	67	94	51
0	48	80	29.05	66
0.9528	60	94	16	81
6	72	37.07	26	96
4	84	20	36	39.10
2	95	34	47	25
0	31.07	47	57	40
0.9518	18	60	68	55
6	30	73	78	69
4	41	86	88	84
2	53	99	98	98
0	64	38.12	30.09	40.12
0.9508	76	25	19	27
6	87	38	29	42
4	99	51	39	56

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
2	32.10	64	50	70
0	21	77	60	85
0.9498	33	90	70	41.00
6	44	39.03	81	14
4	55	15	90	28
2	66	28	31.00	42
0	78	40	10	56
0.9488	89	53	20	71
6	33.00	66	30	86
4	11	78	40	99
2	22	91	50	42.14
0	33	40.04	60	28
0.9478	44	46	70	42
6	55	28	79	56
4	66	41	89	70
2	77	53	99	84
0	88	65	32.08	98
0.9468	99	78	18	43.12
6	34.10	90	28	26
4	21	41.02	38	39
2	32	15	48	54
0	43	27	57	68
0.9458	54	39	67	81
6	65	51	76	95
4	76	63	86	44.08
2	86	75	95	22
0	97	87	33.05	35
0.9448	35.08	99	14	49
6	19	42.11	24	63
4	29	23	33	76
2	40	35	43	90
0	50	46	51	45.03
0.9438	61	58	61	17
6	71	70	70	30
4	82	82	80	44
2	93	94	89	58
0	36.03	43.05	98	71
0.9428	13	17	34.07	84
6	24	28	16	97
4	34	39	25	46.10
2	45	51	34	23
0	55	62	43	36
0.9418	65	74	52	49

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
6	76	85	61	62
4	86	97	70	75
2	96	44.08	79	88
0	37.07	19	88	47.01
0.9408	17	30	96	14
6	27	42	35.06	27
4	37	53	15	41
2	47	64	23	53
0	58	75	32	66
0.9398	68	86	41	79
6	78	98	50	93
4	88	45.09	59	48.06
2	98	20	68	18
0	38.09	31	76	31
0.9388	19	42	85	43
6	29	53	94	56
4	39	64	36.02	69
2	49	75	11	82
0	59	86	20	95
0.9378	69	97	28	49.07
6	79	46.08	37	20
4	89	19	46	33
2	99	30	54	46
0	39.09	41	63	58
0.9368	19	52	72	71
6	29	63	80	84
4	39	73	88	96
2	49	84	97	50.08
0	59	95	37.06	21
0.9358	69	47.06	14	34
6	79	17	23	47
4	89	27	31	59
2	99	38	40	72
0	40.09	49	48	85
0.9348	19	59	56	97
6	29	70	65	51.10
4	38	81	73	22
2	48	92	82	35
0	58	48.02	90	47
0.9338	68	13	99	60
6	78	23	38.07	72
4	88	33	15	84
2	98	44	23	97

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0	41.07	54	31	52.09
0.9328	17	65	40	22
6	27	75	48	34
4	36	86	56	46
2	46	96	64	58
0	56	49.07	73	71
0.9318	65	17	81	83
6	75	27	89	95
4	85	38	97	53.08
2	94	48	39.05	20
0	42.04	58	13	32
0.9308	13	69	22	45
6	23	79	30	56
4	33	89	38	68
2	42	99	46	80
0	52	50.10	54	93
0.9298	61	20	62	54.05
6	71	30	70	17
4	80	40	78	29
2	90	50	86	41
0	43.00	60	94	53
0.9288	09	71	40.02	66
6	18	81	10	78
4	28	91	18	90
2	37	51.01	26	55.02
0	47	11	34	14
0.9278	56	21	42	26
6	66	31	50	38
4	75	41	58	50
2	85	51	66	92
0	94	61	73	74
0.9268	44.04	71	81	86
6	13	81	89	98
4	23	91	97	56.10
2	32	52.00	41.04	21
0	41	10	12	33
0.9258	51	20	20	45
6	60	30	28	57
4	70	40	36	69
2	79	50	44	81
0	88	60	52	93
0.9248	98	69	59	57.04
6	45.07	79	67	16

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	16	89	74	28
2	26	99	82	40
0	35	53.09	90	52
0.9238	44	18	97	63
6	53	28	42.05	75
4	63	38	13	88
2	72	48	21	58.00
0	81	57	28	11
0.9228	91	67	36	23
6	46.00	77	44	35
4	09	86	21	46
2	18	96	59	58
0	28	54.06	67	70
0.9218	37	15	74	81
6	46	25	82	93
4	55	34	89	59.05
2	65	44	97	17
0	74	54	43.05	29
0.9208	83	63	12	40
6	92	73	20	52
4	47.01	82	27	63
2	10	92	35	75
0	20	55.01	42	86
0.9198	29	11	50	98
6	38	20	57	60.10
4	47	30	65	22
2	56	39	72	33
0	65	48	79	44
0.9188	74	58	87	56
6	83	67	94	67
4	93	77	44.02	79
2	48.02	86	09	91
0	11	95	16	61.02
0.9178	20	56.05	24	14
6	29	14	31	25
4	38	23	38	37
2	47	33	46	49
0	56	42	53	60
0.9168	65	51	60	71
6	75	61	68	83
4	84	70	75	95
2	93	79	82	62.06
0	49.02	89	90	18

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0.9158	11	98	97	29
6	20	57.07	45.04	40
4	29	17	12	53
2	38	26	19	64
0	47	35	26	76
0.9148	56	44	34	87
6	65	53	41	98
4	74	62	48	63.09
2	83	72	56	21
0	92	81	63	32
0.9138	50.01	90	70	44
6	10	99	77	55
4	19	58.08	84	66
2	28	17	91	77
0	37	26	98	89
0.9128	46	35	46.05	64.00
6	55	44	12	11
4	64	54	20	23
2	73	63	27	35
0	82	72	35	46
0.9118	91	81	42	57
6	51.00	90	49	68
4	09	99	56	80
2	18	59.08	63	91
0	27	17	70	65.02
0.9108	36	26	77	14
6	45	35	84	25
4	54	44	91	36
2	63	53	99	48
0	71	62	47.06	59
0.9098	80	71	13	70
6	89	80	20	82
4	98	89	27	93
2	52.07	98	34	66.05
0	16	60.07	41	16
0.9088	25	16	48	27
6	34	25	55	39
4	43	34	62	50
2	52	43	70	61
0	60	52	77	72
0.9078	69	60	83	83
6	78	69	90	95
4	87	78	97	67.06

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
2	96	87	48.04	17
0	53.05	96	11	29
0.9068	14	61.05	18	41
6	22	14	26	52
4	31	22	32	62
2	40	31	39	73
0	49	40	46	85
0.9058	58	49	53	97
6	67	57	60	68.07
4	75	66	67	19
2	84	75	74	30
0	93	84	81	41
0.9048	24.02	92	87	52
6	11	62.01	94	63
4	19	10	49.01	75
2	28	19	08	87
0	37	27	15	96
0.9038	46	36	22	69.08
6	54	45	29	19
4	63	53	35	30
2	72	62	42	42
0	81	71	50	53
0.9028	89	79	56	63
6	98	88	63	74
4	55.07	97	70	86
2	16	63.05	76	97
0	25	14	83	70.08
0.9018	33	22	90	19
6	42	31	97	30
4	51	40	50.04	42
2	60	48	10	52
0	68	57	17	64
0.9008	77	65	24	75
6	86	74	31	86
4	95	82	37	97
2	56.03	91	44	71.08
0	12	64.00	51	20
0.8998	21	08	58	30
6	30	17	65	42
4	38	25	71	53
2	47	34	78	64
0	56	42	84	75
0.8988	65	51	92	86

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
6	73	59	99	97
4	82	68	51.05	72.08
2	91	76	11	19
0	57.00	85	18	30
0.8978	08	93	25	41
6	17	65.02	32	53
4	26	10	38	63
2	34	18	44	73
0	43	27	52	85
0.8968	52	35	58	96
6	60	43	64	73.06
4	69	52	71	18
2	78	61	78	30
0	87	69	85	41
0.8958	95	77	91	51
6	58.04	86	99	63
4	13	94	52.05	73
2	21	66.02	11	84
0	30	11	18	95
0.8948	39	19	24	74.06
6	47	27	30	17
4	56	36	38	29
2	65	44	44	39
0	74	53	51	51
0.8938	82	61	57	61
6	91	69	64	72
4	59.00	77	70	83
2	08	86	77	95
0	17	94	83	75.05
0.8928	26	67.02	90	16
6	34	11	97	27
4	43	19	53.03	39
2	52	27	09	49
0	60	36	17	61
0.8918	69	44	23	72
6	77	52	29	83
4	86	61	36	94
2	95	69	43	76.05
0	60.03	77	49	15
0.8908	12	85	55	26
6	21	94	62	38
4	29	68.02	69	49
2	38	10	75	59

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0	47	18	81	70
0.8898	55	26	88	81
6	64	35	95	93
4	72	43	54.01	77.04
2	81	51	07	14
0	90	59	14	25
0.8888	98	67	20	36
6	61.07	75	26	47
4	15	83	33	57
2	24	91	39	68
0	33	69.00	46	80
0.8878	41	08	52	91
6	50	16	59	78.02
4	58	24	65	12
2	67	32	71	23
0	76	40	78	34
0.8868	84	48	84	45
6	93	56	90	56
4	62.01	64	96	66
2	10	72	55.03	77
0	18	80	09	88
0.8858	27	88	15	99
6	36	96	22	79.10
4	44	70.05	29	21
2	53	12	34	31
0	61	20	41	42
0.8848	70	28	47	53
6	79	36	53	64
4	87	45	60	75
2	96	53	67	86
0	63.04	61	73	97
0.8838	13	69	79	80.08
6	21	77	86	19
4	30	85	92	30
2	39	93	98	40
0	47	71.01	56.05	51
0.8828	56	09	11	62
6	64	17	17	73
4	73	25	24	84
2	82	35	30	95
0	90	41	36	81.06
0.8818	99	49	42	17
6	64.07	57	49	28

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	16	65	55	39
2	24	72	61	49
0	33	80	67	60
0.8808	41	88	73	70
6	50	96	80	81
4	59	72.04	86	93
2	67	12	92	82.04
0	76	20	99	15
0.8798	84	28	57.05	25
6	93	36	11	36
4	65.01	44	17	47
2	10	51	23	57
0	18	59	29	68
0.8788	27	67	36	79
6	35	75	42	90
4	44	83	48	83.01
2	52	91	55	12
0	61	98	60	22
0.8778	69	73.06	66	33
6	78	14	73	45
4	86	22	79	56
2	95	29	85	66
0	66.03	37	91	77
0.8768	12	45	97	87
6	20	53	58.03	98
4	29	60	09	84.08
2	37	68	15	19
0	46	76	22	30
0.8758	54	84	28	42
6	63	91	33	51
4	71	99	40	63
2	80	74.07	46	74
0	88	15	52	85
0.8748	97	22	58	95
6	67.05	30	64	85.06
4	14	37	70	16
2	22	45	76	27
0	31	53	82	38
0.8738	39	61	89	49
6	47	68	94	59
4	56	76	59.01	70
2	64	84	07	81
0	73	91	12	91

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0.8728	81	99	19	86.03
6	90	75.06	24	12
4	98	14	31	24
2	68.07	22	37	35
0	15	29	42	45
0.8718	24	37	49	56
6	32	45	55	67
4	41	52	61	77
2	49	60	67	89
0	58	68	73	87.00
0.8708	66	75	79	10
6	75	83	85	21
4	83	90	91	31
2	92	98	97	42
0	69.00	76.06	60.03	53
0.8698	08	13	09	63
6	17	21	15	74
4	25	28	21	85
2	34	36	27	96
0	42	43	32	88.06
0.8688	51	51	39	17
6	59	58	44	27
4	68	66	51	38
2	76	74	57	50
0	84	81	62	60
0.8678	93	89	69	71
6	70.01	96	74	81
4	10	77.04	81	93
2	18	11	86	89.02
0	26	19	92	14
0.8668	35	26	998	24
6	43	33	61.03	34
4	52	41	10	46
2	60	48	15	56
0	68	56	22	67
0.8658	77	63	27	77
6	85	70	33	88
4	94	78	39	99
2	71.02	85	44	90.09
0	10	93	51	21
0.8648	19	78.00	56	31
6	27	07	62	41
4	36	15	68	53

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
2	44	22	74	63
0	52	29	79	73
0.8638	61	37	86	84
6	69	44	91	95
4	77	51	97	91.05
2	86	59	62.03	16
0	94	66	08	26
0.8628	72.03	73	14	36
6	11	81	20	47
4	19	88	26	57
2	28	95	31	68
0	37	79.03	38	79
0.8618	44	10	43	90
6	53	17	49	92.00
4	61	24	54	10
2	69	32	60	22
0	78	39	66	32
0.8608	86	46	72	42
6	95	53	77	52
4	73.03	61	83	64
2	11	68	89	74
0	20	75	94	84
0.8598	28	83	63.01	96
6	36	90	06	93.06
4	45	97	12	16
2	53	80.04	17	27
0	61	11	23	37
0.8588	70	19	29	49
6	78	26	35	59
4	86	33	40	70
2	95	40	46	80
0	74.03	47	51	90
0.8578	11	54	57	94.01
6	20	62	63	13
4	28	69	69	23
2	36	76	74	33
0	44	83	80	43
0.8568	53	90	85	54
6	61	97	91	64
4	69	81.05	97	76
2	78	12	64.03	87
0	86	19	08	97
0.8558	94	26	14	95.07

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
6	75.02	33	19	17
4	11	40	25	28
2	19	47	30	38
0	27	54	36	49
0.8548	35	61	41	59
6	44	68	47	70
4	52	75	52	80
2	60	82	58	90
0	69	89	63	96.01
0.8538	77	96	68	11
6	85	82.03	74	21
4	93	10	80	32
2	76.01	17	85	43
0	10	24	91	53
0.8528	18	31	96	63
6	26	38	65.02	74
4	35	45	08	85
2	43	52	13	95
0	51	59	19	97.06
0.8518	59	66	24	16
6	67	73	30	27
4	76	80	35	38
2	84	87	41	48
0	92	94	46	59
0.8508	77.00	83.01	52	69
6	09	08	57	80
4	17	14	62	89
2	25	21	68	99
0	33	28	73	98.10
0.8498	42	35	79	20
6	50	42	84	31
4	58	49	90	42
2	66	56	95	53
0	74	63	66.01	63
0.8488	83	69	05	73
6	91	76	11	83
4	99	83	16	93
2	78.07	90	22	99.04
0	16	97	28	15
0.8478	24	84.04	33	25
6	32	10	38	34
4	40	17	43	45
2	48	24	49	56

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0	56	31	54	67
0.8468	64	38	60	78
6	73	44	65	87
4	81	51	70	98
2	89	58	76	100.08
0	97	65	81	19
0.8458	79.05	71	86	28
6	13	78	91	39
4	22	85	97	50
2	30	91	67.02	60
0	38	98	07	70
0.8448	46	85.05	13	80
6	54	12	18	91
4	62	18	23	101.01
2	70	25	29	12
0	78	32	34	23
0.8438	87	38	39	32
6	95	45	44	42
4	80.03	51	49	52
2	11	58	55	63
0	19	65	60	74
0.8428	27	71	65	83
6	35	78	70	94
4	43	85	76	102.04
2	51	91	81	14
0	60	98	86	25
0.8418	68	86.05	92	36
6	76	11	96	45
4	84	18	68.02	56
2	92	24	07	65
0	81.00	31	12	76
0.8408	08	37	17	85
6	16	44	22	96
4	24	50	27	103.06
2	32	57	33	17
0	40	63	37	26
0.8398	48	70	43	37
6	56	76	48	47
4	64	83	53	58
2	72	89	58	67
0	80	96	63	78
0.8388	88	87.02	68	87
6	96	09	74	98

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	82.04	15	78	104.08
2	12	21	83	18
0	20	28	89	29
0.8378	28	34	93	38
6	36	41	99	49
4	44	47	69.04	59
2	52	53	08	68
0	60	60	14	79
0.8368	68	66	19	89
6	76	72	23	98
4	84	79	29	105.09
2	92	85	34	19
0	83.00	92	39	30
0.8358	08	98	44	40
6	16	88.04	49	49
4	24	11	54	61
2	32	17	59	70
0	40	23	64	80
0.8348	48	29	68	89
6	56	36	74	106.01
4	64	42	79	10
2	72	48	83	20
0	80	54	88	30
0.8338	88	61	94	42
6	96	67	98	51
4	84.04	73	70.03	61
2	11	79	08	70
0	19	86	13	82
0.8328	27	92	18	91
6	35	98	23	107.01
4	43	89.04	28	10
2	51	10	32	20
0	59	16	37	30
0.8318	67	23	43	42
6	74	29	47	52
4	82	35	52	61
2	90	41	57	71
0	98	47	62	80
0.8308	85.06	53	66	91
6	14	59	71	108.00
4	21	65	76	10
2	29	71	81	20
0	37	77	85	29

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0.8298	45	83	90	39
6	53	90	96	51
4	61	96	71.00	61
2	68	90.02	05	70
0	76	08	10	81
0.8288	84	14	14	90
6	92	20	19	109.00
4	86.00	26	24	10
2	07	32	29	20
0	15	38	33	30
0.8278	23	43	37	38
6	31	49	42	48
4	38	55	47	58
2	46	61	52	68
0	54	67	56	78
0.8268	62	73	61	88
6	69	79	66	98
4	77	85	71	110.08
2	85	91	75	18
0	93	97	80	28
0.8258	87.00	91.03	85	38
6	08	09	89	48
4	16	15	94	58
2	24	20	98	66
0	31	26	72.03	76
0.8248	39	32	08	86
6	47	38	12	96
4	54	44	18	111.06
2	62	50	23	16
0	70	55	27	25
0.8238	78	61	32	35
6	85	67	36	45
4	93	73	41	55
2	88.01	79	45	65
0	08	85	50	75
0.8228	16	90	53	83
6	24	96	58	93
4	31	92.02	63	112.03
2	39	08	68	14
0	47	13	72	22
0.8218	54	19	76	32
6	62	25	81	42
4	69	30	85	51

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
2	77	36	90	61
0	85	42	94	71
0.8208	92	47	98	81
6	89.00	53	73.03	91
4	08	58	07	113.00
2	15	64	12	10
0	23	70	17	20
0.8198	30	75	20	29
6	38	81	25	39
4	45	87	30	49
2	53	92	34	58
0	60	98	39	68
0.8188	68	93.04	43	78
6	75	09	47	86
4	83	14	51	95
2	91	20	56	114.06
0	98	25	60	15
0.8178	90.06	31	65	25
6	13	36	69	33
4	21	42	73	44
2	28	47	77	53
0	35	53	82	63
0.8168	43	58	86	72
6	50	63	90	80
4	58	69	95	91
2	65	74	99	115.00
0	73	80	74.03	10
0.8158	80	85	07	19
6	88	91	12	30
4	95	96	16	38
2	91.03	94.02	21	49
0	10	07	25	58
0.8148	17	12	29	67
6	25	17	33	76
4	32	23	37	86
2	39	28	41	95
0	47	33	45	116.03
0.8138	54	38	49	12
6	61	43	53	21
4	69	49	58	32
2	76	54	62	41
0	83	59	66	50
0.8128	91	64	70	59

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
6	98	70	74	70
4	92.05	75	78	78
2	13	80	82	87
0	20	85	86	96
0.8118	27	91	91	117.07
6	35	96	95	16
4	42	95.01	99	25
2	49	06	75.03	34
0	56	11	07	43
0.8108	64	16	11	52
6	71	21	15	61
4	78	26	19	70
2	85	31	22	80
0	93	36	26	89
0.8098	93.00	41	30	98
6	07	46	34	118.07
4	14	52	39	18
2	22	57	43	27
0	29	62	47	36
0.8088	36	67	51	45
6	43	72	55	54
4	50	77	59	63
2	58	82	63	73
0	65	87	67	82
0.8078	72	92	71	91
6	79	97	75	119.00
4	86	96.02	79	09
2	94	07	83	18
0	94.01	12	86	27
0.8068	08	16	90	35
6	15	21	94	44
4	22	26	98	53
2	29	31	76.02	63
0	36	36	05	72
0.8058	43	41	09	81
6	50	45	13	89
4	57	50	16	98
2	65	55	20	120.08
0	72	60	24	17
0.8048	79	65	28	26
6	86	70	32	35
4	93	74	35	43
2	95.00	79	39	52

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
0	07	84	43	61
0.8038	14	89	47	70
6	21	94	51	80
4	28	99	55	89
2	35	97.03	58	97
0	42	08	62	121.06
0.8028	49	12	65	14
6	56	17	69	23
4	63	22	74	33
2	70	26	77	40
0	77	31	81	50
0.8018	84	35	85	58
6	91	40	88	67
4	98	44	92	75
2	96.04	49	96	84
0	11	54	77.00	94
0.8008	18	58	03	122.01
6	25	63	07	11
4	32	67	10	19
2	39	72	14	29
0	46	76	17	36
0.7998	52	81	21	46
6	59	86	25	55
4	66	90	28	63
2	73	95	32	72
0	80	99	35	80
0.7988	87	98.04	38	90
6	93	08	41	98
4	97.00	12	44	123.06
2	07	17	48	16
0	14	21	53	24
0.7978	20	25	56	332
6	27	29	59	40
4	34	34	63	50
2	41	38	66	58
0	47	42	69	66
0.7968	54	47	73	76
6	61	51	76	84
4	67	55	79	92
2	74	59	83	99
0	81	64	86	124.09
0.7958	88	68	90	17
6	94	72	93	25

Table 4.1.-1. – *Continued*

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
4	98.01	77	97	35
2	08	81	78.00	43
0	14	85	03	51
0.7948	21	89	06	59
6	27	94	09	69
4	34	98	12	77
2	41	99.02	15	85
0	47	06	19	93
0.7938	54	10	22	125.02
6	60	14	25	10
4	67	18	28	18
2	74	22	31	26
0	80	26	34	34
0.7928	87	30	37	43
6	93	34	41	51
4	99.00	38	44	59

Density ρ_{20}	Content of ethanol in an aqueous-alcoholic solution			
	as a percentage		grams per 100 mL at 20 °C	millilitres per 100 g when weighed in air
	mass in mass	volume in volume		
2	06	42	47	67
0	13	46	50	75
0.7918	19	50	53	84
6	26	54	56	92
4	32	58	60	126.01
2	38	62	63	09
0	45	66	66	17
0.7908	51	70	69	25
6	58	74	72	33
4	64	78	75	42
2	70	82	78	50
0	77	86	82	58
0.7898	83	89	84	64
6	89	93	87	72
4	96	97	90	81
0.78927	100.00	100.00	78.93	87

Table 4.1.-2. – *The amount (in grams at a temperature of 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 kg of alcohol with a concentration of 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 92%*

Concentration of alcohol taken, %	30%		40%		50%		60%		70%		80%		90%		92%	
	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water
96	262	738	355	645	452	548	555	445	665	335	783	217	913	87	941	59
95	266	734	360	640	459	541	564	436	675	325	795	205	927	73	955	45
94	270	730	366	634	466	534	572	428	686	314	807	193	941	59	970	30
93	275	725	371	629	473	527	581	419	696	304	820	180	956	44	985	15
92	279	721	377	623	481	519	590	410	707	293	832	168	970	30		
91	283	717	383	617	488	512	599	401	717	283	845	155	985	15		
90	287	713	389	611	495	505	608	392	728	272	858	142				
89	292	708	395	605	503	497	617	383	739	261	871	129				
88	296	704	401	599	511	489	627	373	751	249	884	116				
87	301	699	407	593	518	482	636	364	762	238	898	102				
86	305	695	413	587	526	474	646	354	774	226	911	89				
85	310	690	419	581	534	466	656	344	786	214	925	75				
84	315	685	426	574	543	457	666	334	798	202	940	60				
83	320	680	432	568	551	449	676	324	810	190	954	46				
82	325	675	439	561	560	440	687	313	823	177	969	31				
81	330	670	446	554	568	432	698	302	836	164	984	16				
80	335	665	453	547	577	423	709	291	849	151						
79	340	660	460	540	587	413	720	280	863	137						
78	346	654	468	532	596	404	732	268	876	124						
77	351	649	475	525	605	395	743	257	890	CU						
76	357	643	483	517	615	385	755	245	905	95						
75	363	637	491	509	625	375	768	232	920	80						
74	369	631	499	501	636	364	781	219	935	65						
73	375	625	507	493	646	354	794	206	951	49						
72	381	619	516	484	657	343	807	193	967	33						
71	388	612	525	475	669	331	821	179	983	17						
70	394	606	534	466	680	320	835	165								
69	401	599	543	457	692	308	849	151								
68	408	592	553	447	704	296	864	136								
67	416	584	562	438	716	284	879	121								
66	423	577	572	428	729	271	895	105								
65	431	569	583	417	742	258	911	89								

Table 4.1.-2. – *End*

Concentration of alcohol taken, %	30%		40%		50%		60%		70%		80%		90%		92%	
	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water
64	438	562	593	407	756	244	928	72								
63	447	553	604	396	770	230	945	55								
62	455	545	616	384	784	216	963	37								
61	464	536	627	373	799	201	981	19								
60	472	528	639	361	815	185										
59	482	518	652	348	830	170										
58	491	509	665	335	847	153										
57	501	499	678	322	864	136										
56	511	489	692	308	881	119										
55	522	478	706	294	899	101										
54	532	468	720	280	918	82										
53	544	456	736	264	937	63										
52	555	445	751	249	958	42										
51	567	433	768	232	978	22										
50	580	420	785	215												
49	593	407	803	197												
48	607	393	821	179												
47	621	379	840	160												
46	636	364	860	140												
45	651	349	881	119												
44	667	333	902	98												
43	684	316	925	75												
42	701	299	949	51												
41	720	280	974	26												
40	739	261														
39	759	241														
38	781	219														
37	803	197														
36	826	174														
35	851	149														
34	878	122														
33	905	95														
32	935	65														

Table 4.1.-3. – *The amount (in millilitres at a temperature of 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 litre of alcohol with a concentration of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%*

Concentration of alcohol taken, %	30%		35%		40%		45 %		50%		55%		60%		65 %		70%		75%		80%		85%		90%	
	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water
95	316	707	368	658	421	607	474	556	526	504	579	451	632	397	684	343	737	288	789	233	842	176	895	119	947	61
90	333	687	389	634	444	581	500	526	556	470	611	414	667	357	722	299	778	240	833	182	89	122	944	62		
85	353	665	412	609	471	551	529	493	588	434	647	374	706	313	765	252	824	190	882	127	941	64				
80	375	641	438	581	500	519	262	457	625	394	688	330	750	265	812	200	875	134	938	67						
75	400	614	367	549	533	483	600	417	667	349	733	280	800	211	867	141	933	71								
70	429	584	500	514	571	443	643	371	714	298	786	225	857	150	929	76										
65	462	549	538	473	615	396	692	319	769	240	846	161	923	81												
60	500	509	583	426	667	343	750	258	833	173	916	87														
55	545	462	536	371	727	279	818	187	909	94																
50	600	405	700	305	800	204	900	103																		
45	667	336	778	225	889	113																				
40	750	252	875	126																						
35	857	143																								

Table 4.1.-4. – *The amount (in millilitres at a temperature of 20 °C) of water and alcohol of different concentrations that must be mixed to obtain 1 litre of alcohol with a concentration of 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%*

Concentration of alcohol taken, %	30%		35%		40%		45%		50%		55 %		60%		65%		70%		75%		80%		85%		90%		95%	
	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water
96.5	310.9	713.1	362.7	664.7	414.5	615.3	466.3	565.0	518.1	513.8	569.9	461.8	621.8	409.1	673.6	355.8	725.4	301.8	777.2	247.2	829.0	192.0	880.8	135.8	932.6	78.2	984.5	18.6
96.4	311.2	712.7	363.1	664.2	414.9	614.8	466.8	564.4	518.7	513.1	570.5	461.1	622.4	408.3	674.3	354.9	726.1	300.9	778.0	246.3	829.9	190.9	881.7	134.7	933.6	77.1	985.5	17.3
96.3	311.5	712.3	363.4	663.8	415.4	614.3	467.3	563.8	519.2	512.5	571.1	460.4	623.1	407.6	675.0	354.1	726.9	300.0	778.8	245.3	830.7	189.9	882.7	133.6	934.6	75.9	986.5	16.1
96.2	311.9	712.0	363.8	663.3	415.8	613.7	467.8	563.2	519.8	511.8	571.7	459.7	623.7	406.8	675.7	353.2	727.7	299.1	779.6	244.3	831.6	188.8	883.6	132.4	935.6	74.7	987.5	14.9
96.1	312.2	711.6	364.2	662.9	416.2	613.2	468.2	562.6	520.3	511.2	572.3	458.9	624.3	406.0	676.4	352.4	728.4	298.2	780.4	243.3	832.5	187.8	884.5	131.3	936.5	73.6	988.6	13.6
96.0	312.5	711.2	364.6	662.4	416.7	612.7	468.8	562.0	520.8	510.5	572.9	458.2	625.0	405.2	677.1	351.5	729.2	297.2	781.3	242.4	833.3	186.8	885.4	130.2	937.5	72.4	989.6	12.4
95.9	312.8	710.8	365.0	662.0	417.1	612.2	469.2	561.5	521.4	509.9	573.5	457.5	625.7	404.4	677.8	350.7	729.9	296.3	782.1	241.4	834.2	185.7	886.3	129.1	938.5	71.2	990.6	11.2
95.8	313.2	710.4	365.3	661.5	417.5	611.7	469.7	560.9	521.9	509.2	574.1	456.8	626.3	403.7	678.5	349.8	730.7	295.4	782.9	240.4	835.1	184.7	887.3	128.0	939.5	70.0	991.6	9.9
95.7	313.5	710.0	365.7	661.1	418.0	611.1	470.2	560.3	522.5	508.6	574.7	456.1	627.0	402.9	679.2	349.0	731.5	294.5	783.7	239.4	835.9	183.6	888.2	126.9	940.4	68.9	992.7	8.7
95.6	313.8	709.6	366.1	660.6	418.4	610.6	470.7	559.7	523.0	507.9	575.3	455.4	267.6	402.1	679.9	348.2	732.2	293.6	784.5	238.5	836.8	182.6	889.1	125.8	941.4	67.7	993.7	7.5
95.5	314.1	709.2	366.5	660.1	418.8	610.1	471.2	559.1	523.6	507.3	575.9	454.7	628.3	401.3	680.6	347.3	733.0	292.7	785.3	237.5	837.7	181.6	890.1	124.7	942.4	66.5	994.8	6.2
95.4	314.5	708.8	366.9	659.7	419.3	609.6	471.7	558.5	524.1	506.6	576.5	453.9	628.9	400.5	681.3	346.5	733.7	291.8	786.2	236.5	838.6	180.5	891.0	123.6	943.4	65.4	995.8	5.0
95.3	314.8	708.4	367.3	659.2	419.7	609.1	472.2	558.0	524.7	506.0	577.1	453.2	629.6	399.7	682.1	345.6	734.5	290.9	787.0	235.5	839.5	179.5	891.9	122.5	944.4	64.2	996.8	3.7
95.2	315.1	708.0	367.6	658.8	420.2	608.5	472.7	557.4	525.2	505.3	577.7	452.5	630.3	399.0	682.8	344.8	735.3	290.0	787.8	234.5	840.3	178.4	892.9	121.4	945.4	63.0	997.9	2.5
95.1	315.5	707.6	368.0	658.3	420.6	608.0	473.2	556.8	525.8	504.7	578.3	451.8	630.9	398.2	683.5	343.9	736.1	289.0	788.6	233.6	841.2	177.4	893.8	120.3	946.4	61.8	998.9	1.3

Table 4.1.-5. – *Table for obtaining alcohol of various concentrations at 20 °C*

The concentration of diluted alcohol (1000 volumes), %	Desired concentration of diluted alcohol												
	30%	35%	40%	45 %	50%	55%	60%	65%	70%	75%	80%	85%	90%
35	167												
40	335	144											
45	505	290	127										
50	674	436	255	114									
55	845	583	384	229	103								
60	1017	730	514	344	207	95							
65	1189	878	644	460	311	190	88						
70	1360	1027	774	577	417	285	175	81					
75	1535	1177	906	694	523	382	264	163	76				
80	1709	1327	1039	812	630	480	353	246	153	72			
85	1884	1478	1172	932	738	578	443	329	231	144	68		
90	2061	1630	1306	1052	847	677	535	414	310	218	138	65	
95	2239	1785	1443	1174	957	779	629	501	391	295	209	133	64

Note: The number at the intersection of the horizontal and vertical lines indicates the volume of water at 20 °C, which should be added to 1000 volumes of alcohol of the available concentration at 20 °C, to obtain the dilution.

Table 4.1.-6. – *The AMOUNT (in millilitres at a temperature of 20 °C) of water and alcohol with a concentration from 96.6% to 97.0%, which must be mixed to get 1 litre (at 20 °C) of alcohol with a concentration of 40%, 70%, 80%, 90%, 95%*

Concentration of alcohol taken, %	40%		70%		80%		90%		95%	
	alcohol	water	alcohol	water	alcohol	water	alcohol	water	alcohol	water
96.6	414.1	615.8	724.6	302.7	828.2	193.0	931.7	79.4	983.4	19.8
96.7	413.7	616.3	723.9	303.6	827.3	194.0	930.7	80.6	982.4	21.0
96.8	413.2	616.8	723.1	304.5	826.5	195.7	929.7	81.7	981.4	22.3
96.9	412.8	617.4	722.4	305.4	825.6	196.1	928.8	82.9	980.4	23.5
97.0	412.4	617.9	721.7	306.3	824.7	197.1	927.8	84.0	979.4	24.7