Standard Methods for the Analysis of Oils, Fats and Derivatives

1st Supplement to the 7th Edition

International Union of Pure and Applied Chemistry
Commission on Oils, Fats and Derivatives

FORMER CHAIRMEN

1930–1933: S. Fachini (Italy)
1933–1936: F. Rivals (France)
1936–1938: V. Vesely (Czechoslovakia)
1938–1940: H.P. Kaufmann (Germany)
1946–1949: G.L. Voerman (Netherlands)
1949–1951: K.A. Williams (United Kingdom)
1951–1953: F.D. Snell (United States)
1953–1957: J. Vizern (France)
1957–1961: H.K. Sturm (Switzerland)
1961–1964/65: G. Wolff (France)
1964/65–1969: H.A. Boekenoogen (Netherlands)
1969–1973: E. Heinerth (Fed. Rep. of Germany)
1973–1977: H.J. Vos (Netherlands)
1977–1981: C. Paquot (France)
1981–1983: D. Firestone (United States)
1983–1985: M. Naudet (France)
1985–1987: Ö. Levin (Sweden)
1987–1989: Mrs J. Beare-Rogers (Canada)

COMPOSITION OF THE COMMISSION 1 JANUARY 1991

Titular members

Chairman: W.D. Pocklington (United Kingdom)
Vice-Chairman: A. Dieffenbacher (Switzerland)
Secretary: P.W. Hendrikse (Netherlands)
A. Karleskind (France)
Mrs É. Kurucz (Hungary)
Mrs A. Visapää (Finland)
G. Zwerenz (Austria)

Associate members

Mrs A. Bonekamp [Nasner] (Germany)
H. Brüschweiler (Switzerland)
P. Dysseler (Belgium)
T. Hashimoto (Japan)
Mrs G. Hølmer (Denmark)
F.H. Koelman (Netherlands)
F. Mordret (France)
G. Ostermann (Germany)
M. Pike (United Kingdom)

National representatives

Argentina: R.R. Brenner
Australia: J.R. Sabine
Bulgaria: Mrs N. Yanishlieva
Czechoslovakia: J. Pokorný
Denmark: J.V. Holm
Germany: H. Wessels
Finland: R. Linko
Greece: D. Boscou
Hungary: Miss M. Jeránek
India: H.A. Bhakare
Israel: A. Dolev
Japan: S. Watanabe
Malaysia: S.H. Goh
Netherlands: A. Krab
New Zealand: D. Illingworth
Spain: M. Gassiot Matas
Turkey: T. Tuğlular
United States: A.E. Waltking
USSR: V.L. Roubailo

Observers

T. Asahara (Japan)
D. Firestone (United States)
Ö. Levin (Sweden)
M. Naudet (France)
R. Ohlson (Sweden)
K. Stig-Andersen (Denmark)

IUPAC Secretariat: Bank Court Chambers, 2–3 Pound Way, Templars Square, Cowley, Oxford OX4 3YF, Great Britain

International Union of Pure and Applied Chemistry
Applied Chemistry Division
Commission on Oils, Fats and Derivatives

Standard Methods for the Analysis of Oils, Fats and Derivatives

1st Supplement to the 7th Revised and Enlarged Edition

Prepared for publication by

A. DIEFFENBACHER
Nestec Ltd.
Nestlé Research Centre
Vers-chez-les-Blanc
CH-1000 Lausanne 26
Switzerland

and

W.D. POCKLINGTON
Laboratory of the Government Chemist
Food and Bioscience Division
Queens Road
Teddington, Middlesex, United Kingdom

OXFORD
BLACKWELL SCIENTIFIC PUBLICATIONS
LONDON EDINBURGH BOSTON
MELBOURNE PARIS BERLIN VIENNA

Published by
Blackwell Scientific Publications
Editorial offices:
Osney Mead, Oxford OX2 0EL
25 John Street, London WC1N 2BL
23 Ainslie Place, Edinburgh EH3 6AJ
3 Cambridge Center, Cambridge
Massachusetts 02142, USA
54 University Street, Carlton
Victoria 3053, Australia

Other Editorial Offices:
Librairie Arnette SA
2, rue Casimir-Delavigne
75006 Paris
France

Blackwell Wissenschafts-Verlag
Meinekestrasse 4
D-1000 Berlin 15
Germany

Blackwell MZV
Feldgasse 13
A-1238 Wien
Austria

First published 1987
1st Supplement issued 1992

Set by Excel Typesetters, Hong Kong
Printed in Great Britain at the
Alden Press, Oxford

DISTRIBUTORS

Marston Book Services Ltd
PO Box 87
Oxford OX2 0DT
(*Orders*: Tel: 0865 791155
Fax: 0865 791927
Telex: 837515)

Australia
Blackwell Scientific Publications
(Australia) Pty Ltd
54 University Street
Carlton, Victoria 3053
(*Orders*: Tel: 03 347-0300)

Distributed in the USA
and North America by
CRC Press, Inc.
2000 Corporate Blvd, NW
Boca Raton
Florida 33431

British Library
Cataloguing in Publication Data

Dieffenbacher, A.
Standard methods for the analysis of oils, fats and derivatives.
I. Title II. Pocklington, W.D.
665.0288

ISBN 0-632-03337-1

Contents of the 1st Supplement

* Pages to replace those in the *7th Edition*.
** New pages.

Preface to the 1st Supplement

Since the publication of the 7th edition of the Standard Methods, the Commission has developed and adopted more than 20 new methods. Of the methods now reproduced in this 1st Supplement, 15 have already been published in *Pure and Applied Chemistry*. New sections containing methods for lecithins and emulsifiers are now included. Of the methods for lecithins, four (phospholipids by thin-layer chromatography and phosphorus content, phospholipid profile by high performance liquid chromatography, acetone-insoluble matter, and toluene-insoluble matter) have been specially developed by IUPAC Commission VI.3, the remainder being general methods for oils and fats (published in the 7th edition). The precision of four of the five general methods included has been evaluated by collaborative study and shown to be satisfactory for the analysis of lecithins.

The provision of a new binder with this 1st Supplement to the 7th edition will allow the new material to be added to the original material, and retained in one binder. In some cases, it may not be possible to insert the new material at exactly the right point due to the original pagination sequence. Subscribers are therefore permitted to photocopy the original pages where necessary so that new methods can be inserted in the correct position.

Corrigenda/amendments

It has been pointed out that certain corrections need to be made to the texts of some methods published in the 7th edition. Accordingly, notes on the corrections required have been included with this 1st Supplement. It is recognized that quite a number of improvements could be made to many of the texts in the 7th edition and it is hoped that when preparing a future 8th edition such improvements can be incorporated. The editors welcome comments by users of the methods regarding the improvement of the presentation of any of the methods and would appreciate notice of any textual errors that may be found. The opportunity has also been taken to include in this 1st Supplement certain precision data that were not available when the 7th edition was prepared for publication.

Analytical quality control

An important development since the appearance of the 7th edition has been the publication (1990) of the *IUPAC Harmonized Protocol for the Presentation of the Performance Characteristics of Standardized Analytical Methods*. Unfortunately the time available for preparing the methods in the 1st Supplement for publication has not allowed the presentation of the performance characteristics of the majority of these methods in the recommended IUPAC protocol. However, it is to be hoped that a future 8th edition will see all the methods presented according to the recommended protocol.

Included in this 1st Supplement are the general principles of analytical quality control which have been reproduced from *Pure and Applied Chemistry* (Vol. 62, No. 1, pp. 153–154). It will be found that a limited number of new methods in this 1st Supplement have associated with them specific applications of analytical quality control, presented according to the IUPAC protocol.

Alphabetical index

To meet the request from users of the methods published in the 7th edition, an alphabetical index has now been provided to facilitate the location of the methods in Section 2 (Oils and Fats).

June 1991

Contents

CONTENTS

likewise, the revision of Sections III (Glycerines) and IV (Soaps) proceeds, while the preparation of further Sections is in hand.

So we have good reason to claim that the Commission on Oils, Fats and Derivatives continues its efforts to be useful to all.

C. PAQUOT
President of the Commission on
Oils, Fats and Derivatives

Only eight years have elapsed since the publication of the 6th edition of our Methods. During this period, which though short, has been none the less rich in activities and results, life of the Commission on Oils, Fats and Derivatives may be summed up as follows, by the rhythm of the meetings, which have taken place yearly.

At the 1977 Warsaw meeting, C. Paquot became Chairman, assisted by E. Delvaux, Vice-Chairman, and A. Hautfenne, Secretary. After Brussels in 1978, the Commission met in Davos (Switzerland) in 1979 where E. Delvaux retired having reached the university age limit.

The 1980 Paris meeting gave the opportunity of celebrating the 50th anniversary of the Commission.

In Leuven (Belgium) in 1981, C. Paquot gave up the Chairmanship to D. Firestone; M. Naudet became Vice-Chairman, A. Hautfenne keeping the Secretariat. In order to conform to the Statutes and by-laws of the Union, the Commission was obliged to reduce to six the number of its Titular Members but was authorized to bring to twelve the number of its Associate Members.

After Zeist (The Netherlands) in 1982, came Lyngby (Denmark) in 1983, when M. Naudet succeeded D. Firestone as Chairman, A. Hautfenne became Vice-Chairman and W.D. Pocklington became responsible for the Secretariat.

After its meeting in 1984 in Turku (Finland), the Commission met in Lyon (France) in 1985.

During this period, the Commission has seen the number of represented countries increase very significantly: in 1979, Brazil, Finland and Yugoslavia (until 1981) rejoined the Commission but Norway left; in 1981 Argentina rejoined, after an absence of four years, together with Egypt, Romania, South Africa and the USSR; Israel and Malaysia joined in 1983.

During the same time, several changes arose among the Members of the Commission mainly due to modification or ceasing of activities. Unfortunately one must also mention the death of two very long-serving Members: Mrs E. Lewkowitsch-Morton and K. Williams.

Revision of methods included in Sections III and IV of the 5th edition has been completed and the revised texts have been published in *Pure and Applied Chemistry* (PAC) as Supplement to the 6th edition. Fifteen new methods have been investigated, adopted and drafted. They have also been published in PAC and they are incorporated in the present edition.

About twenty other methods are already at the final draft stage or are only investigated. After their adoption and drafting, they will be published in PAC with the statistical report relating to the collaborative study.

The Commission has strengthened existing bonds and has created new ones with several international organizations by means of a constant collaboration and exchange of observers.

The general objectives assigned to the Commission on Oils, Fats and Derivatives continue to be pursued energetically, thanks to the dedication of all its members.

M. NAUDET
Chairman of the Commission on
Oils, Fats and Derivatives

January 1985

1985–1990

In the 5 years that have elapsed since the 7th edition was prepared for publication there has been a significant number of changes in the membership of the Commission. Unfortunately, both C. Paquot and A. Hautfenne (who together undertook the prodigious task of preparing the 7th edition for publication) have been unable to continue as active members of the Commission. In 1985, M. Naudet (France) relinquished the chairmanship to Ö. Levin (Sweden) who, in turn, was replaced by Mrs J. Beare-Rogers (Canada) who officiated from 1987 until 1989.

Following the 1985 meeting in Lyon (France), 1986 saw the Commission meeting in Vienna. The year of 1987 was noteworthy for the Commission in that two meetings were held that year—the first at Münster (Germany) in July and the second at Boston (USA) in August. The Münster meeting was essentially a meeting for the European members of the Working Groups who could not go to Boston. St. Andrews (Scotland) was the venue for the 1988 meeting, and the 1989 and 1990 meetings took place, respectively, in Lund (Sweden) and Montreux (Switzerland).

It is to be regretted that a few countries have ceased to be represented on our Commission, but the introduction of the IUPAC Affiliate Membership Scheme augurs well for the future since we now have affiliate members from a number of countries not represented as full members, i.e. China, Ethiopia, Kenya, Indonesia, Peru, the Philippines and Sri Lanka.

The Commission continues to enjoy the coveted status of being one of the most active IUPAC Commissions, and this activity is reflected in the standardization during the past 5 years or so of some 20 new methods for the analysis of oils, fats and derivatives. These new methods have been published as the 1st Supplement to the 7th edition of the *Standard Methods for the Analysis of Oils, Fats and Derivatives*.

W.D. POCKLINGTON
Chairman of the Commission on
Oils, Fats and Derivatives

January 1991

Warning

Some of the methods require the utilization of particularly flammable and/or poisonous solvents.

Included in these solvents are: benzene, carbon tetrachloride and chloroform.

In these cases, the safety instructions for the handling of these solvents should be strictly observed.

The procedure must be carried out in a well-ventilated room, preferably in a fume cupboard, to prevent the exposure of laboratory workers to poisonous vapours.

The IUPAC Commission VI.3 has embarked on a programme with the objective of reducing, as far as is practicable, the need for use of such solvents in its standard methods.

General principles

Introduction

The number of test results to be obtained from the analysis of each laboratory sample is related to the purpose for which the analysis is required. Where the analysis is required under the terms of a contractual agreement, or for arbitration purposes, more than one test result for each analyte/parameter may be required. In routine quality control it may be considered adequate to obtain a single test result for each analyte/parameter. However, for analytical quality control purposes, an analysis in duplicate will be necessary for at least one in every ten determinations of each analyte/parameter.

Precision of analyses

When a check on the precision of the analyses is required, two test results for each test sample must be obtained under the conditions of repeatability (i.e. conditions where independent test results are obtained with the same method on identical test material, in the same laboratory by the same operator using the same equipment, within short intervals of time). (*Note*: It may also be desirable to obtain two test results under intermediate precision conditions (e.g. when operator and equipment are the same but time is different).)

Acceptability of test results

When two test results are obtained (under the conditions of repeatability) from two analyses of the same test sample, the *final quoted result* is the mean of the two test results, provided that the requirements of the repeatability clause are met—see the section on *specific applications* of analytical quality control which is appended to the text of the method. If these requirements are not met, reject the results and obtain two further test results (under repeatability conditions). If the requirements of the repeatability clause are again not met, an investigation into the possible sources of error should be carried out. If the source of error cannot be found, the procedure for deriving the *final quoted result*, illustrated in the annexed flow diagram, may be followed. In such a case the final quoted result should be qualified with a statement to the effect that the conditions of repeatability were not met, and the individual test results (with an indication as to how the final quoted result has been calculated) should also be reported.

Utilization of values for the reproducibility limit (R)

When test results obtained by two laboratories (from the analysis of identical laboratory samples) are being compared, it should be noted that the values for (R) [determined according to *ISO 5725*] apply in the particular case when each laboratory obtains one test result only. If it is desired to compare *final quoted results*, which are the mean results from more than one analysis carried out by each laboratory on identical laboratory samples, the appropriate values for (R) can be calculated as outlined in the *1987—IUPAC Harmonized Protocol* or *ISO 5725* Part 6 Section 2. For example, when the *final quoted results* are the mean results from *two* analyses carried out by each laboratory, the formula below applies:

$$R^0 = \sqrt{(R^2 - r^2/2)}$$

where:

R^0 is the adjusted value for the *reproducibility limit*, applicable when the *final quoted results* are the mean results from *two* analyses carried out by each laboratory on identical laboratory samples;
R is the value for the *reproducibility limit** of the method; and
r is the value for the *repeatability limit** for the method.

Trueness of test results

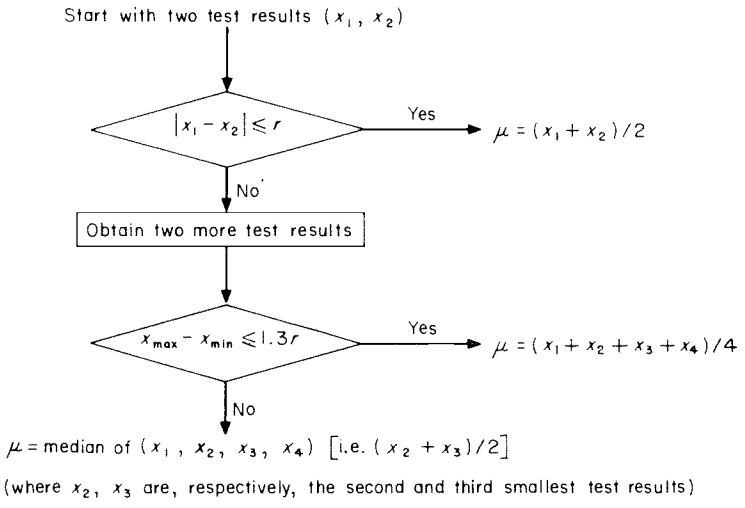
When a check on the trueness of the test results is required, one or more certified (purchased or prepared in-house) reference materials, of similar matrix and analyte concentration to that of the test samples, should be analysed in parallel with the test samples. A decision is then made as to whether the difference, if any, between the expected value(s) for the reference material(s) and that obtained by analysis of the latter, is statistically significant, in the case of:

(a) *certified reference materials*—by reference to the cited confidence limits, or

(b) *prepared in-house reference materials*—by use of plotted data such as that on control charts. In this case a test result obtained for the prepared reference material which falls within ± 2 units of the standard deviation (s)* of the accepted mean value* for the analyte of interest may be considered acceptable. If the difference between the test result and the mean value exceeds $\pm 3s$ the test result must be rejected. *(*Note:* The accepted mean value and standard deviation would be that calculated from the results obtained from not less than ten analyses of the prepared in-house reference material. Lines drawn on the control chart at $2s$ and $3s$ both above (+) and below (−) the line representing the mean value are regarded as warning and action limits, respectively.)

A check on consistent method and individual laboratory *bias* may be made by *spiking* and determining the recovery of added analyte.

Annexe



Flow diagram illustrating the derivation of the final quoted result when test results do not meet the conditions of repeatability. r = repeatability limit for the method; μ = final quoted result. [*Note:* The flow diagram is based on that appearing in Section 7 Part 6 of *ISO 5725* (long-term revision).]

References

Horwitz W. *Pure Appl Chem* 1988; **60**(6): 855–867.
Pocklington WD. *Pure Appl Chem* 1990; **62**(1): 149–162.

* Determined according to the *1987—IUPAC Harmonized Protocol* or *ISO 5725* from a statistical analysis of the results obtained during the collaborative study of the method.

2.201 Determination of the acid value (A.V.) and the acidity

Statistical evaluation of results of study carried out in 1989.

	Palm oil refined	Palm oil crude	Fish oil crude
Number of laboratories	5	5	5
Number of results	10	7	10
Number of laboratories retained after deletion of outliers	5	5	5
Number of accepted results	10	7	10
Mean value (% oleic acid)	**1.12**	**11.5**	**10.4**
Repeatability standard deviation*	0.008	0.03	0.09
Repeatability relative standard deviation (%)	0.79	0.27	0.84
Reproducibility standard deviation*	0.066	0.17	0.30
Reproducibility relative standard deviation (%)	5.9	1.5	2.9
Repeatability (*r*)*	**0.03**	**0.09**	**0.25**
Reproducibility (*R*)*	**0.19**	**0.49**	**0.85**

* Expressed as the percentage of oleic acid; acid value = 2 × acidity.

2.205 Determination of the iodine value: Wijs' method

Statistical evaluation of results of studies carried out in 1989/90.

Table 2.205.1.

Solvent:*	Mean value CTC	Mean value CHX	r CTC	r CHX	R CTC	R CHX	No. of accepted results (CTC, CHX)
Sample							
Sunflower seed oil	133.6	132.9	3.0	3.6	7.2	4.8	(20, 20)
Palm oil, refined	53.1	53.0	0.65	0.82	1.43	1.90	(20, 20)
Fish oil, crude	109.1	108.5	1.67	1.40	4.35	2.80	(18, 14)
Tung oil	164.5	163.1	3.47	2.39	5.27	4.32	(14, 12)
Tallow (beef fat)	47.2	46.9	1.37	1.33	2.75	3.10	(20, 20)
Soyabean oil, hydrogenated	63.3	65.3	1.86	13.8	17.2	14.9	(12, 16)
Palm oil, crude	52.5	52.6	1.29	2.15	2.19	2.89	(18, 20)
Frying oil, used	37.7	37.7	0.97	1.38	1.18	2.48	(14, 14)
Fish oil, hydrogenated	63.3	68.6	3.61	1.69	17.2	11.0	(12, 12)
Palm kernel oil	18.2	18.3	0.17	0.14	0.51	0.64	(16, 14)
Olive oil	82.3	82.2	0.75	1.75	1.86	2.62	(14, 10)

Table 2.205.2.

Sample:† Solvent:*	SBO1 CTC	SBO1 CHX	SBO2 CTC	SBO2 CHX
Number of laboratories	17	17	17	17
Number of results	34	34	34	34
Number of laboratories retained after elimination of outliers	16	17	17	17
Number of accepted results	32	34	34	34
Mean value	102.6	102.3	74.7	74.8
Repeatability limit (r)	1.4	2.2	1.7	1.5
Reproducibility limit (R)	4.8	5.1	3.7	2.1

Table 2.205.3.

Sample:† Solvent:*	HFO CCT	HFO CHX
Number of laboratories	17	17
Number of results	34	34
Number of laboratories retained after elimination of outliers	16	17
Number of accepted results	32	34
Mean value	73.0	72.8
Repeatability limit (r)	1.7	1.6
Reproducibility limit (R)	2.5	2.3

* CTC, carbon tetrachloride; CHX, cyclohexane + glacial acetic acid (1:1).
† SBO, hydrogenated soyabean oil; HFO, hydrogenated fish oil.
Statistical analysis according to *ISO 5725-1986*.

Reference

Pocklington WD. *Pure Appl Chem* 1990; **62**(12): 2339–2343.

2.501 Determination of peroxide value* (P.V.)

Statistical evaluation of results of study carried out in 1971

	Palm oil	Rapeseed oil	Soya oil 1	Soya oil 2	Coconut oil
Number of laboratories	7	7	7	7	7
Number of results	21	21	21	21	21
Number of laboratories retained after deletion of outliers	7	7	7	7	7
Number of accepted results	21	21	21	21	21
Mean value (mEq/kg)	8.56	1.03	3.30	0.80	0.11
Repeatability standard deviation†	0.16	0.22	0.12	0.08	0.08
Repeatability relative standard deviation (%)	1.87	21.6	3.62	9.8	73.3
Reproducibility standard deviation†	0.79	0.25	0.31	0.18	0.14
Reproducibility relative standard deviation (%)	9.21	25.2	9.5	22.2	123.1
Repeatability (r)†	0.45	0.63	0.34	0.22	0.24
Reproducibility (R)†	2.23	0.73	0.89	0.50	0.40

† Expressed as mEq/kg.

* *Note:* Method 2.501 requires the use of chloroform, which is now discouraged in analytical laboratories. Recent collaborative studies organized by the AOCS in which the peroxide values of lard, groundnut, maize oil, linseed oil, rapeseed oil, soybean oil and sunflower seed oil were determined using both chloroform and iso-octane have demonstrated that 'there is good one-to-one correlation between the results for the two solvents'. Subsequently the AOCS published (1990) its method Cd 8b-90 which uses iso-octane in place of chloroform. However, the precision of the method is not stated and it should be noted that the procedure is not technically equivalent to that outlined in the IUPAC method 2.501. A Caution note in the text of the AOCS method advises that iso-octane is toxic by ingestion and inhalation.

Corrigenda/amendments to be made to methods published in the 7th edition

Page	*Method*	*Amendment/correction*
33	2.101	New page 33 containing revised Table 2.101.1 issued with this supplement.
41	2.121	4 *Principle*, line 3: delete 'boiling'.
42		8.1 lines 4/5 from bottom: add after 'sodium chloride solution (6.4)' the words 'which is hot enough to maintain the fatty acids in a liquid state'.
73	2.201	1 *Scope*, line 2: insert 'and' after 'animal'.
74		4.5 *Expression of results*, line 3: insert 'hydroxide' after 'potassium'.
76		6.2 *Expression of results*, line 3: insert 'millilitres' after 'number of'.
88	2.205	4.1 *Principle*, lines 1/2: amend to read 'in an acetic acid and cyclohexane mixture'. Add the following note: 'In view of the carcinogenic nature of carbon tetrachloride it has been recommended that this solvent is replaced by cyclohexane—see *Pure and Applied Chemistry* (1990) Vol. 62, No. 12, pp. 2339–2343.
		4.3.2, line 1: delete 'carbon tetrachloride' and add 'cyclohexane'.
89	2.205	4.4 Add the following Note: 'Wherever possible it is recommended that commercially produced Wijs reagent be used. It should not contain carbon tetrachloride.'
89	2.205	4.4.1, line 7: correct 'thiosulphide' to read 'thiosulphate'.
90	2.205	4.5 *Procedure:* replace, 'carbon tetrachloride' with 'glacial acetic acid–cyclohexane (1:1)'. At the end of the penultimate paragraph add: '*Note:* The starch solution should be added when the yellow colour of the iodine has almost disappeared.'
103	2.208	Title: correct to read '*trans*-octadecenoic acid'.
107	2.209	1 *Scope*, formula: replace the second double bond (=), i.e. that following CH_2, with a single bond (—).
149	2.322	Under *Reagents* add '5.5 Potassium iodide, 100 g/l aqueous solution' and renumber 5.5 and 5.6, respectively, as 5.6 and 5.7.
215	2.506	Clauses 6, 7 and 8 (Procedure, Expression of results, and Notes) were omitted from the 7th edition. These are now provided with this supplement—new pages 215/215A and 216.
242	2.611	7 *Notes*, last line: for 'squalene' read 'squalane'.
272	3.101	New page 272 containing revised table for '1. Water' issued with this supplement.

The volume V_t of the pyknometer is given by:

$$V_t = \frac{m_2 - m_0}{\rho_{H_2O,t_2}} [1 + 0.000025(t - t_2)]$$

where m_0 is the mass, in g, of the empty pyknometer, m_2 is the mass, in g, of the pyknometer filled with water, ρ_{H_2O,t_2} is the density of water at $t_{2°C}$ (Table 2.101.1), 0.000025 is the mean coefficient of cubic expansion of the glass of the pyknometer.

Table 2.101.1. Density of water at $t_{2°C}$

Temperature t_2 (°C)	Density of water ρ_{H_2O,t_2} (g/ml)	Temperature t_2 (°C)	Density of water ρ_{H_2O,t_2} (g/ml)	Temperature t_2 (°C)	Density of water ρ_{H_2O,t_2} (g/ml)
15	0.99805	35	0.99298	55	0.98465
16	0.99789	36	0.99264	56	0.98416
17	0.99772	37	0.99228	57	0.98367
18	0.99754	38	0.99192	58	0.98317
19	0.99735	39	0.99155	59	0.98267
20	0.99715	40	0.99117	60	0.98217
21	0.99694	41	0.99079	61	0.98165
22	0.99672	42	0.99039	62	0.98113
23	0.99649	43	0.98999	63	0.98060
24	0.99624	44	0.98958	64	0.98006
25	0.99599	45	0.98917	65	0.97952
26	0.99573	46	0.98874		
27	0.99546	47	0.98832		
28	0.99518	48	0.98788		
29	0.99490	49	0.98744		
30	0.99460	50	0.98699		
31	0.99429	51	0.98654		
32	0.99398	52	0.98607		
33	0.99365	53	0.98561		
34	0.99332	54	0.98513		

2.102 Determination of the refractive index

1 Scope

This Standard describes a method for the determination of the refractive index of animal and vegetable oils and fats.

2 Field of application

This Standard is applicable to the animal and vegetable oils and fats.

3 Definition

The refractive index of a medium is the ratio of the speed of light at a definite wavelength *in vacuo* to its speed in the medium.

The refractive index of a given substance varies with the wavelength of the light and with the temperature.

Generally the speed of light in air is taken in the place of the speed *in vacuo*, and, unless otherwise specified, the selective wavelength is the mean wavelength of the D-lines of sodium (589.6 nm). In this case the notation is n_D^t at t°C.

In general the refractive index is given at:

−20°C for fats which are liquid at this temperature,

−40 or 60 or 80°C, or above, for fats which are solid at a temperature of 20°C.

4 Principle

Measurement by means of a suitable refractometer of the refractive index of the liquid sample.

5 Apparatus

5.1 Refractometer, e.g. Abbe type, able to measure the refractive index to ±0.0001 within the range n_D = 1.3000–1.7000.

5.2 Light source: sodium vapour lamp. White light can also be used if the refractometer is fitted with an achromatic compensation device.

5.3 Glass plate, of known refractive index.

5.4 100-ml beaker.

5.5 Glass rod, with rounded ends.

5.6 Fluted filter paper.

5.7 Water bath.

5.8 Water bath, thermostatically controlled with circulation pump to maintain the temperature to ±0.5°C.

6 Reagents

6.1 α-Bromonaphthalene.

6.2 Trichlorethylene.

6.3 Sodium sulphate, anhydrous.

7 Preparation of the sample

If the sample is liquid at room temperature, mix it thoroughly with a glass rod (5.5), dry it over anhydrous sodium sulphate (6.3) and filter through a fluted filter paper (5.6).

If the fat sample is solid, heat in a beaker (5.4) on a water bath (5.7) to a temperature 10°C higher than the melting point, dry over anhydrous sodium sulphate, mix thoroughly and filter.

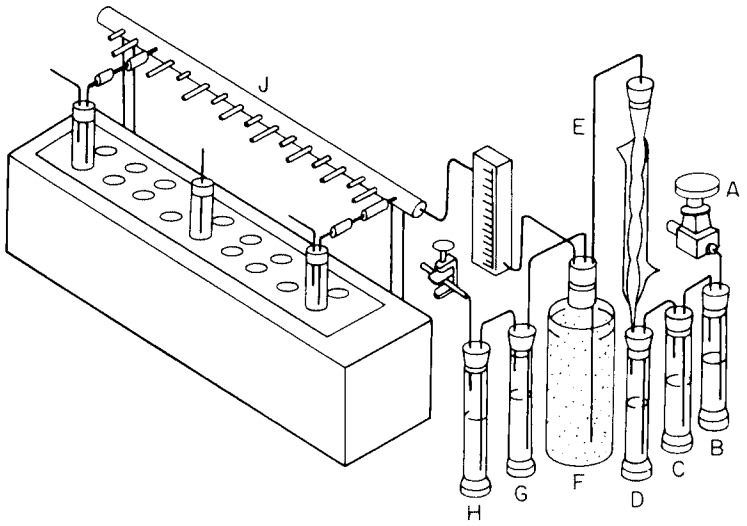


Fig. 2.506.1. Air-purification train.

4.7 25×200-mm test tubes of Pyrex glass. It is advantageous to calibrate these with a mark at the 20-ml level. Each tube is fitted with a neoprene stopper with two holes and an aeration tube (see Fig. 2.506.2). The tube used for temperature control must, additionally, be able to take the thermometer (4.5).

4.8 Oven, regulated at 103±2°C.

4.9 Equipment for the determination of the peroxide value (see 2.501).

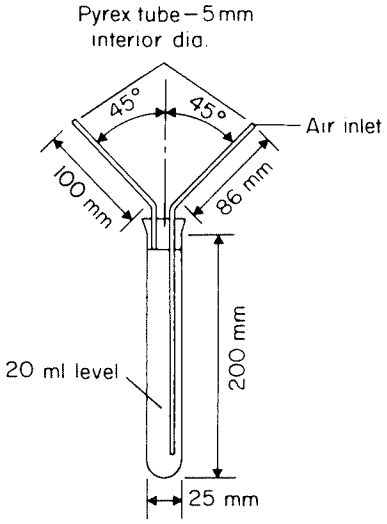


Fig. 2.506.2.

5 Reagents

5.1 Light petroleum, analytical reagent quality, b.p. 40–60°C.

5.2 Acetone, analytical reagent quality.

5.3 Detergent suitable for cleaning the apparatus, which must not leave any contaminating residues.

5.4 Reagents for the determination of the peroxide value (see 2.501).

6 Procedure

6.1 Cleaning of the test tubes and aeration tubes

It is essential that the test tubes and the aeration tubes (4.7) should be most thoroughly cleaned and free from any traces of organic and inorganic matter (especially traces of metals).

After cleaning with light petroleum (5.1) and acetone (5.2), it is advisable to wash all the tubes with near boiling detergent solution (5.3), and to rinse very thoroughly.

Dry in an oven (4.8) at 103 ± 2°C, and store in a dust-free location.

6.2 Determination

Prepare the sample according to 2.001, 'Preparation of the sample'.

Introduce 20 ml of the prepared sample into each of two perfectly clean test tubes (4.7). Do this in such a way that none of the fat comes into contact with the upper part of the tube and consequently the stopper.

Fit the aeration tube and adjust it so that the tip of the air-delivery tube is 5 cm below the surface of the test portion.

Place the assembled tube containing the test portion in a vessel containing boiling water for 5 minutes. Remove the tube, wipe dry and place immediately in the constant-temperature heater (4.1) maintained at 98.0°C. Having previously adjusted the air-flow rate, connect the aeration tube to the capillary on the manifold (4.2) and record the starting time.

A short time before the expected end-point is reached (*note 1*), determine the peroxide value according to 2.501, using a test portion of 0.3 g.

If the peroxide value so determined is above 175 m.e.q., the test portion must be rejected, and another determination started (*note 2*).

If the peroxide value obtained is below 75 m.e.q., estimate the time at which a figure of 75 m.e.q. would be expected, and at that time do a peroxide determination on a fresh test portion of 0.3 g. This will afford the first figure to be recorded.

Determine the peroxide value again on another test portion of 0.3 g taken from the same test tube exactly 1 hour after the first one. This affords the second value to be recorded. The two figures so obtained for the peroxide value must fall between 75 and 175 m.e.q.

7 Expression of results

Taking the reaction times as abscissae, plot the two values of the peroxide value as ordinates on 2-coordinate graph paper.

Join the two points by a straight line. The stability value is then the time (in hours) at which this line cuts the ordinate for 100.

Repeat the whole operation with the second test portion and calculate the average of the results in hours (to the nearest whole number).

8 Notes

1 After some experience, the approach of the end-point can be estimated from the odour of the effluent air from the tubes.

2 It is desirable to heat and aerate the test portions continuously until the end-point. When this is not practicable, remove the tube from the heating bath, chill it immediately, and store it at below 10°C, until the test tube is carried on again, starting as from 6.2, 'Place the assembled tube containing the test portion in a vessel containing boiling water for 5 minutes . . .'.

2.507 Determination of polar compounds in frying fats

1 Scope

This Standard describes a method for the determination of polar compounds in frying fats. Polar compounds are formed during the heating of fats.

2 Field of application

This Standard is applicable to animal and vegetable oils and fats. The method serves to assess the deterioration of used frying fats.

3 Definition

Polar compounds are those compounds in fats which are determined by column chromatography under the conditions specified (*note 1*).

4 Principle

Separation of frying fats by column chromatography into non-polar and polar compounds. Elution of the non-polar compounds. Determination of polar compounds by calculating the difference between weight of the sample added to the column and that of the non-polar fraction eluted.

5 Apparatus

5.1 250- and 500-ml round bottom flasks with ground necks.

5.2 100-ml beakers.

5.3 Ground-glass stoppers to fit the 500-ml flasks (5.1).

5.4 Chromatographic glass column, 21 mm internal diameter, 450 mm in length, with stopcock (preferably in polytetrafluorethylene), and ground-glass joint.

5.5 250-ml dropping funnel, with ground-glass joint to fit the column (5.4).

5.6 Glass funnel, about 8 cm in diameter.

5.7 Glass rod, about 60 cm in length.

5.8 50-ml volumetric flask.

5.9 20-ml volumetric pipette.

5.10 2-μl capillary pipettes for thin-layer chromatography.

5.11 Glass plates for thin-layer chromatography, 20 × 20 cm, coated with silica gel (without fluorescence indicator), 0.25 mm layer thickness.

5.12 Glass developing tank for thin-layer chromatography, with ground-glass lid.

5.13 Spray for thin-layer chromatography.

5.14 Porcelain dish, about 20 cm in diameter.

5.15 Oven regulated at 103 ± 2°C.

5.16 Drying oven controllable between 120 and 160°C.

5.17 Water bath.

5.18 Desiccator, containing a suitable desiccant such as silica gel with moisture indicator (blue gel).

5.19 Apparatus for removing solvent under vacuum, e.g. rotary evaporator.

5.20 Shaking machine.

6 Reagents

6.1 Light petroleum (b.p. 40–60°C), chromatographic quality, redistilled.

6.2 Ethanol, 95 per cent (V/V).

6.3 Chloroform, pure.

The conventional mass per unit volume of glycerines is used to convert volumes to masses (not taking into account buoyancy due to air).

2 The expression 'relative density' (d) and 'specific gravity' are equivalent. When the term 'relative density' includes the symbol 20/4, the measurement is carried out at 4°C for the reference substance (water) and at 20°C for the substance to be examined (glycerines).

The conversion $d_{20/20}$ to $d_{20/4}$ may be calculated using the following formula:

$$d_{20/4} = d_{20/20} \times 0.99823$$

3 If it is necessary to carry out numerous determinations, it may be practical not to calibrate the pyknometer at each measurement, but merely to check that its mass and volume remain constant with time.

4 The volume of the pyknometer (4.1) at a given temperature t (V_t), in ml, is given by the formula:

$$V_t = \frac{m_t}{\rho_t,\ H_2O}$$

where m_t is the conventional mass, in g, of water (5.1) contained in the pyknometer (4.1) at temperature t, as determined in (6), ρ_t, H_2O is the conventional mass per unit volume of water at temperature t, in g/ml.

If the pyknometer is calibrated at 20°C the volume of the pyknometer at t°C can be calculated using the formula:

$$V_t = V_{20}[1 + \alpha(t - 20)]$$

where V_t is the volume of the pyknometer at t°C, in ml, V_{20} is the volume of the pyknometer at 20°C, in ml, α is the cubic expansion coefficient of the glass of the pyknometer, in $°C^{-1}$.

Some values of α:

Boro-silicate glass D50: $10 \times 10^{-6}°C^{-1}$

Boro-silicate glass G20: $16 \times 10^{-6}°C^{-1}$

Sodium glass: $15–30 \times 10^{-6}°C^{-1}$

Appendixes

1. Water

Conventional mass per unit volume (ρ_t, H_2O)—temperature (t).

Temperature t (°C)	Density of water $\rho_{H_2O,t}$ (g/ml)	Temperature t (°C)	Density of water $\rho_{H_2O,t}$ (g/ml)	Temperature t (°C)	Density of water $\rho_{H_2O,t}$ (g/ml)
15	0.99805	35	0.99298	55	0.98465
16	0.99789	36	0.99264	56	0.98416
17	0.99772	37	0.99228	57	0.98367
18	0.99754	38	0.99192	58	0.98317
19	0.99735	39	0.99155	59	0.98267
20	0.99715	40	0.99117	60	0.98217
21	0.99694	41	0.99079	61	0.98165
22	0.99672	42	0.99039	62	0.98113
23	0.99649	43	0.98999	63	0.98060
24	0.99624	44	0.98958	64	0.98006
25	0.99599	45	0.98917	65	0.97952
26	0.99573	46	0.98874		
27	0.99546	47	0.98832		
28	0.99518	48	0.98788		
29	0.99490	49	0.98744		
30	0.99460	50	0.98699		
31	0.99429	51	0.98654		
32	0.99398	52	0.98607		
33	0.99365	53	0.98561		
34	0.99332	54	0.98513		

2. Glycerines

Relative density in air–glycerol concentration (n/m). (From AOCS Book of Official and Tentative Methods.) (Methods Ea 7-50–Bosart and Snoddy Table.)

Glycerol concentration (%)	Relative density in air			
	15/15°C	15.5/15.5°C	20/20°C	25/25°C
100	1.26557	1.26532	1.26362	1.26201
99	1.26300	1.26275	1.26105	1.25945
98	1.26045	1.26020	1.25845	1.25685
97	1.25785	1.25760	1.25585	1.25425
96	1.25525	1.25500	1.25330	1.25165
95	1.25270	1.25245	1.25075	1.24910
94	1.25005	1.24980	1.24810	1.24645
93	1.24740	1.24715	1.24545	1.24380
92	1.24475	1.24450	1.24280	1.24115
91	1.24210	1.24185	1.24020	1.23850
90	1.23950	1.23920	1.23755	1.23585
89	1.23680	1.23655	1.23490	1.23320
88	1.23415	1.23390	1.23220	1.23055
87	1.23150	1.23120	1.22955	1.22790
86	1.22885	1.22855	1.22690	1.22520
85	1.22620	1.22590	1.22420	1.22255
84	1.22355	1.22325	1.22155	1.21990
83	1.22090	1.22055	1.21890	1.21720
82	1.21820	1.21790	1.21620	1.21455
81	1.21555	1.21525	1.21355	1.21190
80	1.21290	1.21260	1.21090	1.20925

Methods included in this 1st Supplement to the 7th edition

Section 1: Oleaginous Seeds and Fruits

Analysis of the Residues Remaining after Oil Extraction

1.171* Determination of total hexane content in extraction meals

1.172* Determination of 'free' hexane content in extraction meals

Section 2: Oils and Fats

2.303* Determination of *n*-3 and *n*-6 unsaturated fatty acids in vegetable oils and fats by capillary gas–liquid chromatography

2.304 Capillary column gas–liquid chromatography of fatty acid methyl esters

2.310* Determination of butyric acid

2.324* Determination of the composition of triglycerides in vegetable oils in terms of their partition number by high performance liquid chromatography (HPLC)

2.325 Determination of individual triglycerides in oils and fats by high performance liquid chromatography

2.326* Determination of mono- and diglycerides by capillary gas chromatography

2.423* Determination of phosphorus by direct graphite furnace atomic absorption spectrometry

2.432* Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography

2.508* Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography

2.531* Determination of the 2-thiobarbituric acid value: direct method

2.607* Determination of 'hexane' residues in fats and oils

2.608* Determination of benzo[*a*]pyrene in edible oils and fats by reversed phase high performance liquid chromatography

2.631* Determination of copper, iron and nickel by direct graphite furnace atomic absorption spectrometry

2.632* Determination of lead by direct graphite furnace atomic absorption spectrometry

2.642* Determination of antioxidants by high performance liquid chromatography

Section 5: Lecithins

Determination of the Chemical Characteristics

5.201 Determination of the acidity of lecithins (ex 2.201)

5.205 Determination of the iodine value (ex 2.205)

Determination of the Principal Constituents

5.301 Determination of phospholipids by thin-layer chromatography and phosphorus analysis

5.302* Determination of the phospholipid profile of lecithins by high performance liquid chromatography

Determination of other Natural Constituents

5.421 Determination of the phosphorus content of lecithins (ex 2.421)

* These methods have already been published in *Pure and Applied Chemistry*.

Determination of the Quality and the Stability

5.501 Determination of the peroxide value (ex 2.501)

5.502 Determination of the acetone-insoluble material in lecithins

Determination of the Foreign Substances and the Additives

5.603 Determination of small quantities of water by modified Karl Fischer method (ex 2.603)

5.604 Determination of the toluene-insoluble material in lecithins

Section 6: Emulsifiers

6.001* Determination of the ester-emulsifiers components after hydrolysis and silylation by gas chromatography

6.002* Determination of mono- and diglycerides by capillary gas chromatography (method 2.326)

* These methods have already been published in *Pure and Applied Chemistry*.

Section 1
Oleaginous Seeds and Fruits

Analysis of the Residues Remaining after Oil Extraction

1.171 Determination of total hexane content in extraction meals

1 Scope and field of application

This Standard describes a method for the determination of the total amount of volatile hydrocarbons, expressed generally in terms of hexane, remaining in oil-seed residues after extraction of the seed with hydrocarbon-based solvents.

2 Definition

The total residual hexane content in extraction meals is the quantity of volatile hydrocarbons determined by the present method and expressed in milligrams per kilogram (p.p.m.) of oil-seed residue.

3 Principle

Desorption of the residual hydrocarbons by heating the sample at 110°C with water in a closed vessel. After calibration, determination of the hydrocarbons contained in the headspace by gas chromatography using capillary or packed columns. Expression of the results as hexane.

4 Apparatus

4.1 Septum vials, 50–60 ml capacity, all with the same volume to within 2 per cent.

4.2 Septa suitable for vials (4.1) about 3 mm in thickness, inert to hexane (*note 1*).

4.3 Metallic foil, e.g. of aluminium.

4.4 Crimping pliers.

4.5 Syringe, 10 μl.

4.6 Gas syringe, graduated, 1 ml, preferably with valve.

4.7 Gas chromatograph with flame ionization detector and integrator and/or recorder. If a capillary column is used, the apparatus shall be equipped with a 1/100 split injector.

4.8 Glass capillary column suitable for gas chromatograph (4.7), approximately 30 m in length and 0.3 mm internal diameter, coated with methylpolysiloxane (*note 2*) (film thickness 0.2 μm) or, failing this, a packed column approximately 1.7 m in length packed with 150–180 μm diatomaceous support, acid washed (*note 3*) and coated with methylpolysiloxane (*note 2*).

4.9 Electric oven regulated at 110°C.

5 Reagents

5.1 Technical hexane or a light petroleum with a composition similar to that used in industrial extraction, or failing these, *n*-hexane.

5.2 Carrier gas: hydrogen, nitrogen or helium, thoroughly dried and containing less than 10 mg/kg of oxygen.

5.3 Auxiliary gases: hydrogen, 99.9 per cent pure, containing no organic impurities; air containing no organic impurities.

6 Sampling and sample storage

It is essential that loss of solvent from the sample be prevented. The laboratory sample shall be in a completely sealed container (preferably in a crimped metal box) and shall be stored at −20°C or below, for example in a deep-freezer.

Plastic containers shall not be used.

The determination of residual hydrocarbons shall be carried out as soon as the container—kept at +4°C for 24 hours before opening—has been opened.

7 Procedure

7.1 Calibration curve

Add 6 ml of water to a vial (4.1) (*note 4*). Add rapidly a specific quantity of solvent (5.1) (*note 5*) measured accurately by means of the syringe (4.5). Seal with a septum (4.2). Cover with a foil (4.3). Crimp with pliers (4.4). Place the vial in the oven (4.9) at 110°C for 15 minutes.

Warm the gas syringe (4.6) to 60°C.

Remove the vial from the oven and leave to cool for 2 minutes.

By means of the warmed gas syringe (4.6), take exactly 0.5 ml of the headspace and inject immediately into the chromatograph (4.7), with the injection and detection temperatures of the chromatograph (4.7) set at 120°C, the oven temperature set at 40°C and the carrier gas pressure at 0.3 bar.

Determine the peak areas obtained for the solvent.

Repeat this operation for different quantities of solvent (5.1) (*note 5*). Determine the peak areas for the solvent in each case.

Plot the calibration curve expressing the relation between the solvent peak areas and the mass of solvent introduced into the vials (*note 6*).

7.2 Test portion analysis

Weigh rapidly, to within 0.1 g, 5 g of the laboratory sample into a vial (4.1). Add 2.5 ml of distilled water. Seal the vial with the septum (4.2). Cover with foil (4.3). Crimp with pliers (4.4). Place the vial in the oven (4.9) at 110°C for exactly 90 minutes (*note 7*).

Warm the gas syringe (4.6) to 60°C.

Remove the vial from the oven. Leave to cool for 2 minutes.

Agitate by inverting. By means of the heated gas syringe, take exactly 0.5 ml of the headspace and inject immediately into the chromatograph (4.7), with the injection and detection temperatures set at 120°C, the oven temperature set at 40°C and the carrier gas (5.2) pressure at 0.3 bar. Determine the sum of the peak areas of hexane and various hydrocarbons which usually make up the technical solvents (*note 8*). Determine from the calibration curve the corresponding quantity, m_1, of solvents present in the vial.

8 Calculation and expression of results

The total hexane content (H) expressed in mg/kg (p.p.m.) is given by the formula:

$$H = \frac{m_1}{m_0}$$

where:

m_0 is the mass, in g, of the test portion

m_1 is the mass of solvents, in μg, of solvents present in the flask.

9 Precision

The results of one interlaboratory test organized at an international level (for different samples) gave the statistical results shown in the table in the Appendix.

9.1 Repeatability

When the mean of duplicate determinations lies within the range of the mean values cited in the table, the difference between the results of two determinations, carried out

in rapid succession by the same operator using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r), which can be deduced from the table, for the level of hexane in the sample examined.

9.2 Reproducibility

When the means of duplicate determinations, obtained in two different laboratories using this standard method for the analysis of the same laboratory sample, lie within the range of the mean values cited in the table, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R), which can be deduced from the table, for the level of hexane in the sample examined.

10 **Notes**

1 A material such as nitrile rubber, for example Perbunan, or butyl rubber with a polytetrafluoroethylene or polychloroprene seal, for example Neoprene, is suitable. These septa often have a very high mechanical resistance. It is therefore preferable to perforate them with a pin before taking the sample from the headspace and using this perforation so that the syringe needle is not damaged on insertion. Re-use of the septa is not recommended.

2 SE 30 is suitable.

3 Chromosorb WAW is suitable.

4 This volume is determined by the fact that the test portion (5 g) hydrated with water (2.5 ml) will occupy a volume of 6 ml.

5 Three additions, for example, with 2, 5 and 10 μl of solvent (5.1) are usually sufficient for constructing the calibration curve. They correspond, respectively, to 268, 670 and 1340 mg/kg of solvent in a test portion of 5 g of meal in the case of technical hexane and to 264, 660 and 1320 mg/kg in the case of *n*-hexane.

6 It may be assumed that 1 μl of solvent introduced into the vials has a mass of 670 μg in the case of technical hexane, and 659 μg in the case of *n*-hexane.

7 It is important to leave the vials in the oven for the same length of time for each sample.

8 Hydrocarbons which usually make up the technical solvents are: 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane etc. A typical chromatogram of these solvents is represented by Fig. 1.171.1. Do not include peaks due to oxidation products. Some of these products may be present in significant amounts.

Reference

Hautfenne A, Pocklington WD, Wolff JP. *Pure Appl Chem* 1987; **59**(10): 1407–1418 (Report on the Collaborative Study).

Appendix

Table 1.171.1. Statistical analysis of results for total hexane (Collaborative Study 1983—duplicate determinations).

	Rapeseed residue	Soyabean residue	Homogenized rapeseed residue
Number of laboratories	15	15	15
Number of results	30	30	30
Number of laboratories after elimination of outliers	15	15	15
Mean value (mg/kg)	452	378	341
Repeatability standard deviation (S_r)	35	19	29
Repeatability coefficient of variation (%)	7.8	5.1	8.6
Repeatability value r ($S_r \times 2.83$)	100	54	83
Reproducibility standard deviation (S_R)	109	129	108
Reproducibility coefficient of variation (%)	24	34	32
Reproducibility value R ($S_R \times 2.83$)	308	365	305

1.172 Determination of 'free' hexane content in extraction meals

1 Scope and field of application

This Standard describes a method for the determination of the 'free' volatile hydrocarbons, expressed generally in terms of hexane, remaining in oil-seed residues after extraction with hydrocarbon-based solvents.

2 Definition

The 'free' hexane content in extraction meals is the quantity of volatile hydrocarbons determined by the present method and expressed in milligrams per kilogram (p.p.m.) of oil-seed residue.

3 Principle

Desorption of volatile hydrocarbons by heating at 80°C in a closed vessel after addition of an internal standard. After calibration, determination of these hydrocarbons in the headspace by gas chromatography using packed or capillary columns. Expression of the results as hexane.

4 Apparatus

4.1 Septum vials, 20 ml capacity, all with the same volume to within 2 per cent.

4.2 Septa suitable for vials (4.1) approximately 3 mm in thickness, inert to hexane (*note 1*).

4.3 Metallic foil, e.g. of aluminium.

4.4 Crimping pliers.

4.5 Syringe, 10 μl.

4.6 Gas syringe, graduated, 1 ml, preferably with valve.

4.7 Gas chromatograph with flame ionization detector and integrator and/or recorder. If a capillary column is used, the apparatus shall be equipped with a 1/100 split injector system.

4.8 Packed columns suitable for gas chromatograph (4.7), approximately 2 m in length, packed with 150–180 μm diatomaceous support, acid washed (*note 2*) and coated with squalane (10 per cent) or methylpolysiloxane (*note 3*), or failing this, a glass capillary column approximately 30 m in length and 0.3 mm internal diameter, coated with methylpolysiloxane (film thickness 0.2 μm) (*note 3*).

4.9 Heating bath fitted with a rack to hold the sealed vials. The bath should be thermostatically controlled at a temperature of 80°C. For continuous use, glycerine is recommended as the heating liquid.

5 Reagents

5.1 Technical hexane or a light petroleum with a composition similar to that used in industrial extraction, or failing these, *n*-hexane.

5.2 *n*-Heptane, analytical reagent quality (*note 4*).

5.3 Cyclohexane, analytical reagent quality (*note 4*).

5.4 Carrier gas: hydrogen, nitrogen or helium thoroughly dried and containing less than 10 mg/kg of oxygen.

5.5 Auxiliary gases: hydrogen, 99.9 per cent pure containing no organic impurities; air containing no organic impurities.

6 Sampling and sample storage

It is essential that loss of solvent from the sample be prevented. The laboratory sample

shall be in a completely sealed container (preferably in a crimped metal box) and shall be stored at +4°C.
Plastic containers shall not be used.
The determination of hydrocarbons shall be carried out as soon as the container has been opened.

7 Procedure

7.1 Determination of the calibration factor (*note 5*)

Weigh to the nearest 0.1 g, 5 g of a meal containing a very low level of extraction solvent (*note 6*) into each of five septum vials (4.1). Seal each vial with a septum (4.2) and an aluminium foil cap (4.3). Add, using the syringe (4.5), variable quantities of technical hexane (5.1) to four of five vials, according to the following table (*note 7*); do not add solvent to one of the vials.

Vial no.	1	2	3	4
μl technical hexane added	1	2	4	7

Allow the sample vials to stand at room temperature for 24 hours (*note 8*).
Then, add 5 μl of *n*-heptane (5.2) or cyclohexane (5.3) as an internal standard, to each sample vial, making the addition with the syringe (4.5) through the septum. At 15-minute intervals, place one of the sample vials in the heating bath, the heating medium coming up to the neck of each vial, and heat at 80°C for exactly 60 minutes.
Warm the gas-tight syringe (4.6) to 60°C.
Without removing the vial from the heating bath, withdraw about 1 ml of the gaseous phase from the headspace through the septum with the heated gas syringe. Inject the sample immediately into the chromatograph (4.7) with the injection and detection temperatures set at 120°C, the oven temperature set at 40°C and the carrier gas (5.4) pressure at 0.3 bar (*note 9*).
For each of the four vials containing added technical hexane (5.1), a calibration factor, F, may be determined by the formula:

$$F = \frac{C_s \times A_i}{C_i \times (A_H - A_B - A_i)}$$

where:

A_B is the peak area of the hydrocarbons present in the oil-seed residue, to which technical hexane has not been added, i.e. in the oil-seed residue present in the fifth vial less the peak area of the internal standard (*note 10*)

A_H is the total peak area of the hydrocarbons including internal standard present in the oil-seed residue of the vial (*note 10*)

A_i is the peak area corresponding to the internal standard

C_i is the concentration of internal standard expressed in milligrams per kilogram of oil-seed residue. For an addition of 5 μg to 5 g of sample, C_i = 680 for *n*-heptane and C_i = 780 for cyclohexane

C_s is the concentration of technical hexane added to the oil-seed residue present in the vial, expressed in milligrams per 1000 g of meal. It is given by the following table:

Vial no.	1	2	3	4
Concentration of technical hexane added (mg/1000 g)	134	268	536	938

If *n*-hexane is used, C_i is given by the following table:

Vial no.	1	2	3	4
Concentration of technical hexane added (mg/1000 g)	132	264	528	924

Calculate the mean calibration factor from the results obtained for the four vials. The mean calibration factor should be 1.0 ± 0.1, and the differences between the individual results should be small.

7.2 Test portion analysis

Weigh to the nearest 0.1 g, 5 g of the test portion into a vial (4.1). Seal it immediately with the septum (4.2). Cover with foil (4.3) and crimp with pliers (4.4). Inject 5 μl of the internal standard (5.2 or 5.3) (*note 4*) into the vial through the septum using the syringe (4.5). Immerse the vial up to the neck in the heating bath (4.9) regulated at 80°C for exactly 1 hour.

Warm the gas-tight syringe (4.6) to 60°C.

Without removing the vial from the heating bath, withdraw about 1 ml of the gaseous phase from the headspace through the septum with the heated gas syringe. Inject the sample immediately into the chromatograph (4.7) with the injection and detection temperatures set at 120°C, the oven temperature set at 40°C and carrier gas (5.4) pressure at 0.3 bar.

Determine the total peak area due to hydrocarbons (including the peak area of the internal standard) and the peak area due to the internal standard.

8 Calculation and expression of results

The 'free' hexane content (H_f) expressed in mg/kg (p.p.m.) is given by the formula:

$$H_f = \frac{(A_H - A_i) \cdot F \cdot C_i}{A_i}$$

where:

A_H is the total peak area due to hydrocarbons (including the peak area of the internal standard) (*note 10*)

A_i is the peak area due to the internal standard

C_i is the concentration of the internal standard expressed in milligrams per kilogram of oil-seed residue. For an addition of 5 μg to 5 g of sample, $C_i = 680$ for *n*-heptane and $C_i = 780$ for cyclohexane

F is the calibration factor determined according to 7.1.

9 Precision

The results of one interlaboratory test organized at an international level (for different samples) gave the statistical results shown in the table in the Appendix.

9.1 Repeatability

When the mean of duplicate determinations lies within the range of the mean values cited in the table, the difference between the results of two determinations, carried out in rapid succession by the same operator using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r), which can be deduced from the table, for the level of hexane in the sample examined.

9.2 Reproducibility

When the means of duplicate determinations, obtained in two different laboratories using this standard method for the analysis of the same laboratory sample, lie within the range of the mean values cited in the table, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R), which can be deduced from the table, for the level of hexane in the sample examined.

10 **Notes**

1 A material such as nitrile rubber, for example Perbunan, or butyl rubber with a polytetrafluoroethylene or polychloroprene seal, for example Neoprene, is suitable. These septa often have a very high mechanical resistance; in this case it is preferable to perforate them with a pin before taking the vapour from the headspace and using this perforation so that the syringe needle is not damaged on insertion. Re-use of the septa is not recommended.

2 Chromosorb WAW is suitable.

3 SE 30 is suitable.

4 If the hexane used for extraction or calibration contains appreciable amounts of cyclohexane, *n*-heptane should be used as the internal standard.

5 Commercial oil-seed residues usually have a water content of 12–14 per cent. For samples having a different water content, the calibration has to be made with an oil-seed residue that has the same water content as the sample.

6 The meal used for the calibration should be of a similar nature as the sample. A meal can be used to determine the calibration factor if it contains only small amounts of extraction solvent (less than 0.01 per cent). If it contains a greater amount of solvent, this should be reduced by leaving the meal in thin layers in the open for some hours in air.

7 When a sample to be tested has a high 'hexane' content, the quantity of technical hexane added to vials 3 and 4 must be sufficiently high so that the levels of hexane contained in the meal of these vials corresponds to the high level of solvent expected in the sample.

8 In the case of shorter contact times, it cannot be certain that the extraction solvent is absorbed completely by the residue and that the absorption–desorption equilibrium of the extraction solvent between meal and air has been attained.

9 For routine analyses, an automatic sampler may be used.

10 Hydrocarbons which usually make up the technical solvents are: 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane etc. A typical chromatogram of these solvents is represented in Fig. 1.172.1. Do not include peaks due to the oxidation products. Some of these products may be present in significant amounts.

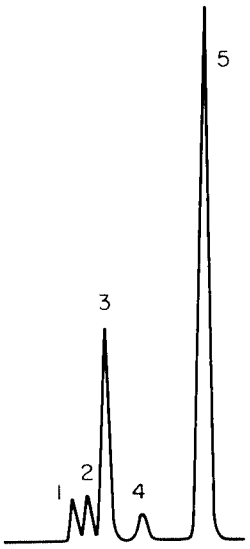


Fig. 1.172.1 Gas chromatogram of hexane hydrocarbons from extraction meal.
1: 2-Methylpentane; *2*: 3-Methylpentane; *3*: *n*-Hexane; *4*: Methylcyclopentane; *5*: Cyclohexane (internal standard) (with other retention times: *n*-heptane)

Appendix

Table 1.172.1. Statistical analysis of results for 'free' hexane (Collaborative Study 1984—duplicate determinations).

	Fresh rapeseed meal	Fresh rapeseed meal	Stored sunflower seed meal
Moisture content (%)	8.8	12.1	9
Number of laboratories	13	13	12
Number of results	26	26	24
Number of laboratories after elimination of outliers	11	11	12
Mean value (mg/kg)	624	599	178
Repeatability standard deviation (S_r)	17	33	12
Repeatability coefficient of variation (%)	2.7	5.5	6.7
Repeatability value r ($S_r \times 2.83$)	48	94	34
Reproducibility standard deviation (S_R)	129	125	55
Reproducibility coefficient of variation (%)	21	21	31
Reproducibility value R ($S_R \times 2.83$)	364	353	156

* Sample 1 (moisture 8.8 per cent) adjusted to moisture level of 12.1 per cent.

References

Arens M, Kroll E. *Fette-Seifen Anstrichmittel* 1983; **85**: 307.
Dupuy HP, Fore SP. *J Am Oil Chem Soc* 1970; **47**: 231.
Hautfenne A, Pocklington WD, Wolff JP. *Pure Appl Chem* 1987; **59**(10): 1407–1418 (Report on the Collaborative Study).
Prevot A, Coustille JL. *Rev Franç Corps Gras* 1981; **28**: 413.

Section 2
Oils and Fats

Alphabetical index to section 2: oils and fats

2.303 Determination of *n*-3 and *n*-6 unsaturated fatty acids in vegetable oils and fats by capillary gas–liquid chromatography

1 Scope

This Standard describes a method for the determination of *n*-3 and *n*-6 polyunsaturated fatty acids in vegetable oils and fats by gas–liquid chromatography.

2 Field of application

This Standard is applicable to the fatty acid methyl esters obtained from vegetable oils and fats according to 2.301.

3 Apparatus

The instructions given apply to normal equipment used for gas–liquid chromatography employing capillary columns and flame-ionization detection.

3.1 Gas–liquid chromatography

3.1.1 **Injection system**

The injection system should be specially designed for use with capillary columns. It should be of the split type or on-column (*note 1*).

3.1.2 **Oven**

The oven should be capable of heating the column to at least 220°C and of maintaining the desired temperature to within 0.1°C (*note 2*).

3.1.3 **Capillary column**

3.1.3.1 *Column*

Fused silica or glass, 25–60 m in length, 0.20–0.35 mm internal diameter.

3.1.3.2 Stationary phase of moderate polarity, mainly of the type polyglycol (polyethylene glycol 20 000), polyester (butanediol polysuccinate) or polar polysiloxane (cyanosilicones), e.g. Carbowax, Durabond 225, FFAP, Silar 5 CP, Supelcowax, CP Sil 88.

3.1.3.3 The coating should be 0.1–0.2 μm.

3.1.3.4 *Assembly and conditioning of the column*

Observe the normal precautions for assembling capillary columns: arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead spaces). Place the column under a carrier gas flow (e.g. 0.3 bar for 25 m of a column of internal diameter 0.3 mm).

Condition the column by temperature programming of the oven at 3°C per minute from ambient temperature to a temperature 10°C below the decomposition limit of the stationary phase. Maintain at this temperature for 1 hour until stabilization of the baseline. Return to 180°C to work under isothermal conditions.

3.2 Syringe: maximum capacity 10 μl, graduated in 0.1 μl.

3.3 Recorder: according to the method 2.302. It should be compatible with the apparatus used.

3.4 Integrator or calculator: specifications of the electronic calculator and integrator according to 2.302.

4 Reagents

4.1 Carrier gas: helium or hydrogen (*note 3*).

4.2 Auxiliary gas: hydrogen 99.9 per cent minimum, free from organic impurities; air or oxygen, free from organic impurities.

4.3 Reference standards: a mixture of methyl esters of *n*-3 and *n*-6 polyunsaturated fatty acids (*note 4*).

5 Procedure

5.1 Test conditions

5.1.1 **Selection of optimum operating conditions**

The properties of efficiency and permeability of capillary columns mean that the separation between constituents and duration of analysis is largely dependent on the flow rate of the carrier gas in the column. It will be necessary to optimize the operating conditions by acting on this parameter (or more simply on the deadspace of the column), according to whether one wishes to improve the separations or to make a rapid analysis.

5.1.2 **Determination of efficiency and resolution**

Use correspondingly the formula given in 2.302. Number of theoretical plates calculated for methyl stearate should be at least 2000 per metre and the resolution at least 1.25.

5.2 Test portion

Use the syringe (3.2) to take 0.1–2.0 μl of the solution of 1–5% of methyl esters prepared according to 2.301.

5.3 Analysis

Operate the oven isothermally at a temperature of 180–210°C or operate by linear heating from 80°C to 220°C (*note 5*).

Set injector and detector temperatures 30–50°C above the column temperature.

6 Calculation and expression of results

6.1 Calculation of results

Obtain the area for each peak by electronic integration and express it as a percentage of the total fatty acid methyl esters. Use formulae given in 2.302. Identify peaks with available standards and material of known composition (*note 4*).

Critical pairs or triplets of fatty acid peaks may be formed depending on the stationary phase (3.1.3.2) used:

— $C_{20:0}$ and $C_{18:3}$ (*n*-6),

— $C_{22:0}$ and $C_{20:3}$ (*n*-6),

— $C_{20:3}$ (*n*-3); $C_{22:1}$ (*n*-9); and $C_{20:4}$ (*n*-3) (*note 6*).

6.2 Expression of results

See 6.2.2 of method 2.302.

7 Precision

The results of interlaboratory studies organized at the international level gave the statistical results (evaluated in accordance with *ISO 5725-1986*) which are summarized in the table in the appendix.

7.1 Repeatability

When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in the table in the appendix, the difference between the two values obtained should not be greater than the repeatability value (r) for the level of fatty acids comparable with those cited in the annexed table.

7.2 Reproducibility

When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in the table in the appendix, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility (R) for the level of fatty acids comparable with those cited in the annexed table.

8 Notes

1 On-column injection is preferred as it generally gives a better resolution.

2 Apparatus equipped with a temperature programmer is recommended.

3 Either helium or nitrogen may be suitable as a carrier gas but these may increase helium elution times with respect to hydrogen.

4 Standards of most known *n*-3 and *n*-6 fatty acids are available from suppliers such as Nuspet: Nu-Check-Prep. Inc., PO Box 172, Elysian, Minnesota 56028 USA; for Europe: Bast of Copenhagen V, Denmark.

Otherwise, oils with specific fatty acids should be used as standards, such as black-currant seed oil containing $C_{18:3}$ (*n*-6) (gamma-linoleic acid; systematic name: 6,9,12-octadecatrienoic acid) and $C_{18:4}$ (*n*-3) (stearidonic acid; systematic name: 6,9,12,15-octadecatetraenoic acid).

5 Operating the oven by linear heating increases the speed of fatty acid elution but does not improve or reduce the resolution of the gas chromatograms. A suitable heating programme is: Start at an oven temperature of 80°C. Hold it for 2 minutes, then heat up at a rate of 20°C per minute until 125°C is reached. Hold it there for 1 minute, then continue to heat up at a rate of 3°C per minute until 220°C is reached. Hold at this temperature for at least 5 minutes until all high carbon number fatty acid methyl esters are eluted.

6 The abbreviated symbol $C_{18:3}$, for example, stands for the number of carbon atoms (C_{18}) and the number of double bonds (:3) in the fatty acid chain; :0 means saturated fatty acids.

Appendix

Table 2.303.1. Statistical results of the 3rd Collaborative Study.

	n-3 fatty acids				*n*-6 fatty acids			
Sample:	A	B	C	D	A	B	C	D
Number of laboratories	21	21	21	21	21	21	21	21
Number of accepted results	18	18	19	20	19	20	19	20
Mean value (g/100 g)	0.04	3.10	0.13	0.25	3.05	41.58	1.61	14.70
Repeatability standard deviation (S_r)	0.0	0.10	0.03	0.11	0.17	0.78	0.12	0.98
Repeatability coefficient of variation (%)	0.0	3.36	25.6	43.6	5.57	1.88	7.45	6.67
Repeatability (r) (2.83 × S_r)	—	0.29	—	0.31	0.48	2.21	0.34	2.77
Reproducibility standard deviation (S_R)	—	0.26	—	0.14	1.53	1.96	0.53	2.48
Reproducibility coefficient of variation (%)	—	8.32	—	55.7	50.16	4.71	32.92	16.87
Reproducibility value (R) (2.83 × S_R)	—	0.73	—	0.39	4.33	5.55	1.50	7.02

References

Beare-Rogers JL, Dieffenbacher A. *Pure Appl Chem* (1990) **62**(4): 795–802 (Report on the Collaborative Study).

IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn. Oxford: Blackwell Scientific Publications, 1987.

WHO/FAO. *The Role of Edible Oils and Fats in Human Nutrition* Rome: WHO/FAO, 1977.

2.304 Capillary column gas–liquid chromatography of fatty acid methyl esters

1 Scope

This Standard describes a general method for the application of capillary column gas–liquid chromatography to determine the qualitative and quantitative composition of a mixture of fatty acid methyl esters.

2 Field of application

This Standard is applicable to the fatty acid methyl esters prepared from oils and fats (*note 1*) according to method 2.301.

3 Apparatus

The instructions given apply to normal equipment used for gas–liquid chromatography employing capillary columns and flame-ionization detection.

3.1 Gas–liquid chromatography

3.1.1 Injection system

The injection system should be specially designed for use with capillary columns. It should be either of the split type or on-column (splitless) type (*note 2*).

3.1.2 Oven

The oven should be capable of heating the column to at least 220°C and of maintaining the desired temperature to within 0.1°C (*note 3*).

3.1.3 Capillary column

3.1.3.1 *Column*

Fused silica or glass, length 25–60 m, internal diameter 0.20–0.35 mm, with a stationary phase coating thickness of 0.1–0.2 μm (*note 4*).

3.1.3.2 *Stationary phase*

This should be of moderate polarity, such as the polyglycol type (e.g. polyethylene glycol 20 000), polyester type (e.g. butanediol succinate) or polar polysiloxane type (e.g. cyanosilicones) (*note 5*).

3.1.3.3 *Assembly and conditioning of the column*

Observe the normal precautions for assembling capillary columns, i.e. ensuring adequate support of the column in the oven, checking joints to injector and detector for leaks, positioning of the ends of the column at the injector and detector such that the dead-space in these areas is reduced to a minimum.

Subject the column to a suitable carrier gas flow and condition the column by heating it to about 10–50°C above the maximum oven temperature to be used for the analysis, but not exceeding the maximum temperature guaranteed for the stationary phase or recommended conditioning temperature for the column. Maintain the column at this temperature for at least 1 hour to achieve stabilization of the baseline. Reduce the oven temperature to 180°C for analysis under isothermal conditions.

3.2

Syringe, maximum capacity 10 μl, graduated in 0.1 μl.

3.3 Recorder, according to method 2.302, and compatible with the apparatus used.

3.4 Integrator, electronic, as specified in method 2.302, or data processor.

4 Reagents

4.1 Carrier gas: helium or hydrogen (*note 6*).

4.2 Auxiliary gases: hydrogen 99.9 per cent minimum, free from organic impurities; air or oxygen, free from organic impurities.

4.3 Reference standards or natural mixtures of lipids of known fatty acid composition corresponding to that of the sample. Otherwise, reference standards of individual fatty acids, or fatty acid methyl esters, or mixtures thereof with a fatty acid composition corresponding to that of the sample (*note 7*).

5 Procedure

5.1 Test conditions

5.1.1 **Selection of optimum operating conditions**

The efficiency of the column and permeability properties of the phase mean that the separation between constituents and duration of the analysis is largely dependent on the type of carrier gas, column dimensions, temperature and type of fatty acid. It will be necessary to optimize the operating conditions of temperature and flow rate according to whether one wishes to improve the separations or to make a rapid analysis. For general purposes a linear gas velocity of 40 cm per minute for hydrogen and 30 cm per minute for helium should be satisfactory (*note 6*).

5.1.2 **Determination of the efficiency and resolution**

Use correspondingly the formula given in method 2.302. The operating conditions selected (5.2) should be those that afford the number of theoretical plates for methyl stearate to be at least 2000 per metre (*note 8*) and the resolution at least 1.25.

5.2 Operating conditions

Operate the oven either isothermally at a temperature of 180–210°C, or operate by temperature-programming from 80–220°C (*note 9*).

Set the injector and detector temperatures about 30–50°C above the column temperature (*note 10*).

5.3 Analysis

Using the syringe (3.2), inject 0.1–0.2 μl of the solution of 1–5 per cent fatty acid methyl esters prepared from the selected reference standard (4.3) according to method 2.301. With on-column injection mode, dilute the solution to 0.05 per cent before injection.

Similarly inject 0.1–0.2 μl of a solution of fatty acid methyl esters prepared from the test sample in exactly the same way.

6 Calculation and expression of results

6.1 Qualitative analysis

See method 2.302 (para. 6.1).

When identifying the peaks of the sample fatty acids by reference to the peak retention times of known fatty acids, it should be realized that critical pairs or triplets of fatty acid peaks may have been formed, depending on the stationary phase (3.1.3.2) used for the analysis, e.g. $C_{20:0}$ and $C_{18:3}$ (*n*-6); $C_{22:0}$ and $C_{20:3}$ (*n*-6); $C_{20:3}$ (*n*-3); $C_{22:1}$ (*n*-9); and $C_{20:4}$ (*n*-3) (*note 11*).

6.2 ## Quantitative analysis

Use the formula given in method 2.302 (para. 6.2).

6.2.1 **Use of internal standards**

In the assay of fatty acids $C_{4:0}$ and $C_{6:0}$ fatty acids in dairy fats (or blends containing dairy fats), $C_{5:0}$ should be used as the internal standard. In cases where not all fatty acid methyl esters are eluted, fatty acid $C_{15:0}$ or $C_{17:0}$ should be used as the internal standard. The content (P_i) of component i, expressed as a percentage (m/m), is given by the formula:

$$P_i = \frac{m_s \times K'_i \times A_i}{m \times K'_s \times A_s}$$

where:

m_s is the mass, in mg, of the internal standard added to the test portion

m is the mass, in mg, of the test portion

K'_i is the correction factor for the component

K'_s is the correction factor for the internal standard

A_i is the area of the peak corresponding to the component i

A_s is the area of the peak corresponding to the internal standard.

For the calculation of the correction factors K'_i and K'_s use the formula given in method 2.302 (para. 6.2.1) (*note 12*).

7 # Quality assurance

7.1 For *general principles* of analytical quality control see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

7.2 For *specific applications* of analytical quality control see the *Annexe* to this standard method.

8 # Notes

1 The precision of the method when applied to butyric and lauric oils and fats has not been determined.

2 On-column injection is preferred as it generally prevents sample injection discrimination involving losses of highly volatile short-chain fatty acid methyl esters. In this case the theoretical response factors can be used instead of the empirical calibration factors. An automatic injection system is recommended as it improves the repeatability of results.

3 Apparatus equipped with a temperature programmer is recommended.

4 For cold on-column injection, a retention gap column must be used to re-concentrate the fatty acid methyl esters at the beginning of the separation column. The retention gap

must be 0.5–1 m in length with an internal diameter of 0.53 mm to allow insertion of the needle into the column.

5 Carbowax, Durabond 225, FFAP, Silar 5 CP, Supelcowax, or CP Sil 88 are suitable stationary phases. The selection of a particular phase depends on the intended separation of fatty acid methyl esters. For separations of unsaturated fatty acid methyl esters, cyanosilicones are preferred to Carbowax columns. If the coating is too thin, this will increase difficulties due to active sites in the column. For this reason a coating of 0.2 μm is to be preferred.

6 Either helium or nitrogen may be suitable as a carrier gas but they will increase elution times. Hydrogen is preferred for the analysis of fatty acid methyl esters derived from fats containing short-chain fatty acids. It is also preferred for the analysis of fats with long-chain fatty acids since the elution time is some two times shorter than with helium—consequently peak broadening is less. Oxygen traces in the carrier gas may damage the column coatings and considerably reduce the life of capillary columns.

7 Standards of most known fatty acids are available from suppliers such as Nuspet: Nu-Check-Prep. Inc., PO Box 172, Elysian, Minnesota 56028 USA; for Europe: Bast of Copenhagen V, Denmark.

Otherwise, natural oils and fats of known fatty acid composition (available from the European Community of Reference (BCR), rue de la Loi 200, B-1049 Brussels, Belgium) or those with a specific fatty acid, such as blackcurrant seed oil for $C_{18:3}$ (*n*-6) (gamma-linolenic acid) and for $C_{18:4}$ (*n*-3) (stearidonic acid), can be used.

8 The separation power in terms of theoretical plate number depends on the length and inside diameter of the column. The longer the column and the thinner the coating of stationary phase, the higher is the theoretical plate number.

9 Operating the oven by temperature programming increases the speed of fatty acid elution but does not improve or reduce the resolution of the peaks. A suitable heating programme is: Start at an oven temperature of 80°C. Hold for 2 minutes, then heat up at a rate of 20°C per minute until 125°C is reached. Hold it there for 1 minute, then continue to heat up at a rate of 3°C per minute until 220°C is reached. Hold at this temperature for at least 5 minutes, until all high carbon number fatty acid methyl esters are eluted.

10 For on-column injection, set the injector and oven 10°C beneath the boiling point of the solvent and start with heating immediately after injection, e.g. 50°C can be used in the case of pentane.

11 In the abbreviated symbols $C_{4:0}$, $C_{18:2}$, the figures 4 and 18 stand for the number of carbon atoms in the fatty acid chain and the figures immediately following the colon (:) refer to the number of unsaturated bonds in the fatty acid chain; *n*-3 and *n*-6 refer to the place of the first double bond in the fatty acid chain counted from the methyl group end of the chain.

12 If a correction factor of 1.000 is taken for $C_{16:0}$, most fatty acid methyl esters will be found to have correction factors close to 1.00, except in the case of the short-chain fatty acids $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$. The correction factors should correspond with the theoretical response factors within certain margins. This is especially important in the case of the analysis of short-chain fatty acids because incorrectly determined correction factors have been found to be a common factor in the obtaining of erroneous results by many laboratories.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the *repeatability limit* (r) deduced by linear interpolation from the data in the table.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the *reproducibility limit* (R) deduced by linear interpolation from the data in the table.

3 Results of the interlaboratory test

An interlaboratory test carried out at the international level in 1988 by the IUPAC Commission on Oils, Fats and Derivatives, in which 16 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in Table 2.304.1.

Table 2.304.1.

	Fatty acid					
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Number of laboratories	13	16	16	16	16	16
Number of results	13	16	16	16	16	16
Number of laboratories retained after elimination of outliers	12	14	14	14	15	14
Number of results retained after elimination of outliers	12	14	14	14	15	14
Mean value (%) (surface/surface)	0.10	10.30	3.51	22.54	53.55	6.70
Repeatability standard deviation (S_r)	0.05	0.21	0.09	0.35	0.29	0.09
Repeatability relative standard deviation	50	2.1	2.6	1.6	0.5	1.3
Repeatability limit (r) ($2.83 \times S_r$)	0.15	0.61	0.26	1.00	0.81	0.25
Reproducibility standard deviation (S_R)	0.06	0.96	0.25	0.85	1.15	0.44
Reproducibility relative standard deviation	55	9.2	7.0	3.8	2.1	6.6
Reproducibility limit (R) ($2.83 \times S_R$)	0.16	2.71	0.70	2.41	3.25	1.25

2.310 Determination of butyric acid

1 Scope and field of application

This Standard describes a method for the determination of the butyric acid content of milkfat or butterfat or mixtures of fats containing milkfat or butterfat.

2 Principle

Saponification of the fat with potassium hydroxide solution followed by acidification with phosphoric acid to liberate the fatty acids. Separation of the water-insoluble and water-soluble fatty acids by filtration. Direct determination of butyric acid by gas–liquid chromatography in the presence of an internal standard.

3 Definition

The butyric acid content of milkfat or butterfat or mixtures of fats containing milkfat and butterfat is the quantity, expressed as a percentage by mass, of butyric acid, determined in this sample by the present method.

4 Apparatus

4.1 50-ml beaker.

4.2 Test tubes, 10 ml, with ground-glass stoppers.

4.3 2–5-ml graduated pipettes.

4.4 1-μl microsyringe.

4.5 Gas–liquid chromatograph, with flame-ionization detector, on-column or all-glass injection system, and recorder.

4.6 Column, glass, approximately 2 m in length and 3 mm internal diameter to fit the chromatograph (4.5), filled with 10–15% stationary phase suitable for free fatty acid analysis (*note 1*) on an 80/100 acid-washed silanized support (*note 2*) conditioned and stabilized for use at an analysis temperature of about 130–135°C (*note 3*).

4.7 Fast filter paper.

4.8 Glass beads.

4.9 Watch-glass.

4.10 Water bath, maintained at boiling temperature.

5 Reagents

5.1 Potassium hydroxide solution, approximately 0.5 N in ethanol: dissolve 4.5 g of potassium hydroxide in 100 ml of ethanol.

5.2 *o*-Phosphoric acid, 5 per cent (m/V) aqueous solution.

5.3 *n*-Butyric acid, reference standard, 0.4 mg/ml aqueous solution: dissolve 400 mg of *n*-butyric acid in 100 ml of water and dilute 10 ml of this solution to 100 ml (*note 4*).

5.4 *n*-Valeric acid, internal standard, 0.25 mg/ml aqueous solution: dissolve 250 mg of *n*-valeric acid in 100 ml of water and dilute 10 ml of this solution to 100 ml (*note 4*).

6 Procedure

6.1 Construction of a calibration curve

By means of graduated pipettes (4.3) transfer to individual test tubes (4.2) 0.2, 0.5, 1.0, 2.0, 3.5 and 5.0 ml of butyric acid solution (5.3). To each test tube add by pipette (4.3) 2.0 ml of valeric acid solution (5.4) and, respectively, 4.8, 4.5, 4.0, 3.0, 1.5 and 0 ml of water. Stopper the test tubes and gently mix the solutions.

The solutions in the test tubes contain respectively 0.08, 0.2, 0.4, 0.8, 1.4 and 2.0 mg of butyric acid and 0.5 mg of valeric acid.

Stabilize the column (4.6) for at least 30 minutes at the analysis temperature (*note 3*).

By means of a microsyringe (4.4) inject about 1 μl of the prepared standard solutions in turn. Measure the heights of the butyric acid and valeric acid peaks to the nearest 0.5 mm. Plot the ratio of these heights (butyric acid/valeric acid) against the corresponding weight of butyric acid (*note 5*).

6.2 Determination of the butyric acid content

Weigh accurately about 100 mg of the sample into a beaker (4.1). Add, by pipette (4.3), 3 ml of the ethanolic potassium hydroxide solution (5.1) and a few glass beads (4.8). Cover the beaker with a watch-glass (4.9) and place in a boiling water bath (4.10). Heat for at least 10 minutes or until fat globules are no longer visible on the surface.

Remove the watch-glass and continue heating until the ethanol has completely evaporated.

Allow the beaker to cool.

Add, by pipette (4.3), 5.0 ml of water. Cover with a watch-glass and swirl gently to completely dissolve the soap (*note 6*).

Add, by pipette (4.3), 5.0 ml of phosphoric acid solution (5.2). Swirl gently to coagulate the precipitated fatty acids. Filter through a small fluted fast filter paper (4.7). By pipette (4.3), transfer 5.0 ml of the filtrate to a test tube (4.2). Add, by pipette (4.3), 2.0 ml of the valeric acid solution (5.3). Stopper and mix.

Stabilize the column (4.6) for at least 30 minutes at the analysis temperature (*notes 3, 7, 8*).

By means of a microsyringe (4.4), inject about 1 μl of the final solution on to the column (4.6). Measure to the nearest 0.5 mm the peak heights of butyric acid from the sample and the valeric acid added as an internal standard (*notes 5, 9*).

7 Calculation and expression of results

7.1 Calculate the peak height ratio of butyric acid/valeric acid obtained from the analysis of the sample and read off from the calibration curve the mass of butyric acid equivalent to the peak height ratio.

7.2 The butyric acid content (B), expressed as a percentage (m/m) of the sample is given by:

$$B = \frac{m_b \times 200}{m}$$

where:

m_b is the mass, in mg, of the butyric acid read from the calibration curve

m is the mass, in mg, of the test portion.

8 Precision

8.1 Repeatability value

When the mean of duplicate determinations lies between any two of the mean values shown in the table in the Appendix, the difference between the results of two determinations, carried out in rapid succession by the same operator using the same apparatus for the analysis of identical test material, should not be greater than the value indicated

in the table for the repeatability value (r) which corresponds to the higher of the two mean values.

8.2 Reproducibility value

When the means of duplicate determinations, obtained in two different laboratories using this standard method for the analysis of identical test material, lie between any two of the mean values shown in the table in the Appendix, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R) which corresponds to the higher of the two mean values.

9 **Notes**

1 FFAP and SP-1220 with 1 per cent phosphoric acid are suitable.

2 Chromosorb W is suitable.

3 The column should be conditioned at about 180°C for at least 48 hours. If on-column injection is not used, the injection port should be set at not less than 175°C. If baseline separation of the butyric acid and valeric acid peaks is not obtained, it may be found that the resolution of columns containing a phosphoric acid stationary phase can be improved by the injection of 1 μl quantities of 2.5 per cent (m/V) phosphoric acid solution on to the column while it is heated at the analysis temperature of about 130–135°C. The carrier-gas flow rate should be adjusted so that the butyric acid has a retention time of about 5 minutes.

If tailing of peaks is experienced, even after conditioning of the column, this can sometimes be reduced or eliminated by injecting 2 μl of trimethyl-chlorosilane (TMCS) on to the column.

4 The solutions of valeric acid and butyric acid must be freshly prepared.

5 The amplifier attenuation should be adjusted so that the height of the butyric acid peak for the highest butyric acid standard (6.1) is about 80 per cent of the full-scale recorder reading.

6 It may be found necessary to warm the mixture gently to achieve complete solution of the soaps.

7 The syringe should be rinsed thoroughly with water between every two analyses and at the completion of analyses should be rinsed with a diluted soap solution in order to minimize any corrosion due to phosphoric acid.

8 After a series of sample injections it is recommended that injections of one or more of the solutions of butyric acid/valeric acid standards (6.1) be made and the calibration curve checked against the corresponding butyric acid/valeric acid peak height ratios obtained from the standard solutions.

9 Peaks for caproic acid and caprylic acid may appear after valeric acid on the chromatogram and interfere with subsequent analyses if care is not taken to ensure that these acids have eluted before another sample solution is injected.

Appendix

Table 2.310.1. Statistical analysis of results for butyric acid.

	Level*			
	A	B	C	D
Number of laboratories	13	7	11	13
Number of accepted results	22	14	22	22
Mean value (percentage m/m)	0.185	0.354	1.79	3.46
Repeatability standard deviation	0.008	0.015	0.044	0.102
Repeatability coefficient of variation (%)	4.5	4.4	2.4	2.95
Reproducibility standard deviation	0.024	0.056	0.155	0.242
Reproducibility coefficient of variation (%)	12.9	15.9	8.7	7.0
Repeatability value r (95) *ISO-5725*	0.02	0.04	0.12	0.29
Reproducibility value R (95) *ISO-5725*	0.07	0.16	0.44	0.69

* Levels A, B, C and D represent the levels of butyric acid to be found in fats containing about 5, 10, 50 and 100 per cent m/m butterfat respectively.

References

Biltcliffe DO, Wood R. *J Assoc Publ Analysts* 1982; **20**: 69–88.

IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn. Oxford: Blackwell Scientific Publications, 1986.

Kuzdzal-Savoie K, Kuzdzal K. *Le Lait* 1968; No. 475–476: 255–260.

Phillips AR, Sanders BJ. *J Assoc Publ Analysts* 1968; **6**: 89–95.

Pocklington, WD, Hautfenne A. *Pure Appl Chem* 1986; **58**(10): 1419–1428 (Report on the Collaborative Study).

2.324 Determination of the composition of triglycerides in vegetable oils in terms of their partition number by high performance liquid chromatography (HPLC)

1 Scope

This Standard describes a method for the separation and quantitative determination of triglycerides in vegetable oils in terms of their molecular weight and degree of unsaturation as a function of their partition number, i.e. their equivalent carbon number (ECN).

2 Field of application

This Standard is applicable to all vegetable oils that contain only triglycerides of long-chain fatty acids (particularly of 16–22 carbon atoms) (*note 1*). The method is especially applicable to the detection of the presence of small quantities of semi-drying oils (rich in linoleic acid) in vegetable oils such as olive oil which contain oleic acid as the predominant unsaturated fatty acid.

3 Principle

Separation of triglycerides according to their partition number by direct high performance liquid chromatography (reversed phase). Identification of the chromatographed triglyceride peaks by reference to external triglyceride standards, and quantitation by internal normalization of the triglyceride peak areas grouped according to their partition numbers.

4 Apparatus

4.1 High performance liquid chromatograph, allowing a thermostatic control of the column temperature.

4.2 Injection unit for 10-μl delivery.

4.3 Detector: differential refractometer. The full-scale sensitivity must be at least 10^{-4} unit of refractive index.

4.4 Column: stainless steel tube, 250 mm in length and internal diameter of about 4.5 mm, packed with microparticulate silica (5 μm) bonded with octadecyldimethylsilyl derivatives (*note 2*).

4.5 Recorder and/or integrator.

5 Reagents

5.1 Chloroform, suitable for HPLC.

5.2 Acetone, suitable for HPLC.

5.3 Acetonitrile, suitable for HPLC.

5.4 HPLC mobile phase: prepare a mixture of acetonitrile (5.3) and acetone (5.2), initially in a proportion of 1:1 (V/V) (see 6.1 below). The mobile phase should be de-gassed before use—it may be recycled several times without effect on the separation of the triglycerides.

5.5 Solubilization solvent: acetone (5.2) alone, or acetone–chloroform (5.2, 5.1) 1:1 (V/V) (*note 3*).

5.6 Reference triglycerides: commercial triglycerides such as tripalmitin, tristearin, triolein, trilinolenein etc. Alternatively, a vegetable oil of known triglyceride composition, e.g. soya oil.

5.7 Reference triglyceride solution 5 per cent (W/V): transfer 0.5 ± 0.01 g of each triglyceride (5.6) to a 10-ml graduated flask and make up to volume with the solubilization solvent (5.5).

6 Procedure

6.1 Optimization of working parameters

Pump the mobile phase (5.4) through the column at a flow rate of about 1.5 ml/min for at least 30 minutes or until a stable baseline is obtained. Adjustment of the relative proportions of the solvents in the mobile phase and the flow rate should be made as required in order to achieve the desired separation of the triglycerides according to their ECN (see Fig. 2.324.1).

6.2 Preparation of test sample solution

Prepare a 5 per cent (W/V) solution by transferring a 0.5 ± 0.01-g sample to a 10-ml graduated flask and making up to volume with the solubilization solvent (5.5). Inject 10 μl of this solution.

Identify the triglyceride peaks (see 6.3 below). Record the retention times of the solvent and triglyceride peaks; also record the peak area of each triglyceride.

6.3 Identification of triglyceride peaks

Analyse the reference triglyceride solution (5.7) by injecting 10 μl of the solution under the same conditions as the test sample solution (6.2), recording retention times and peak areas as in the case of the chromatographed sample (6.2).

Identify, where possible, the triglyceride peaks by comparison of retention times of the triglyceride peaks obtained from the chromatography of vegetable oils of known triglyceride composition or reference triglycerides (*note 4*; see also retention data given by Perrin and Naudet (1983)).

To identify the remaining triglycerides:

(i) Calculate the relative retention time (α_i) of the triglyceride i with respect to the one of triolein as given by the formula:

$$\alpha_i = \frac{RT'_i}{RT'_{\text{triolein}}}$$

where:

RT'_i and RT'_{triolein} are, respectively, the reduced retention times of the triglyceride i and triolein according to:

$$RT'_{i\ or\ \text{triolein}} = RT_{i\ or\ \text{triolein}} - RT_{\text{solvent}}$$

(ii) Plot log α of the reference triglycerides against (n) their number of double bonds. The graph thus obtained allows the relative retention times to be determined for all triglycerides whose component fatty acids are present in the reference triglyceride standards (see Fig. 2.324.2).

Following identification of all the triglyceride peaks, calculate the partition number (ECN) (*note 4*) of each triglyceride. On the chromatogram divide off the triglycerides in groups according to their partition numbers as shown in the example in Fig. 2.324.1.

7 Calculation and expression of results

Using the internal normalization method, the total sum of the peak areas corresponding to all the chromatographed triglycerides is assumed to be 100%. The relative percentage (T_i) of the triglycerides with the same partition number is then given by the formula:

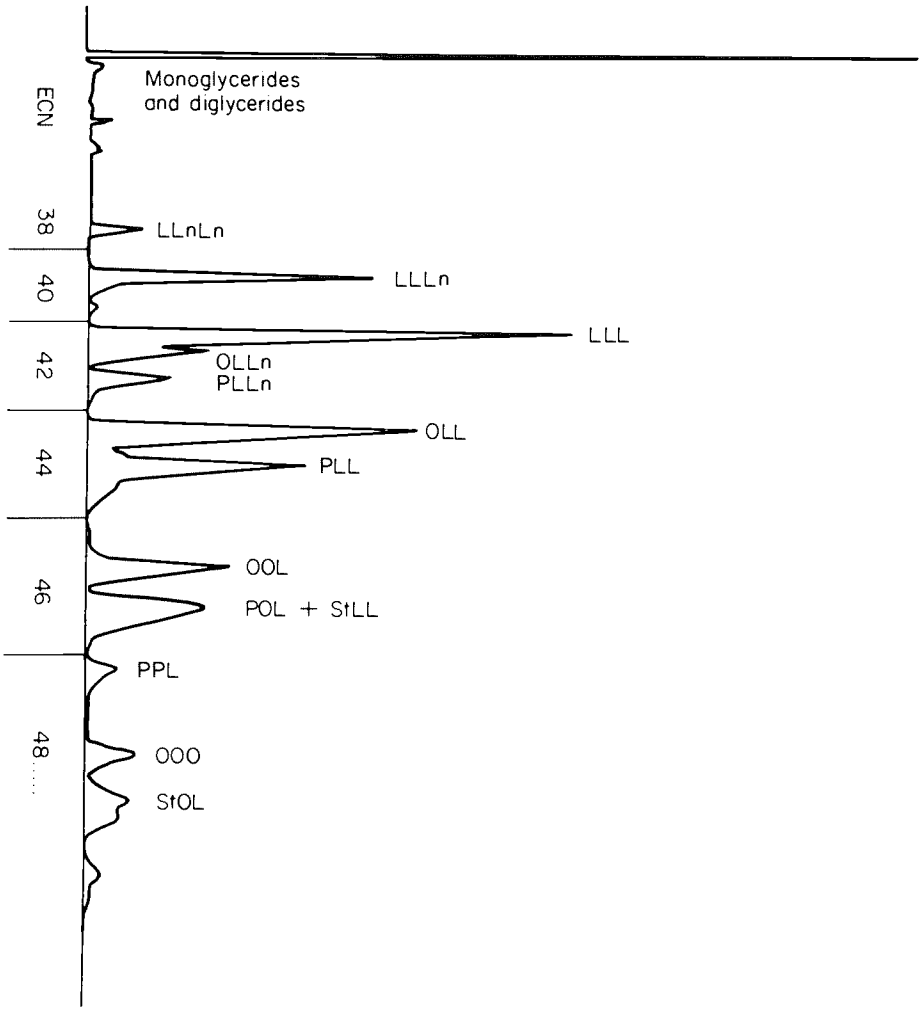


Fig. 2.324.1. Chromatogram of a sample of soya oil. The following abbreviations are used for the fatty acid components of the triglycerides: O, oleic acid; P, palmitic acid; L, linoleic acid; St, stearic acid; Ln, linolenic acid; thus, OOO represents triolein and LLL represents trilinolein.

$$T_i = \frac{\text{Area of peaks } i_{ECN}}{\text{Sum of peak areas}} \times 100$$

where:

i_{ECN} are the triglycerides with the same partition number

Express the results to one decimal place.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 It will be difficult to separate triglycerides containing fatty acids with carbon-chain lengths of less than 16 (particularly those in lauric oils such as coconut and palm kernel oils) according to their partition numbers by the present method.

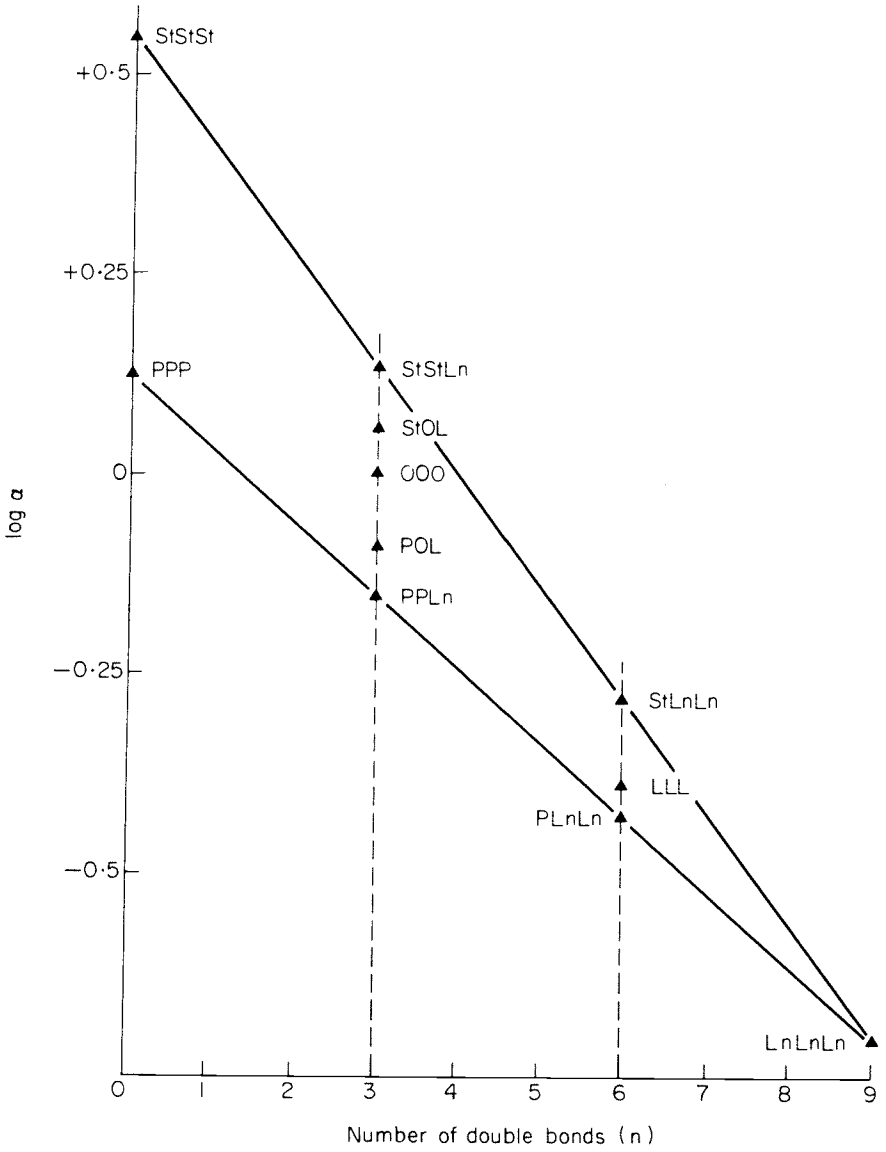


Fig. 2.324.2. Graph log $\alpha = f(n)$. For abbreviations see caption to Fig. 2.324.1.

2 Examples of suitable columns commercially available are: Lichrosphere 100 RP.18 and Lichrosphere 100 CH.18 (Merck).

3 Usually, vegetable oils are readily soluble in acetone with the exception of those containing relatively high amounts of saturated triglycerides, as well as hydrogenated oils and blends thereof. In such cases the proportion of chloroform in the solubilization solvent (5.5) can be increased to advantage.

4 The elution order of the triglycerides can be determined by calculating their partition numbers or equivalent carbon numbers (ECNs) as defined by the relationship ECN = CN − $2n$, where CN is the carbon number and n is the number of double bonds in the triglyceride molecule. It can be calculated much more precisely by taking into account the location of the double bonds. If n_1, n_2 and n_3 are the numbers of double bonds attributable to oleic, linoleic and linolenic acids, respectively, then the ECN can be calculated from the formula:

$$ECN = CN - d_1n_1 - d_2n_2 - d_3n_3$$

where the coefficients d_1, d_2 and d_3 have been calculated by means of reference triglycerides. Under the conditions specified in this method the relationship obtained will be close to:

$$ECN = CN - (2.60\ n_1) - (2.35\ n_2) - (2.17\ n_3)$$

References

Litchfield C. *Analysis of Triglycerides*. New York: Academic Press, 1972.
Pei PTS, Henly RS, Ramachandran S. *Lipids* 1975; **10**: 152.
Perrin JL, Naudet M. *Rev Franç Corps Gras* 1983; **30**: 279.
Podlaha O, Toregard B. *J High Res Chrom* 1982; **5**: 553.
Wolff JP, Mordret FX, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(8): 1173–1182 (Report on the Collaborative Study).

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in Tables 2.324.1 and 2.324.2 below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the tables.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in Tables 2.324.1 and 2.324.2 below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (R) deduced by linear interpolation from the data in the tables.

3 Result of the interlaboratory tests

Two interlaboratory tests carried out at the international level in 1986 and 1987 by the IUPAC Commission on Oils, Fats and Derivatives, in which, respectively, 18 and 16 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in Tables 2.324.1 and 2.324.2.

Table 2.324.1. Statistical analysis of results for sunflower oil (second interlaboratory study).

	Triglyceride groups					
	ECN 40	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	18	18	18	18	18	18
Number of values	36	36	36	36	36	34
Number of accepted laboratories*	16	17	16	14	17	16
Number of values	32	34	32	28	34	32
Mean value (percentage of total triglycerides)	0.3	29.4	38.4	22.5	8.2	1.3
Repeatability						
Standard deviation (S_r)	0.1	0.5	0.4	0.4	0.9	0.5
Coefficient of variation (%)	21.4	1.8	1.2	1.6	10.7	37.0
Repeatability value (r)	0.2	1.5	1.3	1.0	2.5	1.4
Reproducibility						
Standard deviation (S_R)	0.2	0.95	1.3	0.55	1.0	1.2
Coefficient of variation (%)	70.4	3.2	3.4	2.4	11.7	92.6
Reproducibility value (R)	0.6	2.7	3.65	1.55	2.7	3.4

* Elimination on basis of the Cochran and Dixon tests.

Table 2.324.2. Statistical analysis of results for olive oil (third interlaboratory study).

	Triglyceride groups				
	ECN 42	ECN 44	ECN 46	ECN 48	ECN 50
Number of participating laboratories	16	16	16	16	16
Number of values	31	31	31	31	31
Number of accepted laboratories*	12	13	14	14	14
Number of values	24	26	28	28	28
Mean value (percentage of total triglycerides)	0.6	5.85	21.2	64.7	7.15
Repeatability					
Standard deviation (S_r)	0.09	0.09	0.28	0.52	0.26
Coefficient of variation (%)	16.2	1.5	1.3	0.8	3.6
Repeatability value (r)	0.26	0.25	0.78	1.49	0.74
Reproducibility					
Standard deviation (S_R)	0.12	0.18	0.34	1.01	0.61
Coefficient of variation (%)	21.1	3.0	1.6	1.6	8.5
Reproducibility value (R)	0.34	0.50	0.95	2.85	1.73

* Elimination on basis of the Cochran and Dixon tests.

2.325 Determination of the individual triglycerides in oils and fats by high performance liquid chromatography

1 Scope

This Standard describes a method for the separation, identification and determination of individual triglycerides in edible oils and fats. This Standard is complementary to method 2.323 (Determination of triglycerides [according to their carbon number] by gas–liquid chromatography) and method 2.324 (Determination of triglycerides according to their partition number by high performance liquid chromatography).

2 Field of application

This Standard is applicable to vegetable and animal oils and fats which contain only triglycerides of long-chain fatty acids (particularly of 16–22 carbon atoms) (*note 1*).

3 Principle

Separation of the individual triglycerides by direct high performance liquid chromatography (reversed phase). Identification and quantitation by reference to external standards and the determined fatty acid composition of the sample.

4 Apparatus

4.1 High performance liquid chromatograph, as required for method 2.324, equipped as described under 2.324 paras 4.1–4.5.

5 Reagents

5.1 Chloroform, suitable for HPLC.

5.2 Acetone, suitable for HPLC.

5.3 Acetonitrile, suitable for HPLC.

5.4 HPLC mobile phase: prepare a mixture of acetonitrile (5.3) and acetone (5.2), initially in the proportion of 25:75 (V/V) (see para. 6.1 below). The mobile phase should be de-gassed before use—it may be recycled several times without effect on the separation of the triglycerides.

5.5 Solubilization solvent: acetone (5.2) alone, or acetone–chloroform (5.2, 5.1) 1:1 (V/V) (*note 2*).

5.6 Reference triglycerides: commercial triglycerides such as tripalmitin, tristearin, triolein, trilinolenin etc. Alternatively, a vegetable oil of known triglyceride composition, e.g. soya oil.

5.7 Reference triglyceride solution 5 per cent (W/V): transfer 0.5 ± 0.01 g of each triglyceride (5.6) to a 10-ml graduated flask and make up to volume with the solubilization solvent (5.5).

6 Procedure

6.1 Optimization of working parameters

Pump the mobile phase (5.4) through the column at a flow rate of about 1.5 ml/min for at least 30 minutes or until a stable baseline is obtained. Adjustment of the relative proportions of the solvents in the mobile phase and the flow rate should be made as required in order to achieve the desired separation of the individual triglycerides (see Fig. 2.325.1).

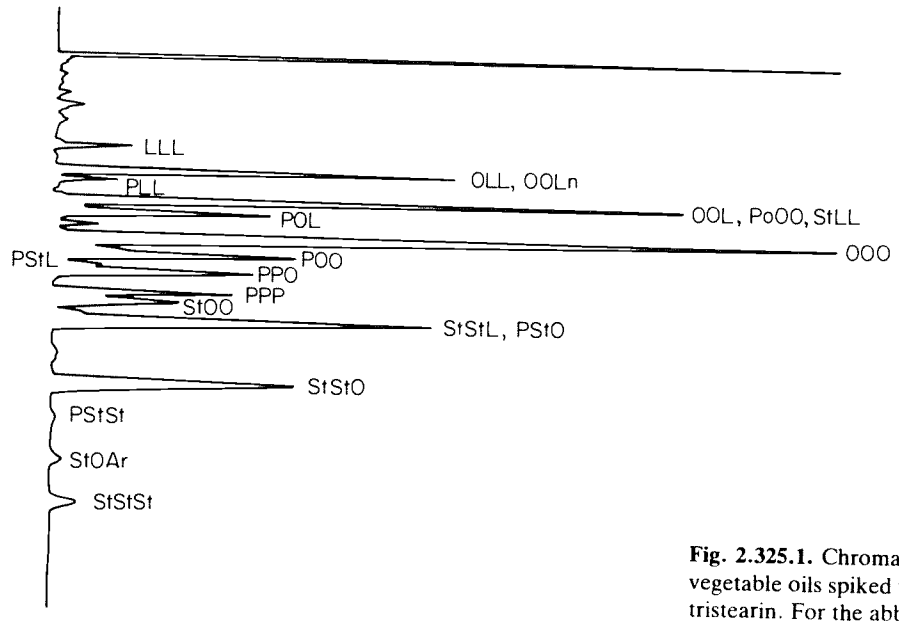


Fig. 2.325.1. Chromatogram of a blend of vegetable oils spiked with tripalmitin and tristearin. For the abbreviations used for the designation of triglycerides see *note 4*.

6.2 ## Preparation of test sample solution

Prepare a 5 per cent (W/V) solution by transferring 0.5 ± 0.01-g sample to a 10-ml graduated flask and making up to volume with the solubilization solvent (5.5). Inject 10 μl of this solution.

Identify the triglyceride peaks (see 6.3 below). Record the retention times of the solvent and triglyceride peaks; also record the peak area of each triglyceride.

6.3 ## Identification of triglyceride peaks

Analyse the reference triglyceride solution (5.7) by injecting 10 μl of the solution under the same conditions as the test sample solution (6.2), recording retention times and peak areas as in the case of the chromatographed sample (6.2). Proceed with the identification of the individual triglyceride peaks as described in para. 6.3 of method 2.324.

7 # Calculation and expression of results

7.1 Using the internal normalization method, the total sum of the peak areas corresponding to all the chromatographed individual triglycerides is assumed to be 100 per cent. The relative percentage (T_i), of each triglyceride, i, is then given by the formula:

$$T_i = \frac{\text{Area of peak i}}{\text{Sum of peak areas}} \times 100$$

Express the results to one decimal place.

7.2 ## Verification of the identification of triglycerides

7.2.1 Determine the fatty acid composition according to methods 2.301 and 2.302 or 2.303 and 2.304.

7.2.2 To verify that the correct identification of triglycerides has been made use a 'balance sheet': for a given fatty acid, compare the sum of the amounts (expressed as a percentage (m/m)) of this fatty acid wherever it occurs in the individual sample triglycerides,

with the amount (expressed as a percentage (m/m)) of this fatty acid shown to be present in the sample when its fatty acid composition is determined using method 2.302, 2.303 or 2.304. For example, the balance sheet applicable in the case of oleic acid is given by the formula:

$$T_O = T_{O\text{-tri}}$$

where:
T_O is the amount, expressed as a percentage (m/m), of the oleic acid in the sample's fatty acid composition
$T_{O\text{-tri}}$ is the sum of the amounts, expressed as a percentage (m/m), of the oleic acid occurring in the sample's triglycerides.
Confirmation that all the individual triglycerides have been identified correctly is indicated when the sum of the amounts (expressed as a percentage (m/m)) of each fatty acid present in the sample's triglycerides corresponds, respectively, to the amount (expressed as a percentage (m/m)) of each fatty acid represented in the fatty acid composition of the sample.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 Triglycerides of hydrogenated fats are generally not very well resolved under the working conditions described, resulting very often in broad triglyceride peaks. The presence of hydrogenated fats in blends may thus be indicated in the chromatogram by a more or less pronounced shoulder on the side of a triglyceride peak, indicating the presence of a partially superimposed peak of another triglyceride. The method does not allow separation of all the individual triglycerides in lauric oils (e.g. coconut and palm kernel oils) because of the complex nature of their glyceride composition.

2 Usually oils and fats are readily soluble in acetone, with the exception of those containing appreciable amounts of saturated triglycerides, such as high melting fats and hydrogenated oils and blends thereof. In such cases the proportion of chloroform in the solubilization solvent (5.5) can be increased to advantage.

3 The elution order of the triglycerides can be determined by calculating their partition numbers or equivalent carbon numbers (ECNs) as described in method 2.324, *note 4* of para. 9.

4 For ease of reference, the abbreviations below are used for those fatty acids that occur in the triglycerides most commonly encountered:

La, lauric acid
My, myristic acid
P, palmitic acid
S, stearic acid
Ar, arachidic acid
Po, palmitoleic acid

O, oleic acid
L, linoleic acid
Ln, linolenic acid

The triglycerides can then be designated as shown in the following examples:

PPP, tripalmitin
OOO, triolein
POP, palmito-oleopalmitin
POS, palmito-oleostearin
PPO, palmitopalmito-olein
PSO, palmitostearo-olein

References

Litchfield C. *Analysis of Triglycerides*. New York: Academic Press, 1972.
Pei PTS, Henly RS, Ramachandran S. *Lipids* 1975: **10**: 152.
Perrin JL, Naudet M. *Rev Franç Corps Gras* 1983: **30**: 279.
Podlaha O, Toregard B. *J High Res Chrom* 1982; **5**: 553.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the *repeatability limit* (r) deduced by linear interpolation from the data in the tables.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the *reproducibility limit* (R) deduced by linear interpolation from the data in the tables below.

3 Result of the interlaboratory tests

An interlaboratory test carried out at the international level in 1990 by the IUPAC Commission on Oils, Fats and Derivatives, in which 13 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following tables.

Table 2.325.1. Triglycerides* in soyabean oil (Collaborative Study 1990).

	LLnLn	OLL	OOL	OOO
Number of laboratories	13	13	12	13
Number of results	26	25	26	26
Number of laboratories retained after eliminating outliers	13	12	12	13
Number of accepted results	26	24	24	26
Mean (percentage of total triglycerides)	1.1	17.3	8.7	3.5
Repeatability limit (r)	0.25	1.57	0.79	0.69
Reproducibility limit (R)	0.45	2.13	0.79	1.26

Table 2.325.2. Triglycerides* in palm oil (Collaborative Study 1990).

	PPL	POO	StOO
Number of laboratories	12	13	13
Number of results	24	26	26
Number of laboratories retained after eliminating outliers	12	13	11
Number of accepted results	24	26	22
Mean (percentage of total triglycerides)	10.8	21.7	2.4
Repeatability limit (r)	0.74	0.61	0.33
Reproducibility limit (R)	1.05	2.58	0.58

Table 2.325.3. Triglycerides* in beef tallow (Collaborative Study 1990).

	POO	PPP	StStSt
Number of laboratories	13	13	9
Number of results	25	25	17
Number of laboratories retained after eliminating outliers	12	12	7
Number of accepted results	24	24	14
Mean (percentage of total triglycerides)	20.2	4.1	1.2
Repeatability limit (r)	1.33	0.94	0.32
Reproducibility limit (R)	5.24	2.03	0.61

* Abbreviations used for the component fatty acids of triglycerides: L, linoleic acid; Ln, linolenic acid; O, oleic acid; P, palmitic acid; St, stearic acid.
Results for r and R are expressed as a percentage of total triglycerides.

2.326 Determination of mono- and diglycerides by capillary gas chromatography

1 Scope

This Standard describes a method for the determination of mono- and diglycerides.

2 Field of application

The Standard is applicable to mono- and diglyceride concentrates and mono- and diglycerides in fats and oils.

3 Principle

Conversion of mono- and diglycerides with *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in pyridine into more volatile trimethylsilyl ether derivatives and quantitative determination by capillary gas chromatography using an internal standard (*n*-tetradecane).

4 Apparatus

4.1 Gas chromatograph, with split injection or on-column injection (*note 1*), oven temperature programming and flame-ionization detector. Recorder and integrator.

4.2 Column, capillary, glass or fused silica, surface fully deactivated by silylation agent (*note 2*), 15–25 m in length, 0.25–0.35 mm internal diameter, coating SE-54 (or phase with similar polarity), film thickness 0.1–0.2 μm.

4.3 Operating conditions, split injection (split ratio 1:10–1:50); direct injection (splitless, hold for 1 minute); temperatures: injection port 320°C, or on-column injection 60°C, column initial 80°C (or 60°C on-column), programme 10°C/min, final 360°C, hold for 15 minutes, detector 350°C, carrier gas flow 5 ml helium/min (at 80°C), injection volume 1–5 μl. An automatic sampler is advantageous.

4.4 Screw-cap vials (2.5 ml) or crimp-top vials for autosampler (e.g. 2.0 ml), with Teflon-faced septa.

4.5 Heating device for vials, 70°C.

5 Reagents

5.1 *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

5.2 Trimethylchlorosilane (TMCS).

5.3 Pyridine, analytical grade, kept over potassium hydroxide.

5.4 *n*-Tetradecane, analytical grade (internal standard).

5.5 *n*-Hexane, analytical grade.

5.6 Reference standards: glycerol, palmitic acid, 1-*O*-palmitoylglycerol, 1-*O*-stearoylglycerol, 1,2-di-*O*-palmitoylglycerol, 1,3-di-*O*-palmitoylglycerol, 1,2-di-*O*-stearoylglycerol.

5.7 Internal standard solution: accurately weigh about 100 mg *n*-tetradecane (5.4) into a 10-ml volumetric flask and dilute to volume with pyridine (5.3).

5.8 Reference solution: accurately weigh about 100 mg of reference standard (e.g. glycerol, fatty acid, mono- and di-*O*-acetylglycerol) and about 100 mg of *n*-tetradecane into a 10-ml volumetric flask. Dilute to volume with pyridine. Alternatively, weigh about 100 mg of a mixture containing several (e.g. five) reference standards and *n*-tetradecane, each component being present in about the same quantities, into a 2-ml volumetric flask and dilute to volume with pyridine.

6 Procedure

6.1 Sample solution

Accurately weigh about 10 mg of homogenized sample of emulsifier concentrates or 50 mg of oils and fats containing emulsifiers into a 2.5 ml screw-cap vial with Teflon-faced septa. Add 0.1 ml of internal standard solution (5.7) containing 1 mg of *n*-tetradecane, 0.2 ml of BSTFA and 0.1 ml of TMCS to the sample (*note 3*).

Humidity is strictly excluded. Close vial and shake vigorously. Heat the reaction mixture in heating device at 70°C for about 20 minutes. Inject 1–5 µl of the reaction mixture into the gas chromatograph showing a stable baseline (*note 1*).

Avoid delay of gas chromatographic analysis. The reaction is carried out twice and duplicate injections are made per reaction.

6.2 Reference solution

Transfer 0.10 ml of reference solution (5.8) to a vial and add the silylating agents, 0.2 ml of BSTFA and 0.1 ml of TMCS (no internal standard solution is added), and inject as described above (*notes 1, 3*).

Use a concentration range of reference standards similar to that of the substances to be quantified in sample solution. A plot of response factor versus concentration of reference standards may be useful to check linearity.

Check response factors periodically. Response factors should be above about 0.5. Lower response factors indicate some loss or decomposition. Use concentration range of 0.5–10 mg/ml of components in reference and sample solutions.

6.3 Identification

Analyse the reference solution under the same operating conditions as the sample solution. Identify peaks by comparison of retention time with known substances or apply coupled GC/MS (gas liquid chromatography/mass spectrometry).

7 Calculation and expression of results

7.1 Response factor

Calculate the response factors of the reference substances versus internal standard using the reference standard chromatogram. The value of the response factor is given by the formula:

$$R_x = (m_{is}/m_x) \times (A_x/A_{is})$$

where:

R_x is the response factor of reference standard x

m_{is} is the mass, in mg, of internal standard

m_x is the mass, in mg, of reference standard x

A_x is the peak area of reference substance x

A_{is} is the peak area of internal standard.

7.2 Calculation of sample component content

Calculate the percentage of mass content of component x in the sample by the formula:

$$m'_x(\%) = 1/R_x \times (m'_{is}/m'_s) \times (A'_x/A'_{is}) \times 100$$

where:
m'_x is the percentage (m/m) of mass of component x in the sample
R_x is the response factor of component x in the sample
m'_{is} is the mass, in mg, of internal standard in the sample
m'_s is the mass, in mg, of the sample
A'_x is the peak area of the component x in the sample
A'_{is} is the peak area of the internal standard in the sample.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 For on-column injection, or direct injection, dilute 50 μl of reaction mixture (6.1; 6.2) with 1 ml of hexane and inject (1 μl).
In order to lengthen the life-time of the columns when applying on-column injections, a pre-column is useful. On-column injection gives better response factors.

2 Use a length of column required to separate 1,2- from 1,3-diglycerides.

3 For automatic samplers with 2-ml crimp-top vials, it is convenient to double the amount of sample and reagents.

4 Beside mono- and diglycerides, other components such as glycerol, fatty acids, sterols etc. can be analysed by the same method.

Annexe

Analytical quality control

1 Repeatability
When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in the tables below, the difference between the two values obtained should not be greater than the repeatability limit (r), which can generally be deduced by linear interpolation from the values in the tables below.

2 Reproducibility
When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in the tables below, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which can generally be deduced by linear interpolation from the values in the tables below.

3 Results of the interlaboratory test
An interlaboratory test carried out at an international level in 1985 by the IUPAC Commission on Oils, Fats and Derivatives, in which eight laboratories participated, each

obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following tables for mono- and diglyceride concentrates (Table 2.326.1) and for mono- and diglycerides in oils (Table 2.326.2).

Table 2.326.1. Statistical analysis of results for mono- and diglyceride concentrates (expressed as percentage of mass of sample).

	1-Myristate	1-Palmitate	1-Stearate	1,3-Dipalmitate	1-Palmitate-3-stearate	1,3-Distearate
Number of laboratories	8	8	8	8	8	8
Number of results	16	16	16	16	16	16
Number of laboratories retained after elimination of outliers	7	8	8	7	7	8
Number of accepted results	14	16	16	14	14	16
Mean value (g/100 g sample)	1.7	27.2	60.1	0.2	0.8	1.1
Repeatability standard deviation (S_r)	0.05	0.9	2.1	0.01	0.05	0.07
Repeatability relative standard deviation	3.0	3.3	3.5	4.6	6.0	6.8
Repeatability limit (r) ($2.83 \times S_r$)	0.14	2.61	5.91	0.31	0.14	0.20
Reproducibility standard deviation (S_R)	0.1	2.4	6.4	0.06	0.1	0.3
Reproducibility relative standard deviation	5.7	8.9	10.7	30.0	17.8	24.8
Reproducibility limit (R) ($2.83 \times S_R$)	0.3	6.8	18.1	0.2	0.4	0.8

Table 2.326.2. Statistical analysis of results for mono- and diglycerides in oils (expressed as percentage of mass of sample).

	1-Palmitate	1-Stearate	1,2-Dipalmitate	1,3-Dipalmitate	1,2-Distearate
Number of laboratories	8	8	8	8	8
Number of results	16	16	16	16	16
Number of laboratories retained after elimination of outliers	8	8	8	8	8
Number of accepted results	16	16	16	16	16
Mean value (g/100 g sample)	0.96	0.98	0.97	0.93	0.97
Repeatability standard deviation (S_r)	0.03	0.03	0.04	0.02	0.06
Repeatability relative standard deviation	3.3	3.4	4.0	2.5	6.2
Repeatability limit (r) ($2.83 \times S_r$)	0.08	0.08	0.11	0.06	0.17
Reproducibility standard deviation (S_R)	0.12	0.14	0.24	0.19	0.19
Reproducibility relative standard deviation	12.0	13.8	24.4	20.2	19.8
Reproducibility limit (R) ($2.83 \times S_R$)	0.34	0.40	0.68	0.54	0.54

References

Brüschweiler H, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(8): 1153–1163. (Report on the Collaborative Study).

D'Alonzo RP, Kozarek WJ, Wharton HW. *J Am Oil Chem Soc* 1981; **58**: 215–227.

Goh EM, Timms RE. *J Am Oil Chem Soc* 1985; **62**: 730–734.

Hammond EW. *J Chromatogr* 1981; **203**: 397–402.

ISO 5725. *Precision of test methods. Determination of repeatability and reproducibility by inter-laboratory tests*. 1986.

IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn.

Prevot A, Coustille J-L. *Rev Franç Corps Gras* 1982; **29**: 17–24.

Riva M, Daghetta A, Galli M *Rivist Ital Sostanze Grasse* 1981; **58**: 432–443.

Shasrabudhe MR. *J Am Oil Chem Soc* 1968; **45**: 379–380.

Shasrabudhe MR. *J Am Oil Chem Soc* 1969; **46**: 8–12.

Soe JB. *Fette Seifen Anstrichmittel* 1983; **85**: 72–76.

Vaver VA, Ushakov AN. *Methods Biol Anal* 1980; **26**: 327–406.

2.423 Determination of phosphorus by direct graphite furnace atomic absorption spectrometry

1 Scope and field of application

This Standard describes a method for the determination of trace amounts (mg/kg) of phosphorus in all types of crude or refined edible oils and fats.

2 Principle

Vaporization of the oil or fat in a suitable graphite furnace with or without a platform connected to an atomic absorption spectrometer, previously calibrated using standard solutions of soya lecithin, and the measurement of the phosphorus content from the observed absorption at a wavelength of 213.5 nm.

3 Apparatus

3.1 Test tubes, 10 ml.

3.2 Micropipettor, 20 μl.

3.3 Pipettor tips.

3.4 Electric oven, regulated at 60 ± 2°C.

3.5 Atomic absorption spectrometer, equipped with either 'peak height' mode and printer, or 'continuous' mode and pen recorder (full scale response in 0.2 seconds), together with the appropriate electrode-less discharge lamp (or hollow cathode lamp) and deuterium background corrector (or Zeeman atomic absorption spectrometer).

3.6 Graphite furnace atomizer, placed in the atomic absorption spectrometer (3.5), equipped with a control unit for temperature programming.

3.7 Graphite tube, normal (uncoated).

3.8 Platform: pyrolytic, in combination with uncoated or pyrolytically coated graphite tube (*note 2*).

4 Reagents

4.1 Cyclohexane, analytical grade.

4.2 Lanthanum organometallic standard: a suitable standard (Conostan, 5000 mg/kg) is available from Continental Oil Company, Ponca City, Oklahoma, USA.

4.3 Matrix modifier (*note 1*): 0.05 per cent (m/V) Lanthanum solution is prepared by dissolving 5 g of lanthanum standard (4.2) in 50 ml of cyclohexane (4.1).

4.4 Blank oil: refined liquid edible oil with a phosphorus content not greater than 1 mg/kg (*note 3*).

4.5 Lecithin: a well-defined lecithin containing 2 per cent phosphorus, e.g. soya lecithin (*note 3*).

4.6 Standard stock solution: a stock solution of 400 mg phosphorus/kg is prepared by dissolving 1 g of lecithin (4.5) in 4 g of cyclohexane (4.1) and 45 g of blank oil (4.4).

4.7 Standard working solutions: 10, 20 and 40 mg phosphorus/kg are prepared by diluting the 400 mg/kg stock solution (4.6) with blank oil (4.4).

4.8 Argon, purity 99.99 per cent minimum.

5 Procedure

5.1 Treatment of samples, blank and standards

5.1.1 Place all samples, blank oil and standard working solutions in the oven (3.4), regulated at 60 ± 2°C.

5.1.2 Shake samples vigorously.

5.1.3 Weigh 1.00 g of sample in a 10-ml test tube, together with 1.00 g of matrix modifier (4.3) and mix thoroughly (*note 4*).

5.1.4 Carry out instructions 5.1.2–5.1.3 also for the three standard working solutions (4.7) and the blank oil (4.4).

5.2 Preparation of apparatus

5.2.1 Switch on the atomic absorption spectrometer and the background correction (Deuterium *or* Zeeman).

5.2.2 In accordance with the manufacturer's instructions supplied with the spectrometer, adjust: lamp current, slit, wavelength and amplification. The required wavelength is 213.5 nm.

5.2.3 Optimize the position of the graphite furnace atomizer (3.6) in the atomic absorption spectrometer (3.5), and set the required programme on the control unit of the furnace. If available, place platform in graphite tube.

5.2.4 Pretreat before each injection, the pipettor tip (3.3) by pipetting and then discarding 20 μl of cyclohexane.

5.2.5 Inject 20 μl of the standard working solution with a content of 40 mg phosphorus/kg (4.7) with the micropipettor (3.2) into the graphite furnace, initiate the temperature programme and record the absorption.

5.2.6 Repeat instruction 5.2.5 until the absorption is constant (*note 5*).

Programme for the graphite furnace atomizer (see *note 6*):

Table 2.423.1.

Step no.	Temp. (°C)	Ramp-time (s)	Hold-time (s)	Int. Gas-flow (ml/min)
1	600	40	20	300
2	1600	50	40	300
3	2800	0	5	0
4	2800	1	3	50

5.3 Determination

5.3.1 Measurement of the graphite tube blank: record the absorption, if any, of the graphite tube as such and autozero this absorption.

5.3.2 Measurement of the blank: inject 20 μl of the blank solution prepared according to 5.1.4 into the graphite furnace, initiate the temperature programme and record the absorption.

5.3.3 Measurement of the working standards: inject 20 μl of the three standard solutions prepared according to 5.1.4 into the graphite furnace and record the absorptions.

5.3.4 Measurement of sample solutions: inject 20 μl of the sample solution prepared according to 5.1.3 into the graphite furnace, initiate the temperature programme and record the absorption.

6 Calculation and expression of results

6.1 Calculation

6.1.1 Measure the peak height on the recorder-chart or take the reading of the display or printer.

6.1.2 Draw a calibration curve by plotting the absorption of the three standards (5.3.3), corrected for the blank (5.3.2), against their respective phosphorus content (*note 3*).

6.1.3 Measure the absorption of the sample and correct for the blank.

6.1.4 Read the phosphorus content of the sample from the calibration curve.

6.2 Expression of results

Express the results as mg/kg (to two significant figures).

7 Quality assurance

7.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

7.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

8 Notes

1 The amount of phosphorus found depends on the types of phosphatide present in the oil. The addition of lanthanum proved to be essential to find the total amount of phosphorus. Instead of lanthanum, calcium may be used.

2 Both atomization off the wall and atomization off the platform can be used. It is not necessary to change the temperature programme for the graphite furnace.

3 The concentration of phosphorus in the blank oil and in the lecithin is determined with method 2.421.

4 If the expected or found concentration of phosphorus is higher than 40 mg/kg, dilute the sample with blank oil (4.4). In that case multiply the observed absorption (5.3.4) with the dilution factor.

5 With a new graphite tube, three to four 'determinations' with a particular working standard have to be carried out in order to obtain an acceptable 'state of equilibrium'.

6 For those with a Varian apparatus, the following temperature programme should be used:

Table 2.423.2.

Step no.	Temp. (°C)	Time (s)	Gas-flow (l/min)
1	120	20	3.0
2	120	20	3.0
3	500	30	3.0
4	500	10	3.0
5	1600	50	3.0
6	1600	20	3.0
7	1600	2	0.0
8	2800	1	0.0
9	2800	2	0.0
10	2800	5	3.0
11	40	20	3.0

Annexe

1 Repeatability limit

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator

using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulae in Table 2.423.3.

Table 2.423.3. Repeatability (r) and reproducibility (R) limits.

P in edible oil	$r = 0.19\,m$	$R = 0.30\,M$

M, corresponding mean concentration value.

2 Reproducibility limit

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulae in Table 2.423.3.

3 Trueness (bias)

The bias of the method was demonstrated in the collaborative study of the method (see Table 2.423.4) to be negligible when used for the determination of concentration levels of phosphorus in the range of 10–30 mg/kg.

4 Sensitivity

The sensitivity of the method is demonstrated by the low values for r and R at the low concentration levels studied (see Table 2.423.4), the limit of detection is 0.1 mg/kg and the limit of determination is 1 mg/kg.

Interference by other elements is not to be expected, provided the measurements are carried out at the wavelength specific for phosphorus (213.5 nm).

5 Statistical and other data derived from the results of the interlaboratory test

The interlaboratory test carried out at the international level in 1989 by the IUPAC Commission on Oils, Fats and Derivatives, in which 21 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in Table 2.423.4.

Table 2.423.4.

Sample:	Sunflower oil		
Batch:	A (high)	B (medium)	C (low)
Number of laboratories retained after eliminating outliers	17	17	17
Number of outliers (laboratories)	2	1	1
Number of accepted results	32	34	34
Mean value (mg/kg sample)	29.55	20.00	10.18
True or accepted value (mg/kg)	28.50	19.00	9.00
Repeatability standard deviation (S_r in mg/kg)	2.30	1.68	0.91
Repeatability relative standard deviation (CV_r in %)	7.8	8.4	9.0
Repeatability limit (r) ($2.8 \times S_r$)	6.45	4.71	2.56
Reproducibility standard deviation (S_R in mg/kg)	3.51	2.33	1.24
Reproducibility relative standard deviation (CV_R in %)	11.9	11.7	12.2
Reproducibility limit (R) ($2.8 \times S_R$)	9.82	6.63	3.48

References

Hendrikse PW, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(8): 1193–1200 (Report on the Collaborative Study).

IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn. Oxford: Blackwell Scientific Publications, 1989.

Slikkerveer FJ, Braad AA, Hendrikse PW. *At Spectr* 1980; **1**: 30.

2.432 Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography

1 Scope and field of application

This Standard describes a procedure for the determination of tocopherols and tocotrienols in vegetable oils and fats. The procedure is not directly applicable to processed products such as margarines containing tocopherol esters, but it may be used to determine tocopherols in the unsaponifiable matter obtained from such products (*note 1*).

2 Principle

Dissolution of the oil or fat (or the unsaponifiable matter obtained from a processed product containing tocopherol esters) in an organic solvent and direct high performance liquid chromatographic (HPLC) separation of the individual tocopherols and tocotrienols. Calibration factors are determined for each tocopherol from the chromatography of solutions of standard tocopherols; calibration factors for tocotrienols are taken to be equivalent to that of the corresponding tocopherols.

3 Definition

The tocopherol and tocotrienol content of an oil or fat is the quantity of tocopherols and tocotrienols, determined in the sample by the described procedure and expressed in micrograms per gram (μg/g).

4 Apparatus

Note: All glassware should be of low actinic activity.

4.1 HPLC system consisting of a high pressure pump, sample injection device, detector, and chart recorder or recording integrator.

A fluorescence detector should preferably be used with the excitation wavelength set at 290 nm and emission wavelength at 330 nm.

A UV detector may be used if a fluorescence detector is not available. The wavelength of the UV detector should be set at 292 nm.

4.2 HPLC analytical column (250 × 4 mm) packed with microparticulate silica having a mean particle size of about 5 μm (*note 2*).

4.3 UV spectrometer capable of absolute measurement of absorbance at precisely defined wavelengths.

4.4 Rotary film evaporator.

5 Reagents

All solvents should be of HPLC grade or equivalent. For additional reagents required when prepared fats containing tocopherol esters are being analysed, see 11.1–11.7.

5.1 α-, β-, γ- and δ-Tocopherol standards (*note 3*).

5.2 Methanol.

5.3 Dichloromethane.

5.4 Hexane.

5.5 Propan-2-ol.

5.6 HPLC mobile phase, propan-2-ol in hexane (0.5/99.5 V/V).

6 Sampling

It is essential that the laboratory sample used for preparing the test sample is taken according to a recognized sampling procedure (such as that described in *ISO 5555*), and that the laboratory sample is subsequently protected from the effects of heat and light.

7 Procedure

7.1 Preparation of solutions of tocopherol standards

7.1.1 α-Tocopherol standard stock solution

Prepare a stock solution of α-tocopherol by accurately weighing about 10 mg of the standard (5.1) into a 100 ml volumetric flask and making up to volume with hexane (5.4). Pipette 10 ml of this solution into an amber glass round-bottomed flask and remove all hexane on a rotary evaporator (4.4) at a temperature not higher than 40°C. Restore atmospheric pressure with nitrogen and remove the flask from the evaporator as soon as all the solvent has been removed. Pipette into the flask 10 ml of methanol (5.2) and swirl to dissolve the tocopherol. Measure the absorbance of this solution at 292 nm and calculate the concentration (as μg/ml α-tocopherol) by dividing the absorbance value by 0.0076 (*note 4*).

7.1.2 β-, γ- and δ-Tocopherol standards stock solutions

Prepare similarly stock solutions and aliquots for UV spectrometry of β-, γ- and δ-tocopherol standards (5.1) as described in 7.1.1 for α-tocopherol. Measure the absorbance of each of these solutions at the following wavelengths and use the corresponding divisor factors (*note 4*) for calculation of concentration:

296 nm β-tocopherol = 0.0089
298 nm γ-tocopherol = 0.0091
298 nm δ-tocopherol = 0.0087

7.1.3 Mixed tocopherol standards working solution

Mix appropriate volumes of the stock solutions of the tocopherol standards to obtain a mixed tocopherol standards working solution, and dilute with hexane to give a solution containing between 1 and 5 μg per ml of each tocopherol. (*Note*: a more concentrated solution may have to be prepared if a UV detector is used. It is important that all standards are protected from light and stored refrigerated—see *note 5*.)

7.2 Optimization of working parameters

Condition the column (4.2) if necessary (*note 6*).

Pump the propan-2-ol/hexane mobile phase (5.6) through the column at a flow rate of 1 ml/min for at least 30 minutes.

Inject about 20 μl of the mixed tocopherol standards working solution (7.1.3) on to the column and if necessary adjust the propan-2-ol content of the mobile phase and the flow rate (*note 7*) to achieve the following conditions:

- α-tocopherol retention time not less than 5 minutes,
- resolution factor (R) for the separation of β- and γ-tocopherol of not less than 1.0, i.e. almost baseline separation (*note 8*).

Select the optimum settings for detector and integrator sensitivity and chart speed. Inject about 20 μl of the mixed tocopherol standards working solution (7.1.3). Repeat the injection and check that reproducible chromatograms are obtained.

7.3 Preparation of the test sample

The test sample should be prepared, in the case of liquid laboratory samples, by homogenization as described in method 2.001, except that filtration should be avoided. In the

case of solid samples, transfer a representative portion (i.e. not less than 10 per cent by weight of the laboratory sample) to a glass beaker and carefully homogenize by melting, with gentle mixing, in a water bath at a temperature not exceeding 40°C. The preparation of the test sample should be carried out, as far as is practicable, in subdued light and in any case out of direct sunlight.

7.4 Preparation of the test solution

Weigh accurately about 2 g of the prepared test sample (*note 9*) into a 25-ml volumetric flask. Add a quantity of hexane (5.4), swirling to dissolve the sample and make up to volume with the same solvent. If a fluorescence detector is used, it may be necessary to make a further dilution of this solution prior to chromatography.

It is important that the test solutions are protected from light prior to analysis, and analysed on the day of preparation.

7.5 HPLC determination of tocopherols in the test solution

Inject 20 μl of the mixed tocopherol standards working solution (7.1.3) on to the column and record the areas of the tocopherol peaks. If an integrator is not available, record peak heights (measured in mm).

Inject 20 μl of the test solution (7.4) on to the column and identify the tocopherols (and tocotrienols) present by reference to the chromatograms obtained from standards (*note 10*). Record the areas of the tocopherol peaks (or peak heights). Record the areas of any tocotrienol peaks if these are present and are to be quantified. Duplicate injections should be made.

Inject a further 20 μl of the mixed tocopherol standards working solution (7.1.3) and record the areas of the tocopherol peaks.

8 Calculation and expression of results

8.1 The α-tocopherol content of the sample is given by:

$$\frac{C \times a \times D \times 25}{A \times m} \mu g/g$$

where:

C is the concentration of the α-tocopherol standard (μg/ml)

A is the mean of the peak areas obtained for the α-tocopherol standard

a is the mean of the peak areas obtained for the α-tocopherol in test sample

m is the mass of test sample taken

D is the dilution factor (e.g. for a test solution prepared from a 1:10 dilution of a 25-ml solution of the test portion, this factor would be 10).

8.2 The β-, γ- and δ-tocopherol contents of the test sample are calculated in the same way, using the data obtained from chromatography of the corresponding tocopherol standard (*note 11*).

8.3 The tocotrienol content of a sample can be estimated using the C and A values for the corresponding tocopherol (*note 12*).

8.4 Report the results for each tocopherol and tocotrienol to the nearest microgram.

9 Precision

The results of an interlaboratory study organized at the international level gave the statistical results which are summarized in Table 2.432.1.

9.1 ## Repeatability

When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in Table 2.432.1, the difference between the two values obtained should not be greater than the repeatability value (r), which can generally be deduced by linear interpolation from Table 2.432.1.

9.2 ## Reproducibility

When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in Table 2.432.1, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility value (R) (*note 13*), which can generally be deduced by linear interpolation from Table 2.432.1.

9.3 **Table 2.432.1.** Statistical analysis of results for tocopherols (expressed as μg/g).

	Level			
	A	B	C	D
Number of laboratories	16	17	16	17
Number of results	32	34	32	34
Number of laboratories retained after elimination of outliers	14	15	13	16
Number of accepted results	28	30	26	32
Mean value (μg/g)	17	69	263	508
Repeatability standard deviation (S_r)	0.8	3.5	5.3	12.6
Repeatability coefficient of variation (%)	5	5	2	2.5
Repeatability value (r) ($2.83 \times S_r$)	2	10	15	36
Reproducibility standard deviation (S_R)	5.1	17.5	44.5	36.0
Reproducibility coefficient of variation (%)	31	25	17	7
Reproducibility value (R) ($2.83 \times S_R$)	15	49	126	102

Statistical analysis according to *ISO 5725-1986*.
Values for reproducibility value (R) are those applicable to results obtained from single determinations (*note 13*).

10 ## Notes

1 It is recommended that the unsaponifiable matter is obtained by a method involving a cold saponification procedure such as that described in the Appendix. Particular attention must be paid to saponification temperature and time, otherwise low recoveries of tocopherols from tocopherol esters may be obtained.

2 Suitable silica column-packing materials are 5 μm LiChrosorb SI 60 or Spherisorb S5W.

3 β-, γ- and δ-Tocopherol standards can be obtained from Merck; α-tocopherol can be obtained from various suppliers. It has been reported that the purity of some commercially available tocopherol standards may vary between 85 and 100 per cent (Ramnell and Hoogenboom, 1985). This confirms the importance of determining the concentration of prepared standard solutions by UV spectrometry.

4 The divisor factors quoted for the tocopherols are derived from their E values (1

per cent/1 cm) quoted by Schudel *et al.* (1972). For example the E value (1 per cent/1 cm) of α-tocopherol is 76 at 292 nm (in methanol); therefore a 1 μg/ml solution of α-tocopherol will have an absorbance of 0.0076 at 292 nm.

5 Stock standard solutions can be satisfactorily stored in amber low actinic glassware for up to a week if refrigerated. Working standard solutions must be prepared each working day. Protection from light is of the utmost importance.

6 If the column (4.2) is new or of unknown history, wash and condition for about 10 minutes with methanol, then dichloromethane, followed by hexane at a flow rate of about 1 ml/min.

7 Mobile phase flow rates in the range 0.7–1.5 ml/min have been found to be satisfactory. Higher flow rates can result in poor chromatography which must be avoided when UV detection is used.

8 The resolution factor (R) is calculated from:

$$R = \frac{R_{d1} - R_{d2}}{0.5(\omega_1 + \omega_2)}$$

where:
R_{d1} is the retention distance of γ-tocopherol
R_{d2} is the retention distance of β-tocopherol
ω_1 is the width at base of γ-tocopherol peak
ω_2 is the width at base of β-tocopherol peak.
It should be possible to achieve an efficiency of 10 000 plates per metre calculated on the δ-tocopherol peak. The efficiency (n), in plates per metre, may be calculated from:

$$n = 5.54(R_{d3}/\omega_h)^2$$

where:
R_{d3} is the retention distance of δ-tocopherol
ω_h is the peak width at half height.

9 When analysing processed products such as margarines, and samples containing added tocopherol esters, a cold saponification procedure must be performed prior to chromatography. (*Note:* When samples contain tocopherol esters, parallel samples spiked with known amounts of α-tocopherol acetate should be analysed to enable a check to be made on the recovery of tocopherols from tocopherol esters.) The saponification procedure is described in the Appendix.

10 If any tocopherol standards are not available, a blend of wheatgerm and soyabean oil can be used to obtain chromatograms which contain α-, β-, γ- and δ-tocopherols. These can be used to assist peak identification in test sample chromatograms.
Palm oil can be used to identify α- and γ-tocotrienols if required. The following relative retention times have been found to be typical:

α-tocopherol = 1.0
β-tocopherol = 1.6
γ-tocopherol = 1.7
δ-tocopherol = 3.0

11 If fluorescence detection is used and the only standard available is α-tocopherol, relate all tocopherols to the α-tocopherol standard, but make this clear when reporting results. If UV detection is used, again relate all tocopherols to the α-tocopherol standard,

but normalize the peak areas to α-tocopherol using the divisor values given in 7.1.1 and 7.1.2.

12 According to the literature, the fluorescence intensity of tocotrienols is the same as the corresponding tocopherol (Thompson *et al.*, 1979), and the UV absorbancies are similar (Schudel *et al.*, 1972).

13 It should be noted that the reproducibility values (R) cited in the table in 9.3 apply in the particular case when the results of single determinations obtained by two laboratories are being compared. When following the method described and it is desired to compare the final results (which have been derived from the means of duplicate determinations) obtained by two laboratories, the values for R should be converted to the 95 per cent probability critical difference values (CrD_{95}) applicable to the means of two determinations, using the formula:

$$CrD_{95} = \sqrt{(R^2 - r^2/2)}$$

Appendix

Cold saponification procedure for samples containing added tocopherol esters.

A1 *Reagents*

A1.1 Ethanol, approx. 96 per cent pure.

A1.2 Ethanol, absolute, 99 per cent pure.

A1.3 Pyrogallol.

A1.4 Potassium hydroxide solution, aqueous, 60 per cent (m/m).

A1.5 Diethyl ether, peroxide-free containing 0.1 per cent (m/m) pyrogallol.

A1.6 Hydrochloric acid, 0.01 mol per litre.

A1.7 Sodium sulphate, anhydrous.

A2 *Procedure*

Weigh accurately about 2 g of the prepared sample (7.3) in to a 100-ml flat-bottomed flask and thoroughly disperse the molten test portion in approximately 8 ml of ethanol (A1.1) by gentle swirling. Add 100 mg of pyrogallol (A1.3) and swirl to dissolve. Purge the flask with nitrogen, add 4 ml of potassium hydroxide solution (A1.4), re-purge the flask with nitrogen, and close with a glass stopper. Place the flask in a 26°C water bath and shake vigorously for 10 minutes. All operations must be performed in the absence of direct sunlight—use amber glassware or shield with aluminium foil.

Add 50 ml of deionized water to the flask and transfer contents quantitatively to a 250-ml separating funnel. Wash the flask with 50 ml of diethyl ether (A1.5) and transfer the washings to the funnel. Shake the separator vigorously for 1 minute, releasing the pressure occasionally. Allow the layers to separate and draw off the lower aqueous layer. Extract the aqueous layer a further four times with 30-ml aliquots of diethyl ether and combine the ether extracts.

Wash the combined diethyl ether extracts with 50 ml of water (shaking carefully to avoid emulsion formation) and then with 30 ml of dilute hydrochloric acid (A1.6). Add about 3 g of anhydrous sodium sulphate (A1.7) with gentle mixing to absorb water. Filter the ether extracts through a phase-separating paper and collect the filtrate in a round-bottomed amber rotary evaporator flask. Remove the ether under reduced pressure (4.4) at a temperature of not more than 40°C. If a liquid residue remains in the flask

add ethanol (A1.2) and re-evaporate to dryness. Wash the sides of the flask with hexane (5.4) and transfer the contents quantitatively to a 50-ml volumetric flask and bulk to volume. Make a suitable dilution of the prepared test solution (as described in 7.4) and proceed to 7.5.

References

Arens M, Kroll S, Muller H. *Fette Seifen Anstrichmittel* 1984; **86**(4): 148.

Pocklington WD, Dieffenbacher A. *Pure Appl Chem* 1988; **60**(6): 877–892 (Report on the Collaborative Study).

Ramnell CG, Hoogenboom JJL. *J Liquid Chromatogr* 1985; **8**(4): 707.

Schudel P, Mayer H, Isler O. Tocopherols, II Chemistry. In: Sebrell WH, Harris RH, eds. *The Vitamins*, 2nd edn, Vol. V. New York: Academic Press, 1972, p. 1658.

Thompson JN, Hatina G. *J Liquid Chromatogr* 1979; **2**(3): 327.

2.508 Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography

1 Scope

This Standard describes a method to determine the polymerized triglycerides content in oils and fats which contain not less than 3 per cent (m/m) of these polymers.

2 Field of application

This method is applicable to any kind of fats and oils, heated or not. It can also be applied to the determination of polymers in animal-feed fats.

3 Principle

Dissolution of the sample in a suitable solvent and separation of the polymerized triglycerides by gel permeation chromatography according to molecular size.

4 Apparatus

4.1 Solvent reservoir of about 250 ml of capacity with a mobile-phase liner filter (pore size 1 μm).

4.2 HPLC pump, pulseless, with a flow of 0.7–1.5 ml/min.

4.3 Injection valve with a 10-μl loop and a suitable syringe with a volume of 50–100 μl (Rheodyne or Valco).

4.4 Stainless steel column: 300 mm in length 7.7 mm internal diameter, packed with a high performance spherical gel made of styrene–divinylbenzene co-polymer; diameter of the particles: 5 μm; pore size: 100 Å (0.1 nm) of the equivalent in terms of exclusion power and resolution.

The storage of such column must be achieved in toluene.

4.5 Detector: refractive index detector with a sensitivity at full scale at least 1×10^{-4} of refractive index.

4.6 Recorder and/or integrator: to allow display and accurate quantification of the peak areas.

5 Reagents

5.1 Tetrahydrofuran, analytical grade.

5.2 Toluene, analytical grade.

6 Procedure

6.1 Starting up HPLC equipment

It is advisable to follow carefully the manufacturer's recommendations. Switch on the system and pump tetrahydrofuran at a rate of 1 ml/min to purge the whole system up to the injection valve. Connect the column to the injection valve and wash it with about 30 ml of tetrahydrofuran. Connect the column to the sample cell of the detector. Fill the reference cell with the tetrahydrofuran. Adjust the mobile phase flow to 0.8–1.0 ml/min. Wait until a convenient stabilization of the system (no appreciable deviation of the baseline) is obtained (*note 1*).

6.2 Preparation of the samples and analysis

As the samples may contain suspended particles, resulting in the blockage of the porous fritted filter at the top of the column, it is advisable to filter them before injection. Filtration can be quickly and effectively realized with a 1-μm pore size filter (*note 2*).

The samples must be anhydrous. If not, they must first be dried (*note 3*).

Weigh about 50 mg of fat and add 1 ml of tetrahydrofuran. Homogenize. Take with the

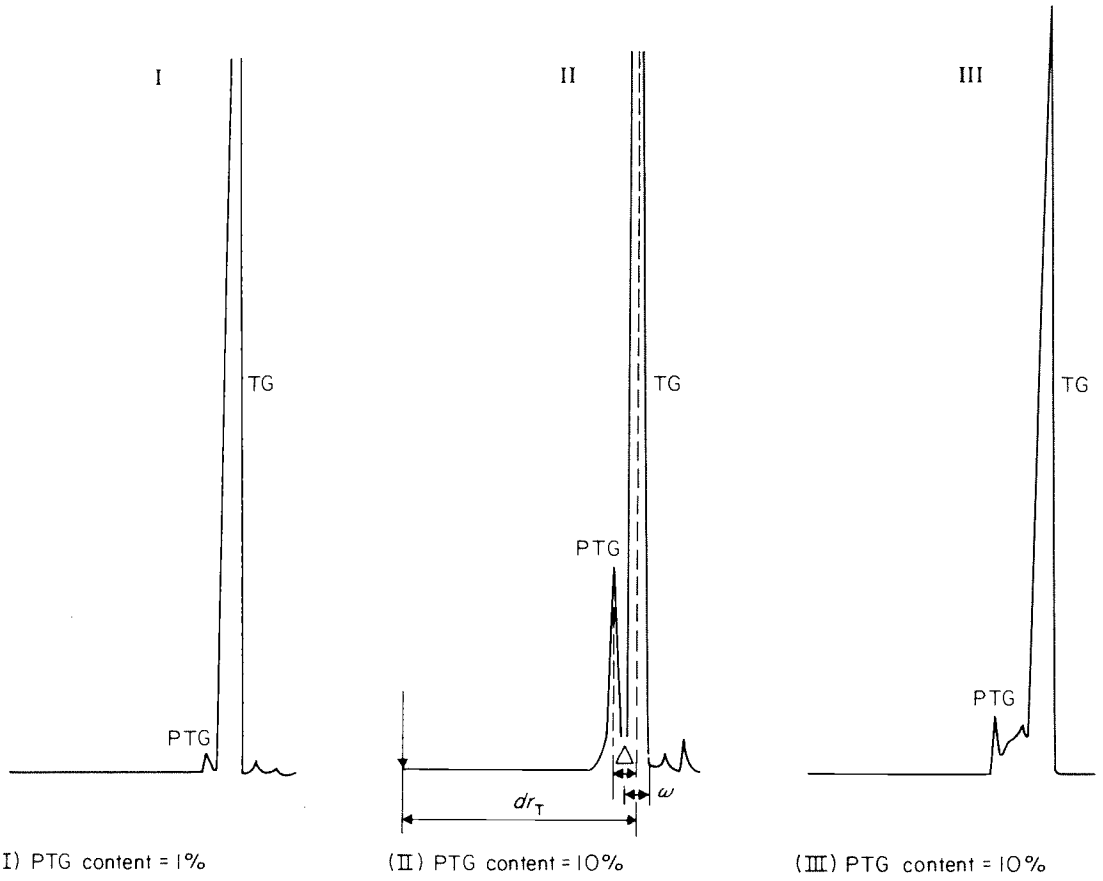


Fig. 2.508.1. HPLC of triglycerides (TG) and polymerized triglycerides (PTG).

syringe 50–100 μl of that solution. Fill the injection loop. Inject and switch on the integrator.

With a mobile phase flow of 1 ml/min, the analysis time is about 10 minutes.

7 Results

7.1 Qualitative analysis

The chromatographic pattern of the determination may show a main peak representative of monomeric triglycerides (mol. wt. about 900) and one or several smaller peaks with a shorter pattern retention distance than triglycerides, representative of polymerized triglycerides (dimers and upper oligomers).

Reference should be made to the three typical chromatograms presented in Fig. 2.508.1. Under suitable conditions, triglycerides and polymerized triglycerides can be separated with good resolution (I and II) even at low levels of polymerized triglycerides (I). However, in some cases, which seem to be connected to the complex degradation phenomena (hydrolysis), the peak pattern preceding the triglycerides peaks may be less clear (III), with consequent difficulties in calculation.

7.2 Quantitative analysis

The calculation is achieved by the internal standard method, assuming that all components of the sample are eluted.

The polymerized triglycerides content can be calculated using the formula:

$$\%\text{PTG} = \frac{A_{\text{PTG}}}{\Sigma A} \times 100$$

where:
%PTG is the percentage of polymerized triglycerides
A_{PTG} is the sum of areas of the polymerized triglycerides peaks
ΣA is the sum of areas of all peaks.
For calculating A_{PTG}, two cases are possible:
(a) *Good resolution between peaks* ($R \geq 1$) (similar to I and II). The general methods of integration (manual and electronic) can be used to calculate individual and total areas.
(b) *Poor resolution between peaks* ($R < 1$) (similar to III).
It is assumed that all components eluted before dr_T (see below) are polymerized triglycerides.
The resolution is calculated from:

$$R = \frac{\Delta}{\omega}$$

where:
Δ is the distance, in mm, between the peak maxima of the non-polymerized triglycerides peak (TG) and the adjacent polymerized triglycerides peak
ω is the width, in mm, of the triglycerides peak at the baseline, measured between the points of the intersection between tangents and baseline
dr_T is the retention distance, in mm, from the beginning of the chromatogram to the peak maximum for triglycerides.
Using electronic integration, the integrator has to be carefully adjusted (backward horizontal integration) to integrate all the surfaces included between the curve and the baseline. If a manual technique is used, it is necessary to determine the triglycerides peak area by triangulation.
Express the results to one decimal place.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 If the composition of the column is as indicated, an acceptable stabilization of the system should be obtained in about 15 minutes. With other column packings, the stabilization of the system may be more difficult: for example, changing the mobile phase should be done stepwise from toluene to tetrahydrofuran, with different mixtures, each time 25 per cent higher in tetrahydrofuran. Acceptable stabilization is normally obtained in about 12 hours.
2 The sample can be filtered with a 1-μm pore size filter made of polytetrafluoroethylene or cellulose esters. Syringe disposable filter units are available from manufacturers of HPLC equipment.

3 Samples that may contain water must first be dried: dissolve 50–100 mg of the sample in 1 ml of tetrahydrofuran. Add 50 mg of anhydrous sodium sulphate, shake, and leave for about 2 minutes. Filter upper layer through a 1-μm pore size filter.

Annexe

1 Repeatability

When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same laboratory sample, lies within the range of the mean values cited in the table below, the difference between the two values obtained should not be greater than the repeatability limit (r), which can generally be deduced by linear interpolation from the values in the table below.

2 Reproducibility

When the values for the final result, obtained by operators in different laboratories using different apparatus under the same conditions for the analysis of the same laboratory sample, lie within the range of the mean values cited in the table below, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which can generally be deduced by linear interpolation from the values in the table below.

3 Results of the interlaboratory tests

Two interlaboratory tests carried out at an international level in 1986–87 by the IUPAC Commission on Oils, Fats and Derivatives, in which 10 and 17 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in Table 2.508.1.

Table 2.508.1.

	Sample							
	A	B	C	D	E	F	G	H
Number of laboratories	17	10	10	10	17	16	10	10
Number of laboratories retained after eliminating outliers	15	9	10	16	10	16	15	10
Number of accepted results	30	18	20	32	20	32	30	20
Mean value (percentage of total triglycerides)	1.8	2.4	3.6	5.2	7.2	9.7	10.0	22.0
Repeatability standard deviation (S_r)*	0.1	0.07	0.12	0.1	0.37	0.3	0.2	0.18
Repeatability relative standard deviation†	0.1	2.8	3.3	2.3	5.2	3.2	2.2	0.8
Repeatability limit (r)* ($2.8 \times S_r$)	0.3	0.19	0.34	0.3	1.06	0.9	0.6	0.51
Reproducibility standard deviation (S_R)*	0.4	0.61	0.45	0.3	2.14	0.4	1.2	1.48
Reproducibility relative standard deviation†	23.7	25.9	12.5	6.7	29.8	4.6	12.2	6.8
Reproducibility limit (R)* ($2.8 \times S_R$)	1.2	1.7	1.25	1.0	6.05	1.3	3.5	4.2

* Expressed as percentage of total triglycerides; † coefficient of variation.

2.531 Determination of the 2-thiobarbituric acid value: direct method

1 Scope

This Standard describes a method for the direct determination of the 2-thiobarbituric acid value (TBA value) in oils and fats without preliminary isolation of secondary oxidation products.

2 Definition

The TBA value is defined as the increase of absorbance measured at 530 nm due to the reaction of the equivalent 1 mg of sample per 1 ml volume with 2-thiobarbituric acid determined by the present method.

3 Field of application

This Standard is applicable to animal and vegetable fats and oils, fatty acids and their esters, partial glycol esters and similar materials (*note 1*).

4 Principle

Secondary oxidation products of oils and fats are reacted with 2-thiobarbituric acid, forming condensation products, the absorbance of which is measured at 530 nm, the wavelength of one of their absorbtion maxima.

5 Material

5.1 Volumetric flask, 25 ml.

5.2 Volumetric flask, 100 ml.

5.3 Pipette, 5 ml.

5.4 Ground test tubes, internal diameter 10–15 mm, with ground-glass stoppers.

5.5 Glass cells, 10 mm, suitable for spectrometric measurements.

5.6 Thermostated bath maintained at 95 ± 0.5°C.

5.7 Spectrometer allowing the reading of absorbance at 530 nm to the nearest 0.001.

6 Reagents

6.1 Pure 1-butanol containing less than 0.5 per cent water (*note 3*).

6.2 Chemically pure 2-thiobarbituric acid (*note 3*).

6.3 The TBA reagent is prepared by dissolving 200 mg of 2-thiobarbituric acid in 100 ml of 1-butanol. Leave the weighed amount with butanol overnight or use an ultrasonic apparatus, filter or centrifuge the suspension to remove the undissolved residue, make up the filtrate to 100 ml with 1-butanol; the reagent should not be stored for more than 1 week in the refrigerator.

7 Procedure

7.1 Weigh accurately 50–200 mg of the sample (*note 4*) into a volumetric flask (5.1). Dissolve it in a small volume of 1-butanol and make up to volume with the same solvent.

7.2 Transfer, using a pipette (5.3) 5.0 ml of the sample solution to a dry test tube (5.4); add by pipette (5.3) 5.0 ml of the reagent solution (6.3). Close the test tube with a ground stopper and mix thoroughly.

7.3 Place the prepared test tube into a thermostated bath (5.6) at 95°C.

7.4 After 120 minutes, remove the test tube from the thermostated bath and cool it under running tap-water for about 10 minutes until it reaches room temperature.

7.5 Measure the absorbance of the reaction solution in a 10-mm cell (5.5) at 530 nm (5.7), using distilled water in the reference cell (*note 5*).

7.6 Run at the same time a reagent blank. The reading of the blank determination should not exceed 0.1 in a 10-mm cell.

8 Calculation and expression of results

8.1 Expression of results

$$\text{TBA value} = \frac{50 \times (A - B)}{m}$$

where:

A is the absorbance of the test solution

B is the absorbance of the reagent blank

m is the mass in mg, of the test portion

50 is a factor valid if the volume of the volumetric flask is 25 ml (5.1) and the cell width 10 mm (5.5) (*note 5*).

9 Precision

9.1 Repeatability

The difference between the results of two determinations, carried out simultaneously or in rapid succession by the same analyst using the same apparatus and the same reagents for the same test material, should not exceed 10 per cent of the mean value of the two determinations in case of oils and 20 per cent in case of milk fat.

10 Notes

1 The method is not applicable to phospholipid concentrates, or to samples containing carbohydrates or proteins which could react either with the reagent or with TBA-active substances. For the analysis of such samples, the lipid fraction should be isolated by extraction before the analysis, or the volatile TBA-active substances should be isolated by steam distillation.

Under the conditions of the method, hydroperoxides and dioxolanes may be partially decomposed with formation of TBA-active compounds, and the decomposition is catalysed by a trace of heavy metals; on the other hand, antioxidants may react with some TBA-active substances, thus decreasing the TBA value. Some substances present in the sample might react with TBA to form other coloured complexes or reaction products, of which the absorbance is not any more at 530 nm. This can be recognized by another colour tone.

2 The absorbance of the reagent blank measured according to procedure 7, but without test portion, should not exceed 0.1. If so, a new reagent solution should be prepared using TBA of better purity. In the case of reagent blanks higher than 0.1, the high blank value may be due to impurities in 1-butanol. They are removed by refluxing the solvent with 0.1 per cent TBA for 2 hours, and distilling. Water may be removed by distillation and rejection of the first, opalescent fraction.

3 Solid samples are melted at not more than 10°C above the melting point and, if not entirely clear, filtered. Butter is melted at 40°C and the water is removed on filtering with a hydrophilic filter.

4 If the absorbance falls outside the range of 0.8–0.2, the determination is repeated with more suitable cells or with more appropriate amount of sample.

5 In the case of low blank values (0.05), the absorbance of the test solution can be measured directly against distilled water. The value is then calculated as follows:

$$\text{TBA value} = \frac{50 \times A}{m}$$

References

Marcuse R, Johansson L. *J Am Oil Chem Soc* 1973; **50**: 387.

Pokorny J, Dieffenbacher A. *Pure Appl Chem* 1989; **61**(6): 1165–1170 (Report on the Collaborative Study).

2.607 Determination of 'hexane' residues in fats and oils

1 Scope and field of application

This Standard describes a method for the determination of free volatile hydrocarbons, expressed generally in terms of hexane, remaining in animal and vegetable fats and oils after their extraction with hydrocarbon-based solvents.

The method is suitable for the determination of quantities of hexane of between 10 and 1500 mg/kg of fat or oil (10–1500 p.p.m.).

2 Definition

The 'hexane' content is the quantity of all volatile hydrocarbons remaining in fats and oils following processing involving the use of solvents and which is determined by the method specified, and expressed in milligrams per kilogram (p.p.m.).

3 Principle

Desorption of volatile hydrocarbons by heating the sample at 80°C in a closed vessel after addition of an internal standard. After determination of the calibration factor, determination of the hydrocarbons in the headspace by gas chromatography using packed or capillary columns. Expression of the results as hexane.

4 Apparatus

4.1 Septum vials, 20-ml capacity.

4.2 Septa and aluminium caps suitable for vials (4.1) together with crimping pliers. The septa must be resistant to oils and solvents (*note 1*).

4.3 Tongs suitable for holding vials (4.1).

4.4 Syringe, 10-μl capacity.

4.5 Syringe, 1-μl capacity.

4.6 Syringe, 1000-μl capacity, gas-tight.

4.7 Gas chromatograph with a flame-ionization detector and integrator/recorder. If a capillary column is used, the apparatus shall have a 1/100 split-injection system (*note 2*). Injector and detector regulated at 100°C. Column oven regulated at 50°C.

4.8 Packed column, made from steel or glass, 2–4 m in length, internal diameter 3.175 mm (1/8 inch), packed with 150–180-μm diatomaceous earth support, acid washed and silanized (*note 3*), coated with squalane (10%) or any other phase permitting the chromatographic separation required; or failing this:

4.9 Glass capillary column, approximately 30 m in length and 0.3 mm internal diameter, coated with methylpolysiloxane (film thickness 0.2 μm).

4.10 Heating bath, equipped with clamps for holding septum vials, regulated thermostatically at 80°C; for continuous operation, glycerol is recommended as the heating medium.

4.11 Shaking machine.

5 Reagents

5.1 Technical hexane, with a composition similar to that likely to have been used in processing or, failing this, *n*-hexane (*note 4*).

5.2 *n*-Heptane for analysis or, failing this, cyclohexane for analysis (*note 5*).

5.3 Carrier gas: hydrogen, or nitrogen, helium etc., thoroughly dried and containing less than 10 mg/kg of oxygen.

5.4 Auxiliary gases: hydrogen 99.9 per cent pure, containing no organic impurities; air, containing no organic impurities.

6 Sampling

It is essential that the sample be protected from loss of solvent residues.

7 Procedure

7.1 Preparation of test sample

Prepare the test sample, taking care to prevent loss of solvent residues.

7.2 Determination of the calibration factor

Weigh, to the nearest 0.01 g, 5 g of vegetable oil free from commercial hexane into each of seven vials (4.1) and close with a septum and a cap (4.2) (*note 6*). By means of the syringe (4.4 or 4.5) add solvent (5.1) to six of seven vials (4.1) according to the following table (*note 7*):

μl/5 g	0.5	1	2	4	7	10
mg/1000 g	67	134	268	536	938	1340

N.B. One vial will remain without the addition of solvent.

Shake vigorously, at room temperature, the six vials in the shaking machine (4.11) for 1 hour.

Using the syringe (4.4), add through the septum exactly 5 μl of *n*-heptane (5.2) as internal standard (*note 5*) to each of the seven vials. Shake by hand for about 1 minute. At intervals of about 15 minutes (*note 8*), place one vial at a time up to its neck in the heating bath (4.10).

Warm the gas-tight syringe (4.6) to 60°C and, after a tempering time of exactly 60 minutes at 80°C, take from each vial (without removing it from the bath) 1000 μl of the headspace above the oil by means of the gas-tight syringe (4.6) and immediately inject into the gas chromatograph (4.7).

Calculate the calibration factor (F) from the chromatogram for the 5-g quantities of vegetable oil 'spiked' with solvent, according to the following formula:

$$F = \frac{C_s \times A_i}{(A_H - A_B - A_i) \times C_i}$$

where:

A_B is the peak area of solvent hydrocarbons of the oil sample without the addition of solvent (however, without the peak area of the internal standard)

A_H is the total peak area of hydrocarbons, including the content of the internal standard

A_i is the peak area corresponding to the internal standard

C_i is the quantity of internal standard added, in mg per 1000 g. (If 5 μl of heptane per 5 g of oil is added, C_i = 680 mg per 1000 g (*note 9*).)

C_s is the quantity of hexane (5.1) added, in mg per 1000 g.

Express the results to the third decimal place.

The calibration factors of the six calibration samples should be approximately the same. Calculate the mean value, which should be about 0.45 for heptane (*notes 10, 11, 12*).

7.3 Determination of the solvent content of the sample

Weigh, to the nearest 0.01 g, 5 g of the test sample into a vial (4.1) as quickly as possible and close immediately with a septum and cap (4.2). Add 5 μl of internal standard (5.2)

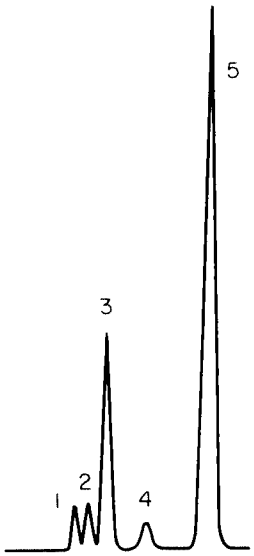


Fig. 2.607.1. Gas chromatogram of hexane hydrocarbons from oil.
1: 2-Methylpentane; **2**: 3-Methylpentane; **3**: *n*-Hexane; **4**: Methylcyclopentane; **5**: Cyclohexane (internal standard) (with other retention times: *n*-heptane).

through the septum by means of the syringe (4.4). Shake vigorously by hand for about 1 minute, and then place the vial up to its neck in the heating bath (4.10) for exactly 60 minutes.

Take 1000 μl from the headspace by means of the gas-tight syringe (4.6), without removing the vial from the heating bath, and immediately inject into the gas chromatograph (4.7). Record the peak areas of the solvent hydrocarbons present in the sample and the peak area of the internal standard from the chromatogram (see Fig. 2.607.1).

8 Calculation and expression of results

The residual solvent content W, expressed in mg/kg (p.p.m.) of the sample, is equal to:

$$W = \frac{(A_H - A_i) \times F \times C_i}{A_i}$$

where:

A_H is the total peak area due to hydrocarbons (including the peak area of the internal standard)

A_i is the peak area corresponding to the internal standard

C_i is the quantity of internal standard added, in mg per 1000 g. (If 5 μl of heptane per 5 g of oil is added, C_i = 680 mg per 1000 g; respectively, C_i = 780 mg if cyclohexane is used.)

F is the calibration factor according to 7.2.

Express the result to the nearest milligram.

9 Precision

The results of one interlaboratory study organized at an international level gave the statistical results shown in Table 2.607.1.

Table 2.607.1. Statistical analysis of results for hexane in oils (duplicate determinations).

	Sample*			
	1	2	3	4
Number of laboratories	16	16	11	11
Number of laboratories after elimination of outliers	16	16	9	10
Mean value (in p.p.m.)	2.95	13.7	104	1048
Repeatability standard deviation (S_r)	0.65	1.70	7.6	71.5
Repeatability coefficient of variation (%)	22.4	12.2	7.3	6.8
Repeatability value r ($S_r \times 2.83$)	1.8	4.8	21	202
Reproducibility standard deviation (S_R)	2.6	4.1	28.4	293
Reproducibility coefficient of variation (%)	89	30	27	28
Reproducibility value R ($S_R \times 2.83$)	7.4	12	80	829

* Sample 1: Fresh edible peanut oil + 3.3 p.p.m. technical hexane.
Sample 2: Fresh peanut oil after storage + 13.2 p.p.m. technical hexane.
Sample 3: Crude rapeseed oil (pressed) + 100 p.p.m. technical hexane.
Sample 4: Crude rapeseed oil (pressed) + 1000 p.p.m. technical hexane.

9.1 ## Repeatability

When the mean of duplicate determinations lies within the range of the mean values cited in Table 2.607.1, the difference between the results of two determinations, carried out in rapid succession by the same operator using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r), which can generally be deduced by linear interpolation from the table.

9.2 ## Reproducibility

When the means of duplicate determinations, obtained in two different laboratories using this standard method for the analysis of the same laboratory sample, lie within the range of the mean values cited in Table 2.607.1, the difference between the mean results obtained by those laboratories should not be greater than the reproducibility value (R) (*note 13*), which can generally be deduced by linear interpolation from the table.

10 ## Notes

1 The septa should be of butyl rubber or red rubber free from hydrocarbon solvent residues.

2 For series analyses a headspace gas chromatograph with automatic sample injection and tempering bath has been shown to be satisfactory. In this case manual injection is omitted.

3 Chromosorb P NAW 60–80 mesh is suitable.

4 For the calibration, technical extraction hexane should be used; this usually has a content of *n*-hexane ≥50 per cent and consists mainly of C_6 isomers.

5 Cyclohexane is also suitable for use as an internal standard provided that the solvent used for the extraction or calibration, respectively, has a negligible content of cyclohexane.

6 Freshly refined and deodorized vegetable oil, the commercial hexane content of which is negligible, is suitable for the calibration.

7 If *n*-hexane is added, the following table applies:

μl/5 g	0.5	1	2	4	7	10
mg/1000 g	66	132	264	528	924	1320

8 The duration of analysis depends on the time of retention of the internal standard. The samples must be placed in the heating bath in such a way that each sample is tempered for exactly 60 minutes.

9 If cyclohexane is used as internal standard, C_i = 780 mg per 1000 g.

10 The factor F so evaluated can be used for determining vial quantities of hexane of less than 60 p.p.m. If the value of F found for the vial containing 0.5 μl of hexane is significantly below the mean value, this deviation is probably due to the difficulty of introducing exactly 0.5 μl, and this determination must be eliminated or repeated.

11 The calibration factor for cyclohexane is normally about 0.57.

12 For quantities of hexane between 10 and 20 p.p.m., it is better to proceed to the calibration (7.2) under the following conditions:

— instead of 'add through the septum exactly 5 μl of *n*-heptane (5.2) as internal standard', add through the septum 2 μl of *n*-heptane (5.2) as internal standard.

— in this case C_i = 272 mg/1000 g.

13 It should be noted that the reproducibility values (R) cited in the table apply in the particular case when the results of single determinations obtained by two laboratories are being compared. When following the method described, and it is desired to compare the final results (which have been derived from the means of duplicate determinations) obtained by two laboratories, the values for R should be converted to the 95% probability critical difference values (CrD_{95}) applicable to the means of two determinations using the following formula:

$$CrD_{95} = \sqrt{(R^2 - r^2/2)}$$

References

Arens M, Kroll E. *Fette Seifen Anstrichmittel* 1985; **87**(1): 80.

Downes MJ. *Leatherhead Food RA Technical Circular No. 799*. 1982.

Hautfenne A, Pocklington WD, Wolff JP. *Pure Appl Chem* 1987; **59**(11): 1561–1570 (Report on the Collaborative Study).

Prevot A, Cabeza F. *Rev. Franç Corps Gras* 1960; **7**(1): 34.

Takagi T, Yamasaki M. *Yukagaku* 1975; **24**(6): 367.

2.608 Determination of benzo[*a*]pyrene in edible oils and fats by reversed phase high performance liquid chromatography

1 Scope and field of application

This Standard describes a method for the determination of benzo[*a*]pyrene in crude or refined edible oils and fats by reversed phase high performance liquid chromatography (HPLC) with fluorimetric detection with a lowest limit of detection of 0.1 μg/kg.

2 Principle

Absorption of a suitable amount of sample on an alumina column. Followed by elution of any benzo[*a*]pyrene present with light petroleum. Final analysis is carried out by reversed phase HPLC using a fluorimetric detector.

3 Apparatus

3.1 Glass column for chromatography, 30 cm in length, 15 mm internal diameter, fitted with sintered glass discs and polytetrafluoroethylene (PTFE) taps.

3.2 Water baths kept at 35°C and 65°C.

3.3 Flash evaporator (*note 1*).

3.4 High performance liquid chromatograph consisting of HPLC pump, injection valve with 10- and 12-μl sample loops, reversed phase column and electronic integrator with chart recorder.

3.5 Crimp-top minivials of about 1-ml volume with Teflon-layered septa and aluminium caps.

3.6 Hand-crimper for crimping the aluminium caps on to the minivials.

3.7 Disposable pipettes.

3.8 Columns for HPLC analysis:

Stainless steel pre-column, 75 mm in length, 4.6 mm internal diameter, packed with Lichrosorb RP-18, 5-μm particle size.

Stainless steel reversed phase HPLC column, Vydac for PAHs, 250 mm in length, 4.6 mm internal diameter.

3.9 Fluorimetric detector with excitation wavelength at 384 nm (slit 10 nm) and emission wavelength at 406 nm (slit 10 nm).

4 Reagents (*note 2*)

4.1 Light petroleum (b.p. range 40–60°C) or hexane, analytical grade, redistilled over potassium hydroxide pellets (4 g/l).

4.2 Acetonitrile, suitable for HPLC, e.g. Lichrosolv.

4.3 Tetrahydrofuran, suitable for HPLC, e.g. Lichrosolv.

4.4 Toluene, suitable for HPLC, e.g. Lichrosolv.

4.5 Sodium sulphate, analytical grade, granular, anhydrous.

4.6 Water, double distilled, filtered over a membrane filter of 0.45-μm pore size.

4.7 Alumina, activity grade 4, prepared as follows: Neutral aluminium oxide, activity super I is deactivated by the addition of 10 ml of distilled water to 90 g of alumina. Be cautious because of the heat of reaction and the build-up of pressure. The vessel is shaken for about 15 minutes and equilibrated for 24 hours before use. The alumina is stored in a closed vessel at ambient temperature.

4.8 Benzo[*a*]pyrene, purity 99.0 per cent (*note 3*).

Caution

Benzo[*a*]pyrene is a known carcinogen. All work using benzo[*a*]pyrene should be carried out in a fume hood and gloves should be worn to minimize exposure.

4.9 Preparation of benzo[*a*]pyrene—standard solutions

4.9.1 **Stock solution**

Weigh accurately about 12.5 mg of benzo[*a*]pyrene in a graduated flask of 25 ml. Dissolve the benzo[*a*]pyrene in toluene and fill up to the mark. The benzo[*a*]pyrene concentration in the stock solution is about 0.5 mg/ml. When stored in the dark at 4°C, this standard stock solution is stable for at least 6 months.

4.9.2 **Standard solutions**

Prepare benzo[*a*]pyrene standard solutions of about 0.2 μg/ml and 0.01 μg/ml by diluting aliquots of the standard stock solution (4.9.1) with acetonitrile.

5 Procedure

5.1 Clean-up

5.1.1 Weigh to the nearest mg about 2 g of the oil sample into a graduated flask of 10 ml. Dissolve the oil in light petroleum and make up to the mark with light petroleum.

5.1.2 Fill the chromatography column (3.1) to half its height with light petroleum. Rapidly weigh 22 g of alumina, activity grade 4 (4.7), into a small beaker and transfer the alumina immediately to the column. Promote setting of the alumina by gently tapping the column.

5.1.3 Add anhydrous sodium sulphate on the top of the column in a layer of about 3 cm.

5.1.4 Open the tap and allow the light petroleum to become level with the top of the sodium sulphate layer.

5.1.5 Place a 20-ml graduated flask under the column.

5.1.6 Pipette 2.00 ml of the oil solution (5.1.1) on to the column. Rinse the column with minimal amounts of light petroleum, allowing the solvent layer to run into the sodium sulphate layer between rinsings.

5.1.7 Elute the column with light petroleum with a flow of about 1 ml/min.

5.1.8 Discard the first 20 ml of eluate.

5.1.9 Collect a further 60 ml of eluate into a 100-ml round-bottomed flask.

5.1.10 Concentrate the eluate in a water bath of 65°C to about 0.5–1.0 ml, and transfer the concentrated solution into a pre-weighed (to the nearest 0.1 mg) crimp-top minivial of about 1 ml (*note 1*).

5.1.11 Continue evaporation from the minivial in water of 35°C under a gentle stream of nitrogen (about 25 ml/min) until nearly dry.

5.1.12 Rinse the round-bottomed flask twice with about 1 ml of light petroleum and collect these rinsings quantitatively into the minivial.

5.1.13 Continue evaporation at 35°C under a gentle stream of nitrogen till dryness.

5.1.14 Weigh the minivial to the nearest 0.1 mg and calculate the weight of the residue.

5.1.15 Stopper the minivial with the Teflon-layered septum and the aluminium cap, and store at 4°C.

5.2 High performance liquid chromatography

5.2.1 Elution solvent: acetonitrile/water 88/12 (V/V).

Degas the eluant solvent to remove oxygen in order to avoid fluorescence quenching of the benzo[*a*]pyrene. Use helium purging.

5.2.2 Elute at a flow of about 1 ml/min.

5.2.3 **Calibration curve** (*note 4*)

Prepare a four-point concentration curve as follows: inject 4 and 20 μl of the 0.01 μl/ml benzo[*a*]pyrene standard solution (4.9.2), corresponding to 0.04 and 0.2 ng benzo[*a*]pyrene, and then inject 5 and 10 μl of the 0.2 μl/ml benzo[*a*]pyrene standard solution (4.9.2), corresponding to 1 and 2 ng of benzo[*a*]pyrene. Use the peak area obtained by means of the integrator and chart recorder (3.4) to construct the calibration curve.

5.3 Sample analysis (*note 5*)

5.3.1 Inject 20 μl of tetrahydrofuran (4.3) into the minivial containing the cleaned residue (5.1.15).

5.3.2 Dissolve the residue in the vial in tetrahydrofuran (4.3) by careful swirling, avoiding contact of the solvent with the septum.

5.3.3 Inject an accurately known volume of about 10 μl of the dissolved residue into the HPLC column and start the running of the chromatogram.

6 Calculation and expression of results

6.1 Calculation

Calculate from the calibration curve (5.2.3) the amount of benzo[*a*]pyrene in the injected volume of the residue dissolved in tetrahydrofuran (5.3.2).

The benzo[*a*]pyrene content (BaP), in μg/kg, is given by the formula:

$$\text{BaP} = \frac{5 \times A \times (20 + 1.25\,G)}{V \times M}$$

where:

A is the amount of benzo[*a*]pyrene, in ng, read from the calibration curve (5.2.3)

G is the mass of residue in the minivial (5.1.14), in mg, with an assumed density of 0.8 and therefore a volume of 1.25 G μl.

V is the volume of tetrahydrofuran injected into the chromatograph.

M is the mass of test portion, in g, weighed in (*note 6*).

6.2 Expression of results

Express the results as μg/kg (to two significant figures).

7 Quality assurance

7.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

7.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

8 Notes

1 A rotary evaporator with vacuum and a waterbath of 40°C may be used. Care should be taken to prevent cross-contamination. Clean the system thoroughly between subsequent determinations.

2 If analytical grade solvents other than the recommended ones are used, a full blank analysis has to be carried out and the results of this blank analysis have to be reported.

3 Supplied by the Commission of the European Community Bureau of Reference (BCR), Rue de la Loi 200, B-1049 Brussels, Belgium.

4 If an autosampler is used, the sample loop should be flushed with acetonitrile between two subsequent injections.
5 Tetrahydrofuran proved to be the optimal solvent for residue analysis of oils and fats following the clean-up procedure described in 5.1. Injection of volumes in excess of the prescribed 10 μl will give rise to problems. Do not store the samples in tetrahydrofuran for a prolonged period because benzo[*a*]pyrene is not stable in this solvent.
6 With the calibration curve (5.2.3) benzo[*a*]pyrene levels of 0.1–10 μg/kg can be determined. For benzo[*a*]pyrene concentrations above 10 μg/kg, the residue solution (5.3.2) should be diluted further with tetrahydrofuran, or a smaller volume than 10 μl (5.3.3) should be injected.

Annexe

1 Repeatability limit
The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as given in the table with statistical and other data derived from the results of the interlaboratory test.

2 Reproducibility limit
The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as given in the table with statistical and other data derived from the results of the interlaboratory test.

3 Trueness (bias)
The bias of the method was demonstrated in the collaborative study of the method (see table of statistical data below) to be negligible when used for the determination of concentration levels of benzo[*a*]pyrene in the range 1–5 μg/kg.

4 Sensitivity
The sensitivity of the method is demonstrated by the low values for r and R at the low concentration levels studied (see table of statistical data below), the limit of detection is 0.1 μg/kg, and the limit of determination is 1 μg/kg.

Interference by other poly-aromatic hydrocarbons is not to be expected provided the measurements are carried out at the wavelengths specific for benzo[*a*]pyrene: excitation wavelength 384 nm and emission wavelength 406 nm.

5 Statistical and other data derived from the results of the interlaboratory test
The interlaboratory test carried out at the international level in 1989 by the IUPAC Commission on Oils, Fats and Derivatives, in which 23 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following table:

Table 2.608.1.

Sample:	Fish oil			Rapeseed oil		
Batch:	A	B	C	A	B	C
Number of laboratories retained after eliminating outliers	10	10	10	11	11	11
Number of outliers (laboratories)	2	2	2	1	1	1
Number of accepted results	240	240	240	264	264	264
Mean value (μg/kg sample)	4.87	3.08	0.98	4.39	2.78	0.99
True or accepted value (μg/kg)	5.35	3.45	1.15	4.85	2.95	1.15
Repeatability standard deviation (S_r in μg/kg)	0.64	0.20	0.24	0.73	0.35	0.27
Repeatability relative standard deviation (CV_r in %)	13.1	6.5	24.6	16.6	12.6	27.4
Repeatability limit (r) ($2.8 \times S_r$)	1.79	0.56	0.67	2.04	0.98	0.76
Reproducibility standard deviation (S_R in μg/kg)	1.09	0.54	0.29	0.77	0.67	0.41
Reproducibility relative standard deviation (CV_R in %)	22.4	17.6	29.4	17.5	24.0	41.0
Reproducibility limit (R) ($2.8 \times S_R$)	3.05	1.52	0.80	2.16	1.87	1.14

References

Applied Chemistry Division: Commission VI.3. Minutes of the Meeting in St. Andrews, Scotland on 16–18 August 1988, pp. A13–A18.

Hendrikse PW, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(11): 1659–1666 (Report of the Collaborative Study).

2.631 Determination of copper, iron and nickel by direct graphite furnace atomic absorption spectrometry

1 Scope and field of application

This Standard describes a method for the determination of trace amounts (μg/kg) of copper, iron and nickel in all types of crude or refined oils and fats, including contaminants from bleaching earth and/or catalysts.

2 Principle

Vaporization of the oil/fat in a suitable graphite furnace connected to an atomic absorption spectrometer, previously calibrated using standard solutions of organocompounds of copper, iron and nickel. Measurement of the metal content from the observed absorptions at selected wavelengths. The elements are analysed sequentially.

3 Apparatus

3.1 Polyethylene or polypropylene-capped bottles, 20 and 50 ml, metal-free (*note 1*).

3.2 Micropipettors, 20 and 50 μl.

3.3 Pipettor tips.

3.4 Atomic absorption spectrometer, equipped with either 'peak height' mode and printer, or 'continuous' mode and pen-recorder (full-scale response in 0.2 seconds), together with appropriate hollow cathode tubes and deuterium background corrector. The spectrometer should be located in a dust-free atmosphere.

3.5 Graphite furnace atomizer (equipped with facilities).

3.6 Graphite tubes for use in a furnace atomizer (3.5).

3.7 Chromatographic columns (diameter/height ratio 1/10) (*note 2*).

3.8 Electric oven, regulated at 60 ± 2°C and suitable for heating at 150°C.

3.9 Water bath.

4 Reagents

4.1 *n*-Heptane, analytical grade.

4.2 Light petroleum, b.p. 40–60°C, analytical grade.

4.3 Sunflower oil, refined, or a similar, stable liquid oil with a low metal content (*note 2*).

4.4 Aluminium oxide, chromatographic grade (*note 3*).

4.5 Nitric acid, 2 M, free from traces of iron, nickel and copper.

4.6 Niobium nitrate ($Nb(NO_3)_5$), aqueous solution 1000 mg/l (*note 4*).

4.7 Standard stock solutions: Fe 10 mg/kg, Ni 10 mg/kg and Cu 2 mg/kg, prepared by appropriate dilution of organometallic standards with sunflower oil (4.3) (*note 5*).

4.8 Standard working solutions: prepare daily the following working solutions by diluting the stock solutions (4.7) with oil with a low metal content (4.3):

Copper	0.05,	0.1,	0.2 mg/kg
Iron	0.25,	0.5,	1.0 mg/kg
Nickel	0.25,	0.5,	1.0 mg/kg

4.9 Argon, purity 99.99 per cent minimum (*note 6*).

5 Procedure

5.1 Treatment of samples and solutions

Place all samples and working solutions in the oven (3.8) during the period of determination.

Shake samples vigorously before analysis.
If the metal content of a crude oil is known to be outside the range specified for the standards in 4.8, dilute the sample with low metal content oil (4.3) to bring the metal content within that range.

5.2 Preparation of apparatus

Switch on the atomic absorption spectrometer (3.4) and the D_2 corrector. In accordance with the instructions of the manufacturer, adjust: lamp current, slit, wavelength and amplification. The required wavelengths are (nm):

Copper	324.7
Iron	302.1
Nickel	232.0

Optimize the position of the graphite furnace atomizer (3.5) in the atomic absorption spectrometer (3.4) and set the required programme for the furnace as follows:

Table 2.631.1.

	Step no.	Temp. (°C)	Ramp-time (s)	Hold-time (s)	Int. gas-flow (ml/min)
For Cu	1	900	50	30	300
	2	2700	1	5	50
For Fe, Ni	1	1200	50	30	300
	2	2700	1	5	50

If it is not possible to programme the graphite furnace exactly as above, use a comparative programme suitable for the equipment (*note 7*).
Use a normal graphite tube (*note 8*).
Pretreat the pipettor tip (3.3) by pipetting (using a micropipettor, 3.2) and then discarding 20 μl of heptane. The film of heptane remaining on the wall of the tip facilitates a reproducible transfer of the oil sample. The tip has to be pretreated before each injection of an oil sample.

5.3 Determination

5.3.1 Graphite tube blank

Record the absorption, if any, of the graphite tube (3.6) as such and autozero this absorption.

5.3.2 Liquid oil blank

By means of a micropipettor (3.2), inject 20 μl of low-metal oil (4.3) into the graphite furnace (3.5), initiate the temperature programme and record the absorption.

5.3.3 Standardization of apparatus

By means of a micropipettor (3.2), inject 20 μl of the three standard working solutions of the metal under investigation (4.8) into the graphite furnace (3.5) and record the absorption.

5.3.4 Sample for analysis.

5.3.4.1 **Oil (liquid) samples**

By means of a micropipettor (3.2), inject 20 μl of the oil sample into the graphite furnace (3.5), initiate the temperature programme and record the absorption.

5.3.4.2 **Fat samples (m.p. 40°C and higher)**

By means of a micropipettor (3.2), introduce an extra temperature programming step: hold-time 20 seconds, temperature 60°C, internal gas flow 0 ml/min. Initiate the temperature programme. Within the first programme step, introduce by means of a micropipettor (3.2) 20 μl of the melted fat into the graphite furnace (3.5), allow the tip to remain in the injection opening to liquefy the fat and then inject. Record the absorption (*note 9*).

6 Calculation and expression of results

6.1 Calculation

Measure the peak height on the recorder chart or read from the display or printer.
Draw a calibration curve by plotting the absorption of the three standards (5.3.3), corrected for the blank (5.3.2), against their respective metal contents (*note 10*).
Read the metal content of the sample from the relevant calibration curve.

6.2 Repeatability value

The differences between the values obtained from two single determinations, carried out in rapid succession by the same operator using the same apparatus for the analysis of the same test sample, should not be greater than the repeatability value (r) as calculated from the formulae in Table 2.631.2, which express the precision in relation to the determined mean value.

Table 2.631.2. Repeatability (r) and reproducibility values (R).

Metal	Substrate	r	R
Cu	Oil	$0.0102 + 0.140\,M$	$0.0085 + 0.358\,M$
	Fat	$0.0076 + 0.109\,M$	$0.0028 + 0.492\,M$
Fe	Oil	$0.077 + 0.081\,M$	$0.040 + 0.480\,M$
	Fat	$0.026 + 0.196\,M$	$0.031 + 0.543\,M$
Ni	Oil	$0.056 + 0.127\,M$	$0.027 + 0.442\,M$

M = corresponding mean concentration value.

6.3 Reproducibility value

The difference between the values for the final result, obtained by two (or more) laboratories using this standard method for the analysis of the same laboratory sample, should not be greater than the reproducibility value (R) as calculated from the formulae in Table 2.631.2, which express the precision in relation to the determined mean value.

7

Notes

1 The polyethylene- or polypropylene-capped bottles are made metal-free in the following way: clean the bottles thoroughly with warm nitric acid (4.5), rinse with distilled water and dry the bottles in a drying oven (3.8) at about 80°C.

2 A sample of a low metal content oil is obtained by the following procedure: dissolve 1 part of oil (4.3) (m) in 3 parts (V) of light petroleum (4.2). Prepare an aluminium oxide column (3.7) using twice the mass of aluminium oxide (4.4), activated by heating in an oven (3.8) at 150°C for 14 hours, as the mass of the oil to be purified. Add the oil solution to the column and elute with 5 parts (V) of light petroleum. Evaporate the light petroleum of the eluate on a heated water bath (3.9) using a gentle stream of nitrogen (2–5 l/min) (4.9). Remove final traces of light petroleum under vacuum.

3 Merck product no. 1077 is suitable.

4 Solution (code 88083) is available from Alfa Division, 152 Andover Street, Danvers, MA 01923, USA.

5 Suitable standards are available from, for example, Continental Oil Company, Ponca City, Oklahoma, USA (Conostan, 5000 mg/kg) or Merck, D-6100 Darmstadt, Germany (metal in standard oil, 1000 mg/kg).

6 If argon is not available, nitrogen may be used as purge gas. At temperatures above 2300°C, nitrogen forms toxic cyanogen gas; therefore, continuous ventilation in the furnace area should be provided.

7 If in this case the background correction fails, dilute blank, standards and samples with an organic fat solvent, e.g. heptane (4.1) to a maximum of 1:2 (m/m) and work at ambient temperature.

8 The graphite tube for the determination of iron has to be coated with niobium to ensure that the total amount of iron is determined. With an uncoated tube the result will vary according to the type of iron compound present in the oil. A suitable coating procedure is: by means of a micropipettor (3.2), inject 100 μl of niobium nitrate solution (4.6) into the furnace. Start the temperature programme to dry at 100°C for 60 seconds and then atomize at 2700°C for 5 seconds. Repeat this procedure until 300 μl of niobium nitrate solution has been injected. Atomize at 2700°C until constant absorbance (to remove any iron contamination).

9 The normal, minimum limit of detection can be improved by either a greater scale expansion or by repeated injections of the sample at the end of the ashing operation, then allowing the programme to proceed to completion. If the metal content is too high (i.e. exceeds the calibration curve), measure the absorption after a further dilution of the sample with sunflower oil.

10 With the use of sophisticated equipment, autocalibration can be applied.

References

Hendrikse PW, Slikkerveer FJ, Zaalberg J, Hautfenne A. *Pure Appl Chem* 1988; **60**(6): 893–900 (Report on the Collaborative Study).
King AE, Roschen HL, Irwin WM. *Oil Soap* 1933; **10**: 204.
List GR, Evans CD, Kwolek WF. *J Am Oil Chem Soc* 1971; **48**: 438.
List GR, Evans CD, Moser HA. *J Am Oil Chem Soc* 1972; **49**: 287.
List GR, Evans CD, Black LT, Mounts TL. *J Am Oil Chem Soc* 1978; **55**: 275.
List GR, Mounts TL, Heakin AJ. *J Am Oil Chem Soc* 1978; **55**: 280.
Marcuse R, Fredrikson PO. *J Am Oil Chem Soc* 1971; **48**: 448.
Mertens WG, Swindells CF, Teasdale BF. *J Am Oil Chem Soc* 1971; **48**: 544.
Notevarp O, Chanine MH. *J Am Oil Chem Soc* 1972; **49**: 247.

2.632 Determination of lead by direct graphite furnace atomic absorption spectrometry

1 Scope and field of application

This Standard describes a method for the determination of trace amounts (μg/kg) of lead in all types of crude or refined edible oils and fats.

2 Principle

Vaporization of the oil or fat in a suitable graphite furnace with or without a platform connected to an atomic absorption spectrometer, previously calibrated using standard solutions of organocompound of lead, and the measurement of the metal content from the observed absorption at a wavelength of 283.3 nm.

3 Apparatus

3.1 Polyethylene or polypropylene capped bottles, 20 ml, metal-free (*note 1*).

3.2 Micropipettor, 20 μl.

3.3 Pipettor tips.

3.4 Electric oven, regulated at 60 ± 2°C.

3.5 Atomic absorption spectrometer, equipped with either 'peak height' mode and printer, or 'continuous' mode and pen-recorder (full-scale response in 0.2 seconds), together with the appropriate electrode-less discharge lamp (or hollow cathode lamp) and deuterium background corrector (or Zeeman atomic absorption spectrometer).

3.6 Graphite furnace atomizer, placed in the atomic absorption spectrometer (3.5), equipped with a control unit for temperature programming.

3.7 Graphite tube, normal (uncoated).

3.8 Platform: pyrolytic, in combination with uncoated or pyrolytically coated graphite tube (*note 2*).

4 Reagents

4.1 Cyclohexane, analytical grade.

4.2 Lecithin, well-defined, containing 2 per cent phosphorus.

4.3 Matrix modifier: 2 per cent (m/V) lecithin solution is prepared by dissolving 2 g of lecithin (4.2) in 100 ml of cyclohexane (4.1).

4.4 Blank oil, refined. Any edible oil is suitable, to be stored in a metal free polyethylene bottle. Lead content of oil not greater than 1 μg/kg.

4.5 Standard stock solution: a stock lead solution of 10 mg/kg is prepared by appropriate dilution of an organometallic standard with the blank oil (4.4). A suitable standard (Conostan, 5000 mg/kg) is available from Continental Oil Company, Ponca City, Oklahoma, USA.

4.6 Standard working solutions: 0.02, 0.05 and 0.100 mg lead/kg to be prepared daily by diluting the 10 mg/kg stock solution (4.5) with blank oil (4.4).

4.7 Argon, purity 99.99 per cent minimum.

5 Procedure

5.1 Treatment of samples, blank and standards

5.1.1 Place all samples and standard working solutions in the oven (3.4), regulated at 60 ± 2°C.

5.1.2 Shake samples vigorously.

5.1.3 Weigh 5.0 g sample in a 20-ml bottle (3.1) together with 5.0 g of matrix modifier (4.3), and mix thoroughly.

5.1.4 Carry out instructions 5.1.2 and 5.1.3 also for the three standard working solutions (4.6) and the blank oil (4.4).

5.2 Preparation of apparatus

5.2.1 Switch on the atomic absorption spectrometer and the background correction (D_2 of Zeeman).

5.2.2 In accordance with the manufacturer's instructions supplied with the spectrometer, adjust: lamp current, slit, wavelength and amplification. The required wavelength is 283.3 nm.

5.2.3 Optimize the position of the graphite furnace atomizer (3.6) in the atomic absorption spectrometer (3.5) and set the required programme on the control unit of the furnace. If available, place platform in graphite tube (*note 2*).

5.2.4 Pretreat before each injection, the pipettor tip (3.3) by pipetting and then discarding 20 µl of cyclohexane.

Programmes for the graphite furnace atomizer are (for Varian spectrometer, see *note 4*):

Table 2.632.1. Uncoated tube (3.7) off the wall; maximum power heating; gas-stop.

Step no.	Temp. (°C)	Ramp-time (s)	Hold-time (s)	Int. gas-flow (ml/min)
1	100	10	20	300
2	650	60	40	300
3	1900	0	5	0
4	2700	1	3	50

Table 2.632.2. Pyro-coated tube (3.8) with platform; maximum power heating; gas-stop.

Step no.	Temp. (°C)	Ramp-time (s)	Hold-time (s)	Int. gas-flow (ml/min)
1	200	10	20	300
2	650	60	40	300
3	1700	0	5	0
4	2700	1	3	50

5.3 Determination

5.3.1 **Measurement of the blank**

Inject 20 µl of the blank solution prepared according to 5.1.4 into the graphite furnace, initiate the temperature programme and record the absorption.

5.3.2 **Measurement of standards**

Inject 20 µl of the three standard solutions prepared according to 5.1.4 into the graphite furnace and record the absorptions.

5.3.3 **Measurement of sample solutions**

Inject 20 μl of the sample solution prepared according to 5.1.3 into the graphite furnace, initiate the temperature programme and record the absorption.

6 Calculation and expression of results

6.1 Calculation

6.1.1 Measure the peak height on the recorder-chart or take the reading of the display or printer.

6.1.2 Draw a calibration curve by plotting the absorption of the three standards (5.3.2), corrected for the blank (5.3.1), against their respective metal content (*note 3*).

6.1.3 Read the metal content of the sample from the calibration curve.

6.2 Expression of results

Express the results as mg/kg (to two significant figures)

7 Quality assurance

7.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

7.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

8 Notes

1 The polyethylene or polypropylene capped bottles are made metal-free in the following way: clean the bottles thoroughly with warm nitric acid, rinse with distilled water and dry the bottles in a drying oven at about 80°C.

2 Both atomization off the wall and atomization off the platform can be used. The accuracy and the sensitivity off the platform is two times higher than off the wall.

3 With the use of sophisticated equipment, autocalibration can be applied.

4 Modification for Varian spectrometers. For those having a Varian apparatus, the following modifications should be made (5.2.3):

Table 2.632.3. Varian programme for uncoated tube (3.7), off the wall.

Step no.	Temp. (°C)	Time (s)	Gas-flow (l/min)	Gas-type	Read command
1	100	20.0	3.0	Normal	No
2	100	40.0	3.0	Normal	No
3	550	60.0	3.0	Normal	No
4	550	40.0	3.0	Normal	No
5	550	1.0	0.0	Normal	No
6	2500	1.0	0.0	Normal	Yes
7	2500	5.0	0.0	Normal	Yes
8	2500	3.0	3.0	Normal	No

Table 2.632.4. Varian programme for pyro-coated tube with platform.

Step no.	Temp. (°C)	Time (s)	Gas-flow (l/min)	Gas-type	Read command
1	200	10.0	3.0	Normal	No
2	200	20.0	3.0	Normal	No
3	650	60.0	3.0	Normal	No
4	650	40.0	3.0	Normal	No
5	650	1.0	0.0	Normal	No
6	2000	0.7	0.0	Normal	Yes
7	2000	5.0	0.0	Normal	Yes
8	2000	3.0	3.0	Normal	No

Clause 5.3: use 10 μl for injection.

Annexe

1 Repeatability limit

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time, should not be greater than the repeatability limit (r) as calculated from the formulae in Table 2.632.5.

Table 2.632.5. Repeatability (r) and reproducibility limits (R).

Pb in edible oil	$r = 0.19\,M$	$R = 0.30\,M$
Pb in cocoa butter	$r = 0.15\,M$	$R = 0.68\,M$

M = corresponding mean concentration value.

2 Reproducibility limit

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (R) as calculated from the formulae in Table 2.632.5.

3 Trueness (bias)

The bias of the method was demonstrated in the collaborative study of the method (see Table 2.632.6) to be negligible when used for the determination of concentration levels of lead in the range 0.01–0.10 mg/kg.

4 Sensitivity

The sensitivity of the method is demonstrated by the low values for r and R at the low concentration levels studied (see Table 2.632.6), the limit of detection is 0.001 mg/kg, and the limit of determination is 0.01 mg/kg.

Interference by other elements is not to be expected provided the measurements are carried out at the wavelength specific for lead (283.3 nm).

5 Statistical and other data derived from the results of the interlaboratory test

The interlaboratory test carried out at the international level in 1988 by the IUPAC Commission on Oils, Fats and Derivatives, in which 20 laboratories participated, each

obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following table:

Table 2.632.6

Sample:	Soyabean oil			Cocoa butter		
Batch:	A	B	C	A	B	C
Number of laboratories retained after eliminating outliers	14	14	14	16	16	16
Number of outliers (laboratories)	6	6	6	4	4	4
Number of accepted results	112	112	112	128	128	128
Mean value (mg/kg sample)	0.087	0.053	0.022	0.087	0.054	0.026
True or accepted value (mg/kg)	0.085	0.050	0.020	0.085	0.050	0.020
Repeatability standard deviation (S_r in mg/kg)	0.0059	0.0034	0.0022	0.0039	0.0031	0.0013
Repeatability relative standard deviation (%)	6.8	6.4	10.1	4.5	5.7	5.0
Repeatability limit (r) ($2.8 \times S_r$)	0.017	0.0095	0.0062	0.011	0.0087	0.004
Reproducibility standard deviation (S_R in mg/kg)	0.0089	0.0057	0.0034	0.0180	0.0117	0.0073
Reproducibility relative standard deviation (%)	10.2	10.9	15.4	20.6	21.7	27.7
Reproducibility limit (R) ($2.8 \times S_R$)	0.025	0.016	0.009	0.050	0.033	0.020

References

Hendrikse PW, Slikkerveer FJ, Zaalberg J, Hautfenne A. *Pure Appl Chem* 1988; **60**: 893.

Hendrikse PW, Slikkerveer FJ, Folkersma A, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(8): 1185–1192 (Report on the Collaborative Study).

Slikkerveer FJ, Braad AA, Hendrikse PW. *At Spectr* 1980; **1**: 30.

2.642 Determination of antioxidants by high performance liquid chromatography

1 Scope

This Standard describes a method for the quantitative determination of phenolic antioxidants.

2 Field of application

This Standard is applicable to the determination of: propylgallate (PG), octylgallate (OG), dodecylgallate (DG), 2,4,5-trihydroxybutyrophenone (THBP), *tert*-butylhydroquinone (TBHQ), nordihydroguiaretic acid (NDGA), butylated hydroxyanisole (BHA, a mixture of 2-*tert*-butyl- and 3-*tert*-butyl-4-hydroxy anisole), 2,6-di-*tert*-butyl-4-hydroxymethylphenol (Ionox-100), and butylated hydroxytoluene (BHT, or 3,5-di-*tert*-butyl-4-hydroxytoluene) in edible oils and fats.

3 Principle

Dissolution of the oil or fat in hexane, extraction of the antioxidants by acetonitrile and then determination by high performance liquid chromatography using UV detection at 280 nm.

4 Apparatus

Note: All glassware must be rinsed with chloroform, acetone and methanol, then blown dry with nitrogen.

4.1 High performance liquid chromatograph consisting of an HPLC pump and injector valve with 10-μl sample loop.

4.2 UV detector system to measure absorbance at 280 nm.

4.3 Integrator, electronic, with chart-recorder.

4.4 Column: stainless steel tube, 150 mm in length and internal diameter about 4.6 mm, packed with microparticulate spherical silica (5 μm) bonded with octadecyldimethylsilyl derivatives (*note 1*). A guard column is recommended. The column should be capable of allowing baseline separation of all seven antioxidants.

4.5 Flash evaporator or rotary film evaporator.

5 Reagents

5.1 *n*-Hexane, HPLC grade or glass-distilled.

5.2 Acetonitrile, HPLC grade or glass-distilled.

5.3 Acetic acid, glacial, analytical-grade.

5.4 2-Propanol, HPLC grade or glass-distilled.

5.5 Extracting solvents

Saturate hexane (5.1) and acetonitrile (5.2) by shaking equal volumes together and allowing to separate.

The upper layer is formed by hexane saturated with acetonitrile and the lower layer by acetonitrile saturated with hexane. These layers are used for the extraction of the antioxidants from the sample.

5.6 Mobile phase solutions

Solution A: Prepare a 5 per cent (V/V) solution of acetic acid (5.3) in water (*note 2*).

Solution B: Prepare a 5 per cent (V/V) solution of acetic acid (5.3) in acetonitrile (5.2) (*note 2*).

5.7 Antioxidants reference solution

5.7.1 **Stock solutions**

Weigh accurately about 25 mg of each antioxidant to be determined into separate 25-ml volumetric flasks. Dissolve the antioxidant in 1:1 (V/V) 2-propanol (5.4)/acetonitrile (5.2) and make up to volume with the same solvent. The concentration of the antioxidants in the stock solutions is about 1 mg/ml. These stock solutions are stable for at least 2 weeks if stored in the dark at 4°C.

5.7.2 **Standard solutions**

Prepare standard solutions of about 10 μg/ml by pipetting 1 ml of each stock solution into a 50-ml volumetric flask. Make up to volume with 1:1 (V/V) 2-propanol/acetonitrile. Prepare fresh standard solutions each day immediately before use.

6 Procedure

6.1 Extraction of antioxidants from oil or fat

6.1.1 Accurately weigh about 20 g of sample into a 50-ml beaker and quantitatively transfer to a 100-ml volumetric flask, rinsing the beaker with hexane (upper layer of extracting solvent (5.5)). Dilute to volume with the same solvent and mix.

6.1.2 Pipette 25 ml of the prepared sample solution into a 125-ml separator and extract with three 50-ml portions of acetonitrile (lower layer of extracting solvent (5.5)). If emulsions form, break them by holding the separator under hot water.

6.1.3 Collect the extracts in a 250-ml separator. Allow the combined extracts to drain slowly (to aid the removal of hexane–oil droplets (*note 3*)) into a 250-ml round-bottomed evaporator flask. Evaporate the extracts down to 3–4 ml using the evaporator (4.6) with the bath water temperature not exceeding 40°C, completing the evaporation in less than 10 minutes (*note 4*). Use an efficient vacuum source and ice-water cooling of the distillate to decrease evaporation time.

6.1.4 Using a disposable pipette, transfer the acetonitrile–oil droplets mixture to a 10-ml volumetric flask. Rinse the evaporator flask with small portions of hexane saturated with acetonitrile (upper layer of (5.5)) and, using the disposable pipette, transfer the rinsings to the volumetric flask until a volume of 5 ml is collected. Rinse the pipette and evaporator flask with small portions of 2-propanol (5.4), transferring all rinsings to the volumetric flask until the volume is completed.

6.1.5 Mix the contents of the volumetric flask and filter the solution through a disposable Teflon filter (pore size 0.45 μm) (*note 4*).

6.2 Chromatography

6.2.1 **Elution programme**

Use a mobile-phase flow rate of 2 ml/min and a linear gradient, from 30 per cent Solution B in Solution A (5.6) to 100 per cent Solution B over 7 minutes, with a 5-minute hold at 100 per cent Solution B.

For the sample only: increase the flow rate to 6 ml at 100 per cent Solution B for 5 minutes, until non-polar lipids have been eluted (*note 4*).

For both sample and standard: return to 30 per cent Solution B in Solution A over

1 minute at 2 ml per minute and allow to stabilize for about 5 minutes. Run a blank gradient (*note 5*).

6.2.2 Inject, in duplicate, 10 μl of filtrate (6.1.5) on to the column and apply the solvent programme described above (6.2.1), both before and after each sample solution injection. For sample peaks off-scale, quantitatively dilute sample solutions (6.1.5) with 2-propanol/acetonitrile 1:1 (V/V) and make further injections as required. Identify the antioxidant peaks by comparison with the retention times of the chromatographed standards (6.2.3) (*note 6*).

6.2.3 Similarly, inject (also in duplicate) 10-μl antioxidant standard solution (5.7.2) and apply the solvent programme as described (6.2.1).

6.2.4 Carry out a reagent blank determination by following the procedure above (6.1) but substituting 25 ml of hexane for 25 ml of the prepared sample solution. Use the solvent programme as described above (6.2.1) and inject 10 μl of reagent blank solution. Peaks which could interfere with the determination of any antioxidant should not be observed in the resulting chromatogram.

6.2.5 Determine the mean peak height or peak area of each antioxidant obtained from the duplicate sample injections. Determine the mean peak height or peak area of the antioxidant standards from duplicate injections and correct for reagent and gradient blank.

7 Calculation and expression of results

The antioxidant content (A), expressed in mg per kg, is given by the formula:

$$A = \frac{C_{st} \times R_s \times D \times 10}{R_{st} \times m}$$

where:

C_{st} is the concentration of the standard solution (5.7.2)
D is the dilution factor, applied if necessary according to (6.2.2)
R_s is the peak height or area of the sample antioxidant
R_{st} is the peak height or area of the standard antioxidant
m is the mass of the sample in g in the final extract (6.1.5).

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 Examples of suitable commercially available columns are: Lichrosorb RP-18 (Merck) and OCD C-18 (Supelco).

2 Acetic acid may induce interfering signals. It is possible partially to crystallize glacial acetic acid at a temperature related to its water content but below its theoretical melting point of 17°C. Impurities will then accumulate in the liquid phase. If this crystallization does not reduce impurities of the acetic acid to an acceptable level, the amount of acetic acid in the mobile phase solutions A and B (5.6) can be reduced down to 1 per cent provided that the resolution of chromatograms is not affected.

3 At this point the acetonitrile extract (6.1.3) may be stored overnight under refrigeration.
4 Losses of antioxidants, especially of TBHQ, may occur if evaporation time, or time of analysis, is prolonged.
5 If resulting back pressure is excessive, use reduced flow rates and longer rinsing time. Peaks interfering with the determination should not be present. If small peaks are present which cannot be eliminated, all relevant peaks are to be corrected for interference.
6 Octylgallate (OG) may co-elute with Ionox-100. It can be separated with a water–methanol gradient as follows: 30 per cent methanol with 5 per cent acetic acid (Solution C) in Solution B (water with 5 per cent acetic acid) to 100 per cent Solution C over 10 minutes. If both Ionox-100 and OG are present, accurate quantitative analysis may not be possible.

References

Page BD. *J Off Anal Chem* 1979; **62**: 1239–1246.
Page BD. *J Off Anal Chem* 1983; **66**: 727–745.

Annexe

Analytical quality control

1 Repeatability
When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the tables.

2 Reproducibility
When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (R) deduced by linear interpolation from the data in the tables.

3 Results of the interlaboratory test
An interlaboratory test carried out at the international level in 1987 by the IUPAC Commission on Oils, Fats and Derivatives, in which 11 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in Tables 2.642.1–2.642.4.

Table 2.642.1. Sunflower-seed oil.

	Sample reference						
	BHA	BHT	PG	OG	DG	TBHQ	NDGA
Number of laboratories	11	11	11	11	10	10	10
Number of results	41	41	—	—	41	—	—
Number of laboratories retained after elimination of outliers	8	9	—	—	9	—	—
Number of results retained after elimination of outliers	32	36	—	—	36	—	—
Mean value (mg/kg sample)	173.3	64.4	—	—	57.9	—	—
Repeatability standard deviation (S_r)	4.75	5.88	—	—	4.22	—	—
Repeatability relative standard deviation	2.7	9.1	—	—	7.3	—	—
Repeatability limit (r) ($2.83 \times S_r$)	13.44	16.64	—	—	11.94	—	—
Reproducibility standard deviation (S_R)	9.18	7.58	—	—	10.29	—	—
Reproducibility relative standard deviation	5.3	11.8	—	—	7.8	—	—
Reproducibility limit (R) ($2.83 \times S_R$)	25.99	21.44	—	—	29.12	—	—

Table 2.642.2. Groundnut oil (1).

	Sample reference						
	BHA	BHT	PG	OG	DG	TBHQ	NDGA
Number of laboratories	11	11	11	11	10	10	10
Number of results	35	35	35	35	35	35	35
Number of laboratories retained after elimination of outliers	8	7	8	7	7	8	5
Number of results retained after elimination of outliers	32	28	32	28	28	32	20
Mean value (mg/kg sample)	179.0	49.5	85.5	86.9	88.2	78.7	86.3
Repeatability standard deviation (S_r)	9.77	4.49	4.24	0.85	11.19	3.2	0.55
Repeatability relative standard deviation	5.5	8.8	4.9	2.0	8.5	3.9	0.4
Repeatability limit (r) ($2.83 \times S_r$)	27.64	12.41	12.00	2.40	31.67	9.07	1.55
Reproducibility standard deviation (S_R)	17.19	7.98	8.34	3.28	14.88	7.79	12.08
Reproducibility relative standard deviation	9.6	16.1	9.7	7.8	11.20	9.4	9.3
Reproducibility limit (R) ($2.83 \times S_R$)	48.64	22.6	23.61	9.22	42.11	22.04	34.17

Table 2.642.3. Groundnut oil (2).

	Sample reference						
	BHA	BHT	PG	OG	DG	TBHQ	NDGA
Number of laboratories	12	12	—	12	—	12	—
Number of results	45	41	—	45	—	45	—
Number of laboratories retained after elimination of outliers	11	10	—	10	—	10	—
Number of results retained after elimination of outliers	44	40	—	40	—	40	—
Mean value (mg/kg sample)	51.6	146.0	—	103.7	—	105.2	—
Repeatability standard deviation (S_r)	1.71	5.51	—	3.27	—	4.24	—
Repeatability relative standard deviation	3.3	3.8	—	3.1	—	4.0	—
Repeatability limit (r) ($2.83 \times S_r$)	4.83	15.61	—	9.24	—	11.99	—
Reproducibility standard deviation (S_R)	4.79	19.62	—	6.22	—	15.87	—
Reproducibility relative standard deviation	9.3	13.6	—	6.0	—	15.1	—
Reproducibility limit (R) ($2.83 \times S_R$)	13.54	56.37	—	17.61	—	44.92	—

Table 2.642.4. Lard.

	Sample reference						
	BHA	BHT	PG	OG	DG	TBHQ	NDGA
Number of laboratories	—	—	11	11	11	11	11
Number of results	—	—	41	45	41	41	41
Number of laboratories retained after elimination of outliers	—	—	9	10	8	9	9
Number of results retained after elimination of outliers	—	—	36	40	32	36	32
Mean value (mg/kg sample)	—	—	139.2	76.9	189.1	44.5	71.9
Repeatability standard deviation (S_r)	—	—	7.54	1.50	4.14	2.33	3.36
Repeatability relative standard deviation	—	—	5.4	2.0	2.2	5.2	4.7
Repeatability limit (r) ($2.83 \times S_r$)	—	—	21.34	4.23	11.73	6.58	9.50
Reproducibility standard deviation (S_R)	—	—	8.24	2.77	15.21	9.07	10.57
Reproducibility relative standard deviation	—	—	5.9	3.6	8.0	20.4	14.7
Reproducibility limit (R) ($2.83 \times S_R$)	—	—	23.32	7.84	43.05	25.66	29.91

Section 5
Lecithins

Determination of the Chemical Characteristics

5.201 (ex 2.201) Determination of the acidity of lecithins

Use the method 2.201 outlined in *Section 2: Oils and Fats*.

An indication of the precision of this method, when applied to the analysis of lecithins, is given in the tables below. The data presented have been derived from the statistical analysis of results obtained during collaborative studies carried out at an international level in 1984 and 1985.

Table 5.201.1. Interlaboratory study 1984.

	Sample			
	A	B	C	D
Number of laboratories	8	8	8	8
Number of laboratories used*	3	3	—	3
Number of results accepted	6	6	—	6
Mean	23.1	21.8	—	27.7
Repeatability limit (r)	0.53	0.78	—	1.65
Reproducibility limit (R)	3.32	5.84	—	1.84

* Of the eight laboratories submitting results, only four reported duplicates. One set of duplicates was found to be about 10 units for acidity lower when compared with the mean of the other three, and accordingly only three sets of results have been included in the statistical analysis.

Table 5.201.2. Interlaboratory study 1985.

	Sample			
	E	F	G	H
Number of laboratories	12	12	12	12
Number of laboratories used*	8	8	8	8
Number of results accepted	16	16	16	16
Mean	23.3	24.5	23.2	23.8
Repeatability limit (r)	0.49	0.89	0.73	0.50
Reproducibility limit (R)	5.03	3.87	4.17	4.32

* Two laboratories reported single results; two were rejected as outliers.

5.205 (ex 2.205) Determination of the iodine value of lecithins

Use the method 2.205 (Wijs) outlined in *Section 2: Oils and Fats*.

An indication of the precision of this method, when applied to the analysis of lecithins, is given in the tables below. The data presented has been derived from the statistical analysis of results obtained during collaborative studies carried out at an international level in 1984 and 1985.

The method requires the use of carbon tetrachloride which is now considered to have carcinogenic properties. Although cyclohexane with acetic acid has been shown to be a satisfactory replacement for carbon tetrachloride when determining the iodine value of oils and fats, precision data for the analysis of lecithins when using cyclohexane/acetic acid are not yet available.

Table 5.205.1. Interlaboratory study 1984.

	Sample			
	A	B	C	D
Number of laboratories	9	9	9	9
Number of laboratories used*	6	6	—	6
Number of results accepted	12	12	—	12
Mean	96.1	93.6	—	101.7
Repeatability limit (r)	2.33	2.18	—	2.86
Reproducibility limit (R)	4.07	7.04	—	8.23

* Three of the laboratories reported single results for each sample and accordingly these results have been omitted from the statistical analysis.

Table 5.205.2. Interlaboratory study 1985.

	Sample			
	E	F	G	H
Number of laboratories	10	10	10	10
Number of laboratories used*	7	6	6	6
Number of results accepted	14	12	12	12
Mean	95.9	98.0	97.2	98.9
Repeatability limit (r)	2.24	1.03	1.87	1.59
Reproducibility limit (R)	5.18	4.22	4.79	4.21

* Two laboratories reported single results; one laboratory was rejected as an outlier for sample E; and two laboratories were rejected as outliers for samples F, G and H.

Determination of the Principal Constituents

5.301 Determination of phospholipids by thin-layer chromatography and phosphorus analysis

1 Scope

This Standard describes a semi-quantitative procedure for the estimation of the phospholipids content of commercial preparations which may be labelled as 'lecithin'.

2 Fields of application

This Standard is applicable to animal or vegetable lipid preparations containing phospholipids. It is suitable for obtaining an indication of the distribution and amounts of phospholipids on the basis of phosphorus content.

3 Principle

Application of a prepared solution of the phospholipid concentrate to a thin-layer plate followed by two-dimensional thin-layer chromatography. Visualization of the phospholipid spots and determination of the phosphorus content of each spot fraction by molybdate complex formation and its measurement by ultraviolet spectrometry.

4 Apparatus (*note 1*)

4.1 Flasks, volumetric, stoppered, 100, 250 and 500 ml.

4.2 Micropipettes, 40, 80, 120, 160, 200 µl.

4.3 Pipettes, 1, 5, 10 ml.

4.4 Glass beads.

4.5 Thin-layer chromatography plates, 200 × 200 mm, precoated with a 0.25-mm silica gel layer (*note 2*).

4.6 Chromatography tank, glass, suitable for plates (4.5).

4.7 Digestion tubes, glass, 19 mm in diameter, 100 mm in length, and marked at the 15-ml level. A glass marble may be used on the top of each tube.

4.8 Centrifuge to give 5700 r.p.m.

4.9 Centrifuge tubes, plastic, with stoppers, 16–20 ml (*note 3*) suitable for centrifuge (4.8).

4.10 UV spectrometer, suitable for reading at 310 nm.

4.11 Cells, quartz, 10-mm path length, suitable for spectrometer (4.10).

4.12 Block-heater, suitable for digestion tubes (4.7).

4.13 Fume hood, suitable for perchloric acid operations.

5 Reagents

5.1 *n*-Hexane.

5.2 Methanol.

5.3 Ethanol.

5.4 Acetic acid, glacial.

5.5 *n*-Butyl acetate.

5.6 Chloroform.

5.7 Ammonium hydroxide 7 N (*note 4*).

5.8 Iodine crystals.

5.9 Hydrochloric acid, concentrated.

5.10 Nitric acid, concentrated.

5.11 Perchloric acid, 70 per cent.

5.12 Ammonium heptamolybdate $(NH_4)_2Mo_7O_{24}$).

5.13 Potassium dihydrogeno-orthophosphate (KH_2PO_4).

5.14 Molybdate reagent

Weigh 29.0 g of ammonium heptamolybdate (5.12) and transfer quantitatively to a 500-ml flask (4.1). Make up to the mark with re-distilled water and mix. This reagent, which should be aged for at least 24 hours, is stable for several months when stored refrigerated.

5.15 Development solvent A

Mix 130 ml of chloroform (5.6), 60 ml of methanol (5.2) and 8 ml of ammonium hydroxide (5.7).

5.16 Development solvent B

Mix 170 ml of chloroform (5.6), 25 ml of methanol (5.2), 25 ml of acetic acid (5.4) and 6 ml of re-distilled water.

5.17 Inorganic phosphorus, 1 mg P/ml, stock solution

Weigh 1.0982 g of potassium dihydrogeno-orthophosphate (5.13) and transfer quantitatively to a 250-ml flask (4.1). Make up to the mark with re-distilled water and mix.

5.18 Inorganic phosphorus, 50 μg P/ml, working solution

Using a pipette (4.3), transfer 5 ml of the stock solution (5.17) to a 100-ml flask (4.1). Make up to the mark with re-distilled water and mix.

6 Procedure

Caution

Perchloric acid is a potentially hazardous chemical which should be used only in an efficient fume hood (4.13).

6.1 Standard curve

Set up duplicate digestion tubes (3.7) containing 2, 4, 6, 8 and 10 μg of inorganic phosphorus by pipetting with micropipettes (4.2) 40, 80, 120, 160 and 200 μl of working solution (5.18). Set up also in duplicate a digestion tube (4.7) without inorganic phosphorus.

Add two glass beads (4.4) to these tubes. Under the fume hood (4.13) add, using a pipette (4.3), 1 ml of perchloric acid (5.11) and 2–3 drops of nitric acid (5.10). Heat the tubes in a block-heater (4.12), pre-heated to 140°C, for 60 minutes. Allow to cool.

Add re-distilled water to the 15-ml mark. Mix contents. Using a pipette (4.3) transfer 10 ml of the solution to a centrifuge tube (4.9). Using a pipette (4.3), add 1 ml molybdate reagent (5.14). Mix vigorously. Add 5 ml of *n*-butyl acetate (5.5). Mix well. By means of the centrifuge (4.8), regulated at 5700 r.p.m. for about 3 minutes, obtain a clear separation of phases. Transfer the upper phase to a quartz cell (4.11). Using the spectrometer (4.10), measure the absorption at 310 nm.

Construct a calibration curve by plotting recorded absorption values against the corresponding quantity, in μg, of inorganic phosphorus introduced.

6.2 Preparation of sample solution

Weigh accurately an amount of about 500 mg of sample. Transfer quantitatively to a 50-ml flask (4.1). Make up to the mark with *n*-hexane (5.1). Mix.

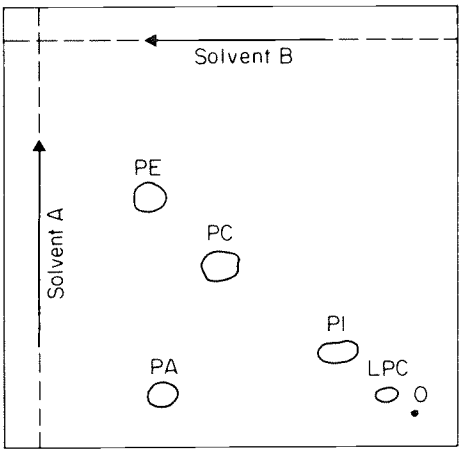


Fig. 5.301.1. Chromatogram showing separation of phospholipids. See footnote to tables for key.

6.3 Thin-layer chromatography

Using a micropipette (4.2), apply 100 μl of sample solution (6.2) to the lower right-hand corner of a TLC plate (4.5), about 20 mm from either edge. Develop the plate in a tank (4.6) containing development solvent A (5.15) for 90 minutes. Allow the plate to dry for about 30 minutes. Turn the plate 90° to the right and develop it in another tank (4.6) containing development solvent B (5.16) for 90 minutes. Remove the plate and allow to dry.

Place the plate in a third tank (4.6) containing iodine crystals (5.8) in order to obtain iodine vapour. Visualize the spots, identifying the individual phospholipids by reference to the chromatogram in Fig. 5.301.1. Outline the spots with a sharp needle or a pencil. Moisten the silica gel with water or ethanol (5.3) to make it pliable, then remove each spot individually, transferring the material to separate digestion tubes (4.7).

6.4 Quantitation of phosphorus

Carry out the determination as indicated in 6.1 standard curve from 'Add two glass beads . . .' to '. . . measure the absorption at 310 nm'.

From the calibration graph, read off the phosphorus content of the sample, expressed in micrograms.

7 Calculation and expression of results

The phosphorus content (P) of the individual phospholipids, expressed as mg/g (or μg/mg) of sample, is given by the formula:

$$P = \frac{p}{m}$$

where:

p is the mass of phosphorus content, in μg, read from the calibration curve

m is the mass of the sample, expressed in mg.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 ## Notes

1 Glassware should not be washed in detergent.
2 Merck Silicagel G or equivalent is suitable.
3 Sorvall No. 00771 is suitable.
4 Concentrated ammonium hydroxide (SG 0.88) is 14.8 M.

References

Dittmer JC, Lester RL. *J Lipid Res* 1964; **5**: 126.
Erdahl WL, Stolyhwo A, Privett OS. *J Am Oil Chem Soc* 1973; **50**: 513.
Lowry RR, Tinsley IJ. *Lipids* 1974; **9**: 491.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *Repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the tables.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (R) deduced by linear interpolation from the data in the tables.

3 Results of the interlaboratory studies

Interlaboratory tests carried out at the international level in 1982, 1983 and 1984 by the IUPAC Commission on Oils, Fats and Derivatives, in which respectively 7, 9 and 14 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following tables.

Table 5.301.1. Collaborative Study 1982.

	Phospholipid*				
	PC	PE	PI	PA	LPC
Number of laboratories	7	7	7	7	—
Number of results	14	14	14	14	—
Number of laboratories retained after eliminating outliers	5	5	4	5	—
Number of accepted results	10	10	8	10	—
Mean (mg/g)	6.1	3.5	2.6	1.2	—
Repeatability limit (r)	1.16	0.82	0.23	0.51	—
Reproducibility limit (R)	3.62	1.90	2.12	1.64	—

Table 5.301.2. Collaborative Study 1983.

	Phospholipid*				
	PC	PE	PI	PA	LPC
Number of laboratories	9	9	9	9	9
Number of results	18	18	18	18	18
Number of laboratories retained after eliminating outliers	9	9	8	8	9
Number of accepted results	18	18	16	16	18
Mean (mg/g)	4.7	3.0	3.0	1.8	1.2
Repeatability limit (r)	0.40	0.54	0.34	0.45	0.82
Reproducibility limit (R)	3.20	2.49	0.88	0.48	2.77

Table 5.301.3. Collaborative Study 1983.

	Phospholipid*				
	PC	PE	PI	PA	LPC
Number of laboratories	14	14	14	14	14
Number of results	28	28	28	28	28
Number of laboratories retained after eliminating outliers	12	13	12	13	12
Number of accepted results	24	26	24	26	24
Mean (mg/g)	5.2	4.3	2.9	1.5	1.0
Repeatability limit (r)	0.45	1.19	0.76	0.51	0.40
Reproducibility limit (R)	2.12	5.63	2.52	1.30	2.55

Values for r and R are expressed as mg/g.

* PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; LPC, lysophosphatidylcholine.

Note: The number of samples provided for the 1982, 1983 and 1984 interlaboratory tests was, respectively, three, four and four. For reasons of space, the statistical data reproduced above have been confined to those derived from the results obtained for one sample only in each of the three interlaboratory tests. In view of the precision achieved by the participating laboratories the method can only be viewed as semi-quantitative.

5.302 Determination of the phospholipid profile of lecithins by high performance liquid chromatography

1 Scope

This standard describes a method for the quantitative determination of individual phospholipids in commercial soyabean lecithin preparations.

2 Field of application

The method allows the determination of phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylcholine (PC) in commercial soyabean lecithin preparations.

3 Principle

Separation of the individual phospholipids by direct high performance liquid chromatography (HPLC) and their detection by ultraviolet light at 206 nm. Quantitation using external standards.

4 Apparatus

4.1 High performance liquid chromatograph, consisting of an HPLC pump and an injection valve with 10-μl sample loop.

4.2 UV detector system to measure absorbance at 206 nm.

4.3 *Column:* stainless steel tube, 200 mm in length and internal diameter of about 4.6 mm, packed with microparticulate spherical silica (5 μm) (*note 1*).

4.4 Recorder and/or integrator.

4.5 Syringe, 25 μl, graduated in 1 μl.

4.6 Flasks, volumetric, 10, 500 and 1000 ml.

5 Reagents

5.1 *n*-Hexane, HPLC grade.

5.2 2-Propanol, HPLC grade.

5.3 Water, double distilled, filtered through a 0.45 μm pore size membrane filter.

5.4 Sodium acetate, 0.2 M. Weigh 27.22 g of sodium acetate ($3H_2O$), analytical grade, into a 1000-ml flask (4.6) and make up to volume with water. Mix.

5.5 Acetic acid, 0.2 M. Weigh 6.006 g of glacial acetic acid, analytical grade, into a 500-ml flask (4.6) and make up to volume with water. Mix.

5.6 Acetate buffer (Walpole), pH 4.2. Prepare by mixing 2.65 ml of sodium acetate solution (5.4) and 7.35 ml of acetic acid solution (5.5).

5.7 HPLC mobile phase. Prepare by mixing *n*-hexane (5.1), 2-propanol (5.2) and acetate buffer (5.6) in the proportion 8:8:1 (V/V).

5.8 *Reference phospholipids:* phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylcholine (PC).

6 Procedure

6.1 Stabilization of the column

To achieve a stable operating performance of the column it must be charged with a suitable quantity of buffer. This is carried out by passing the mobile phase (5.7) through the column for at least 3 days at a flow rate of 0.2 ml/min until a stable baseline is obtained. A stable baseline will result in the retention times of peaks also being stable.

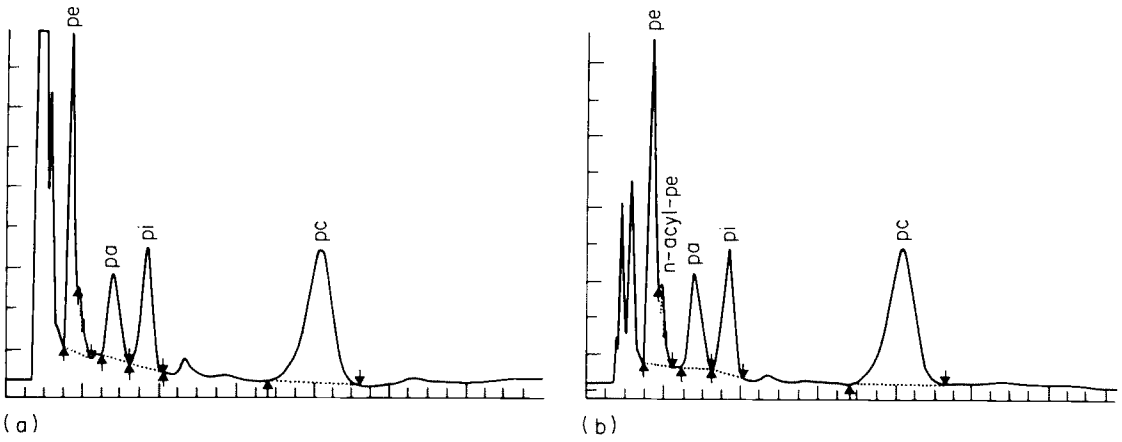


Fig. 5.302.1. Chromatograms of soyabean phospholipids. (a) Crude soyabean lecithin. (b) De-oiled soyabean lecithin.

6.2 ## HPLC chromatography

Use the mobile phase (5.7) at a flow rate of 2 ml/min.

6.3 ## Calibration curve

Accurately weigh 10, 20, 30 and 40 mg quantities of each reference phospholipid (5.8) into separate 10-ml flasks (4.6). Dissolve the phospholipid in about 3 ml of mobile phase (5.7), make up to volume with the same solvent, and mix well (*note 2*).

Construct a calibration curve for each phospholipid by injecting exactly 10 μl of each of the calibration solutions, recording the peak areas, and plotting the latter against the corresponding quantity of phospholipid.

6.4 ## Sample analysis

Weigh accurately 50 to 250 mg of the lecithin sample into a small beaker and dissolve in about 3 ml of the mobile phase (5.7). Transfer quantitatively to a 10-ml flask (4.6), make up to volume with the same solvent, and mix well. In the case of some lecithins, a sediment may form after some time. Inject 10 μl of the upper layer. The sediment has no influence on the analysis but can affect the column's performance (*note 3*).

Record the individual peak areas of the eluted phospholipids, identifying them from the chromatogram (Fig. 5.302.1) or by comparison with the retention times observed during the chromatography (6.3) of the reference phospholipids (5.8).

7 # Calculation and expression of results

7.1 Read off from the calibration curves the amount of phospholipids in the injected volume of the solution of the lecithin sample (6.4).

7.2 The phospholipid content (P), expressed as a percentage (m/m), is given by the formula:

$$P = \frac{A \times 100}{m}$$

where:

A is the amount of phospholipid, in mg, read from the calibration curve (7.1)

m is the mass of sample weight, in mg (6.4).

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 Example of a suitable commercially available column: Nucleosil 50-5 (Macherey-Nagel).

2 An exposure of a few minutes in an ultrasonic water bath of the standard and sample solutions may facilitate complete homogenization of phospholipids and lecithins, respectively.

3 A deterioration in the performance of the column is indicated by a poor separation of the phosphatidylethanolamine and *N*-acylphosphatidylethanolamine peaks. The column should be refilled with fresh support to ensure good reproducibility of chromatograms. After completion of analyses, the pump/head system should be rinsed, first with acetone, then with water, and again with water at a very low flow rate.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the tables.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the tables below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (R) deduced by linear interpolation from the data in the tables.

3 Results of the interlaboratory test

An interlaboratory test carried out at the international level in 1988 by the IUPAC Commission on Oils, Fats and Derivatives, in which 11 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following tables.

Table 5.302.1. Phosphatidic acid.

	Sample					
	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	11	10	10	10	11
Mean value (g/100 g sample)	12.58	11.68	4.68	12.18	12.09	7.18
Repeatability standard deviation (S_r)	0.27	0.17	0.21	0.28	0.27	0.17
Repeatability relative standard deviation	2.14	1.45	4.58	2.34	2.23	2.37
Repeatability limit (r) ($2.83 \times S_r$)	0.74	0.48	0.59	0.79	0.74	0.48
Reproducibility standard deviation (S_R)	1.55	1.12	0.96	2.51	2.54	0.61
Reproducibility relative standard deviation	12.28	9.56	20.61	20.63	20.01	8.46
Reproducibility limit (R) ($2.83 \times S_R$)	4.39	3.16	2.72	7.10	7.19	1.73

Table 5.302.2. Phosphatidylethanolamine.

	Sample					
	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	10	11	10	10	11
Mean value (g/100 g sample)	14.04	15.80	10.24	9.45	9.49	11.18
Repeatability standard deviation (S_r)	0.17	0.15	0.23	0.09	0.09	0.17
Repeatability relative standard deviation	1.18	0.98	2.29	0.98	1.00	1.54
Repeatability limit (r) ($2.83 \times S_r$)	0.48	0.42	0.65	0.25	0.25	0.48
Reproducibility standard deviation (S_R)	0.74	1.10	0.88	0.77	0.59	1.19
Reproducibility relative standard deviation	5.29	6.98	8.55	8.10	6.18	10.65
Reproducibility limit (R) ($2.83 \times S_R$)	2.09	3.11	2.49	2.18	1.67	3.37

Table 5.302.3. Phosphatidylcholine.

	Sample					
	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	11	11	10	10	10	11
Mean value (g/100 g sample)	17.24	19.01	14.44	9.68	9.33	14.74
Repeatability standard deviation (S_r)	0.20	0.49	0.35	0.13	0.41	0.40
Repeatability relative standard deviation	1.19	2.60	2.44	1.40	4.35	2.68
Repeatability limit (r) ($2.83 \times S_r$)	0.57	1.39	0.99	0.37	1.16	1.13
Reproducibility standard deviation (S_R)	0.97	1.12	0.86	0.22	0.66	0.83
Reproducibility relative standard deviation	5.61	5.89	5.95	2.25	7.04	5.66
Reproducibility limit (R) ($2.83 \times S_R$)	4.39	3.16	2.72	7.10	7.19	1.73

Table 5.302.4. Phosphatidylinositol.

	Sample					
	1	2	3	4	5	6
Number of laboratories	11	11	11	11	11	11
Number of laboratories retained after elimination of outliers	9	10	9	9	10	8
Mean value (g/100 g sample)	11.60	12.43	7.67	7.84	8.86	9.16
Repeatability standard deviation (S_r)	0.49	0.52	0.27	0.32	0.56	0.19
Repeatability relative standard deviation	4.20	4.22	3.52	4.08	6.28	2.11
Repeatability limit (r) ($2.83 \times S_r$)	1.39	1.47	0.76	0.91	1.58	0.54
Reproducibility standard deviation (S_R)	1.07	1.47	1.55	0.85	3.45	0.63
Reproducibility relative standard deviation	9.20	11.80	20.18	10.88	38.89	6.89
Reproducibility limit (R) ($2.83 \times S_R$)	3.03	4.16	4.39	2.41	9.76	1.78

Determination of other Natural Constituents

5.421 (ex 2.421) Determination of the phosphorus content of lecithins

Use the method 2.421 outlined in *Section 2: Oils and Fats*.

The precision of this method, when applied to the analysis of lecithins, has not been determined by interlaboratory study.

Determination of the Quality and the Stability

5.501 (ex 2.501) Determination of the peroxide value

Use the method 2.501 outlined in *Section 2: Oils and Fats*.

An indication of the precision of this method, when applied to the analysis of lecithins, is given in the tables below. The data presented have been derived from the statistical analysis of results obtained during collaborative studies carried out at an international level in 1984 and 1985.

It should be noted that the use of chloroform (required for the method) is now discouraged in analytical laboratories. However, at present there are no precision data available for the peroxide value determination when applied to the analysis of lecithins and using a more acceptable solvent.

Table 5.501.1. Interlaboratory study 1984.

	Sample			
	A	B	C	D
Number of laboratories	10	10	10	10
Number of laboratories used*	6	—	6	6
Number of results accepted	12	—	12	12
Mean (mEq/kg)	2.69	—	0.59	1.86
Repeatability limit (r)	1.10	—	0.18	0.24
Reproducibility limit (R)	5.67	—	1.54	4.68

* Four laboratories reported only single results for the analysis of each sample and accordingly these results have not been included in the statistical analysis.

Table 5.501.2. Interlaboratory study 1985.

	Sample			
	E	F	G	H
Number of laboratories	12	12	12	12
Number of laboratories used*	9	8	9	9
Number of results accepted	18	16	18	18
Mean (mEq/kg)	4.13	7.67	6.56	9.25
Repeatability limit (r)	1.18	1.32	3.30	2.97
Reproducibility limit (R)	4.23	4.66	7.39	9.87

* Two laboratories reported single results; two laboratories were rejected as outliers for sample F; one laboratory was rejected as an outlier for samples E, G and H.

The comparatively poor reproducibility (in both studies) is possibly explained by a change in the peroxide value during transit of the samples.

5.502 Determination of the acetone-insoluble material in lecithins

1 Scope

This Standard describes a method for the determination of material in commercial lecithin preparations which is not soluble in acetone (phospholipids).

2 Field of application

This Standard is especially applicable to commercial lecithins.

3 Principle

Treatment of the sample with acetone to dissolve oil, water and free fatty acids, whilst leaving phospholipids undissolved. Evaporation of the filtered acetone and weighing of the residue. Acetone-insoluble material is defined as the difference between the weight of the sample and the weight of the residue plus weight of the water in the sample.

4 Apparatus

4.1 Ice-water bath.

4.2 Rotary film evaporator.

4.3 Water bath at temperature of 80°C.

5 Reagent

5.1 Acetone, analytical reagent quality.

6 Procedure

6.1 Determine the water content of the sample according to method 2.603: Determination of small quantities of water (modified Karl Fischer method).

6.2 Weigh 5 ± 0.01 g of sample into a 100-ml beaker. Cool the sample in the ice-water bath (4.1) and add 20-ml acetone (5.1) cooled to 0°C. Break up the sample into small particles using a glass rod and allow the particles of lecithin to settle.

6.3 Transfer the supernatant solution through a filter paper to a tared flask. Repeat the extraction five times. Check for complete extraction by placing a drop of the last acetone extract on a filter paper: if a greasy stain appears, repeat the extraction as necessary.

6.4 Finally, wash the filter paper with acetone and collect the acetone in the flask. Connect the flask to the evaporator (4.2), keeping the flask immersed in the water bath (4.3) maintained at 80°C, and allow the acetone to evaporate until constant weight of the flask is achieved.

7 Calculation and expression of results

7.1 The acetone-soluble matter (S), expressed as a percentage (m/m) of the sample, is given by the formula:

$$S = \frac{A \times 100}{m}$$

where:

A is the mass, in g, of the residue in the flask after evaporation

m is the mass, in g, of the test portion.

7.2 The acetone-insoluble matter (IS), expressed as a percentage (m/m) of the sample, is given by the formula:

$$IS = 100 - \left(\frac{A \times 100}{m} + \%\ \text{water}\right)$$

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the table.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (R) deduced by linear interpolation from the data in the table.

3 Results of the interlaboratory test

An interlaboratory test carried out at the international level in 1986 by the IUPAC Commission on Oils, Fats and Derivatives, in which seven laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following table.

Table 5.502.1.

	Sample							
	85/11	85/12	85/13	85/14	85/15	85/16	85/17	85/18
Number of laboratories	7	7	7	7	7	7	7	7
Number of results	14	14	14	14	14	14	14	14
Number of laboratories retained after elimination of outliers	6	6	7	7	7	7	7	7
Number of results retained after elimination of outliers	12	12	14	14	14	14	14	14
Mean value (g/100 g sample)	35.81	36.42	65.83	65.77	66.68	97.37	97.14	96.84
Repeatability standard deviation (S_r)	0.42	0.17	0.51	0.39	0.79	0.13	0.09	0.09
Repeatability relative standard deviation	1.16	0.46	0.78	0.59	1.18	0.13	0.25	0.26
Repeatability limit (r) ($2.83 \times S_r$)	1.18	0.47	1.45	1.10	2.21	0.36	0.09	0.10
Reproducibility standard deviation (S_R)	2.43	2.56	2.09	2.01	2.03	0.60	0.60	0.94
Reproducibility relative standard deviation	6.80	7.03	3.18	3.06	3.04	0.61	0.62	0.97
Reproducibility limit (R) ($2.83 \times S_R$)	6.89	7.24	5.93	5.69	5.74	1.69	1.70	2.66

Determination of the Foreign Substances and the Additives

5.603 (ex 2.603) Determination of small quantities of water by modified Karl Fischer method

Use the method 2.603 outlined in *Section 2: Oils and Fats*.

An indication of the precision of this method, when applied to the analysis of lecithins, is given in the tables below. The data presented have been derived from the statistical analysis of results obtained during collaborative studies carried out at an international level in 1984 and 1985.

Table 5.603.1. Interlaboratory study 1984.

	Sample			
	A	B	C	D
Number of laboratories	8	8	8	8
Number of laboratories used*	3	—	3	3
Number of results accepted	6	—	6	6
Mean	0.62	—	0.54	0.48
Repeatability limit (r)	0.05	—	0.20	0.07
Reproducibility limit (R)	0.15	—	0.21	0.28

* Five of the laboratories submitted single results for each sample. Accordingly these results have not been included in the statistical analysis.

Table 5.603.2. Interlaboratory study 1985.

	Sample			
	E	F	G	H
Number of laboratories	10	10	10	10
Number of laboratories used*	7	7	8	7
Number of results accepted	14	14	16	14
Mean	0.41	0.40	0.49	0.46
Repeatability limit (r)	0.03	0.07	0.07	0.06
Reproducibility limit (R)	0.41	0.23	0.22	0.14

* Two laboratories submitted single results; one laboratory was rejected as an outlier for samples E, F and H.

5.604 Determination of the toluene-insoluble material in lecithins

1 Scope

This Standard describes a method for the determination of material in commercial lecithin preparations which is not soluble in toluene. Such extraneous material is commonly referred to as 'dirt'.

2 Field of application

This Standard is applicable to commercial lecithins containing toluene-insoluble matter not exceeding 0.3 per cent (*note 1*).

3 Principle

Dissolution of the sample in toluene followed by filtration. Drying and weighing of the residue retained by the filter.

4 Apparatus

4.1 Filter, sintered glass, porosity 16–40 μm (ISO designation P40; other designations are G3 or D3), capacity 30–50 ml (*note 2*).

4.2 Water pump and low pressure filtration unit suitable for the filter (4.1).

4.3 Oven, for operation at 105°C.

5 Reagent

5.1 Toluene, analytical reagent quality.

6 Procedure

Weigh 10 ± 0.01 g of well-mixed sample (*note 3*) into a 250-ml flask. Add 100 ml of toluene (5.1) and stir well until maximum solution of the sample is achieved.

Filter (4.2) the solution through a tared, cleaned and dried filter (*note 4*), and wash the filter with two 25-ml portions of toluene (5.1).

Dry the filter in the oven (4.3) for 60 minutes. Allow to cool in a desiccator and weigh (*note 5*).

7 Calculation and expression of results

7.1 The toluene-insoluble matter (T), expressed as a percentage (m/m) of the sample, is given by the formula:

$$T = \frac{A \times 100}{m}$$

where:

A is the mass, in g, of the dried residue on the filter

m is the mass, in g, of the test portion.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9

Notes

1 For samples containing greater than 0.3 per cent toluene-insoluble matter the precision (repeatability) of the method has been found to be unsatisfactory.

2 It is essential to check that the porosity of the filter is within specification before use. It is recommended that this check is carried out by the procedure described in the Annexe to *ISO 4793-1980*.

3 If required, the sample may be heated to a maximum of 60°C to facilitate mixing.

4 The filter must be thoroughly cleaned using potassium dichromate/sulphuric acid, then rinsed thoroughly with distilled or deionized water, and finally dried at 105°C.

5 After repeated use, the porosity of the filter may eventually exceed that of the specified value (for checking the porosity see *note 2*) and it will then be necessary to replace the filter with a new one.

Annexe

Analytical quality control

1 Repeatability

When the mean value of two single test results obtained under *repeatability conditions* (conditions where independent test results are obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time) lies within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the repeatability limit (r) deduced by linear interpolation from the data in the table.

2 Reproducibility

When the values of two single test results obtained under *reproducibility conditions* (conditions where test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment) lie within the range of the values shown in the table below, the absolute difference between the two test results obtained should not be greater than the reproducibility limit (r) deduced by linear interpolation from the data in the table.

3 Results of the interlaboratory test

An interlaboratory test carried out at the international level in 1985 by the IUPAC Commission on Oils, Fats and Derivatives, in which 13 laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725-1986*) summarized in the following table.

Table 5.604.1

	Sample			
	A	B	C	D
Number of laboratories	13	13	13	13
Number of results	26	26	26	26
Number of laboratories retained after eliminating outliers	13	13	13	12
Number of accepted results	26	26	26	24
Mean (% m/m)	0.12	0.07	0.33	0.28
Repeatability standard deviation*	0.014	0.012	0.029	0.025
Repeatability coefficient of variation† (%)	11.9	19.1	8.8	13.7
Repeatability limit (r)*	0.04	0.035	0.08	0.07
Reproducibility standard deviation*	0.056	0.048	0.084	0.079
Reproducibility coefficient of variation† (%)	47.4	73.3	25.6	43.3
Reproducibility limit (R)*	0.16	0.14	0.24	0.23

* Expressed as m/m.
† relative standard deviation.

Section 6
Emulsifiers

6.001 Determination of the ester-emulsifiers components after hydrolysis and silylation by gas chromatography

1 Scope

This Standard describes a method for the determination by gas–liquid chromatography of ester-emulsifiers components after hydrolysis (saponification) and silylation.

By this procedure the components determined are:

Acids, or their sodium and potassium salts: fatty acids, hydroxy fatty acids, dicarboxylic acids (lactic, tartaric, citric acid), glycerol phosphoric acids and inorganic acids (phosphoric, sulphuric acid), and *alcohols and polyalcohols*: alcohols (fatty alcohols), glycols, polyglycols, glycerols, polyglycerols, sorbitol and its anhydrides, carbohydrates (mono- and disaccharides).

2 Field of application

This Standard is applicable to hydrolysis products of ester-emulsifiers concentrates and to emulsifiers separated from oils and fats (*note 1*).

The components of the following classes of emulsifiers may be determined:

- esters of fatty acids with hydroxy carboxylic acids (e.g. sodium stearoyl-2-lactylate),
- esters of fatty alcohols with hydroxy carboxylic acids (e.g. sodium stearylcitrate),
- fatty acid esters of mono- and polyglycols (e.g. 1,2-propylene glycol mono- and diesters of fatty acids, polyoxyethylene-(8)-stearate),
- glycerol esters of fatty acids (mono- and diglycerides, monoglycerides esterified with acetic, citric, tartaric, and lactic acid, esters of polyglycerol),
- sorbitan esters of fatty acids,
- sugar esters of fatty acids,
- lecithin.

3 Definition

The contents of a component of an emulsifier is a quantity determined by the present method and expressed as a percentage by mass relative to the sample.

4 Principle

Hydrolysis (saponification) of the emulsifiers with alcoholic potassium hydroxide solution (*note 1*). Evaporation of the alcohol. Silylation of the components. Identification and determination of the trimethylsilyl derivatives by gas–liquid chromatography with packed or capillary column.

5 Apparatus

5.1 Screw cap vials, 2 ml, with conical bottom.

5.2 Screw caps suitable for vials (5.1) with inert plastic-faced septa (*notes 2, 3*).

5.3 Volumetric flasks, 10 ml.

5.4 Pipettes, 0.1, 0.2, 0.5 ml.

5.5 Microsyringes, 1–5 μl.

5.6 Gas–liquid chromatograph with flame-ionization detector, temperature programming and integrator.

5.7 Packed column, metal or glass, suitable for chromatograph (5.6), about 2–3 m in length and 2–4 mm internal diameter, filled with either methylpolysiloxane, phenylmethylpolysiloxane or methylphenylvinylpolysiloxane (*note 4*) on an acid-washed, silylated, fully deactivated diatomaceous earth (*notes 5, 6*), or

5.8 Capillary column, glass or fused silica, fully deactivated, suitable for chromatograph

(5.6), about 15–25 m in length and 0.2–0.4 mm inside diameter. Film thickness 0.1–0.2 μm. Coating: methylpolysiloxane, phenylmethylpolysiloxane or methylphenylpolysiloxane (*notes 4, 7*).

5.9 Heating device for vials (5.1).

5.10 Needles for blowing a gentle stream of nitrogen into the vials (5.1).

6 Reagents

6.1 Tetradecane, pure.

6.2 Pyridine, pure, kept over potassium hydroxide (*note 8*).

6.3 Trimethylchlorosilane (TMCS).

6.4 Bis(trimethylsilyl)trifluoroacetamide (BSTFA).

6.5 Potassium hydroxide, 0.5 N ethanolic solution.

6.6 Tetradecane in pyridine, internal standard solution.

Accurately weigh about 100 mg of tetradecane (6.1) into a volumetric flask (5.3). Dilute to volume with pyridine (6.2).

6.7 Reference solutions with internal standard.

Accurately weigh about 100 mg of reference substance(s) (alcohols, polyalcohols, fatty acids, hydroxycarboxylic acids, or their sodium or potassium salts) into volumetric flasks (5.3). Add about 100 mg of accurately weighed tetradecane (6.1). Dilute to volume with pyridine (6.2). Several substances (e.g. fatty acids) may be present in a reference solution.

6.8 Carrier gas: helium, dry, free from impurities.

6.9 Auxiliary gases: hydrogen, free from organic impurities, air or oxygen.

6.10 Nitrogen.

7 Procedure

7.1 Hydrolysis (saponification) of sample solutions

Accurately weigh about 10 mg of the homogenized sample into a vial (5.1).

By means of a pipette (5.4) add 0.25 ml of the ethanolic potassium hydroxide solution (6.5) (*note 9*). Put the reaction vial into a heating device (5.9) at a temperature of 70°C. The hydrolysis is completed after about 3 hours. By means of a needle (5.10) evaporate carefully all the ethanol in a stream of nitrogen (6.10).

7.2 Silylation of sample solutions

By means of a pipette (5.4) or syringe add to the dried hydrolysed product 0.1 ml of internal standard in pyridine (6.6), 0.2 ml of BSTFA (6.4) and 0.1 ml of TMCS (6.3) (*note 10*). Humidity must be strictly excluded. Close the vial. Shake vigorously. If necessary, destroy skin formed and remove substances from the glass wall with a small glass rod which is broken off after use. Heat the reaction mixture for about 20 minutes at 70°C.

7.3 Chromatography of sample solutions

The supernatant liquor of the silyl derivatives should be injected soon after the derivatization process. Carry out two determinations, each consisting of duplicate injections of the test solutions, and record the areas of the peaks.

The operating conditions with gas chromatograph (5.6) and columns (5.7 and 5.8) should be the following:

Injector temperature:	310°C
Detector temperature:	310°C
Column temperature:	initial 80°C programme rate 5–10°C/min final 275°C
With packed column:	flow rate of carrier gas 30 ml/min
With capillary column:	flow rate of carrier gas: 1–3 ml/min (measured at 80°C)
Injection by means of a microsyringe (5.5):	1–5 µl (*note 11*).

7.4 Identification

Identify peaks by comparison of retention time of known substances in reference solution (6.7) after silylation and chromatography under the same conditions as the sample or apply coupled gas chromatography and mass spectrometry.

7.5 Silylation and chromatography of reference solution

By means of a pipette (5.4) transfer 0.1 ml of the reference solution (6.7) into a vial (5.1).

With a pipette (5.4) add 0.2 ml of BSTFA (6.4) and 0.1 ml of TMCS (6.3) (*note 10*).

Humidity must strictly be excluded. Close the vial. Shake vigorously.

If necessary, remove substances from the glass wall. Heat the reaction mixture for about 20 minutes at 70°C.

The supernatant liquor of the silylated reference solution should be injected soon after the derivatization process.

Operate under the same conditions as for the sample solution (7.3). Use duplicate injections and concentration ranges of reference substances as to be quantified in sample. Check response factors periodically.

8 Calculation and expression of results

8.1 Response factor

Calculate response factor of the reference substance versus internal standard using the reference solution chromatogram. The value of the response factor is given by the formula:

$$R_x = (m_{is}/m_x) \times (A_x/A_{is})$$

where:

R_x is the response factor of reference substance x

m_{is} is the mass, in mg, of internal standard

m_x is the mass, in mg, of reference substance x

A_x is the peak area of reference substance x

A_{is} is the peak area of internal standard.

8.2 Calculation of sample component content

Calculate the percentage of mass content of component x in the sample by the formula:

$$m'_x(\%) = 1/R_x \times (m'_{is}/m'_s) \times (A'_x/A'_{is}) \times 100$$

where:
m'_x is the percentage of mass of component x in sample
R_x is the response factor of component x in sample
m'_{is} is the mass, in mg, of internal standard in sample
m'_s is the mass, in mg, of sample
A'_x is the peak area of the component x in sample
A'_{is} is the peak area of the internal standard in sample.

9 **Notes**

1 If emulsifiers are present in oils and fats, the polar emulsifiers are first separated from the triglycerides by method 2.507 'Determination of polar compounds in frying fats', or by method 2.321 'Determination of mono-, di- and triglycerides by column chromatography'. The triglycerides are removed with five 60-ml portions of benzene and the emulsifiers are eluted with five 60-ml portions of dichloromethane/methanol (2/1, V/V) solvents. Finally, phospholipids are eluted with three 60-ml portions of methanol. It is recommended that the triglycerides extracted from the column are also hydrolysed and silylated in order to recognize the difference between the polar and apolar fractions.

2 Screw cap vials with magnetic stirrer, or ultrasound may be applied.

3 Teflon-faced septa are suitable.

4 Silicones OV-1, OV-17, SE-52, SE-54 are suitable.

5 Chromosorbs G, AW-DMCS is suitable.

6 The stationary phase has to be fully deactivated using an excess of silylating agents, otherwise adsorption of trimethylsilyl fatty acid derivatives occurs. The deactivation can be tested with a mixture of hydrocarbons such as hexadecane (C_{16}), eicosane (C_{20}), tetracosane (C_{24}), octacosane (C_{28}) and silylated fatty acids such as decanoic acid (C_{10}), tetradecanoic acid (C_{14}), octadecanoic acid (C_{18}) and docosanoic acid (C_{22}). The peak of the corresponding hydrocarbons and TMS-esters of fatty acids should have about the same height (Donike test). Injection of silylating agents may lead to the required deactivation of the columns.
Higher fatty acids and unsaturated fatty acids may show greater discrimination. Faster heating rates or higher initial temperatures may reduce these effects. Fatty acid methyl esters are to be preferred.

7 Capillary columns are preferred, as the $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ fatty acids are well separated and can be quantified.

8 Instead of pyridine solvents such as dimethylformamide, acetonitrile, tetrahydrofurane, hexane or mixtures of them may be used.

9 The amount of ethanolic potassium hydroxide solution specified is enough to hydrolyse (saponify) an acidic diacetyl tartaric acid glyceride with a saponification value of 490. For better dissolution of some emulsifiers, an addition of 0.1 ml of water is advantageous.

10 Salts of hydroxylic acids such as lactic, tartaric and citric acids are better and more completely silylated with the BSFTA + TMCS combination than with hexamethyldisilazane (HDMS) and TMCS.

11 For on-column injection, or direct injection, dilute 50 µl of reaction mixture (7.2; 7.5) with 1 ml of hexane and, by means of a microsyringe (5.5), inject supernatant (1 µl).

In order to lengthen the life-time of the column, applying on-column injections, a pre-column is useful.

Warning

Pyridine

The danger from non-purified pyridine is greater than from the pure reagent, the associated homologues and impurities being generally more toxic than pyridine itself. Odour and irritation furnish clear warning of a vapour concentration likely to be dangerous.

Appendix

In some cases, names commonly used in emulsifier terminology do not conform with IUPAC recommendations. The following table lists IUPAC equivalents for some names used in the present document.

Table 6.001.1.

	IUPAC name
Behenic acid	Docosanoic acid
Stearoyl-2-lactylate	2-*O*-(2-*O*-stearoyllactoyl)lactate
Capric acid	Decanoic acid
Diacetyl tartaric acid	2,3-Di-*O*-acetyltartaric acid
Polyoxyethylene(20)sorbitan	Mixture of ethoxylated glucitol and anhydroglucitols (molar ratio glucitol + anhydroglucitols/oxirane = 1/20)
Polyoxyethylene(8)stearate	Mixture of ethoxylated stearic acid (molar ratio stearic acid/oxirane = 1/8)
Propyleneglycol	Propane-1,2-diol
Ricinoleic acid	12-Hydroxy-*cis*-octadec-9-enoic acid
Sorbitan	Mixture of glucitol, 1,4-, 2,5-, 3,6-anhydroglucitol, and dianhydroglucitols

References

Brüschweiler H, Hautfenne A. *Pure Appl Chem* 1990; **62**(4): 781–793 (Report on the Collaborative Study).
Brüschweiler H, Felber H, Schwager F. *Fat Sci Technol* 1989; **91**(2): 73–79.
Buxtorf UP, Brüschweiler H, Martin E. *Z Lebensm Unters Forschung* 1982; **175**: 25–28.
IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th edn.* Oxford: Blackwell Scientific Publications, 1987.
Sahasrabudhe MR. *J Am Oil Chem Soc* 1967; **44**: 376–378.
Sahasrabudhe MR. *J Am Oil Chem Soc* 1969; **46**: 8–12.
Schütze I. *Die Nahrung* 1977; **21**: 405–415.
Tsuda T, Nakanishi H. *J Ass Off Anal Chem* 1983; **66**: 1050–1052.
Tsuda T, Nakanishi H, Kobayashi S, Morita T. *J Ass Off Anal Chem* 1984; **67**: 1149–1151.

6.002 (ex 2.326) Determination of mono- and diglycerides by capillary gas chromatography

1 Scope

This Standard describes a method for the determination of mono- and diglycerides.

2 Field of application

The Standard is applicable to mono- and diglyceride concentrates and mono- and diglycerides in fats and oils.

3 Principle

Conversion of mono- and diglycerides with *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) in pyridine into more volatile trimethylsilyl ether derivatives and quantitative determination by capillary gas chromatography using an internal standard (*n*-tetradecane).

4 Apparatus

4.1 Gas chromatograph, with split injection or on-column injection (*note 1*), oven temperature programming and flame-ionization detector. Recorder and integrator.

4.2 Column, capillary, glass or fused silica, surface fully deactivated by silylation agent (*note 2*), 15–25 m in length, 0.25–0.35 mm internal diameter, coating SE-54 (or phase with similar polarity), film thickness 0.1–0.2 μm.

4.3 Operating conditions, split injection (split ratio 1:10–1:50); direct injection (splitless, hold for 1 minute); temperatures: injection port 320°C, or on-column injection 60°C, column initial 80°C (or 60°C on-column), programme 10°C/min, final 360°C, hold for 15 minutes, detector 350°C, carrier gas flow 5 ml helium min (at 80°C), injection volume 1–5 μl. An automatic sampler is advantageous.

4.4 Screw cap vials (2.5 ml) or crimp-top vials for autosampler (e.g. 2.0 ml), with Teflon-faced septa.

4.5 Heating device for vials, 70°C.

5 Reagents

5.1 *N*,*N*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

5.2 Trimethylchlorosilane (TMCS).

5.3 Pyridine, analytical grade, kept over potassium hydroxide.

5.4 *n*-Tetradecane, analytical grade (internal standard).

5.5 *n*-Hexane, analytical grade.

5.6 Reference standards: glycerol, palmitic acid, 1-*O*-palmitoylglycerol, 1-*O*-stearoylglycerol, 1,2-di-*O*-palmitoylglycerol, 1,3-di-*O*-palmitoylglycerol, 1,2-di-*O*-stearoylglycerol.

5.7 Internal standard solution: accurately weigh about 100 mg of *n*-tetradecane (5.4) into a 10-ml volumetric flask and dilute to volume with pyridine (5.3).

5.8 Reference solution: accurately weigh about 100 mg of reference standard (e.g. glycerol, fatty acid, mono- and di-*O*-acetylglycerol) and accurately weigh about 100 mg of *n*-tetradecane into a 10-ml volumetric flask. Dilute to volume with pyridine. Alternatively, weigh about 100 mg of a mixture containing several (e.g. five) reference standards and *n*-tetradecane, each component being present in about the same quantities, into a 2-ml volumetric flask and dilute to volume with pyridine.

6 Procedure

6.1 Sample solution

Accurately weigh about 10 mg of homogenized sample of emulsifier concentrates or 50 mg of oils and fats containing emulsifiers into a 2.5-ml screw-cap vial with Teflon-faced septa. Add 0.1 ml of internal standard solution (5.7) containing 1 mg of *n*-tetradecane, 0.2 ml of BSTFA and 0.1 ml of TMCS to the sample (*note 3*).

Humidity is strictly excluded. Close vial and shake vigorously. Heat the reaction mixture in heating device at 70°C for about 20 minutes. Inject 1–5 μl of the reaction mixture into the gas chromatograph, showing a stable baseline (*note 1*).

Avoid delay of gas chromatograph analysis. The reaction is carried out twice, and duplicate injections are made per reaction.

6.2 Reference solution

Transfer 0.10 ml of reference solution (5.8) to a vial and add the silylating agents, 0.2 ml of BSTFA and 0.1 ml of TMCS (no internal standard solution is added), and inject as described above (*notes 1*, *3*).

Use a concentration range of reference standards similar to that of the substances to be quantified in sample solution. A plot of response factor versus concentration of reference standards may be useful to check linearity.

Check response factors periodically. Response factors should be above about 0.5. Lower response factors indicate some loss or decomposition. Use concentration range of 0.5–10 mg/ml of components in reference and sample solutions.

6.3 Identification

Analyse the reference solution under the same operating conditions as the sample solution. Identify peaks by comparison of retention time with known substances or apply coupled GC/MS (gas liquid chromatography/mass spectrometry).

7 Calculation and expression of results

7.1 Response factor

Calculate the response factors of the reference substances versus internal standard using the reference standard chromatogram. The value of the response factor is given by the formula:

$$R_x = (m_{is}/m_x) \times (A_x/A_{is})$$

where:

R_x is the response factor of reference standard x

m_{is} is the mass, in mg, of internal standard

m_x is the mass, in mg, of reference standard x

A_x is the peak area of reference substance x

A_{is} is the peak area of internal standard.

7.2 Calculation of sample component content

Calculate the percentage of mass content of component x in the sample by the formula:

$$m'_x\ (\%) = 1/R_x \times (m'_{is}/m'_s) \times (A'_x/A'_{is}) \times 100$$

where:
m'_x is the percentage (m/m) of mass of component x in the sample
R_x is the response factor of component x in the sample
m'_{is} is the mass, in mg, of internal standard in the sample
m'_s is the mass, in mg, of the sample
A'_x is the peak area of the component x in the sample
A'_{is} is the peak area of the internal standard in the sample.

8 Quality assurance

8.1 For *general principles* of analytical quality control, see the section on *Quality Assurance* in the introductory part of the *Compendium of Standard Methods*.

8.2 For *specific applications* of analytical quality control, see the Annexe to this standard method.

9 Notes

1 For on-column injection, or direct injection, dilute 50 μl of reaction mixture (6.1; 6.2) with 1 ml of hexane and inject (1 μl).
In order to lengthen the life-time of the columns when applying on-column injections, a pre-column is useful. On-column injection gives better response factors.
2 Use a length of column required to separate 1,2- from 1,3-diglycerides.
3 For automatic samplers with 2-ml crimp-top vials, it is convenient to double the amount of sample and reagents.
4 Beside mono- and diglycerides, other components such as glycerol, fatty acids, sterols, etc. can be analysed by the same method.

Annexe

Analytical quality control

1 Repeatability
When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in the tables below, the difference between the two values obtained should not be greater than the repeatability limit (r), which can generally be deduced by linear interpolation from the values in the tables below.

2 Reproducibility
When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions from the analysis of the same laboratory sample, lie within the range of mean values cited in the tables below, the difference between the values for the final result obtained by those operators should not be greater than the reproducibility limit (R), which can generally be deduced by linear interpolation from the values in the tables below.

3 Results of the interlaboratory test

An interlaboratory test carried out at an international level in 1985 by the IUPAC Commission on Oils, Fats and Derivatives, in which eight laboratories participated, each obtaining two test results for each sample, gave the statistical results (evaluated in accordance with *ISO 5725–1986*) summarized in the following tables for mono- and diglyceride concentrates (Table 6.002.1) and for mono- and diglycerides in oils (Table 6.002.2).

Table 6.002.1. Statistical analysis of results for mono- and diglyceride concentrates (expressed as percentage of mass of sample).

	1-Myristate	1-Palmitate	1-Stearate	1,3-Dipalmitate	1-Palmitate-3-stearate	1,3-Distearate
Number of laboratories	8	8	8	8	8	8
Number of results	16	16	16	16	16	16
Number of laboratories retained after elimination of outliers	7	8	8	7	7	6
Number of accepted results	14	16	16	14	14	16
Mean value (g/100g sample)	1.7	27.2	60.1	0.2	0.8	1.1
Repeatability standard deviation (S_r)	0.05	0.9	2.1	0.01	0.05	0.07
Repeatability relative standard deviation	3.0	3.3	3.5	4.6	6.0	6.8
Repeatability limit (r) ($2.85 \times S_r$)	0.14	2.61	5.91	0.31	0.14	0.20
Reproducibility standard deviation (S_R)	0.1	2.4	6.4	0.06	0.1	0.3
Reproducibility relative standard deviation	5.7	8.9	10.7	30.0	17.8	24.8
Reproducibility limit (R) ($2.83 \times S_R$)	0.3	6.8	18.1	0.2	0.4	0.8

Table 6.002.2. Statistical analysis of results for mono- and diglycerides in oil (expressed as percentage of mass of sample).

	1-Palmitate	1-Stearate	1,2-Dipalmitate	1,3-Dipalmitate	1,2-Distearate
Number of laboratories	8	8	8	8	8
Number of results	16	16	16	16	16
Number of laboratories retained after elimination of outliers	8	8	8	8	8
Number of accepted results	16	16	16	16	16
Mean value (g/100g sample)	0.96	0.98	0.97	0.93	0.97
Repeatability standard deviation (S_r)	0.03	0.03	0.04	0.02	0.06
Repeatability relative standard deviation	3.3	3.4	4.0	2.5	6.2
Repeatability limit (r) ($2.83 \times S_r$)	0.08	0.08	0.11	0.06	0.17
Reproducibility standard deviation (S_R)	0.12	0.14	0.24	0.19	0.19
Reproducibility relative standard deviation	12.0	13.8	24.4	20.2	19.8
Reproducibility limit (R) ($2.83 \times S_R$)	0.34	0.40	0.68	0.54	0.54

References

Brüschweiler H, Dieffenbacher A. *Pure Appl Chem* 1991; **63**(8): 1153–1163 (Report on the Collaborative Study).

D'Alonzo RP, Kozarek WJ, Wharton HW. *J Am Oil Chem Soc* 1981; **58**: 215–227.

Goh EM, Tims RE. *J Am Oil Chem Soc* 1985; **62**: 730–734.

Hammond EW. *J Chromatogr* 1981; **203**: 397–402.

ISO 5725. *Precision of test methods. Determination of repeatability and reproducibility by inter-laboratory tests.* 1986.

IUPAC. *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th edn. Oxford: Blackwell Scientific Publications, 1986.

Prevot A, Coustille J-L. *Rev Franc Corps Gras* 1982; **29**: 17–24.

Riva M, Daghetta A, Galli M. *Rivist Ital Sostanze Grasse* 1981; **58**: 432–443.

Shasrabudhe MR. *J Am Oil Chem Soc* 1968; **45**: 379–380.

Shasrabudhe MR. *J Am Oil Chem Soc* 1969; **46**: 8–12.

Soe JB. *Fette Seifen Anstrichmittel* 1983; **85**: 72–76.

Vaver VA, Ushakov AN. *Methods Biol Anal* 1980; **26**: 327–406.