⟨561⟩ ARTICLES OF BOTANICAL ORIGIN

SAMPLING

In order to reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled or more samples per container.

Gross Sample

Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each one as a homogeneous batch. It is recommended to include samples from the first, middle, and last containers where the *No. of Containers in Batch* (*N*) is 11 or more and each container in the batch is numbered or lettered in order.

No. of Containers in Batch (*N*)	No. of Containers to Be Sampled (*n*)
1–10	All
11–19	11
>19	*n* = 10 + (*N*/10)

(Round calculated "*n*" to next highest whole number.)

Samples are taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts that are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, not less than two cores being taken from different angles. For materials with component parts over 1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

For articles in containers holding less than 1 kg, mix the contents, and withdraw a quantity sufficient for the tests. For articles in containers holding between 1 and 5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing not less than 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

Laboratory Sample

Prepare the laboratory sample by repeated quartering of the gross sample.

NOTE—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.

The laboratory sample should be of a size sufficient for performing all the necessary tests.

Test Sample

Unless otherwise directed in the individual monograph or test procedure below, prepare the test sample as follows.

Decrease the size of the laboratory sample by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer, and withdraw the portion for analysis.

METHODS OF ANALYSIS

Foreign Organic Matter

Test Sample—Unless otherwise specified in the individual monograph, weigh the following quantities of the laboratory sample, taking care that the withdrawn portion is representative (quartering if necessary).

Roots, rhizomes, bark, and herbs	500 g
Leaves, flowers, seeds, and fruit	250 g
Cut vegetable drugs (average weight of the pieces is less than 0.5 g)	50 g

Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

Total Ash

Accurately weigh a quantity of the *Test Sample*, representing 2–4 g of the air-dried material, in a tared crucible, and inciner-ate, gently at first, and gradually increase the temperature to $675 \pm 25°$, until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of $675 \pm 25°$. If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 mL of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of $675 \pm 25°$. Cool in a desiccator, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

Acid-Insoluble Ash

Boil the ash obtained as directed in *Total Ash* with 25 mL of 3 N hydrochloric acid for 5 min, collect the insoluble matter on a tared filtering crucible or ashless filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

Water-Soluble Ash

Boil the ash obtained as directed in *Total Ash* with 25 mL of water for 5 min. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper. Wash with hot water, and ignite for 15 min at a temperature not exceeding 450°. Sub-tract the weight of this residue, in mg, obtained in *Total Ash*, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined in *Total Ash*.

Alcohol-Soluble Extractives

Method 1 (hot extraction method)—Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, and weigh the flask. Shake, and allow to stand for 1 h. Attach a reflux condenser to the flask, boil gently for 1 h, cool, and weigh. Readjust to the original weight with alcohol. Shake, and filter rap-idly through a dry filter. Transfer 25 mL of the filtrate to a tared flat-bottomed dish, and evaporate on a water bath to dryness. Dry at 105° for 6 h, cool in a desiccator for 30 min, and weigh without delay. Calculate the content, in mg/g, of alcohol-extractable matter in the test specimen.

Method 2 (cold extraction method)—Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, insert a stopper into the flask, and macerate for 24 h, shaking frequently during the first 8 h, and then allowing to stand. Filter rapidly, taking precautions against loss of alcohol. Evaporate 25 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish, and dry at 105° to constant weight. Calculate the content, in mg/g, of alcohol-extractable matter in the test specimen.

Water-Soluble Extractives

Method 1 (hot extraction method)—Proceed as directed in *Method 1* (hot extraction method) in *Alcohol-Soluble Extractives*, except use water in place of alcohol.

Method 2 (cold extraction method)—Proceed as directed in *Method 2* (cold extraction method) in *Alcohol-Soluble Extrac-tives*, except use water in place of alcohol.

Crude Fiber

Exhaust a weighed quantity of the *Test Sample*, representing about 2 g of the drug, with ether. Add 200 mL of boiling dilute sulfuric acid (1 in 78) to the ether-exhausted marc, in a 500-mL flask, and connect the flask to a reflux condenser. Reflux the mixture for 30 min, accurately timed, then pass through a linen or hardened-paper filter, and wash the residue on the filter with boiling water until the effluent washing is no longer acid. Rinse the residue back into the flask with 200 mL of boiling sodium hydroxide solution, adjusted to 1.25% by titration and free from sodium carbonate. Again reflux the mixture for 30 min, accurately timed, then rapidly pass through a tared filter, wash the residue with boiling water until the last washing is neutral, and dry it at 110° to constant weight. Incinerate the dried residue, ignite to constant weight, cool in a desiccator, and weigh the ash: the difference between the weight obtained by drying at 110° and that of the ash represents the weight of the crude fiber.

NOTE—The boiling with acid and alkali should continue for 30 min, accurately timed, from the time that the liquid (which is cooled below the boiling point by being added to the cold flask) again boils. After the solution has been brought to boiling, the heat should be turned low enough just to maintain boiling. During the boiling, the flask should be gently rotated from time to time to wash down any particles that may adhere to the walls of the flask. A slow current of air introduced into the flask during the boiling operation aids in preventing excessive frothing.

Starch Content

Method 1—The following is a general procedure for all reducing sugars and may be used to determine the starch content in botanical articles.

Malt Extract—Use clean new barley malt of known efficacy, and grind just before use. Prepare malt extract just prior to use. For every 80 mL of malt extract needed, digest 5 g of ground malt with 100 mL of water at room temperature for 2 h. [NOTE— If an electric mixer is used, stir the mixture for 20 min.] Filter to obtain a clear extract, filtering again, if necessary, and mix the infusion well.

Test Solution—Extract about 5 g of the finely ground test specimen with five 10-mL portions of ether, using a filter that will completely retain the smallest starch granule. Allow the ether to evaporate from the residue, and wash with 250 mL of aqueous alcohol solution (10 in 100). Carefully wash the residue from the paper into a 500-mL beaker with about 100 mL of water. Heat to about 60° (avoiding, if possible, gelatinizing starch), and allow to stand for about 1 h, stirring frequently to effect complete solution of sugars. Transfer to a wide-mouth bottle, rinse the beaker with a little warm water, and cool. Add an equal volume of alcohol, mix, and allow to stand for NLT 1 h.

Centrifuge until the precipitate is closely packed on the bottom of the bottle, and decant the supernatant. Wash the precipitate with successive 50-mL portions of alcohol solution (50 in 100) by centrifuging and decanting through a suitable filter until the washings are sugar-free. [NOTE—To test for the presence of sugar, transfer a few drops of the washings to a test tube, add 3 or 4 drops of a 20% solution of 1-naphthol in alcohol, prepared by dissolving 200 mg of 1-naphthol in 1 mL of alcohol and 2 mL of water. Shake the test tube well to allow uniform mixing, allow 2–4 mL of sulfuric acid to flow down the sides of the test tube, and hold the test tube upright. If sugar is present, the interface of the two liquids is colored faint to deep violet, and on shaking, the whole solution becomes blue-violet.]

Transfer the residue from the bottle and hardened filter to a beaker with about 50 mL of water. Immerse the beaker in boiling water, and stir constantly for 15 min or until all of the starch is gelatinized. Cool the beaker to 55°, add 20 mL of *Malt Extract*, and hold at this temperature for 1 h. Heat again to boiling for a few min, cool to 55°, add 20 mL of *Malt Extract*, and hold at this temperature for 1 h or until the residue when treated with iodine TS shows no blue tinge upon microscopic examination. Cool, dilute with water to 250 mL, and filter.

General Procedure—Transfer 200 mL of the *Test Solution* to a flask fitted with a reflux condenser, add 20 mL of hydrochloric acid, and heat in a boiling water bath for $2^1/_2$ h. Cool, nearly neutralize with sodium hydroxide TS, complete neutralization with sodium carbonate TS, dilute with water to 500 mL, mix, and filter. The volume of aliquot taken depends on the starch content of the specimen under test (see *Table 1*). The aliquot should contain between 100 and 200 mg of dextrose. Transfer 50 mL of the filtrate to a 400-mL alkali-resistant glass beaker, add 50 mL of alkaline cupric tartrate TS, cover the beaker with a water glass, and heat. Adjust the flame in the burner so that the contents of the flask begin to boil in 4 min, and continue boiling for exactly 2 min. Filter the hot solution at once through a sintered-glass filter. Wash the precipitate of cuprous oxide thoroughly with water at about 60°, then with 10 mL of alcohol, and finally with 10 mL of ether.

Table 1. Determination of the Optimum Aliquot

Expected Starch Content (%)	Aliquot (mL)
60	25
50	35
40	50
30	50
20	50

For solutions of reducing sugars of comparatively high purity, proceed as directed in *Method 1A* to determine the amount of reduced copper obtained by weighing the dried cuprous oxide. For solutions of reducing sugars containing large amounts of organic impurities, including sucrose, proceed as directed in *Method 1B* to determine the amount of reduced copper obtained by titration with sodium thiosulfate.

METHOD 1A—Dry the precipitate obtained in *General Procedure* for 30 min in an oven at $110 \pm 2°$, cool to room temperature in a desiccator, and weigh. Refer to *Table 2* to find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide found. Determine the percentage of dextrose and then the content of starch by the following formula:

Percentage of dextrose = (wt. of dextrose in mg × 0.1 × 500)/(wt. of sample in g × aliquot in mL)

Content of starch = % dextrose × 0.9

Table 2. Calculating Dextrose (Applicable when Cu$_2$O is weighed directly) (Expressed in mg)

Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu$_2$O)	Dextrose (D-Glucose)
10	4.0	90	38.9	170	75.1	250	112.8	330	152.2	410	193.7
12	4.9	92	39.8	172	76.0	252	113.7	332	153.2	412	194.7
14	5.7	94	40.6	174	76.9	254	114.7	334	154.2	414	195.8
16	6.6	96	41.5	176	77.8	256	115.7	336	155.2	416	196.8
18	7.5	98	42.4	178	78.8	258	116.6	338	156.3	418	197.9
20	8.3	100	43.3	180	79.7	260	117.6	340	157.3	420	199.0
22	9.2	102	44.2	182	80.6	262	118.6	342	158.3	422	200.1
24	10.0	104	45.1	184	81.5	264	119.5	344	159.3	424	201.1
26	10.9	106	46.0	186	82.5	266	120.5	346	160.3	426	202.2
28	11.8	108	46.9	188	83.4	268	121.5	348	161.4	428	203.3
30	12.6	110	47.8	190	84.3	270	122.5	350	162.4	430	204.4
32	13.5	112	48.7	192	85.3	272	123.4	352	163.4	432	205.5
34	14.3	114	49.6	194	86.2	274	124.4	354	164.4	434	206.5
36	15.2	116	50.5	196	87.1	276	125.4	356	165.4	436	207.6
38	16.1	118	51.4	198	88.1	278	126.4	358	166.5	438	208.7
40	16.9	120	52.3	200	89.0	280	127.3	360	167.5	440	209.8
42	17.8	122	53.2	202	89.9	282	128.3	362	168.5	442	210.9
44	18.7	124	54.1	204	90.9	284	129.3	364	169.6	444	212.0
46	19.6	126	55.0	206	91.8	286	130.3	366	170.6	446	213.1
48	20.4	128	55.9	208	92.8	288	131.3	368	171.6	448	214.1
50	21.3	130	56.8	210	93.7	290	132.3	370	172.7	450	215.2
52	22.2	132	57.7	212	94.6	292	133.2	372	173.7	452	216.3
54	23.0	134	58.6	214	95.6	294	134.2	374	174.7	454	217.4
56	23.9	136	59.5	216	96.5	296	135.2	376	175.8	456	218.5
58	24.8	138	60.4	218	97.5	298	136.2	378	176.8	458	219.6
60	25.6	140	61.3	220	98.4	300	137.2	380	177.9	460	220.7
62	26.5	142	62.2	222	99.4	302	138.2	382	178.9	462	221.8
64	27.4	144	63.1	224	100.3	304	139.2	384	180.0	464	222.9
66	28.3	146	64.0	226	101.3	306	140.2	386	181.0	466	224.0
68	29.2	148	65.0	228	102.2	308	141.2	388	182.0	468	225.1
70	30.0	150	65.9	230	103.2	310	142.2	390	183.1	470	226.2
72	30.9	152	66.8	232	104.1	312	143.2	392	184.1	472	227.4
74	31.8	154	67.7	234	105.1	314	144.2	394	185.2	474	228.3
76	32.7	156	68.6	236	106.0	316	145.2	396	186.2	476	229.6

Table 2. Calculating Dextrose (Applicable when Cu₂O is weighed directly) (Expressed in mg) *(Continued)*

Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)	Cuprous Oxide (Cu₂O)	Dextrose (D-Glucose)
78	33.6	158	69.5	238	107.0	318	146.2	398	187.3	478	230.7
80	34.4	160	70.4	240	108.0	320	147.2	400	188.4	480	231.8
82	35.3	162	71.4	242	108.9	322	148.2	402	189.4	482	232.9
84	36.2	164	72.3	244	109.9	324	149.2	404	190.5	484	234.1
86	37.1	166	73.2	246	110.8	326	150.2	406	191.5	486	235.2
88	38.0	168	74.1	248	111.8	328	151.2	408	192.6	488	236.3

METHOD 1B—

Sodium Thiosulfate Solution—Transfer 3.9 g of sodium thiosulfate, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Potassium Iodide Solution—Dissolve 42 g of potassium iodide in 100 mL of water.

Sodium Acetate Solution—Dissolve 5.74 g of sodium acetate in 10 mL of water.

Copper Solution—Transfer about 0.3 g of pure electrolytic copper, accurately weighed, to a 250-mL flask, add 5 mL of nitric acid to dissolve the copper, add about 25 mL of water, and boil to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, add 10 mL of *Sodium Acetate Solution* followed by 10 mL of *Potassium Iodide Solution*, and titrate with *Sodium Thiosulfate Solution* to a light yellow color. Add enough starch TS to produce a marked blue color, and continue the titration. As the endpoint nears, add 2 g of potassium thiocyanate, and stir until completely dissolved. Continue titration until the precipitate is completely white. One mL of sodium thiosulfate solution is equivalent to about 10 mg of copper. [NOTE—It is essential that the concentration of *Potassium Iodide Solution* be carefully regulated. If the solution contains less than 320 mg of copper at the completion of titration, add 4.2–5 g of potassium iodide to make a total solution of 100 mL. If greater amounts of Cu are present, add *Potassium Iodide Solution* slowly, with constant agitation, from the buret in amounts proportionately greater.]

Procedure—Wash the precipitated cuprous oxide obtained in *General Procedure* with water, cover this filter with a watch glass, and dissolve the cuprous oxide with 5 mL of nitric acid directed under the watch glass with a pipet. Collect the filtrate in a 250-mL flask, wash the watch glass, and the filter with water. Collect all the washings in the flask. Boil the contents of the flask to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, and proceed as directed in *Copper Solution* beginning with "add 10 mL of *Sodium Acetate Solution*." From the volume of *Sodium Thiosulfate Solution* consumed, obtain the weight of copper, in mg, and multiply the weight of copper by 1.1259 to obtain the weight, in mg, of cuprous oxide. From *Table 2*, find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide. The content of starch is equivalent to the weight, in mg, of dextrose obtained times 0.9. Conduct a blank determination, using 50 mL of alkaline cupric tartrate TS and 50 mL of *Malt Extract*. If the weight of the cuprous oxide so obtained exceeds 0.5 mg, correct the result of the determination accordingly. [NOTE—The alkaline cupric tartrate TS deteriorates on standing, and the quantity of cuprous oxide obtained in the blank determination increases.]

Method 2—The following method is specific for dextrose (glucose), and because of its extreme sensitivity it may account for differences noted between values obtained from the same specimen. Duplicate determinations do not vary more than 2%.

Glucoamylase Solution—Prepare a solution of glucoamylase in water containing 30 International Units (IU)/mL. Use glucoamylase obtained preferably from *Rhizopus delemar*. The total glucoamylase activity of the test specimen being used should be NLT 150 IU.

Acetate Buffer Solution—Dissolve 16.4 g of sodium acetate in 100 mL of water, add 12.0 mL of glacial acetic acid, and mix. The pH of this solution is 4.8.

Phosphate Buffer—Dissolve 3.63 g of tris (hydroxymethyl) aminomethane and 5.0 g of monobasic sodium phosphate in 50.0 mL of water. At 37°, adjust with phosphoric acid to a pH of 7.0, dilute with water to 100.0 mL, and mix. [NOTE—The pH of the buffer medium is sensitive to temperature and should be adjusted to the desired pH at the temperature to be used during incubation.]

Enzyme Solution—Dissolve 30 mg of glucose oxidase (Type II from *Aspergillus niger*), 3 mg of peroxidase (Type I from horseradish), and 10 mg of potassium ferrocyanide in 100 mL of *Phosphate Buffer*. [NOTE—This mixture can be stored in a refrigerator for up to 10 days.]

18 N Sulfuric Acid—Add slowly, while stirring, 54 mL of sulfuric acid to 102 mL of water, allow to cool to 25°, and mix.

Standard Solutions—Dissolve an accurately weighed quantity of USP Dextrose RS in water to obtain a solution containing 1.0 mg of USP Dextrose RS per mL. Quantitatively dilute a known volume of this solution with water to obtain *Standard Solutions A, B, C, D,* and *E,* having known concentrations of 10, 20, 25, 40, and 50 μg/mL of USP Dextrose RS, respectively. [NOTE—Allow 4 h for complete mutarotation before use.]

Test Solutions—Extract about 5 g of finely ground test specimen with five 25-mL portions of 80% alcohol, and filter. Remove all the alcohol from the residue by drying in an air oven at 105° for about 8 h. [NOTE 1—Any traces of alcohol remaining in the

residue will inhibit glucoamylase.] Cool, and transfer the flask containing the dried test specimen to a desiccator. Transfer about 1 g, accurately weighed, of the test specimen to a previously tared flask, add 25 mL of water, and adjust with phosphoric acid to a pH of 5.0–7.0, if necessary. Boil the suspension for about 3 min, transfer the flask to an autoclave, and heat to 135° for 2 h. Remove the flask from the autoclave, maintain the temperature near 55°, and add 2.5 mL of *Acetate Buffer Solution* and sufficient water to adjust the total weight of the solution to 45 ± 1 g. Immerse the flask in a water bath maintained at 55 ± 1°, and add 5 mL of *Glucoamylase Solution*. Continuously swirl the flask for 2 h to effect hydrolysis, pass through filter paper into a 250-mL volumetric flask, wash quantitatively with water, and collect all the washings in the flask. Dilute the contents of the flask with water to volume, and mix. Transfer 1 mL of an aliquot containing 20–60 μg of D-glucose to each of five test tubes. [NOTE 2—In order to obtain the range of concentration of glucose in the hydrolysate, quantitatively dilute, if necessary, with water to volume.] Add 2 mL of *Enzyme Solution* to each of the five test tubes, and place the test tubes in the dark at 37 ± 1° for exactly 30 min to develop the color. At the end of 30 min, add 2 mL of *18 N Sulfuric Acid* to each of the test tubes to stop the reaction, and mix.

   Control Solution—Transfer an accurately weighed quantity of about 0.4 g of starch to a previously tared flask, and proceed as directed in *Test Solutions* beginning with "add 25 mL of water, and adjust the pH with phosphoric acid."
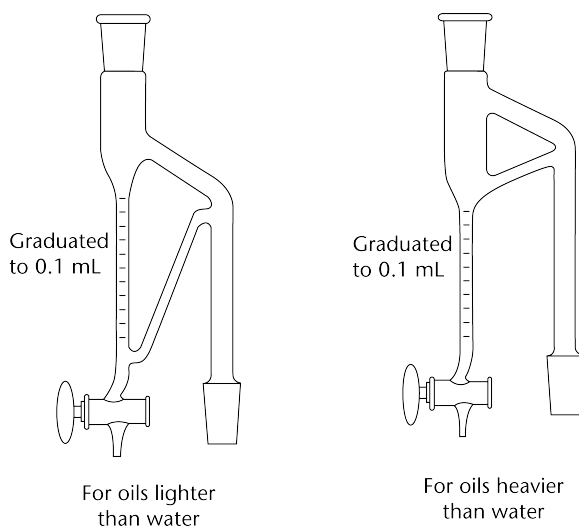
   Procedure—Concomitantly determine the absorbances of the *Standard Solutions* and the *Test Solutions* at the wavelength of maximum absorbance at about 540 nm, with a suitable spectrophotometer, using the *Control Solution* as the blank to set the instrument. Plot the absorbance values of the *Standard Solutions* versus concentration, in μg/mL, of dextrose, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in μg/mL, of dextrose in each of the *Test Solutions*, calculate the average concentration, in μg/mL, of the solution under test. The percentage of starch content in the weight of the test specimen taken is calculated by the formula:

$$(0.9C/10^6)(V_1)(250/V_0)(100/E)(100/W) = 2.25CV_1/V_0EW$$

in which E is the weight, in g, of the test specimen taken; V_0 is the volume, in mL, of the aliquot taken from the 250-mL volumetric flask; W is the percentage of dry weight of the test specimen; and V_1 is the volume, in mL, if extra dilution is done (see *Note 2* in *Test Solutions*). [NOTE—V_0 is 1.0 when no extra dilution is done.]

Volatile Oil Determination

   Set up a round-bottom, shortneck, 1-L flask in a heating mantle set over a magnetic stirrer. Insert an egg-shaped stirring bar magnet in the flask, and attach a cold-finger condenser and an appropriate volatile oil trap of the type illustrated.

Graduated to 0.1 mL

Graduated to 0.1 mL

For oils lighter than water

For oils heavier than water

Traps for Volatile Oil Apparatus

   Coarsely comminute a sufficient quantity of the drug to yield from 1 to 3 mL of volatile oil. Small seeds, fruits, or broken leaves of herbs ordinarily do not need comminution. Very fine powders are to be avoided. If this is not possible, it may be necessary to mix them with purified sawdust or purified sand. Place a suitable quantity of the drug, accurately weighed, in the flask, and fill it one-half with water. Attach the condenser and the proper separator. Boil the contents of the flask, using a suitable amount of heat to maintain gentle boiling for 2 h, or until the volatile oil has been completely separated from the drug and no longer collects in the graduated tube of the separator.

   If a proper quantity of the volatile oil has been obtained in the graduated tube of the separator, it can be read to tenths of 1 mL, and the volume of volatile oil from each 100 g of drug can be calculated from the weight of the drug taken. The graduations on the separator "for oils heavier than water" are so placed that oil remains below the aqueous condensate that automatically flows back into the flask.

Water Content

For unground or unpowdered drugs, prepare about 10 g of the *Laboratory Sample* by cutting, granulating, or shredding, so that the parts are about 3 mm in thickness. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care that no appreciable amount of moisture is lost during the preparation and that the portion taken is representative of the *Laboratory Sample*. Determine the water content as directed for *Procedure for Articles of Botanical Origin* in *Water Determination* ⟨921⟩, *Method III* (*Gravimetric*).

TEST FOR AFLATOXINS

[*Caution—Aflatoxins are highly dangerous, and extreme care should be exercised in handling aflatoxin materials.*]

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for aflatoxin B_1 (AFB_1) and NMT 20 ppb for the sum of aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2). The extent of testing may be determined using a risk-based approach that considers the likelihood of contamination. The presence of unexpected contamination with aflatoxins is to be considered in determining compliance. The following analytical procedures are provided for determining compliance. Unless otherwise specified in the individual monograph, use *Method I*. If system suitability fails, use either *Method II* or *Method III*.

Method I

This TLC test is provided to detect the possible presence of AFB_1, AFB_2, AFG_1, and AFG_2 in any material of plant origin.

Zinc Acetate–Aluminum Chloride Reagent—Dissolve 20 g of zinc acetate and 5 g of aluminum chloride in sufficient water to make 100 mL.

Sodium Chloride Solution—Dissolve 5 g of sodium chloride in 50 mL of water.

Test Solution 1—Grind about 200 g of plant material to a fine powder. Transfer about 50 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for NLT 30 min, and filter. [NOTE—If the solution has interfering plant pigments, proceed as directed for *Test Solution 2*.] Discard the first 50 mL of the filtrate, and collect the next 40-mL portion. Transfer the filtrate to a separatory funnel. Add 40 mL of *Sodium Chloride Solution* and 25 mL of solvent hexane, and shake for 1 min. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 25 mL of methylene chloride, by shaking for 1 min. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 125-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup Procedure* in *Test Solution 2*. Otherwise, dissolve the residue obtained above in 0.2 mL of a mixture of chloroform and acetonitrile (9.8: 0.2), and shake by mechanical means if necessary.

Test Solution 2—Collect 100 mL of the filtrate from the start of the flow, and transfer to a 250-mL beaker. Add 20 mL of *Zinc Acetate–Aluminum Chloride Reagent* and 80 mL of water. Stir, and allow to stand for 5 min. Add 5 g of a suitable filtering aid, such as diatomaceous earth, mix, and filter. Discard the first 50 mL of the filtrate, and collect the next 80-mL portion. Proceed as directed for *Test Solution 1*, beginning with "Transfer the filtrate to a separatory funnel."

Cleanup Procedure—Place a medium-porosity sintered-glass disk or a glass wool plug at the bottom of a 10-mm × 300-mm chromatographic tube. Prepare a slurry of 2 g of silica gel with a mixture of ethyl ether and solvent hexane (3:1), pour the slurry into the column, and wash with 5 mL of the same solvent mixture. Allow the absorbent to settle, and add to the top of the column a layer of 1.5 g of anhydrous sodium sulfate. Dissolve the residue obtained above in 3 mL of methylene chloride, and transfer it to the column. Rinse the flask twice with 1-mL portions of methylene chloride, transfer the rinses to the column, and elute at a rate not greater than 1 mL/min. Add successively to the column 3 mL of solvent hexane, 3 mL of ethyl ether, and 3 mL of methylene chloride; elute at a rate not greater than 3 mL/min; and discard the eluates. Add to the column 6 mL of a mixture of methylene chloride and acetone (9:1), and elute at a rate not greater than 1 mL/min, preferably without the aid of vacuum. Collect this eluate in a small vial, add a boiling chip if necessary, and evaporate to dryness on a water bath. Dissolve the residue in 0.2 mL of a mixture of chloroform and acetonitrile (9.8: 0.2), and shake by mechanical means if necessary.

Test Solution 3—If interferences still exist in the residue, proceed as directed for *Cleanup Procedure with IAC* in *Test Solution* in *Method II*.

Aflatoxin Solution—[*Caution—Aflatoxins are highly toxic. Handle with care.*] Dilute the USP Aflatoxins RS 1:5 with acetonitrile to obtain a solution having a concentration of 0.4 μg/mL each of AFB_1 and AFG_1, and 0.1 μg/mL each of AFB_2 and AFG_2.

Procedure—Separately apply 2.5, 5, 7.5, and 10 μL of the *Aflatoxin Solution* and three 10-μL applications of either *Test Solution 1*, *Test Solution 2*, or *Test Solution 3* to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩) coated with a 0.25-mm layer of chromatographic silica gel mixture. Superimpose 5 μL of the *Aflatoxin Solution* on one of the three 10-μL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and isopropyl alcohol (85:10:5) until the solvent front

has moved NLT 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.

System Suitability—The four applications of the *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of the *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*.

Acceptance Criteria—No spot from any of the other applications of the *Test Solution* corresponds to any of the spots obtained from the applications of the *Aflatoxin Solution*. If any spot of aflatoxins is obtained in the *Test Solution*, match the position of each fluorescent spot of the *Test Solution* with those of the *Aflatoxin Solution* to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the *Test Solution*, when compared with that of the corresponding aflatoxin in the *Aflatoxin Solution* will give an approximate concentration of aflatoxin in the *Test Solution*. Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for AFB_1 and NMT 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2, except when otherwise indicated.

Method II

Sodium Chloride Solution—See *Method I*.

Phosphate Buffered Saline Solution—Prepare 10 mM phosphate buffer solution containing 0.138 M sodium chloride and 0.0027 M potassium chloride in water, and adjust with 2 M sodium hydroxide to a pH of 7.4.[1]

Immunoaffinity Column (IAC)—Prior to conditioning, adjust the IAC to room temperature. For conditioning, apply 10 mL of *Phosphate Buffered Saline Solution* onto the column and let it flow through the column by gravity force at a rate of 2–3 mL/min. Leave 0.5 mL of the *Phosphate Buffered Saline Solution* on top of the column until the *Test Solution* is applied.

Test Solution—

Sample Extraction—Transfer about 5 g of a representative powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for NLT 30 min, and filter. Discard the first 5 mL of the filtrate, and collect the next 4-mL portion. Transfer the filtrate to a separatory funnel. Add 4 mL of *Sodium Chloride Solution* and 2.5 mL of hexane, and shake for 1 min. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 2.5 mL of methylene chloride, by shaking for 1 min. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 50-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup Procedure with IAC*. Otherwise, dissolve the residue obtained above in 200 µL of acetonitrile, and shake by mechanical means if necessary.

Cleanup Procedure with IAC—The residue is dissolved in 5 mL of a mixture of methanol and water (60:40) and then diluted with 5 mL of water. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 mL of *Phosphate Buffered Saline Solution*, and the elution is performed slowly with 2 mL of methanol. Evaporate the eluate with nitrogen, and dissolve the residue in 200 µL of acetonitrile.

Aflatoxin Solution—[*Caution—Aflatoxins are highly toxic. Handle with care.*] Dilute quantitatively the USP Aflatoxins RS 1:50 with acetonitrile to obtain a solution containing 0.04 µg/mL each of AFB_1 and AFG_1, and 0.01 µg/mL each of AFB_2 and AFG_2.

Analysis—Separately apply 5, 7.5, and 10 µL of *Aflatoxin Solution* and three 10-µL applications of the *Test Solution* to a suitable HPTLC plate (see *Chromatography* ⟨621⟩) coated with a 200-µm layer of chromatographic silica gel mixture. Superimpose 5 µL of *Aflatoxin Solution* on one of the three 10-µL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in a saturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and water (140: 20: 0.3) until the solvent front has moved NLT 72 mm from the origin (80 mm from the lower edge of the plate). Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry for 5 min. Locate the spots on the plate by scanning fluorescence density (>400 nm) under UV light at 366 nm. Match the position of each fluorescent spot of the *Test Solution* with those of *Aflatoxin Solution* to identify the type of aflatoxin present. The concentration of aflatoxins in the *Test Solution* can be calculated from the calibration curve obtained from the scan data with *Aflatoxin Solution*.

System Suitability—The four applications of *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*. The mean recovery of spiked AFB_1 and AFG_1 is NLT 70%.

Acceptance Criteria— Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for AFB_1 and NMT 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2, except when otherwise indicated.

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[1] A suitable powder mixture is available from Sigma as PBS P-3813.

Method III

This test method is provided as an example for the detection of the possible presence of AFB_1 and total aflatoxins (AF: sum of AFB_1, AFB_2, AFG_1, and AFG_2). It has been shown to be suitable for powdered ginseng and ginger. Its suitability to other articles of botanical origin must be demonstrated.

0.1 M Phosphate Buffer Solution—Dissolve 8.69 g of anhydrous disodium phosphate and 4.66 g of anhydrous monosodium phosphate or 5.36 g of monosodium phosphate monohydrate in 800 mL water, adjust with 2 M sodium hydroxide to a pH of 7.4, add 10 mL of polysorbate 20, and dilute to 1 L.

Phosphate Buffered Saline Solution—Prepare as directed in *Method II*.

Working Aflatoxin Standard Solutions—Prepare six solutions in separate 10-mL volumetric flasks according to *Table 3*. Dilute with methanol and water (1:1, v/v) to volume. Store in a refrigerator, and equilibrate to room temperature before use. Prepare the solutions daily.

Table 3. Preparation of Working Aflatoxin Standard Solutions

Working Aflatoxin Standard Solutions	USP Aflatoxins RS (µL)	Final Aflatoxin Concentration of Working Aflatoxin Standard Solution (ng/mL)				
		AFB_1	AFB_2	AFG_1	AFG_2	ΣAF
1	0	0	0	0	0	0
2	12.5	0.25	0.0625	0.25	0.0625	0.625
3	25	0.5	0.125	0.5	0.125	1.25
4	50	1	0.25	1	0.25	2.5
5	100	2	0.5	2	0.5	5
6	200	4	1	4	1	10

Immunoaffinity Column (IAC)[2]—Use an immunoaffinity column that contains monoclonal antibodies cross reactive toward AFB_1, AFB_2, AFG_1, and AFG_2. The immunoaffinity columns have a minimum capacity of NLT 100 ng of total aflatoxin and give a recovery of NLT 80% for AFB_1, AFB_2, AFG_1, and AFG_2 when 5 ng of each AFB_1, AFB_2, AFG_1, and AFG_2 is applied in 10 mL of 10% methanol in *Phosphate Buffered Saline Solution* (v/v).

Test Solution—

Extraction—Weigh 5 g of a representative test sample in a 50-mL centrifuge tube. Add 1 g of sodium chloride and 25 mL of a mixture of methanol and 0.5% sodium bicarbonate (700:300). Mix on a vortex mixer until sample particles and extract solvent are well mixed. Shake at 400 rpm for 10 min. Centrifuge for 10 min at 7000 rpm (*g* value = 5323 mm/s^2) or at a speed that can result in a firm pellet of residues. Immediately pipet 7 mL into a 50-mL centrifuge tube, add 28 mL of *0.1 M Phosphate Buffer Solution*, mix, and filter through glass microfiber paper. Collect 25 mL of filtrate (equivalent to 1 g of test sample) into a 25-mL graduated cylinder, and proceed immediately with IAC chromatography.

IAC Cleanup—[NOTE—For IAC cleanup, columns must be kept at room temperature for at least 15 min before use.] Remove the top cap from the column, and connect it with the reservoir. Remove the end cap from the column, and attach it to the column manifold (the fit must be tight). Let the liquid in the column pass through until the liquid is about 2–3 mm above the column bed. Pass 25 mL of filtrate into the reservoir. Let the filtrate flow through the column by gravity force. Let the column run dry. In order to start the flow easily again, remove the column from the manifold, add about 2 mL of *Phosphate Buffered Saline Solution* into the column, reattach the column to the reservoir, and wash the column with an additional 3 mL of *Phosphate Buffered Saline Solution* and then with 5 mL of water (the 5 mL of *Phosphate Buffered Saline Solution* can be added directly to the column reservoir if other techniques are used to dislodge the air bubble at the end of the column and to start flow easily again). Let the column run dry, then force 3 mL of air through the column with a syringe. Elute with 1 mL of methanol, and collect the analytes in a 3-mL volumetric flask, letting the eluate drip freely. Let the column run dry. Let stand for 1 min, then elute with an additional 1 mL of methanol, and collect in the same volumetric flask. Let the column run dry, and force 10 mL of air through the column. Dilute the eluate with water to volume. Use this as the *Test Solution*, and perform the analysis of aflatoxins immediately.

System Suitability Solution—Prepare a spiked sample by adding 5 mL of *Working Aflatoxin Standard Solution 5* to a 5-g sample and repeating the procedure for the *Test Solution*, using 20 mL instead of 25 mL of the mixture of methanol and 0.5% sodium bicarbonate (700:300).

Chromatographic System—

Flow rate—0.8 mL/min

Detection—Fluorescence detector set at excitation wavelength (Ex) 362 nm and emission wavelength (Em) 440 nm

Column—4.6-mm × 15-cm containing 3-µm packing L1

Mobile phase—Isocratic

FOR POST-COLUMN DERIVATIZATION WITH PHRED CELL[3]— Water, methanol, and acetonitrile (600:250:150)

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[2] AflaOchraTest column (G1017; Vicam, Watertown, MA, USA) or equivalent. Aflatoxin/OTA immunoaffinity columns are suitable.
[3] PHRED™ Photochemical Reactor (AURA Industries, New York, NY, USA) or equivalent. Avoid looking at the UV lamp.

FOR POST-COLUMN DERIVATIZATION WITH KOBRA CELL[4]—A solution prepared by mixing 1 L of a mixture of water, methanol, and acetonitrile (600:250:150); 350 μL of 4 M nitric acid; and 120 mg of potassium bromide

Post-Column Derivatization (PCD) Systems—
PHRED CELL: Post-column photochemical derivatization cell
KOBRA CELL: Electrochemical cell, post-column bromination derivatization cell
Analysis—
Post-Column Derivatization for Aflatoxins—Use a UV or Kobra cell. Inject 50 μL of reagent blank *(Working Aflatoxin Standard Solution 1)*, the *Working Aflatoxin Standard Solutions 2–6*, or the *Test Solution* into the LC column. Identify the aflatoxin peaks in the *Test Solution* by comparing the retention times with those of the working standards. The aflatoxins elute in the order AFG_2, AFG_1, AFB_2, and AFB_1. After passing through the PHRED or Kobra cell, the AFG_1 and AFB_1 have been derivatized to form AFG_{2a} (derivative of AFG_1) and AFB_{2a} (derivative of AFB_1). [NOTE—The chemical structures of the derivatives resulting from electrochemical bromination and photolysis are not the same. The structures of AFB_1 and AFG_1 photolysis products have not been established.] The retention times of AFG_2, AFG_{2a}, AFB_2, and AFB_{2a} are between about 14 and 27 min using the PHRED cell; retention times are shorter using the Kobra cell. The peaks should be baseline resolved. Construct standard curves for each aflatoxin. Determine the concentration of each aflatoxin in the *Test Solution* from the calibration curve.
Aflatoxins Calibration Curves—Calibration curves are prepared for each of the aflatoxins using the *Working Aflatoxin Standard Solutions* containing the four aflatoxins described. These solutions cover the range of 0.25–4 ng/mL for AFB_1 and AFG_1, and the range of 0.0625–1 ng/mL for AFB_2 and AFG_2. Make the calibration curves prior to analysis according to *Table 3*, and check the plot for linearity. If the test portion area response is outside (higher) the calibration range, then the *Test Solution* should be diluted with a mixture of methanol and water (1:1, v/v) and reinjected into the LC column.
Quantitation of Aflatoxins—Quantitation of aflatoxins is performed by measuring peak areas at each aflatoxin retention time and comparing them with the corresponding calibration curve.
System Suitability—The mean recovery of spiked AFB_1 (2 μg/kg) and the total of aflatoxins (5 μg/kg) is NLT 68% and 70%, respectively. The relative standard deviation (RSD) is NMT 10% for AFB_1 and for the total of aflatoxins.
Calculations—Plot the peak area (response, *y*-axis) of each of the toxin standards against the concentration (ng/mL, *x*-axis) and determine the slope (*S*) and *y*-intercept (*a*). Calculate the level of toxin in the sample by the following formula:

$$\text{Toxin (μg/kg)} = \{[(R - a)/S] \times V/W\} \times F$$

where *R* is the *Test Solution* peak area; *V* is the final volume of the injected *Test Solution* (mL); and *F* is the dilution factor. *F* = 1 when *V* = 3 mL. *W* is 1 g of test sample passed through the immunoaffinity column. The total of aflatoxins is the sum of AFG_2, AFG_1, AFB_2, and AFB_1.
Acceptance Criteria—Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are NMT 5 ppb for AFB_1 and NMT 20 ppb for the sum of AFB_1, AFB_2, AFG_1, and AFG_2, except when otherwise indicated.

GENERAL METHOD FOR PESTICIDE RESIDUES ANALYSIS

Definition—Where used in this Pharmacopeia, the designation *pesticide* applies to any substance or mixture of substances intended to prevent, destroy, or control any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

Limits—Within the United States, many botanicals are treated as dietary supplements and are subject to the statutory provisions that govern foods but not drugs in the Federal Food, Drug, and Cosmetic Act. Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA) as indicated in the Code of Federal Regulations (40 CFR Part 180) or the Federal Register (FR). For pesticide chemicals without EPA-established tolerance levels, the limits should be below the detection limit of the specified method. Results less than the EPA detection limits are considered zero values. The limits contained herein, therefore, are not applicable in the United States when articles of botanical origins are labeled for food purposes. The limits, however, may be applicable in other countries where the presence of pesticide residues is permitted. Unless otherwise indicated in the monograph, the article to be examined complies with the limits indicated in *Table 4*. The limits for suspected pesticides that are not listed in *Table 4* must comply with the regulations of the EPA. For instances in which a pesticide is not listed in *Table 4* or in EPA regulations, calculate the limit by the formula:

$$\text{Limits (mg/kg)} = AM/100B$$

where *A* is the acceptable daily intake (ADI), as published by FAO-WHO, in mg/kg of body weight; *M* is body weight, in kg (60 kg); and *B* is the daily dose of the article, in kg.

If the article is intended for the preparation of extracts, tinctures, or other pharmaceutical forms of which the preparation method modifies the content of pesticides in the finished product, calculate the limits by the formula:

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[4] Kobra Cell™ (R-Biopharm Inc., Marshall, MI, USA) or equivalent. Set at 100 mA. Do not turn on the current until the LC pump is operating to avoid overheating the cell membrane.

Limits (mg/kg) = $AME/100B$

where E is the extraction factor of the preparation method, determined experimentally; and A, M, and B are as defined above.

A total or partial exemption from the test may be granted when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

Table 4

Substance	Limit (mg/kg)
Acephate	0.1
Alachlor	0.05
Aldrin and dieldrin (sum of)	0.05
Azinphos-ethyl	0.1
Azinphos-methyl	1
Bromide, inorganic (calculated as bromide ion)	125
Bromophos-ethyl	0.05
Bromophos-methyl	0.05
Brompropylate	3
Chlordane (sum of *cis*-, *trans*-, andoxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyriphos-ethyl	0.2
Chlorpyriphos-methyl	0.1
Chlorthal-dimethyl	0.01
Cyfluthrin (sum of)	0.1
λ-Cyhalothrin	1
Cypermethrin and isomers (sum of)	1
DDT (sum of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, *o,p'*-TDE, and *p,p'*-TDE)	1
Deltamethrin	0.5
Diazinon	0.5
Dichlofluanid	0.1
Dichlorvos	1
Dicofol	0.5
Dimethoate and omethoate (sum of)	0.1
Dithiocarbamates (expressed as CS2)	2
Endosulfan (sum of isomers and endosulfan sulphate)	3
Endrin	0.05
Ethion	2
Etrimphos	0.05
Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon)	0.1
Fenitrothion	0.5
Fenpropathrin	0.03
Fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon, and fensulfothion-sulfon)	0.05
Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon-sulfon, fenthion-oxon-sulfoxid, fenthion-sulfon, and fenthion-sulfoxid)	0.05
Fenvalerate	1.5
Flucytrinate	0.05
τ-Fluvalinate	0.05
Fonophos	0.05
Heptachlor (sum of heptachlor, *cis*-heptachlorepoxide, and *trans*-heptachlorepoxide)	0.05
Hexachlorbenzene	0.1
Hexachlorocyclohexane (sum of isomers α-, *β* -, δ-, and ε-)	0.3
Lindan (γ-hexachlorocyclohexane)	0.6
Malathion and malaoxon (sum of)	1
Mecarbam	0.05

Table 4 *(Continued)*

Substance	Limit (mg/kg)
Methacriphos	0.05
Methamidophos	0.05
Methidathion	0.2
Methoxychlor	0.05
Mirex	0.01
Monocrotophos	0.1
Parathion-ethyl and Paraoxon-ethyl (sum of)	0.5
Parathion-methyl and Paraoxon-methyl (sum of)	0.2
Pendimethalin	0.1
Pentachloranisol	0.01
Permethrin and isomers (sum of)	1
Phosalone	0.1
Phosmet	0.05
Piperonyl butoxide	3
Pirimiphos-ethyl	0.05
Pirimiphos-methyl (sum of pirimiphos-methyl and *N*-desethyl-pirimiphos-methyl)	4
Procymidone	0.1
Profenophos	0.1
Prothiophos	0.05
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3
Quinalphos	0.05
Quintozene (sum of quintozene, pentachloraniline, and methylpentachlorphenyl sulfide)	1
S-421	0.02
Tecnazene	0.05
Tetradifon	0.3
Vinclozolin	0.4

Reagents—Use reagents and solvents that are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special grade solvents suitable for pesticide residue analysis or solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be performed.

Preparation of Apparatus—Clean all equipment, especially glassware, to ensure that it is free from pesticides. Soak all glassware for a minimum of 16 h in a solution of phosphate-free detergent, rinse with copious quantities of distilled water, and then wash with acetone, followed by hexane or heptane.

Qualitative and Quantitative Analysis of Pesticide Residues—Use validated analytical procedures (e.g., FDA Pesticide Analytical Manual (PAM) [http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm2006955.htm], or other analytical procedures validated in accordance with EU guideline [Note—Document No. SANCO/10232/2006, http://ec.europa.eu/food/plant/resources/qualcontrol_en.pdf] or *Validation of Compendial Procedures* 〈1225〉) that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives. Measure the limits of detection and quantification for each pesticide matrix combination to be analyzed: the method is shown to recover between 70% and 110% of each pesticide; the repeatability and reproducibility of the method are NLT the appropriate values indicated in *Table 5*; and the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Table 5

Concentration Range of the Pesticide (mg/kg)	Repeatability (RSD) (%)	Reproducibility (RSD) (%)
0.001–0.01	30	60
>0.01–0.1	20	40
>0.1–1	15	30
>1	10	20

TEST FOR PESTICIDES

Unless otherwise specified in the individual monograph, the following methods may be used for the analysis of pesticides. Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another column having a different polarity, another detection method (e.g., mass spectrometry), or a different method (e.g., immunochemical method) to confirm the results.

Extraction—[NOTE—Use the following procedure for the analysis of samples of articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.] To 10 g of the coarsely powdered substance under test add 100 mL of acetone, and allow to stand for 20 min. Add 1 mL of a solution in toluene containing 1.8 µg of carbophenothion per mL. Mix in a high-speed blender for 3 min. Filter this solution, and wash the residue with two 25-mL portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely evaporated. To the residue add a few mL of toluene, and heat again until the acetone is completely removed. Dissolve the residue in 8 mL of toluene. Pass through a membrane filter of 45-µm pore size, rinse the flask and the filter with toluene, dilute with toluene to 10.0 mL (*Solution A*), and mix.

Purification—

Organochlorine, Organophosphorus, and Pyrethroid Insecticides—The size-exclusion chromatograph is equipped with a 7.8-mm × 30-cm stainless steel column containing 5-µm packing L21. Toluene is used as the mobile phase at a flow rate of about 1 mL/min.

Performance of the Column—Inject 100 µL of a solution in toluene containing, in each mL, 0.5 mg of methyl red and 0.5 mg of oracet blue or equivalent. The column is not suitable unless the color of the eluate changes from orange to blue at an elution volume of about 10.3 mL. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

Purification of the Test Solution—Inject a suitable volume (100 to 500 µL) of *Solution A* into the chromatograph. Collect the fraction (*Solution B*) as determined above under *Performance of the Column*. Organophosphorus pesticides elute between 8.8 and 10.9 mL. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 mL.

Organochlorine and *Pyrethroid Insecticides*—Into a 5-mm × 10-cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in an oven at 150° for at least 4 h. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 h. Condition the column with 1.5 mL of hexane. [NOTE—Prepacked columns containing about 0.50 g of a suitable silica gel may also be used, provided they have been previously validated.] Concentrate *Solution B* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200 µL to 1 mL, according to the volume injected in the preparation of *Solution B*). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 mL of toluene as the mobile phase. Collect the eluate (*Solution C*).

Quantitative Analysis of Organophosphorus Insecticides—

Test Solution—Concentrate *Solution B* almost to dryness, with the aid of a stream of helium, dilute with toluene to 100 µL, and mix.

Standard Solution—Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System—The gas chromatograph is equipped with an alkali flame-ionization detector or a flame-photometric detector and a 0.32-mm × 30-m fused silica column coated with a 0.25-µm layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 250°, and the detector is maintained at 275°. The column temperature is maintained at 80° for 1 min, then increased to 150° at a rate of 30°/min, maintained at 150° for 3 min, then increased to 280° at a rate of 4°/min, and maintained at this temperature for 1 min. Use carbophenothion as the internal standard. [NOTE—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Quantitative Analysis of Organochlorine and Pyrethroid Insecticides—

Test Solution—Concentrate *Solution C* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, dilute with toluene to 500 µL, and mix.

Standard Solution—Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System—The gas chromatograph is equipped with an electron-capture detector, a device allowing direct on-column cold injection, and a 0.32-mm × 30-m fused silica column coated with a 0.25-µm layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 275°, and the detector is maintained at 300°. The column temperature is maintained at 80° for 1 min, then increased to 150° at a rate of 30°/min, maintained at 150° for 3 min, then increased to 280° at a rate of 4°/min, and maintained at this

temperature for 1 min. Use carbophenothion as the internal standard. [Note—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solutions.

⟨616⟩ BULK DENSITY AND TAPPED DENSITY OF POWDERS

BULK DENSITY

This general chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. ⬥The portion that is not harmonized is marked with symbols (⬥₎) to specify this fact.₎

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per mL (g/mL) although the international unit is kilograms per cubic meter (1 g/mL = 1000 kg/m^3) because the measurements are made using cylinders. It may also be expressed in grams per cubic centimeter (g/cm^3). The bulking properties of a powder are dependent upon the preparation, treatment, and storage of the sample, i.e., how it was handled. The particles can be packed to have a range of bulk densities; however, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made. The bulk density of a powder is determined by measuring the volume of a known weight of powder sample, that may have been passed through a sieve, into a graduated cylinder (*Method I*), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (*Method II*) or a measuring vessel (*Method III*).

Method I and *Method III* are favored.

Method I—Measurement in a Graduated Cylinder

Procedure—Pass a quantity of material sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry graduated 250-mL cylinder (readable to 2 mL) introduce, without compacting, approximately 100 g of test sample, *M*, weighed with 0.1% accuracy. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume (V_0) to the nearest graduated unit. Calculate the bulk density in g/mL by the formula m/V_0. Generally, replicate determinations are desirable for the determination of this property. If the powder density is too low or too high, such that the test sample has an untapped apparent volume of either more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. Therefore, a different amount of powder has to be selected as the test sample, such that its untapped apparent volume is 150–250 mL (apparent volume greater than or equal to 60% of the total volume of the cylinder); the weight of the test sample is specified in the expression of results. For test samples having an apparent volume between 50 mL and 100 mL, a 100-mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

Method II—Measurement in a Volumeter

Apparatus—The apparatus (*Figure 1*) consists of a top funnel fitted with a 1.0-mm sieve.[1] The funnel is mounted over a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup of specified capacity mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an inside diameter of 30.00 ± 2.00 mm) or cubical (16.39 ± 0.20 mL volume with inside dimensions of 25.400 ± 0.076 mm).

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[1] The apparatus (the Scott Volumeter) conforms to the dimensions in ASTM B329–06(2012).
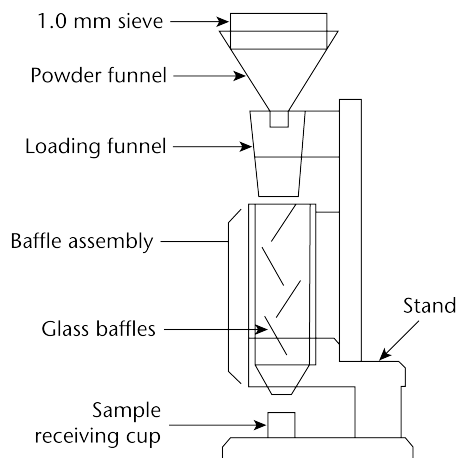
Figure 1.

   Procedure—Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm³ of powder with the square cup and 35 cm³ of powder with the cylindrical cup. Carefully scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the sides of the cup, and determine the weight, M, of the powder to the nearest 0.1%. Calculate the bulk density, in g/mL, by the formula:

$$(M)/(V_0)$$

in which V_0 is the volume, in mL, of the cup. Record the average of three determinations using three different powder samples.

Method III—Measurement in a Vessel

   Apparatus—The apparatus consists of a 100-mL cylindrical vessel of stainless steel with dimensions as specified in *Figure 2*.
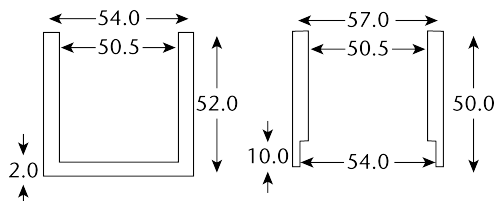

Figure 2.

   Procedure—Pass a quantity of powder sufficient to complete the test through a 1.0-mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described for *Method II*. Determine the weight (M_0) of the powder to the nearest 0.1% by subtraction of the previously determined mass of the empty measuring vessel. Calculate the bulk density (g/mL) by the formula $M_0/100$, and record the average of three determinations using three different powder samples.

TAPPED DENSITY

   The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample. Tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing a powder sample. After observing the initial powder volume or weight, the measuring cylinder or vessel is mechanically tapped, and volume or weight readings are taken until little further volume or weight change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop under its own weight a specified distance by either of three methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimize any possible separation of the mass during tapping down.

Method I

   Apparatus—The apparatus (*Figure 3*) consists of the following:

• A 250-mL graduated cylinder (readable to 2 mL with a mass of 220 ± 44 g)
• A settling apparatus capable of producing, in 1 min, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.
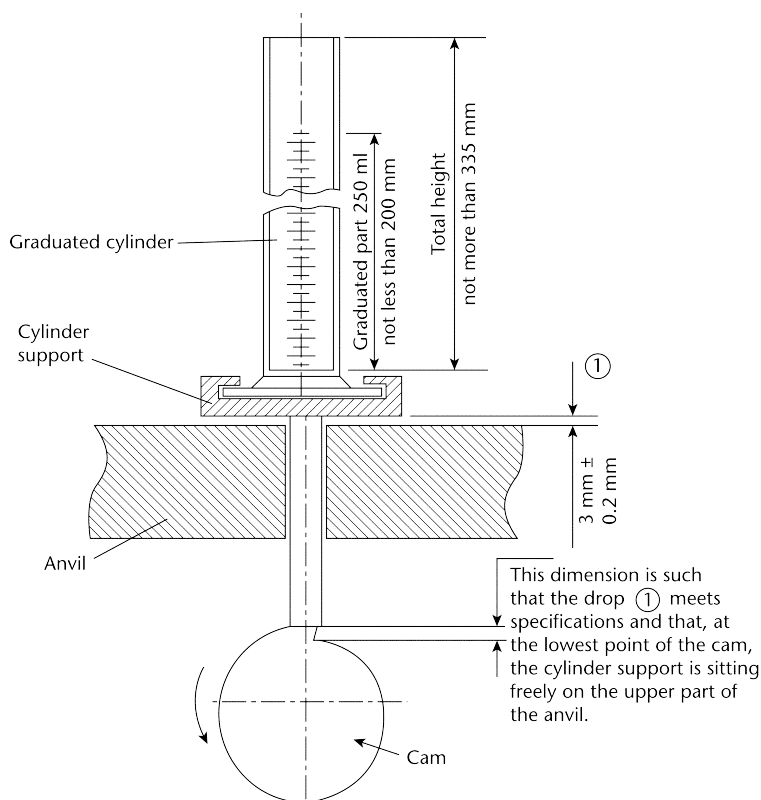

Figure 3.

Procedure—Proceed as described above for the determination of the bulk volume (V_0). Secure the cylinder in the holder. Carry out 10, 500, and 1250 taps on the same powder sample and read the corresponding volumes V_{10}, V_{500}, and V_{1250} to the nearest graduated unit. If the difference between V_{500} and V_{1250} is less than or equal to 2 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 2 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density (g/mL) using the formula m/V_F, in which V_F is the final tapped volume. Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results. If it is not possible to use a 100-g test sample, use a reduced amount and a suitable 100-mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a holder weighing 240 ± 12 g. If the difference between V_{500} and V_{1250} is less than or equal to 1 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 1 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 1 mL. The modified test conditions are specified in the expression of the results.

Method II

Apparatus and **Procedure**—Proceed as directed under *Method I* except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per min.

Method III

Apparatus and **Procedure**—Proceed as directed in *Method III—Measurement in a Vessel* for measuring bulk density using the measuring vessel equipped with the cap shown in *Figure 2*. The measuring vessel with the cap is lifted 50–60 times per min by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap, and carefully scrape excess powder from the top of the measuring vessel as described in *Method III—Measurement in a Vessel* for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the two masses obtained after 200 and 400 taps exceeds 2%, carry out a test using 200 additional taps until the difference between succeeding measurements is less than 2%. Calculate the tapped density (g/mL) using the formula $M_F/100$, where M_F is the mass of powder in the measuring vessel. Record the average of three

determinations using three different powder samples. The test conditions including tapping height are specified in the expression of the results.

MEASURES OF POWDER COMPRESSIBILITY

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the *Compressibility Index* or the *Hausner Ratio* as described below.

The *Compressibility Index* and *Hausner Ratio* are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the *Compressibility Index* and the *Hausner Ratio*.

Compressibility Index—Calculate by the formula:

$$100(V_0 - V_F)/V_0$$

V_0 = unsettled apparent volume
V_F = final tapped volume

Hausner Ratio—

$$V_0/V_F$$

Depending on the material, the compressibility index can be determined using V_{10} instead of V_0. [NOTE—If V_{10} is used, it will be clearly stated in the results.]

⟨1010⟩ ANALYTICAL DATA—INTERPRETATION AND TREATMENT

INTRODUCTION

This chapter provides information regarding acceptable practices for the analysis and consistent interpretation of data obtained from chemical and other analyses. Basic statistical approaches for evaluating data are described, and the treatment of outliers and comparison of analytical procedures are discussed in some detail.
[NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.]

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including robust formulation design, validation, testing of starting materials, in-process testing, and final-product testing. Each of these practices is dependent on reliable test procedures. In the development process, test procedures are developed and validated to ensure that the manufactured products are thoroughly characterized. Final-product testing provides further assurance that the products are consistently safe, efficacious, and in compliance with their specifications.

Measurements are inherently variable. The variability of biological tests has long been recognized by the USP. For example, the need to consider this variability when analyzing biological test data is addressed in *Analysis of Biological Assays* ⟨1034⟩. The chemical analysis measurements commonly used to analyze pharmaceuticals are also inherently variable, although less so than those of the biological tests. However, in many instances the acceptance criteria are proportionally tighter, and thus, this smaller allowable variability has to be considered when analyzing data generated using analytical procedures. If the variability of a measurement is not characterized and stated along with the result of the measurement, then the data can only be interpreted in the most limited sense. For example, stating that the difference between the averages from two laboratories when testing a common set of samples is 10% has limited interpretation, in terms of how important such a difference is, without knowledge of the intralaboratory variability.

This chapter provides direction for scientifically acceptable treatment and interpretation of data. Statistical tools that may be helpful in the interpretation of analytical data are described. Many descriptive statistics, such as the mean and standard deviation, are in common use. Other statistical tools, such as outlier tests, can be performed using several different, scientifically valid approaches, and examples of these tools and their applications are also included. The framework within which the results from a compendial test are interpreted is clearly outlined in *General Notices and Requirements 7. Test Results*. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in *Appendix G* at the end of the chapter. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to laboratory data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

Sound Record Keeping

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should generally be obtained with more decimal places than the specification requires and rounded only after final calculations are completed as per the *General Notices and Requirements*.

Sampling Considerations

Effective sampling is an important step in the assessment of a quality attribute of a population. The purpose of sampling is to provide representative data (the sample) for estimating the properties of the population. How to attain such a sample depends entirely on the question that is to be answered by the sample data. In general, use of a random process is considered the most appropriate way of selecting a sample. Indeed, a random and independent sample is necessary to ensure that the resulting data produce valid estimates of the properties of the population. Generating a nonrandom or "convenience" sample risks the possibility that the estimates will be biased. The most straightforward type of random sampling is called *simple random sampling*, a process in which every unit of the population has an equal chance of appearing in the sample. However, sometimes this method of selecting a random sample is not optimal because it cannot guarantee equal representation among factors (i.e., time, location, machine) that may influence the critical properties of the population. For example, if it requires 12 hours to manufacture all of the units in a lot and it is vital that the sample be representative of the entire production process, then taking a simple random sample after the production has been completed may not be appropriate because there can be no guarantee that such a sample will contain a similar number of units made from every time period within the 12-hour process. Instead, it is better to take a *systematic random sample* whereby a unit is randomly selected from the production process at

systematically selected times or locations (e.g., sampling every 30 minutes from the units produced at that time) to ensure that units taken throughout the entire manufacturing process are included in the sample. Another type of random sampling procedure is needed if, for example, a product is filled into vials using four different filling machines. In this case it would be important to capture a random sample of vials from each of the filling machines. A *stratified random sample*, which randomly samples an equal number of vials from each of the four filling machines, would satisfy this requirement. Regardless of the reason for taking a sample (e.g., batch-release testing), a sampling plan should be established to provide details on how the sample is to be obtained to ensure that the sample is representative of the entirety of the population and that the resulting data have the required sensitivity. The optimal sampling strategy will depend on knowledge of the manufacturing and analytical measurement processes. Once the sampling scheme has been defined, it is likely that the sampling will include some element of random selection. Finally, there must be sufficient sample collected for the original analysis, subsequent verification analyses, and other analyses. Consulting a statistician to identify the optimal sampling strategy is recommended.

Tests discussed in the remainder of this chapter assume that simple random sampling has been performed.

Use of Reference Standards

Where *USP* or *NF* tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive for purposes of demonstrating conformance to such USP or NF standards. While USP standards apply at all times in the life of an article from production to expiration, USP does not specify when testing must be done, or any frequency of testing. Accordingly, users of *USP* and *NF* apply a range of strategies and practices to assure articles achieve and maintain conformance with compendial requirements, including when and if tested. Such strategies and practices can include the use of secondary standards traceable to the USP Reference Standard, to supplement or support any testing undertaken for the purpose of conclusively demonstrating conformance to applicable compendial standards. Because the assignment of a value to a standard is one of the most important factors that influences the accuracy of an analysis, it is critical that this be done correctly.

System Performance Verification

Verifying an acceptable level of performance for an analytical system in routine or continuous use can be a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals, or using other means, such as, variation among the standards, background signal-to-noise ratios, etc. Attention to the measured parameter, such as charting the results obtained by analysis of a control sample, can signal a change in performance that requires adjustment of the analytical system. An example of a controlled chart is provided in *Appendix A*.

Procedure Validation

All analytical procedures are appropriately validated as specified in *Validation of Compendial Procedures* ⟨1225⟩. Analytical procedures published in the *USP–NF* have been validated and meet the Current Good Manufacturing Practices regulatory requirement for validation as established in the Code of Federal Regulations. A validated procedure may be used to test a new formulation (such as a new product, dosage form, or process intermediate) only after confirming that the new formulation does not interfere with the accuracy, linearity, or precision of the method. It may not be assumed that a validated procedure could correctly measure the active ingredient in a formulation that is different from that used in establishing the original validity of the procedure. [NOTE ON TERMINOLOGY—The definition of *accuracy* in ⟨1225⟩ and in ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), *accuracy* has a different meaning. In ISO, *accuracy* combines the concepts of unbiasedness (termed *trueness*) and precision. This chapter follows the definition in ⟨1225⟩, which corresponds only to trueness.]

MEASUREMENT PRINCIPLES AND VARIATION

All measurements are, at best, estimates of the actual ("true" or "accepted") value for they contain random variability (also referred to as random error) and may also contain systematic variation (bias). Thus, the measured value differs from the actual value because of variability inherent in the measurement. If an array of measurements consists of individual results that are representative of the whole, statistical methods can be used to estimate informative properties of the entirety, and statistical tests are available to investigate whether it is likely that these properties comply with given requirements. The resulting statistical analyses should address the variability associated with the measurement process as well as that of the entity being measured. Statistical measures used to assess the direction and magnitude of these errors include the mean, standard deviation, and expressions derived therefrom, such as the percent coefficient of variation (%CV; also called the percent relative standard deviation, %RSD). The estimated variability can be used to calculate confidence intervals for the mean, or measures of variability, and tolerance intervals capturing a specified proportion of the individual measurements.

The use of statistical measures must be tempered with good judgment, especially with regard to representative sampling. Data should be consistent with the statistical assumptions used for the analysis. If one or more of these assumptions appear to

be violated, alternative methods may be required in the evaluation of the data. In particular, most of the statistical measures and tests cited in this chapter rely on the assumptions that the distribution of the entire population is represented by a normal distribution and that the analyzed sample is a representative subset of this population. The normal (or Gaussian) distribution is bell-shaped and symmetric about its center and has certain characteristics that are required for these tests to be valid. The data may not always be expected to be normally distributed and may require a transformation to better fit a normal distribution. For example, there exist variables that have distributions with longer right tails than left. Such distributions can often be made approximately normal through a log transformation. An alternative approach would be to use "distribution-free" or "nonparametric" statistical procedures that do not require that the shape of the population be that of a normal distribution. When the objective is to construct a confidence interval for the mean or for the difference between two means, for example, then the normality assumption is not as important because of the central limit theorem. However, one must verify normality of data to construct valid confidence intervals for standard deviations and ratios of standard deviations, perform some outlier tests, and construct valid statistical tolerance limits. In the latter case, normality is a critical assumption. Simple graphical methods, such as dot plots, histograms, and normal probability plots, are useful aids for investigating this assumption.

A single analytical measurement may be useful in quality assessment if the sample is from a whole that has been prepared using a well-validated, documented process and if the analytical errors are well known. The obtained analytical result may be qualified by including an estimate of the associated errors. There may be instances when one might consider the use of averaging because the variability associated with an average value is always reduced as compared to the variability in the individual measurements. The choice of whether to use individual measurements or averages will depend upon the use of the measure and its variability. For example, when multiple measurements are obtained on the same sample aliquot, such as from multiple injections of the sample in an HPLC method, it is generally advisable to average the resulting data for the reason discussed above.

Variability is associated with the dispersion of observations around the center of a distribution. The most commonly used statistic to measure the center is the sample mean ($\bar{x}$):

$$\bar{X} = \frac{\sum_{i=1}^{n} x_1}{n} = \frac{x_1 + x_2 + \ldots + x_n}{n}$$

Analytical procedure variability can be estimated in various ways. The most common and useful assessment of a procedure's variability is the determination of the standard deviation based on repeated independent[1] measurements of a sample. The sample standard deviation, *s*, is calculated by the formula:

$$S = \sqrt{\sum_{i=1}^{n} \left(x_i - \bar{x} \right)^2 / (n-1)}$$

in which x_i is the individual measurement in a set of *n* measurements; and $\bar{x}$ is the mean of all the measurements. The percent relative standard deviation (%RSD) is then calculated as:

$$\%RSD = \frac{S}{\bar{X}} \cdot 100\%$$

and expressed as a percentage. If the data requires log transformation to achieve normality (e.g., for biological assays), then alternative methods are available.[2]

A precision study should be conducted to provide a better estimate of procedure variability. The precision study may be designed to determine intermediate precision (which includes the components of both "between run" and "within-run" varia-

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[1] Multiple measurements (or, equivalently, the experimental errors associated with the multiple measurements) are independent from one another when they can be assumed to represent a random sample from the population. In such a sample, the magnitude of one measurement is not influenced by, nor does it influence the magnitude of, any other measurement. Lack of independence implies the measurements are correlated over time or space. Consider the example of a 96-well microtiter plate. Suppose that whenever the unknown causes that produce experimental error lead to a low result (negative error) when a sample is placed in the first column and these same causes would also lead to a low result for a sample placed in the second column, then the two resulting measurements would not be statistically independent. One way to avoid such possibilities would be to randomize the placement of the samples on the plate.

[2] When data have been log (base e) transformed to achieve normality, the %RSD is:

$$\%RSD = 100\% \cdot \sqrt{e^{s^2} - 1}$$

This can be reasonably approximated by:

$$\%RSD = 100\% \cdot (e^s - 1)$$

where *s* is the standard deviation of the log (base e) transformed data.

bility) and repeatability ("within-run" variability). The intermediate precision studies should allow for changes in the experimental conditions that might be expected, such as different analysts, different preparations of reagents, different days, and different instruments. To perform a precision study, the test is repeated several times. Each run must be completely independent of the others to provide accurate estimates of the various components of variability. In addition, within each run, replicates are made in order to estimate repeatability. See an example of a precision study in *Appendix B*.

A confidence interval for the mean may be considered in the interpretation of data. Such intervals are calculated from several data points using the sample mean ($\bar{x}$) and sample standard deviation(s) according to the formula:

$$\left(\overline{X} - t_{\alpha/2,\, n-1} \frac{s}{\sqrt{n}} \,, \overline{X} + t_{\alpha/2,\, n-1} \frac{s}{\sqrt{n}} \right)$$

in which $t_{\alpha/2,\, n-1}$ is a statistical number dependent upon the sample size (n), the number of degrees of freedom ($n-1$), and the desired confidence level ($1-\alpha$). Its values are obtained from published tables of the Student *t*-distribution. The confidence interval provides an estimate of the range within which the "true" population mean (μ) falls, and it also evaluates the reliability of the sample mean as an estimate of the true mean. If the same experimental set-up were to be replicated over and over and a 95% (for example) confidence interval for the true mean is calculated each time, then 95% of such intervals would be expected to contain the true mean, μ. One cannot say with certainty whether or not the confidence interval derived from a specific set of data actually collected contains μ. However, assuming the data represent mutually independent measurements randomly generated from a normally distributed population, the procedure used to construct the confidence interval guarantees that 95% of such confidence intervals contain μ. Note that it is important to define the population appropriately so that all relevant sources of variation are captured. [NOTE ON TERMINOLOGY—In the documents of the International Organization for Standardization (ISO), different terminology is used for some of the concepts described here. The term $s/\sqrt{n}$, which is commonly called the standard error of the mean, is called the standard uncertainty in ISO documents. The term $t_{\alpha/2,\, n-1}\, S/\sqrt{n}$ is called the expanded uncertainty, and $t_{\alpha/2,\, n-1}$ is called the coverage factor, by ISO. If the standard deviation is found by combining estimates of variability from multiple sources, it is called the combined standard uncertainty. Some of these sources could have nonstatistical estimates of uncertainty, called Type B uncertainties, such as uncertainty in calibration of a balance.]

OUTLYING RESULTS

Occasionally, observed analytical results are very different from those expected. Aberrant, anomalous, contaminated, discordant, spurious, suspicious or wild observations; and flyers, rogues, and mavericks are properly called outlying results. Like all laboratory results, these outliers must be documented, interpreted, and managed. Such results may be accurate measurements of the entity being measured, but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the entity being measured is typical. When an outlying result is obtained, systematic laboratory and, in certain cases, process investigations of the result are conducted to determine if an assignable cause for the result can be established. Factors to be considered when investigating an outlying result include—but are not limited to—human error, instrumentation error, calculation error, and product or component deficiency. If an assignable cause that is not related to a product or component deficiency can be identified, then retesting may be performed on the same sample, if possible, or on a new sample. The precision and accuracy of the procedure, the USP Reference Standard, process trends, and the specification limits should all be examined. Data may be invalidated, based on this documented investigation, and eliminated from subsequent calculations.

If no documentable, assignable cause for the outlying laboratory result is found, the result may be tested, as part of the overall investigation, to determine whether it is an outlier.

However, careful consideration is warranted when using these tests. Two types of errors may occur with outlier tests: (a) labeling observations as outliers when they really are not; and (b) failing to identify outliers when they truly exist. Any judgment about the acceptability of data in which outliers are observed requires careful interpretation.

"Outlier labeling" is informal recognition of suspicious laboratory values that should be further investigated with more formal methods. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values. Outlier labeling is most often done visually with graphical techniques. "Outlier identification" is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed statistical model.

When used appropriately, outlier tests are valuable tools for pharmaceutical laboratories. Several tests exist for detecting outliers. Examples illustrating three of these procedures, the Extreme Studentized Deviate (ESD) Test, Dixon's Test, and Hampel's Rule, are presented in *Appendix C*.

Choosing the appropriate outlier test will depend on the sample size and distributional assumptions. Many of these tests (e.g., the ESD Test) require the assumption that the data generated by the laboratory on the test results can be thought of as a random sample from a population that is normally distributed, possibly after transformation. If a transformation is made to the data, the outlier test is applied to the transformed data. Common transformations include taking the logarithm or square root of the data. Other approaches to handling single and multiple outliers are available and can also be used. These include tests that use robust measures of central tendency and spread, such as the median and median absolute deviation and exploratory data analysis (EDA) methods. "Outlier accommodation" is the use of robust techniques, such as tests based on the order or

rank of each data value in the data set instead of the actual data value, to produce results that are not adversely influenced by the presence of outliers. The use of such methods reduces the risks associated with both types of error in the identification of outliers.

"Outlier rejection" is the actual removal of the identified outlier from the data set. However, an outlier test cannot be the sole means for removing an outlying result from the laboratory data. An outlier test may be useful as part of the evaluation of the significance of that result, along with other data. Outlier tests have no applicability in cases where the variability in the product is what is being assessed, such as content uniformity, dissolution, or release-rate determination. In these applications, a value determined to be an outlier may in fact be an accurate result of a nonuniform product. All data, especially outliers, should be kept for future review. Unusual data, when seen in the context of other historical data, are often not unusual after all but reflect the influences of additional sources of variation.

In summary, the rejection or retention of an apparent outlier can be a serious source of bias. The nature of the testing as well as scientific understanding of the manufacturing process and analytical procedure have to be considered to determine the source of the apparent outlier. An outlier test can never take the place of a thorough laboratory investigation. Rather, it is performed only when the investigation is inconclusive and no deviations in the manufacture or testing of the product were noted. Even if such statistical tests indicate that one or more values are outliers, they should still be retained in the record. Including or excluding outliers in calculations to assess conformance to acceptance criteria should be based on scientific judgment and the internal policies of the manufacturer. It is often useful to perform the calculations with and without the outliers to evaluate their impact.

Outliers that are attributed to measurement process mistakes should be reported (i.e., footnoted), but not included in further statistical calculations. When assessing conformance to a particular acceptance criterion, it is important to define whether the reportable result (the result that is compared to the limits) is an average value, an individual measurement, or something else. If, for example, the acceptance criterion was derived for an average, then it would not be statistically appropriate to require individual measurements to also satisfy the criterion because the variability associated with the average of a series of measurements is smaller than that of any individual measurement.

COMPARISON OF ANALYTICAL PROCEDURES

It is often necessary to compare two procedures to determine if their average results or their variabilities differ by an amount that is deemed important. The goal of a procedure comparison experiment is to generate adequate data to evaluate the equivalency of the two procedures over a range of values. Some of the considerations to be made when performing such comparisons are discussed in this section.

Precision

Precision is the degree of agreement among individual test results when the analytical procedure is applied repeatedly to a homogeneous sample. For an alternative procedure to be considered to have "comparable" precision to that of a current procedure, its precision (see *Analytical Performance Characteristics* in ⟨1225⟩, *Validation*) must not be worse than that of the current procedure by an amount deemed important. A decrease in precision (or increase in variability) can lead to an increase in the number of results expected to fail required specifications. On the other hand, an alternative procedure providing improved precision is acceptable.

One way of comparing the precision of two procedures is by estimating the variance for each procedure (the sample variance, s^2, is the square of the sample standard deviation) and calculating a one-sided upper confidence interval for the ratio of (true) variances, where the ratio is defined as the variance of the alternative procedure to that of the current procedure. An example, with this assumption, is outlined in *Appendix D*. The one-sided upper confidence limit should be compared to an upper limit deemed acceptable, *a priori*, by the analytical laboratory. If the one-sided upper confidence limit is less than this upper acceptable limit, then the precision of the alternative procedure is considered acceptable in the sense that the use of the alternative procedure will not lead to an important loss in precision. Note that if the one-sided upper confidence limit is less than one, then the alternative procedure has been shown to have improved precision relative to the current procedure.

The confidence interval method just described is preferred to applying the two-sample *F*-test to test the statistical significance of the ratio of variances. To perform the two-sample *F*-test, the calculated ratio of sample variances would be compared to a critical value based on tabulated values of the *F* distribution for the desired level of confidence and the number of degrees of freedom for each variance. Tables providing *F*-values are available in most standard statistical textbooks. If the calculated ratio exceeds this critical value, a statistically significant difference in precision is said to exist between the two procedures. However, if the calculated ratio is less than the critical value, this does not prove that the procedures have the same or equivalent level of precision; but rather that there was not enough evidence to prove that a statistically significant difference did, in fact, exist.

Accuracy

Comparison of the accuracy (see *Analytical Performance Characteristics* in ⟨1225⟩, *Validation*) of procedures provides information useful in determining if the new procedure is equivalent, on the average, to the current procedure. A simple method for making this comparison is by calculating a confidence interval for the difference in true means, where the difference is estimated by the sample mean of the alternative procedure minus that of the current procedure.

The confidence interval should be compared to a lower and upper range deemed acceptable, *a priori*, by the laboratory. If the confidence interval falls entirely within this acceptable range, then the two procedures can be considered equivalent, in the sense that the average difference between them is not of practical concern. The lower and upper limits of the confidence interval only show how large the true difference between the two procedures may be, not whether this difference is considered tolerable. Such an assessment can be made only within the appropriate scientific context. This approach is often referred to as TOST (two one-sided tests; see *Appendix F*).

The confidence interval method just described is preferred to the practice of applying a *t*-test to test the statistical significance of the difference in averages. One way to perform the *t*-test is to calculate the confidence interval and to examine whether or not it contains the value zero. The two procedures have a statistically significant difference in averages if the confidence interval excludes zero. A statistically significant difference may not be large enough to have practical importance to the laboratory because it may have arisen as a result of highly precise data or a larger sample size. On the other hand, it is possible that no statistically significant difference is found, which happens when the confidence interval includes zero, and yet an important practical difference cannot be ruled out. This might occur, for example, if the data are highly variable or the sample size is too small. Thus, while the outcome of the *t*-test indicates whether or not a statistically significant difference has been observed, it is not informative with regard to the presence or absence of a difference of practical importance.

Determination of Sample Size

Sample size determination is based on the comparison of the accuracy and precision of the two procedures[3] and is similar to that for testing hypotheses about average differences in the former case and variance ratios in the latter case, but the meaning of some of the input is different. The first component to be specified is δ, the largest acceptable difference between the two procedures that, if achieved, still leads to the conclusion of equivalence. That is, if the two procedures differ by no more than δ, on the average, they are considered acceptably similar. The comparison can be two-sided as just expressed, considering a difference of δ in either direction, as would be used when comparing means. Alternatively, it can be one-sided as in the case of comparing variances where a decrease in variability is acceptable and equivalency is concluded if the ratio of the variances (new/current, as a proportion) is not more than $1.0 + \delta$. A researcher will need to state δ based on knowledge of the current procedure and/or its use, or it may be calculated. One consideration, when there are specifications to satisfy, is that the new procedure should not differ by so much from the current procedure as to risk generating out-of-specification results. One then chooses δ to have a low likelihood of this happening by, for example, comparing the distribution of data for the current procedure to the specification limits. This could be done graphically or by using a tolerance interval, an example of which is given in *Appendix E*. In general, the choice for δ must depend on the scientific requirements of the laboratory.

The next two components relate to the probability of error. The data could lead to a conclusion of similarity when the procedures are unacceptably different (as defined by δ). This is called a false positive or Type I error. The error could also be in the other direction; that is, the procedures could be similar, but the data do not permit that conclusion. This is a false negative or Type II error. With statistical methods, it is not possible to completely eliminate the possibility of either error. However, by choosing the sample size appropriately, the probability of each of these errors can be made acceptably small. The acceptable maximum probability of a Type I error is commonly denoted as α and is commonly taken as 5%, but may be chosen differently. The desired maximum probability of a Type II error is commonly denoted by β. Often, β is specified indirectly by choosing a desired level of $1 - \beta$, which is called the "power" of the test. In the context of equivalency testing, power is the probability of correctly concluding that two procedures are equivalent. Power is commonly taken to be 80% or 90% (corresponding to a β of 20% or 10%), though other values may be chosen. The protocol for the experiment should specify δ, α, and power. The sample size will depend on all of these components. An example is given in *Appendix E*. Although *Appendix E* determines only a single value, it is often useful to determine a table of sample sizes corresponding to different choices of δ, α, and power. Such a table often allows for a more informed choice of sample size to better balance the competing priorities of resources and risks (false negative and false positive conclusions).

APPENDIX A: CONTROL CHARTS

Figure 1 illustrates a control chart for individual values. There are several different methods for calculating the upper control limit (UCL) and lower control limit (LCL). One method involves the moving range, which is defined as the absolute difference between two consecutive measurements ($x_i - x_{i-1}$). These moving ranges are averaged ($\overline{MR}$) and used in the following formulas:

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[3] In general, the sample size required to compare the precision of two procedures will be greater than that required to compare the accuracy of the procedures.

$$UCL = \bar{x} + 3\frac{\overline{MR}}{d_2}$$

$$LCL = \bar{x} - 3\frac{\overline{MR}}{d_2}$$

where $\bar{x}$ is the sample mean, and d_2 is a constant commonly used for this type of chart and is based on the number of observations associated with the moving range calculation. Where $n = 2$ (two consecutive measurements), as here, $d_2 = 1.128$. For the example in *Figure 1*, the $\overline{MR}$ was 1.7:

$$UCL = 102.0 + 3\frac{1.7}{1.128} = 106.5$$

$$LCL = 102.0 - 3\frac{1.7}{1.128} = 97.5$$

Other methods exist that are better able to detect small shifts in the process mean, such as the cumulative sum (also known as "CUSUM") and exponentially weighted moving average ("EWMA").
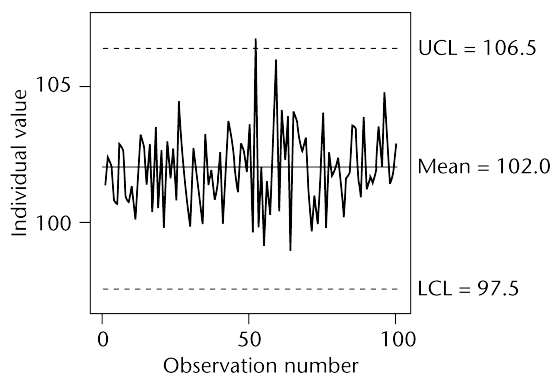

Figure 1. Individual X or individual measurements control chart for control samples. In this particular example, the mean for all the samples ($\bar{x}$) is 102.0, the UCL is 106.5, and the LCL is 97.5.

APPENDIX B: PRECISION STUDY

Table 1 displays data collected from a precision study. This study consisted of five independent runs and, within each run, results from three replicates were collected.

Table 1. Data from a Precision Study

Replicate Number	Run Number				
	1	2	3	4	5
1	100.70	99.46	99.96	101.80	101.91
2	101.05	99.37	100.17	102.16	102.00
3	101.15	99.59	101.01	102.44	101.67
Mean	100.97	99.47	100.38	102.13	101.86
Standard deviation	0.236	0.111	0.556	0.321	0.171
%RSD[a]	0.234%	0.111%	0.554%	0.314%	0.167%

[a] %RSD (percent relative standard deviation) = 100% × (standard deviation/mean)

Table 1A. Analysis of Variance Table for Data Presented in *Table 1*

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Squares[a] (MS)	$F = MS_B/MS_W$
Between runs	4	14.200	3.550	34.886
Within runs	10	1.018	0.102	
Total	14	15.217		

[a] The Mean Squares Between (MS_B) = $SS_{Between}/df_{Between}$ and the Mean Squares Within (MS_W) = SS_{Within}/df_{Within}

Performing an analysis of variance (ANOVA) on the data in *Table 1* leads to the ANOVA table (*Table 1A*). Because there were an equal number of replicates per run in the precision study, values for Variance$_{Run}$ and Variance$_{Rep}$ can be derived from the ANOVA table in a straightforward manner. The equations below calculate the variability associated with both the runs and the replicates where the MS$_{within}$ represents the "error" or "within-run" mean square, and MS$_{between}$ represents the "between-run" mean square.

$$\text{Variance}_{Rep} = \text{MS}_{within} = 0.102$$

$$\text{Variance}_{Run} = \frac{\text{MS}_{between} - \text{MS}_{within}}{\# \text{ of reps per run}} = \frac{3.550 - 0.102}{3} = 1.149$$

[NOTE—It is common practice to use a value of 0 for Variance$_{Run}$ when the calculated value is negative.] Estimates can still be obtained with unequal replication, but the formulas are more complex. Many statistical software packages can easily handle unequal replication. Studying the relative magnitude of the two variance components is important when designing and interpreting a precision study. The insight gained can be used to focus any ongoing procedure improvement effort and, more important, it can be used to ensure that procedures are capable of supporting their intended uses. By carefully defining what constitutes a result (i.e., reportable value), one harnesses the power of averaging to achieve virtually any desired precision. That is, by basing the reportable value on an average across replicates and/or runs, rather than on any single result, one can reduce the %RSD, and reduce it in a predictable fashion.

Table 2 shows the computed variance and %RSD of the mean (i.e., of the reportable value) for different combinations of number of runs and number of replicates per run using the following formulas:

$$\text{Variance of the mean} =$$

$$\frac{\text{Variance}_{Run}}{(\# \text{ of runs})} + \frac{\text{Variance}_{rep}}{(\# \text{ of runs})(\# \text{ of reps per run})}$$

$$\text{Standard deviation of the mean} = \sqrt{\text{Variance of the mean}}$$

$$\text{RSD} = \frac{\text{Standard deviation of the mean}}{\text{Average of all results}} \times 100\%$$

For example, the *Variance of the mean, Standard deviation of the mean*, and %RSD of a test involving two runs and three replicates per each run are 0.592, 0.769, and 0.76% respectively, as shown below.

$$\text{Variance of the mean} = \frac{1.149}{2} + \frac{0.102}{(2 \cdot 3)} = 0.592$$

$$\text{Standard deviation of the mean} = \sqrt{0.592} = 0.769$$

$$\text{RSD} = (0.769/100.96) \times 100\% = 0.76\%$$

where 100.96 is the mean for all the data points in *Table 1*. As illustrated in *Table 2*, increasing the number of runs from one to two provides a more dramatic reduction in the variability of the reportable value than does increasing the number of replicates per run.

No distributional assumptions were made on the data in *Table 1*, as the purpose of this Appendix is to illustrate the calculations involved in a precision study.

Table 2. The Predicted Impact of the Test Plan (No. of Runs and No. of Replicates per Run) on the Precision of the Mean

No. of Runs	No. of Replicates per Run	Variance of the Mean	SD of the Mean	% RSD[a]
1	1	1.251	1.118	1.11
1	2	1.200	1.095	1.09
1	3	1.183	1.088	1.08
2	1	0.625	0.791	0.78
2	2	0.600	0.775	0.77
2	3	0.592	0.769	0.76

[a] A mean value of 100.96, based on the 15 data points presented in *Table 1,* was used (as the divisor) to compute the %RSD.

APPENDIX C: EXAMPLES OF OUTLIER TESTS FOR ANALYTICAL DATA

Given the following set of 10 measurements: 100.0, 100.1, 100.3, 100.0, 99.7, 99.9, 100.2, 99.5, 100.0, and 95.7 (mean = 99.5, standard deviation = 1.369), are there any outliers?

Generalized Extreme Studentized Deviate (ESD) Test

This is a modified version of the ESD Test that allows for testing up to a previously specified number, r, of outliers from a normally distributed population. For the detection of a single outlier ($r = 1$), the generalized ESD procedure is also known as Grubb's test. Grubb's test is not recommended for the detection of multiple outliers. Let r equal 2, and n equal 10.

Stage 1 (n = 10)—Normalize each result by subtracting the mean from each value and dividing this difference by the standard deviation (see *Table 3*).[4]

Table 3. Generalized ESD Test Results

	n = **10**		*n* = **9**	
	Data	**Normalized**	**Data**	**Normalized**
	100.3	+0.555	100.3	+1.361
	100.2	+0.482	100.2	+0.953
	100.1	+0.409	100.1	+0.544
	100.0	+0.336	100.0	+0.136
	100.0	+0.336	100.0	+0.136
	100.0	+0.336	100.0	+0.136
	99.9	+0.263	99.9	−0.272
	99.7	+0.117	99.7	−1.089
	99.5	−0.029	99.5	−1.905
	95.7	−2.805		
Mean =	99.54		99.95	
SD =	1.369		0.245	

Take the absolute value of these results, select the maximum value ($R_1 = 2.805$), and compare it to a previously specified tabled critical value λ_1 (2.290) based on the selected significance level (for example, 5%). The maximum value is larger than the tabled value and is identified as being inconsistent with the remaining data. Sources for λ-values are included in many statistical textbooks. Caution should be exercised when using any statistical table to ensure that the correct notations (i.e., level of acceptable error) are used when extracting table values.

Stage 2 (n = 9)—Remove the observation corresponding to the maximum absolute normalized result from the original data set, so that n is now 9. Again, find the mean and standard deviation (*Table 3*, right two columns), normalize each value, and take the absolute value of these results. Find the maximum of the absolute values of the 9 normalized results ($R_2 = 1.905$), and compare it to λ_2 (2.215). The maximum value is not larger than the tabled value.

Conclusion—The result from the first stage, 95.7, is declared to be an outlier, but the result from the second stage, 99.5, is not an outlier.

Dixon-Type Tests

Dixon's Test can be one-sided or two-sided, depending on an a priori decision as to whether outliers will be considered on one side only. As with the ESD Test, Dixon's Test assumes that the data, in the absence of outliers, come from a single normal population. Following the strategy used for the ESD Test, we proceed as if there were no a priori decision as to side, and so use a two-sided Dixon's Test. From examination of the example data, we see that it is the two smallest that are to be tested as outliers. Dixon provides for testing for two outliers simultaneously; however, these procedures are beyond the scope of this Appendix. The stepwise procedure discussed below is not an exact procedure for testing for the second outlier, because the result of the second test is conditional upon the first. And because the sample size is also reduced in the second stage, the end result is a procedure that usually lacks the sensitivity of Dixon's exact procedures.

Stage 1 (n = 10)—The results are ordered on the basis of their magnitude (i.e., X_n is the largest observation, X_{n-1} is the second largest, etc., and X_1 is the smallest observation). Dixon's Test has different ratios based on the sample size (in this example, with $n = 10$), and to declare X_1 an outlier, the following ratio, r_{11}, is calculated by the formula:

$$r_{11} = \frac{X_2 - X_1}{X_{n-1} - X_1}$$

A different ratio would be employed if the largest data point was tested as an outlier. The r_{11} result is compared to an $r_{11,\,0.05}$ value in a table of critical values. If r_{11} is greater than $r_{11,\,0.05}$, then it is declared an outlier. For the above set of data, $r_{11} = (99.5 - 95.7)/(100.2 - 95.7) = 0.84$. This ratio is greater than $r_{11,\,0.05}$, which is 0.52979 at the 5% significance level for a two-sided Dixon's Test. Sources for $r_{11,\,0.05}$ values are included in many statistical textbooks.[5]

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[4] The difference between each value and the mean is termed the residual. Other Studentized residual outlier tests exist where the residual, instead of being divided by the standard deviation, can be divided by the standard deviation times the square root of $n - 1$ divided by n.
[5] The critical values for r in this example are taken from Reference 2 in *Appendix G, Outlier Tests*.

Stage 2—Remove the smallest observation from the original data set, so that *n* is now 9. The same r_{11} equation is used, but a new critical $r_{11,\,0.05}$ value for *n* = 9 is needed ($r_{11,\,0.05}$ = 0.56420). Now r_{11} = (99.7 − 99.5)/(100.2 − 99.5) = 0.29, which is less than $r_{11,\,0.05}$ and not significant at the 5% level.

Conclusion—Therefore, 95.7 is declared to be an outlier but 99.5 is not an outlier.

Hampel's Rule

Step 1—The first step in applying Hampel's Rule is to normalize the data. However, instead of subtracting the mean from each data point and dividing the difference by the standard deviation, the median is subtracted from each data value and the resulting differences are divided by MAD (see below). The calculation of MAD is done in three stages. First, the median is subtracted from each data point. Next, the absolute values of the differences are obtained. These are called the *absolute deviations*. Finally, the median of the absolute deviations is calculated and multiplied by the constant 1.483 to obtain MAD.[6]

Step 2—The second step is to take the absolute value of the normalized data. Any such result that is greater than 3.5 is declared to be an outlier. *Table 4* summarizes the calculations.

The value of 95.7 is again identified as an outlier. This value can then be removed from the data set and Hampel's Rule re-applied to the remaining data. The resulting table is displayed as *Table 5*. Similar to the previous examples, 99.5 is not considered an outlier.

Table 4. Test Results Using Hampel's Rule

		Deviations from the Median	Absolute Deviations	Absolute Normalized
	Data			
	100.3	0.3	0.3	1.35
	100.2	0.2	0.2	0.90
	100.1	0.1	0.1	0.45
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	−0.1	0.1	0.45
	99.7	−0.3	0.3	1.35
	99.5	−0.5	0.5	2.25
	95.7	−4.3	4.3	19.33
Median =	100		0.15	
MAD =			0.22	

n = **10** (header spanning)

Table 5. Test Results of Re-Applied Hampel's Rule

		Deviations from the Median	Absolute Deviations	Absolute Normalized
	Data			
	100.3	0.3	0.3	2.02
	100.2	0.2	0.2	1.35
	100.1	0.1	0.1	0.67
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	−0.1	0.1	0.67
	99.7	−0.3	0.3	2.02
	99.5	−0.5	0.5	3.37
Median =	100		0.1	
MAD =			0.14	

n = **9** (header spanning)

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[6] Assuming an underlying normal distribution, 1.483 is a constant used so that the resulting MAD is a consistent estimator of the population standard deviation. This means that as the sample size gets larger, MAD gets closer to the population standard deviation.

APPENDIX D: COMPARISON OF PROCEDURES—PRECISION

The following example illustrates the calculation of a 90% confidence interval for the ratio of (true) variances for the purpose of comparing the precision of two procedures. It is assumed that the underlying distribution of the sample measurements are well-characterized by normal distributions. For this example, assume the laboratory will accept the alternative procedure if its precision (as measured by the variance) is no more than four-fold greater than that of the current procedure.

To determine the appropriate sample size for precision, one possible method involves a trial and error approach using the following formula:

$$\text{Power} = \Pr\left[F > \frac{1}{4}F_{\alpha,\, n-1,\, n-1}\right]$$

where n is the smallest sample size required to give the desired power, which is the likelihood of correctly claiming the alternative procedure has acceptable precision when in fact the two procedures have equal precision; α is the risk of wrongly claiming the alternative procedure has acceptable precision; and the 4 is the allowed upper limit for an increase in variance. F-values are found in commonly available tables of critical values of the F-distribution. $F_{\alpha,\, n-1,\, n-1}$ is the upper α percentile of an F-distribution with $n-1$ numerator and $n-1$ denominator degrees of freedom; that is, the value exceeded with probability α. Suppose initially the laboratory guessed a sample size of 11 per procedure was necessary (10 numerator and denominator degrees of freedom); the power calculation would be as follows:[7]

$$\Pr\left[F > {}^1\!/_4 F_{\alpha,\, n-1,\, n-1}\right] = \Pr\left[F > {}^1\!/_4 F_{.05,\, 10,\, 10}\right]$$
$$= \Pr\left[F > (2.978/4)\right] = 0.6751$$

In this case the power was only 68%; that is, even if the two procedures had exactly equal variances, with only 11 samples per procedure, there is only a 68% chance that the experiment will lead to data that permit a conclusion of no more than a four-fold increase in variance. Most commonly, sample size is chosen to have at least 80% power, with choices of 90% power or higher also used. To determine the appropriate sample size, various numbers can be tested until a probability is found that exceeds the acceptable limit (e.g., power >0.90). For example, the power determination for sample sizes of 12–20 are displayed in *Table 6*. In this case, the initial guess at a sample size of 11 was not adequate for comparing precision, but 15 samples per procedure would provide a large enough sample size if 80% power were desired, or 20 per procedure for 90% power.

Table 6. Power Determinations for Various Sample Sizes (Specific to the Example in *Appendix D***)**

Sample Size	$\Pr[F > {}^1\!/_4\, F_{0.05,\, n-1,\, n-1}]$
12	0.7145
13	0.7495
14	0.7807
15	0.8083
16	0.8327
17	0.8543
18	0.8732
19	0.8899
20	0.9044

Typically the sample size for precision comparisons will be larger than for accuracy comparisons. If the sample size for precision is so large as to be impractical for the laboratory to conduct the study, there are some options. The first is to reconsider the choice of an allowable increase in variance. For larger allowable increases in variance, the required sample size for a fixed power will be smaller. Another alternative is to plan an interim analysis at a smaller sample size, with the possibility of proceeding to a larger sample size if needed. In this case, it is strongly advisable to seek professional help from a statistician.

Now, suppose the laboratory opts for 90% power and obtains the results presented in *Table 7* based on the data generated from 20 independent runs per procedure.

$$\text{Ratio} = \text{alternative procedure variance/current procedure variance} = 45.0/25.0 = 1.8$$

$$\text{Lower limit of confidence interval} = \text{ratio}/F_{.05} = 1.8/2.168 = 0.83$$

$$\text{Upper limit of confidence interval} = \text{ratio}/F_{.95} = 1.8/0.461 = 3.90$$

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[7] This could be calculated using a computer spreadsheet. For example, in Microsoft® Excel the formula would be: FDIST((R/A)*FINV(alpha, $n-1$, $n-1$), $n-1$, $n-1$), where R is the ratio of variances at which to determine power (e.g., $R = 1$, which was the value chosen in the power calculations provided in *Table 6*) and A is the maximum ratio for acceptance (e.g., $A = 4$). Alpha is the significance level, typically 0.05.

Table 7. Example of Measures of Variance for Independent Runs (Specific to the Example in *Appendix D***)**

Procedure	Variance (standard deviation)	Sample Size	Degrees of Freedom
Alternative	45.0 (6.71)	20	19
Current	25.0 (5.00)	20	19

For this application, a 90% (*two*-sided) confidence interval is used when a 5% *one*-sided test is sought. The test is one-sided, because only an increase in standard deviation of the alternative procedure is of concern. Some care must be exercised in using two-sided intervals in this way, as they must have the property of equal tails—most common intervals have this property. Because the one-side upper confidence limit, 3.90, is less than the allowed limit, 4.0, the study has demonstrated that the alternative procedure has acceptable precision. If the same results had been obtained from a study with a sample size of 15— as if 80% power had been chosen—the laboratory would not be able to conclude that the alternative procedure had acceptable precision (upper confidence limit of 4.47).

APPENDIX E: COMPARISON OF PROCEDURES—DETERMINING THE LARGEST ACCEPTABLE DIFFERENCE, δ, BETWEEN TWO PROCEDURES

This Appendix describes one approach to determining the difference, δ, between two procedures (alternative-current), a difference that, if achieved, still leads to the conclusion of equivalence between the two procedures. Without any other prior information to guide the laboratory in the choice of δ, it is a reasonable way to proceed. Sample size calculations under various scenarios are discussed in this Appendix.

Tolerance Interval Determination

Suppose the process mean and the standard deviation are both unknown, but a sample of size 50 produced a mean and standard deviation of 99.5 and 2.0, respectively. These values were calculated using the last 50 results generated by this specific procedure for a particular (control) sample. Given this information, the tolerance limits can be calculated by the following formula:

$$\bar{x} \pm K_s$$

in which $\bar{x}$ is the mean; s is the standard deviation; and K is based on the level of confidence, the proportion of results to be captured in the interval, and the sample size, n. Tables providing K values are available. In this example, the value of K required to enclose 95% of the population with 95% confidence for 50 samples is 2.382.[8] The tolerance limits are calculated as follows:

$$99.5 \pm 2.382 \times 2.0$$

hence, the tolerance interval is (94.7, 104.3).

Comparison of the Tolerance Limits to the Specification Limits

Assume the specification interval for this procedure is (90.0, 110.0) and the process mean and standard deviation have not changed since this interval was established. The following quantities can be defined: the lower specification limit (LSL) is 90.0, the upper specification limit (USL) is 110.0, the lower tolerance limit (LTL) is 94.7, and the upper tolerance limit (UTL) is 104.3. Calculate the acceptable difference, (δ), in the following manner:

$$A = LTL - LSL \text{ for } LTL \geq LSL$$

$$(A = 94.7 - 90.0 = 4.7);$$

$$B = USL - UTL \text{ for } USL \geq UTL$$

$$(B = 110.0 - 104.3 = 5.7); \text{ and}$$
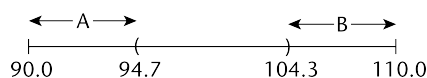
$$\delta = \text{minimum } (A, B) = 4.7$$

Figure 2. A graph of the quantities calculated above.

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[8] There are existing tables of tolerance factors that give approximate values and thus differ slightly from the values reported here.

With this choice of δ, and assuming the two procedures have comparable precision, the confidence interval for the difference in means between the two procedures (alternative-current) should fall within −4.7 and +4.7 to claim that no important difference exists between the two procedures.

Quality control analytical laboratories sometimes deal with 99% tolerance limits, in which cases the interval will widen. Using the previous example, the value of *K* required to enclose 99% of the population with 99% confidence for 50 samples is 3.390. The tolerance limits are calculated as follows:

$$99.5 \pm 3.390 \times 2.0$$

The resultant wider tolerance interval is (92.7, 106.3). Similarly, the new LTL of 92.7 and UTL of 106.3 would produce a smaller δ:

$$A = LTL - LSL \text{ for } LTL \geq LSL$$

$$(A = 92.7 - 90.0 = 2.7);$$

$$B = USL - UTL \text{ for } USL \geq UTL$$

$$(B = 110.0 - 106.3 = 3.7); \text{ and}$$

$$\delta = \text{minimum } (A, B) = 2.7$$

Though a manufacturer may choose any δ that serves adequately in the determination of equivalence, the choice of a larger δ, while yielding a smaller *n*, may risk a loss of capacity for discriminating between procedures.

Sample Size

Formulas are available that can be used for a specified δ, under the assumption that the population variances are known and equal, to calculate the number of samples required to be tested per procedure, *n*. The level of confidence and power must also be specified. [NOTE—Power refers to the probability of correctly concluding that two identical procedures are equivalent.] For example, if δ = 4.7, and the two population variances are assumed to equal 4.0, then, for a 5% level test[9] and 80% power (with associated *z*-values of 1.645 and 1.282, respectively), the sample size is approximated by the following formula:

$$n \geq \frac{2\sigma^2}{\delta^2}\left(z_\alpha + z_{\beta/2}\right)^2$$

$$n \geq \frac{2(4)}{(4.7)^2}(1.645 + 1.282)^2 = 3.10$$

Thus, assuming each procedure has a population variance, σ^2, of 4.0, the number of samples, *n*, required to conclude with 80% probability that the two procedures are equivalent (90% confidence interval for the difference in the true means falls between −4.7 and +4.7) when in fact they are identical (the true mean difference is zero) is 4. Because the normal distribution was used in the above formula, 4 is actually a lower bound on the needed sample size. If feasible, one might want to use a larger sample size. Values for *z* for common confidence levels are presented in *Table 8*. The formula above makes three assumptions: 1) the variance used in the sample size calculation is based on a sufficiently large amount of prior data to be treated as known; 2) the prior known variance will be used in the analysis of the new experiment, or the sample size for the new experiment is sufficiently large so that the normal distribution is a good approximation to the *t* distribution; and 3) the laboratory is confident that there is no actual difference in the means, the most optimistic case. It is not common for all three of these assumptions to hold. The formula above should be treated most often as an initial approximation. Deviations from the three assumptions will lead to a larger required sample size. In general, we recommend seeking assistance from someone familiar with the necessary methods.

Table 8. Common Values for a Standard Normal Distribution

Confidence Level	z-Values	
	One-sided (α)	Two-sided (α/2)
99%	2.326	2.576
95%	1.645	1.960
90%	1.282	1.645
80%	0.842	1.282

When a log transformation is required to achieve normality, the sample size formula needs to be slightly adjusted as shown below. Instead of formulating the problem in terms of the population variance and the largest acceptable difference, δ, be-

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[9] When testing equivalence, a 5% level test corresponds to a 90% confidence interval.

tween the two procedures, it now is formulated in terms of the population %RSD and the largest acceptable proportional difference between the two procedures.

$$n \geq \frac{2\sigma_L^2}{\delta_L^2} \left(z_\alpha + z_{\beta/2} \right)^2$$

where

$$\sigma_L^2 = \log\left((\%RSD/100)^2 + 1 \right)$$

$$\delta_L^2 = \left(\log(\rho + 1) \right)^2$$

and ρ represents the largest acceptable proportional difference between the two procedures ((alternative-current)/current), and the population %RSDs are assumed known and equal.

APPENDIX F: EQUIVALENCE TESTING AND TOST

In classical statistical hypothesis testing, there are two hypotheses, the null and the alternative. For example, the null may be that two means are equal and the alternative that they differ. With this classical approach, one rejects the null hypothesis in favor of the alternative if the evidence is sufficient against the null. A common error is to interpret failure to reject the null as evidence that the null is true. Actually, failure to reject the null just means the evidence against the null was not sufficient. For example, the procedure used could have been too variable or the number of determinations too small.

The consequence of this understanding is that, when one seeks to demonstrate similarity, such as results from two laboratories, then one needs similarity as the alternative hypothesis. A statistical test for an alternative hypothesis of similarity is referred to as an equivalence test. It is important to understand that "equivalence" does not mean "equality." Equivalence should be understood as "sufficiently similar" for the purposes of the laboratory(ies). As noted earlier in this chapter, how close is close enough is something to be decided a priori.

As a specific example, suppose we are interested in comparing average results, such as when transferring a procedure from one laboratory to another. (Such an application would also likely include a comparison of precision; see *Appendix D*.) A priori, we determine that the means need to differ by no more than some positive value, δ, to be considered equivalent or sufficiently similar. (*Appendix E* provides some guidance on choosing δ.) Our hypotheses are then:

Alternative (H_1): $|\mu_1 - \mu_2| \leq \delta$

Null (H_0): $|\mu_1 - \mu_2| > \delta$

where μ_1 and μ_2 are the two means being compared.

The two one-sided tests (TOST) approach is to convert the above equivalence hypotheses into two one-sided hypotheses. The rationale is that one can conclude $|\mu_1 - \mu_2| \leq \delta$ if one can demonstrate *both*

$$\mu_1 - \mu_2 \leq +\delta \text{ and } \mu_1 - \mu_2 \geq -\delta$$

As one-sided tests, they can be addressed with standard one-sided *t*-tests. In order for the test of the equivalence hypotheses to be of level α, both one-sided tests are conducted at level α (typically, but not necessarily, 0.05). Often, the two one-sided test is performed using a confidence interval. In this case, reject the null in favor of the equivalence hypothesis if the $100(1 - 2\alpha)\%$ two-sided confidence interval is entirely contained in $(-\delta, +\delta)$. This is the approach described earlier in the *Accuracy* section.

APPENDIX G: ADDITIONAL SOURCES OF INFORMATION

There may be a variety of statistical tests that can be used to evaluate any given set of data. This chapter presents several tests for interpreting and managing analytical data, but many other similar tests could also be employed. The chapter simply illustrates the analysis of data using statistically acceptable methods. As mentioned in the *Introduction*, specific tests are presented for illustrative purposes, and USP does not endorse any of these tests as the sole approach for handling analytical data. Additional information and alternative tests can be found in the references listed below or in many statistical textbooks.

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⟨1092⟩ THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

INTRODUCTION

Purpose

The Dissolution Procedure: Development and Validation ⟨1092⟩ provides a comprehensive approach covering items to consider for developing and validating dissolution procedures and the accompanying analytical procedures. It addresses the use of automation throughout the test and provides guidance and criteria for validation. It also addresses the treatment of the data generated and the interpretation of acceptance criteria for immediate- and modified-release solid oral dosage forms.

Scope

Chapter⟨1092⟩ addresses the development and validation of dissolution procedures, with a focus on solid oral dosage forms. Many of the concepts presented, however, may be applicable to other dosage forms and routes of administration. General recommendations are given with the understanding that modifications of the apparatus and procedures as given in *USP* general chapters need to be justified.

The organization of ⟨1092⟩ follows the sequence of actions often performed in the development and validation of a dissolution test. The sections appear in the following sequence.

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/DISSOLUTION METHOD DEVELOPMENT)
    1.1 Performing Filter Compatibility
    1.2 Determining Solubility and Stability of Drug Substance in Various Media
    1.3 Choosing a Medium and Volume
    1.4 Choosing an Apparatus
2. METHOD DEVELOPMENT
    2.1 Deaeration
    2.2 Sinkers
    2.3 Agitation
    2.4 Study Design
        2.4.1 Time Points
        2.4.2 Observations
        2.4.3 Sampling
        2.4.4 Cleaning
    2.5 Data Handling
    2.6 Dissolution Procedure Assessment
3. ANALYTICAL FINISH
    3.1 Sample Processing
    3.2 Filters
    3.3 Centrifugation
    3.4 Analytical Procedure
    3.5 Spectrophotometric Analysis
    3.6 HPLC
4. AUTOMATION
    4.1 Medium Preparation
    4.2 Sample Introduction and Timing
    4.3 Sampling and Filtration
    4.4 Cleaning
    4.5 Operating Software and Computation of Results
    4.6 Common Deviations from the Compendia Procedures That May Require Validation
5. VALIDATION
    5.1 Specificity/Placebo Interference
    5.2 Linearity and Range
    5.3 Accuracy/Recovery
    5.4 Precision
        5.4.1 Repeatability of Analysis
        5.4.2 Intermediate Precision/Ruggedness
        5.4.3 Reproducibility

1. PRELIMINARY ASSESSMENT (FOR EARLY STAGES OF PRODUCT DEVELOPMENT/ DISSOLUTION METHOD DEVELOPMENT)

Before method development can begin, it is important to characterize the molecule so that the filter, medium, volume of medium, and apparatus can be chosen properly in order to evaluate the performance of the dosage form.

1.1 Performing Filter Compatibility

Filtration is a key sample-preparation step in achieving accurate test results. The purpose of filtration is to remove undissolved drug and excipients from the withdrawn solution. If not removed from the sample solution, particles of the drug will continue to dissolve and can bias the results. Therefore, filtering the dissolution samples is usually necessary and should be done immediately if the filter is not positioned on the cannula.

Filtration also removes insoluble excipients that may otherwise interfere with the analytical finish. Selection of the proper filter material is important and should be accomplished, and experimentally justified, early in the development of the dissolution procedure. Important characteristics to consider when choosing a filter material are type, filter size, and pore size. The filter that is selected based on evaluation during the early stages of dissolution procedure development may need to be reconsidered at a later time point. Requalification has to be considered after a change in composition of the drug product or changes in the quality of the ingredients (e.g. particle size of microcrystalline cellulose).

Examples of filters used in dissolution testing can be cannula filters, filter disks or frits, filter tips, or syringe filters. The filter material has to be compatible with the media and the drug. Common pore sizes range from 0.20 to 70 μm, however, filters of other pore sizes can be used as needed. If the drug substance particle size is very small (e.g., micronized or nanoparticles), it can be challenging to find a filter pore size that excludes these small particles.

Adsorption of the drug(s) by the filter may occur and needs to be evaluated. Filter materials will interact with dissolution media to affect the recovery of the individual solutes and must be considered on a case-by-case basis. Different filter materials exhibit different drug-binding properties. Percentage of drug loss from the filtrate due to binding may be dependent on the drug concentration. Therefore the adsorptive interference should be evaluated on sample solutions at different concentrations bracketing the expected concentration range. Where the drug adsorption is saturable, discarding an initial volume of filtrate may allow the collection of a subsequent solution that approaches the original solution concentration. Alternative filter materials that minimize adsorptive interference can usually be found. Prewetting of the filter with the medium may be necessary. In addition, it is important that leachables from the filter do not interfere with the analytical procedure. This can be evaluated by analyzing the filtered dissolution medium and comparing it with the unfiltered medium.

The filter size should be based on the volume to be withdrawn and the amount of particles to be separated. Use of the correct filter dimensions will improve throughput and recovery, and also reduce clogging. Use of a large filter for small-volume filtration can lead to loss of sample through hold-up volume, whereas filtration through small filter sizes needs higher pressures and longer times, and the filters can clog quickly.

Filters used for USP Apparatus 4 need special attention because they are integrated in the flow-through process. Undissolved particles may deposit on the filters, creating resistance to the flow.

In the case of automated systems, selection of the filter with regard to material and pore size can be done in a similar manner to manual filtration. Flow rate through the filter and clogging may be critical for filters used in automated systems. Experimental verification that a filter is appropriate may be accomplished by comparing the responses for filtered and unfiltered standard and sample solutions. This is done by first preparing a suitable standard solution and a sample solution. For example, prepare a typical dissolution sample in a beaker and stir vigorously with a magnetic stirrer to dissolve the drug load completely. For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

1.2 Determining Solubility and Stability of Drug Substance in Various Media

Physical and chemical characteristics of the drug substance need to be determined as part of the process of selecting the proper dissolution medium. When deciding the composition of the medium for dissolution testing, it is important to evaluate the influence of buffers, pH, and if needed, different surfactants on the solubility and stability of the drug substance. Solubility of the drug substance is usually evaluated by determining the saturation concentration of the drug in different media at 37° using the shake-flask solubility method (equilibrium solubility). To level out potential ion effects between the drug and the buffers used in the media, mixtures of hydrochloric acid and sodium hydroxide are used to perform solubility investigations; this is in addition to the typical buffer solutions. In certain cases, it may be necessary to evaluate the solubility of the drug at temperatures other than 37° (i.e., 25°). The pH of the clear supernatant should be checked to determine whether the pH changes during the solubility test. Alternative approaches for solubility determination may also be used.

Typical media for dissolution may include the following (not listed in order of preference): diluted hydrochloric acid, buffers (phosphate or acetate) in the physiologic pH range of 1.2–7.5, simulated gastric or intestinal fluid (with or without enzymes), and water. For some drugs, incompatibility of the drug with certain buffers or salts may influence the choice of buffer. The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

Aqueous solutions (acidic or buffer solutions) may contain a percentage of a surfactant [e.g., sodium dodecyl sulfate (SDS), polysorbate, or lauryldimethylamine oxide] to enhance the solubility of the drug. The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC). *Table 1* shows a list of some of the surfactants used in dissolution media. Approximate CMC values are provided with references when available. The list is not comprehensive and is not intended to exclude surfactants that are not listed. Other substances, such as hydroxypropyl β-cyclodextrin, have been used as dissolution media additives to enhance dissolution of poorly soluble compounds. The U.S. Food and Drug Administration (FDA) maintains a database of dissolution methods, including information on dissolution media that have been used [1]. Typically, the amount of surfactant added is sufficient to achieve sink conditions in the desired volume of dissolution medium.

It is important to control the grade and purity of surfactants because use of different grades could affect the solubility of the drug. For example, SDS is available in both a technical grade and a high-purity grade. Obtaining polysorbate 80 from different sources can affect its suitability when performing high-performance liquid chromatography (HPLC) analysis.

There may be effects of counter-ions or pH on the solubility or solution stability of the surfactant solutions. For example, a precipitate forms when the potassium salt for the phosphate buffer is used at a concentration of 0.5 M in combination with SDS. This can be avoided by using the sodium phosphate salt when preparing media with SDS.

Table 1. Commonly Used Surfactants with Critical Micelle Concentrations

	Surfactant	CMC (% wt/volume)	Reference
Anionic	Sodium dodecyl sulfate (SDS), Sodium lauryl sulfate (SLS)	0.18%–0.23%	(2–4)
	Taurocholic acid sodium salt	0.2%	(3)
	Cholic acid sodium salt	0.16%	(3)
	Desoxycholic acid sodium salt	0.12%	(3)
Cationic	Cetyltrimethyl ammonium bromide (CTAB, Hexadecyltrimethylammonium bromide)	0.033%–0.036% (0.92–1.0 mM)	(5,6)
	Benzethonium chloride (Hyamine 1622)	0.18% (4 mM)	(2)
Nonionic	Polysorbate 20 (Polyoxyethylene (20) sorbitan monolaurate, Tween 20)	0.07%–0.09%	(3,7)
	Polysorbate 80 (Polyoxyethylene (20) sorbitan monooleate, Tween 80)	0.02%–0.08%	(3,7)
	Caprylocaproyl polyoxyl-8 glycerides (Labrasol)	0.01%	(4)
	Polyoxyl 35 castor oil (Cremophor EL)	0.02%	(8)
	Polyoxyethylene 23 lauryl ether (Brij 35)	0.013%	(9)
	Octoxinol (Triton X-100)	0.01%–0.03%	(3,10)
Zwitterion	Lauryldimethylamine *N*-oxide (LDAO)	0.023%	(11)

Routinely, the dissolution medium is buffered; however, the use of purified water as the dissolution medium is suitable for products with a dissolution behavior independent of the pH of the medium. There are several reasons why purified water may not be preferred. The water quality can vary depending on its source, and the pH of the water is not as strictly controlled as the pH of buffer solutions. Additionally, the pH can vary from day to day and can also change during the run, depending on the drug substance and excipients. Use of an aqueous–organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Investigations of the stability of the drug substance should be carried out, when needed, in the selected dissolution medium with excipients present, at 37°. This elevated temperature has the potential to decrease solution stability (degradation). Stabili-

ty should allow for sufficient time to complete or repeat the analytical procedure. Physical stability may be of concern when precipitation occurs because of lower solubility at room or refrigerated temperature.

1.3 Choosing a Medium and Volume

When developing a dissolution procedure, one goal is to have sink conditions, which are defined as having a volume of medium at least three times the volume required to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is appropriately justified. The composition and volume of dissolution medium are guided by the solubility investigations. For example, the choice and concentration of a surfactant need to be justified from the solubility data and the dissolution profiles.

The use of enzymes in the dissolution medium is permitted, in accordance with *Dissolution* ⟨711⟩, when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products. A discussion of the phenomenon of cross-linking and method development using enzymes can be found in *Capsules–Dissolution Testing and Related Quality Attributes* ⟨1094⟩. Validation should be performed with the method using enzymes according to section *5. Validation*.

Another option is to use media that follow more closely the composition of fluids in the stomach and intestinal tract. These media may contain physiological surface-active ingredients, such as taurocholates. The media also may contain emulsifiers (lecithin) and components such as saline solution that increase osmolality. Also, the ionic strength or molarity of the buffer solutions may be manipulated. The media are designed to represent the fed and fasted state in the stomach and small intestine. These media may be very useful in modeling in vivo dissolution behavior of immediate-release (IR) dosage forms, in particular those containing lipophilic drug substances, and may help in understanding the dissolution kinetics of the product related to the physiological make-up of the digestive fluids. Results of successful modeling of dissolution kinetics have been published, mainly for IR products. In the case of extended-release dosage forms with reduced effect of the drug substance on dissolution behavior, the use of such media needs to be evaluated differently. In vitro performance testing does not necessarily require media modeling the fasted and postprandial states *(12,13)*.

An acid stage is part of the testing of delayed-release products by *Method A* or *Method B* in ⟨711⟩. For drugs with acid solubility less than 10% of the label claim or drugs that degrade in acid the usefulness of the acid stage in detecting a coating failure is compromised. This would be handled on a case-by-case basis. Possible resolutions include the addition of surfactant to the acid stage, or adjustment of the specifications.

During selection of the dissolution medium, care should be taken to ensure that the drug substance is suitably stable throughout the analysis. In some cases, antioxidants such as ascorbic acid may be used in the dissolution medium to stabilize the drug. There are occasions where such actions are not sufficient. For compounds that rapidly degrade to form a stable degradant, monitoring the degradant alone or in combination with a drug substance may be more suitable than analyzing only the drug substance. In situ spectroscopic techniques tend to be less affected by degradation when compared with HPLC analysis (including UHPLC and other liquid chromatographic approaches).

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium can vary from 500 to 1000 mL. Usually, the volume needed for the dissolution test can be determined in order to maintain sink conditions. In some cases, the volume can be increased to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this approach is expected. In practice, the volume of the dissolution medium is usually maintained within the compendial range given above. Alternatively, it may be preferable to switch to other compendial apparatus, such as a reciprocating cylinder (Apparatus 3), reciprocating holder (Apparatus 7), or flow-through cell (Apparatus 4). Certain applications may require low volumes of dissolution media (e.g., 100–200 mL) when the use of a paddle or basket is preferred. In these cases, an alternative, noncompendial apparatus (e.g., small-volume apparatus) may be used.

1.4 Choosing an Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. In general, a compendial apparatus should be selected.

For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used. Apparatus 3 (reciprocating cylinder) has been found especially useful for chewable tablets, soft gelatin capsules, delayed-release dosage forms, and nondisintegrating-type products, such as coated beads. Apparatus 4 (flow-through cell) may offer advantages for modified-release dosage forms and immediate-release dosage forms that contain active ingredients with limited solubility. In addition, Apparatus 4 may have utility for multiple dosage form types such as soft gelatin capsules, beaded products, suppositories, or depot dosage forms, as well as suspension-type extended-release dosage forms. Apparatus 5 (paddle over disk) and Apparatus 6 (rotating cylinder) are useful for evaluating and testing transdermal dosage forms. Apparatus 7 (reciprocating holder) has application to non-disintegrating, oral modified-release dosage forms, stents, and implants, as well as transdermal dosage forms. For semisolid dosage forms, the generally used apparatus include the vertical diffusion cell, immersion cell, and flow-through cell apparatus with the insert for topical dosage forms (see *Semisolid Drug Products—Performance Tests* ⟨1724⟩).

Some changes can be made to the compendial apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10-, 20-, or 80-mesh) may be used when the need is clearly documented by supporting data. Care must be taken that baskets are uniform and meet the dimensional requirements specified in ⟨711⟩.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. A rotating bottle or dialysis tubes may have utility for microspheres and implants, peak vessels, and modified flow-through cells for special dosage forms including powders and stents.

2. METHOD DEVELOPMENT

A properly designed test should yield data that are not highly variable, and should be free of significant stability problems. High variability in the results can make it difficult to identify trends or effects of formulation changes. Sample size can affect the observed variability. One guidance defines dissolution results as highly variable if the relative standard deviation (RSD) is more than 20% at time points of 10 min or less and more than 10% at later time points for a sample size of 12 *(14)*. However, during method development, smaller sample sizes may be used, and the analyst will need to make a judgment accordingly. Most dissolution results, however, exhibit less variability. In the development of a dissolution procedure the source of the variability should be investigated, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing any of the following factors: the apparatus type, speed of agitation, level of deaeration, sinker type, or composition of the medium.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences.

2.1 Deaeration

The significance of deaeration of the dissolution medium should be determined because air bubbles can act as a barrier to the dissolution process if present on the dosage unit or basket mesh and can adversely affect the reliability of the test results. Furthermore, bubbles can cause particles to cling to the apparatus and vessel walls. Bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. Poorly soluble drugs are most sensitive to interference from air bubbles; therefore, deaeration may be needed when testing these types of products. A deaeration method is described as a footnote in the *Procedure* section of ⟨711⟩. Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and are in routine use throughout the industry. Once a suitable deaeration process is identified, it should be documented as part of the dissolution procedure. The extent of deaeration can be evaluated by measuring the total dissolved gas pressure or by measuring the concentration of dissolved oxygen in water. For example, an oxygen concentration below 6 mg/L has been found effective as a marker for adequate deaeration of water for the Performance Verification Test with USP Prednisone Tablets RS.

Media containing surfactants usually are not deaerated because the process results in excessive foaming, and usually the effect of dissolved air on the dissolution process is mitigated by the reduced surface tension of the medium. Sometimes, deaerating the medium before adding surfactants can be effective.

To determine whether deaeration of the medium is necessary, compare results from dissolution samples run in non-deaerated medium and medium deaerated using a compendial technique, as described above. If no effect of deaeration is detected, this experiment could serve as justification that deaeration is not required in the future. If there is an effect, however, then it is necessary to carefully control this parameter, and it is prudent to characterize the robustness of the deaeration process. The dissolved gas content of deaerated media under atmospheric pressure is unstable and will tend toward saturation. Manipulations of the deaerated medium such as stirring or pouring can increase the rate at which atmospheric gases are redissolved.

2.2 Sinkers

Sinkers are often used to adjust the buoyancy of dosage forms that would otherwise float during testing with Apparatus 2. When sinkers are used, a detailed description of the sinker must be provided in the written procedure. It may be useful to evaluate different sinker types, recognizing that sinkers can significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the same sinkers should be used, or if a different design is used, it should be shown to produce equivalent results. There are several types of commercially available sinkers. In ⟨711⟩, a harmonized sinker is described in *Figure 2a*.

Table 2. Wire Sinkers Used With Common Capsule Shell Sizes

Capsule Shell Size	Length of Wire (cm)	Diameter Size (cm)	Cork Bore Number
#0, elongated	12	0.8	4
#1 and #2	10	0.7	3
#3 and #4	8	0.55	2

A standard sinker can be made by using the appropriate length of wire and coiling it around a cylinder. For materials, use 316 stainless steel wire, typically 0.032 inch/20 gauge, or other inert material and wind the wire around cylinders of appropriate diameter (e.g., cork borers) for an appropriate number of turns to fit the capsule shell type. Sizes are shown in *Table 2*. The ends of the coil can be curved to retain the capsule within the sinker when they are immersed. Because the ends of the wire may be rough, they may need to be filed. If the sinker is handmade, the sinker material and construction procedure instructions should be documented (e.g., dimension, design, number of coils); if a commercial sinker is used, the vendor part number should be reported if available.

Although sinkers are typically used to keep the dosage form at the bottom of the vessel, they can also be used to keep dosage forms from sticking to the vessel (e.g., film-coated tablets). The sinker should be appropriate to the dosage form; therefore, the same sinker size may not be suitable for all dosage-form sizes. The sinker should not be too tight around the dosage form because this may restrict interaction with the medium. Conversely, if wrapped too loosely, the dosage form may escape soon after the test begins. The sinker should be small enough that the capsule does not change its orientation within the sinker. Care should be taken when testing capsules that have some cross-linking present, to keep the sticky shell from attaching to the vessel bottom. In this case, the harmonized sinker design provided in *Figure 2a* of ⟨711⟩ will be advantageous.

2.3 Agitation

For immediate-release capsule or tablet formulations, Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are used commonly. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used with Apparatus 2, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if to achieve an in-vitro–in-vivo correlation (IVIVC) the resulting profiles better reflect in vivo performance, or if the method results in better discrimination without adversely affecting method variability.

Apparatus 3 (reciprocating cylinder) can be used at dip rates ranging from 5 to 30 dips/min. The hydrodynamics are influenced by the cylinder's reciprocating motion and the resulting movement of the sample in the medium. The reciprocating motion of the cylinder and screen may cause foaming if the medium contains surfactants. Addition of an anti-foaming agent such as simethicone or *n*-octanol may be useful for avoiding foaming from surfactants.

Apparatus 4 (flow-through cell) is described in ⟨711⟩ with standard flow rates of 4, 8, and 16 mL/min. Other flow rates for Apparatus 4 can be used if justified and if within the capacity of the pump to conform with the requirements in ⟨711⟩. Agitation in Apparatus 4 is not only related to the pump speed but can also be affected by cell diameter. At a set flow rate, as measured by volume, the 12-mm cell will develop a greater linear fluid velocity than is achieved in the 22.6-mm cell. Apparatus 4 can be configured with the addition of glass beads in the entry cone of the flow-through cell (packed column) or without glass beads (open column).

The flow characteristics of the flow-through cell are discussed in the scientific literature (*15*). The placement of the sample in the flow-through cell will influence the flow patterns that occur and thus should be a consideration in the attempt to reduce variability of the results.

2.4 Study Design

Selection of the agitation rate and other study design elements for the dosage form, whether immediate release or modified release, should conform to the requirements and specifications (i.e., apparatus, procedures, and interpretation) given in ⟨711⟩.

2.4.1 TIME POINTS

For immediate-release dosage forms, the duration of the dissolution procedure is typically 30–60 min; in most cases, a single time point specification is adequate for pharmacopeial purposes. For method development, however, a sufficient number of time points should be selected to adequately characterize the ascending and plateau phases of the dissolution curve. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. According to the Biopharmaceutics Classification System referred to in several FDA Guidances, highly soluble, highly permeable drugs formulated into very rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the drug substance within 15 min. For these types of

products, a one-point test or disintegration will suffice. However, most products do not fall into this category. Dissolution pro-files of immediate-release products typically show a gradual increase reaching 85%–100% at about 30–45 min. Thus, sufficient dissolution time points are chosen to characterize the performance for most immediate-release products. For some products, including suspensions, useful information may be obtained from earlier points, e.g., 5–10 min. For slower-dissolving products, time points later than 60 min may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

The f_2 similarity factor may not be useful when more than 85% is dissolved at 15 min. If the f_2 similarity factor is to be used, multiple time points for the dissolution test are required, with at least two time points with mean percent dissolved (typically for $n = 12$) below 85% dissolved and only one point above 85% for both products *(16)*. Therefore, the addition of early time points may be useful.

For testing an extended-release dosage form, at least three time points are chosen, to guard against dose dumping, to define the in vitro release profile, and to show that essentially complete release (>80%) of the drug is achieved. Additional sampling times may be useful. Certain IVIVC criteria, such as level B correlation (according to *In Vitro and In Vivo Evaluation of Dosage Forms* ⟨1088⟩), require the experimental determination of the time to dissolve 100% of the label claim. Selection of the final time points is reflective of the data from the drug release profile that are generated during development. For products containing more than a single active ingredient, determine the drug release for each active ingredient.

Delayed-release dosage forms usually require specifications for at least two time points; therefore, it is important during development to evaluate the entire dissolution profile. In the case of enteric-coated dosage forms, the functionality of the coating is usually proven by challenge in an acid medium, followed by a demonstration of dissolution in a higher-pH medium. Chapter ⟨711⟩ gives a standard buffer medium for that stage of testing but other media may be used if justified. The timing of the acid stage is typically 2 h, and release in the buffer is similar to the timing for immediate-release forms. For delayed-release dosage forms that are not enteric coated, setting of specifications is different. Unlike delayed release, the onset of release is not determined by the experimental design, which is the pH change; multivariate specifications, therefore, may be needed to define time ranges and corresponding percentage ranges.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run (after the last time point) for a sustained period (typically, 15–60 min), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can be compared to content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

2.4.2 OBSERVATIONS

Visual observations and recordings of product dissolution and disintegration behavior are useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. For visual observation, proper lighting (with appropriate consideration of photo-degradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real-time dissolution test. Observations are especially useful during method development and formulation optimization. It is important to record observations of all six vessels to determine if the observation is seen in all six vessels, or just a few. If the test is performed to assist with formulation development, provide any unique observations to the formulator. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus (e.g., below the basket or paddle), when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit, especially at the surface of the media.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products, [e.g., the partial opening and splitting apart (similar to a clamshell) or incomplete opening of the shell], accompanied by the release of air bubbles and excipients.
9. Whether the dosage form lands in the vessel center or off-center, and if off-center, whether it sticks there.
10. Time required for the complete dissolution of the capsule shell or for tablet disintegration.

Observations also help to document that the proper procedure has been followed, or more importantly, that a deviation has occurred. Examples include the confirmation that a dosage form is actually in the vessel during the test or that more than one dosage form are inadvertently in the same vessel, or that a filter from the autosampler has dropped into the vessel.

2.4.3 SAMPLING

Manual: For manual sampling, use chemically inert devices (e.g., polymeric or glass syringes, and polymeric or stainless steel cannula), a filter, and/or a filter holder. The sampling site must conform to specifications in 〈711〉. When the agitation conditions are very slow, e.g., a 50-rpm basket, care should be taken to sample consistently in the same location in the vessel because there may be a concentration gradient; avoid sampling very close to the shaft or vessel wall. During method development, a decision should be made regarding whether to replace the media after each time point. Replacement is not preferred because the dosage unit may be disturbed during delivery of the media. However, replacement may be necessary if maintaining sink conditions is a challenge. With replacement, the volume used in the calculations remains the same throughout the time points, but there is some drug substance withdrawn with each sample that will need to be accounted for in the calculations.

Metal surfaces may interact with the sample. For example, adsorption onto metal surfaces may occur, or the metal surfaces may release metal ions into aqueous media. The ions can then catalyze degradation reactions, leading to artifacts during the analytical procedures. The surfaces of stirring elements and metal locks of syringes may be sources of interference to accurate sampling.

Autosampling: Autosampling is discussed in section *4. Automation*.

2.4.4 CLEANING

Importance is placed on evaluation of the cleaning process between tests. Changes of dissolution medium and/or product necessitate the need for cleaning. Residues on the vessels can affect the results (e.g., adsorbed residues may dissolve and alter subsequent media properties or interfere with the sample analysis), and effective cleaning will return them to a suitable state. Automated systems are discussed in section *4.4 Cleaning*.

2.5 Data Handling

Dissolution rates are calculated from the change in drug concentration in the dissolution medium. For procedures in which the volume of medium is fixed, such as for Apparatus 1 and Apparatus 2 testing of immediate-release dosage forms with only one sampling time, the concentration of the sample is multiplied by the medium volume to arrive at the mass of drug dissolved usually expressed as percentage of label claim. When multiple time points are taken, the total amount of drug removed at earlier time points should be assessed and may be part of the calculation of the amount dissolved, if considered important. Similarly, if the medium volume is not fixed, for example when the sample volume is not replaced in testing extended-release products, the change in medium volume must be part of the calculation for successive sampling points. Dissolution tests performed with Apparatus 4 in the closed-loop configuration with in situ detection provide a convenient control of the medium volume. For testing with Apparatus 4 in the open configuration, the test time and flow rate will determine the volume of medium used in the dissolution calculations.

Dissolution results can be evaluated as either cumulative rates or fractional rates. Cumulative rates represent the sum of all drug dissolution that occurs during an interval (*Figure 1*). Fractional rates are assessed at a specific time point or during a portion of the total test time (*Figure 2*). Typically, the rate of release will be expressed as either mass or percentage of label claim per unit time. For most compendial dissolution testing, the dissolution rate is expressed as a percentage of the label claim dissolved at the indicated test time.
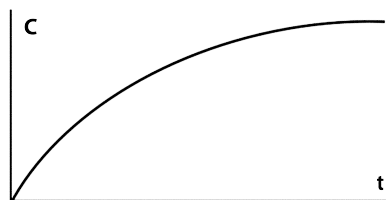

Figure 1. An example of a plot of dissolution as a cumulative process. Concentration, C, is the amount of drug released per volume of medium, and t represents time. This type of plot is readily observed in constant-volume dissolution systems, such as Apparatus 1 or Apparatus 2, or Apparatus 4 in closed-loop configuration.
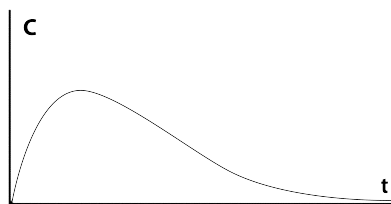
Figure 2. An example of a plot of the observed concentration of the sample taken for an interval that is negligibly small in relation to the time of the overall dissolution process. This concentration is propostional to the instantaneous or fractional dissolution rate (dc/dt). This type of plot is readily observed in continuous-flow dissolution systems, such as Apparatus 4 in open-loop configuration.

Cumulative dissolution profiles represent the total amount of drug dissolved from the formulation over time. When cumulative dissolution is measured in a constant-volume system, no correction for the amount lost in sampling needs to be made. If sample is removed from the system, the amount consumed in analysis must be accounted for in the calculation. Recirculated sampling with Apparatus 1 or Apparatus 2, or with Apparatus 4 in the closed-loop configuration (*Figure 3*), are all examples of systems that will produce cumulative dissolution rates. With Apparatus 4 in the open configuration (*Figure 4*), cumulative rates accounting for the total amount of drug dissolved across the testing interval are obtained by collecting and analyzing the entire outflow from each individual flow-through cell. With Apparatus 3 (*Figure 5*), the medium in each tube is sampled at the end of the programmed interval, and the analyzed concentration represents the cumulative dissolution rate during that interval.
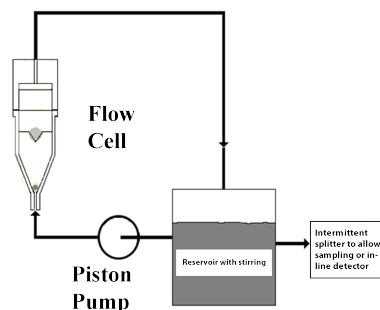

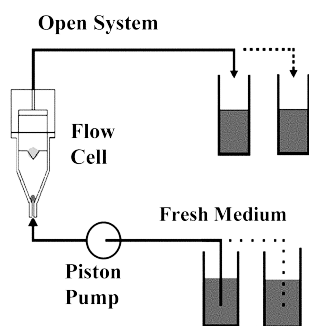Figure 3. Apparatus 4 in the closed-loop configuration.

Figure 4. Apparatus 4 in the open-loop configuration. The sample can be collected in fractions, as shown at the top. The medium can be changed by using successive reservoirs.
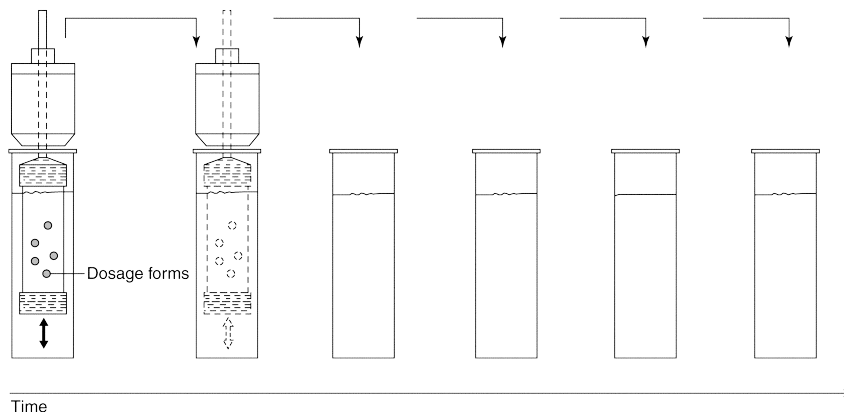
Figure 5. The progression that is possible for one reciprocating cylinder from Apparatus 3. The reciprocating cylinder can move from vessel to vessel. This feature facilitates changing the dissolution medium and testing for different intervals in successive tubes.

Fractional dissolution rates are typically measured for a discrete interval. A series of such rates will produce a step function as the dissolution profile. At any time, the cumulative dissolution rate from this type of profile is the sum of the preceding intervals. This type of profile is represented by Apparatus 3 using multiple tubes or Apparatus 4 in the open-loop configuration where the total outflow is collected and analyzed for successive intervals.

A number of algebraic and numerical methods exist for transforming cumulative and fractional dissolution results. The difference in amount released for successive time points can be calculated, and the average release rate is determined by the formula:

$$\text{Result} = (M_2 - M_1)/(t_2 - t_1)$$

M = mass or percentage of label claim
t = time

As the difference of t_2 from t_1 is reduced, the average rate can be considered to approach an instantaneous rate. Sampling considerations and physical constraints on measurement of the mass transfer at the medium interface of the dosage form make the measurement of true instantaneous dissolution impractical for routine determination in the laboratory. Fractional dissolution is measured for intervals where the difference between t_2 and t_1 is small, relative to the total test time. The design of Apparatus 4 in the open configuration permits a direct measurement of the fractional dissolution over small time intervals. For example, if a 4-mL fraction of outflow for Apparatus 4 running 16 mL/min is sampled, either by in situ detection or offline, the amount of drug detected represents the dissolution occurring in a 15-s interval.

Pooled dissolution has been used in a number of monographs. The pooled dissolution procedure produces an average release rate for the units tested by combining equal volumes from each vessel or cell and performing analysis of only the one resulting solution. Because this approach uses only the average release rate for comparison with the acceptance table, the pooled dissolution procedure has been viewed as reducing the amount of data available from the dissolution test and, thus, reducing its value. However, it should be noted that the pooling of equal sample volumes is equivalent, from a calculation standpoint, to determining the arithmetic mean of the individual sample results.

The use of the f_2 similarity factor in the comparison of dissolution profiles is discussed in *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* 〈1090〉.

For the purpose of correlation with in vivo data, parameters of mathematical models are obtained by fitting to dissolution data to establish a continuous functional relationship called IVIVC (see 〈1088〉).

2.6 Dissolution Procedure Assessment

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that together provide a method that is sensitive to changes in critical quality attributes, yet sufficiently rugged and reproducible for day-to-day operation. The method should be able to be transferred between laboratories.

The ideal dissolution procedure will not contribute an unacceptable degree of variability and will provide a profile with adequate points below 85% dissolved. If 85% dissolved occurs before 15 min, then f_2 comparisons may not be appropriate.

There are many ways to challenge the sensitivity of the method. One option is to compare dissolution profiles of formulations that are intentionally manufactured with meaningful variations for the most relevant critical manufacturing variable, for example, ±10%–20% change to the ranges of these variables. Similarly, samples that have been stressed may be used to demonstrate sensitivity to changes on stability. This concept may be used to establish the factors that are most significant in their influence on the dissolution rate. These studies can focus on either the dissolution parameters (e.g., media concentration, agitation rate, and deaeration) or the product attributes (e.g., excipient ratios, particle size, compression). The ultimate goal is to

understand the release mechanisms and determine whether the dissolution procedure can show change in the critical quality attributes of a drug product.

3. ANALYTICAL FINISH

The dissolution step has been described as an involved sample preparation. The sample handling and analytical procedure that are used to determine the amount of drug substance dissolved during the dissolution procedure are termed the "analytical finish." Although spectrophotometric determinations and HPLC are used most commonly and are discussed in this chapter, any suitable analytical technology may be used. Section *5. Validation* describes criteria for the methods.

3.1 Sample Processing

After the samples are withdrawn from the dissolution medium, they may require additional processing to make them suitable for the analytical methodology used to determine the amount released. For example, filtration may be used to remove undissolved particulate matter, or samples may need to be protected from exposure to light or may need refrigerated storage. In addition, samples may have to be diluted to a level that is within the linear range of the method. With analysis by HPLC, dilution of the sample with mobile phase may be necessary to reduce the effect on the separation of injecting dissolution medium. Other types of treatment may be necessary depending on the product formulation, such as the inactivation or elimination of interference caused by components of the formulation by the addition of appropriate reagents. However, separation may not be possible or needed in all cases. In some cases, in situ measurements obtained with methods such as fiber optics or electrochemical determination may be useful.

3.2 Filters

The topic of filtration is discussed in section *1.1 Performing Filter Compatibility.*

3.3 Centrifugation

Centrifugation of samples is not preferred, for several reasons: dissolution can continue to occur until the solids are removed, a concentration gradient may form in the supernatant, and energy imparted may lead to increased dissolution of the drug substance particles. Possible exceptions, when centrifugation could be preferred, might include the use with compounds that adsorb onto all common filters, or situations when the potential filter leachables and extractables might interfere in the quantitative step of the dissolution test (e.g., when fluorescence procedures are used in quantitation). Centrifugation may prove useful during method development for evaluating the suitability of the filter material.

3.4 Analytical Procedure

The usual assay for a dissolution sample employs either a spectrophotometric procedure or a liquid chromatographic procedure. Spectrophotometric determination may be direct or may provide the detection for HPLC. Spectrophotometric determination is used often because results can be obtained faster, the analysis is simpler, it is easier to automate, and fewer solvents are needed. The use of direct spectrophotometric determination typically requires confirmation of specificity. HPLC is preferred for a number of reasons such as providing a wide dynamic range that reduces the need to dilute some samples while also providing sensitivity in the analysis of dilute samples, and greater selectivity when excipients or multiple drugs in the formulation present a significant interference. Modern HPLC systems employ autosamplers that provide speed and simplicity advantages comparable to spectrophotometric analysis.

3.5 Spectrophotometric Analysis

Direct spectrophotometric analysis may be performed on samples that are manually introduced to the cuvette. Alternatively, samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance, as described in the standard operating procedures or metrology documents, help to ensure reliable operation of these instruments. Cells with path lengths ranging from 0.02 cm to 1 cm are typically used, and longer path-length cuvettes can be used to increase the range for quantification of dilute samples. Cell alignment and air bubbles could be sources of error. The shorter path-length cells are used to avoid diluting the sample; in all cases, however, acceptable linearity and standard error need to be demonstrated.

The choice of wavelength for the determination should be based on the spectrum of the drug in solution. In some cases, where the drug substance can degrade in the dissolution medium (e.g., dosage forms containing aspirin), it is useful to carry out the measurements at the isosbestic point. Excipients can also have effects, but performing analysis at multiple wavelengths can minimize their effects. The contribution of the absorbance from an excipient at the analytical wavelength can sometimes be determined by ratio from its absorbance at a wavelength where the absorbance of the drug substance is minimal.

Using a validated analytical finish, standard solutions are typically prepared in dissolution media and analyzed at just one concentration, either at 100% of the dosage strength or the selected Q value because linearity of the analytical finish has been established. Prior to validation, dissolution profile analysis, or analysis of products of various strengths, requires using multiple standard solutions covering the expected range of concentration. A typical media blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis. The standard and sample solutions should both be prepared in the dissolution medium in the linear concentration range and measured at the same wavelength. However, small amounts of an organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg/mL, divided by the cell path length in cm. A rearrangement of the Beer-Lambert expression gives the absorptivity, *a*, as:

$$a = A/bc$$

A = absorbance
b = path length (cm)
c = concentration (mg/mL)

Typical units for absorptivity that are used for dissolution testing are in terms of AU · mL/mg, where AU is absorbance unit. Historical data may be used to provide an acceptable absorptivity range for the analyte (using the appropriate path-length cell). This value may be useful in troubleshooting aberrant data.

Fiber optics as a sampling and determinative method, with proper validation, are an option.

3.6 HPLC

For HPLC analysis, the effect on the chromatogram of peaks resulting from injection of dissolution media require enumeration. A large solvent disturbance may affect accuracy and precision of response if it is poorly resolved from the peak of interest. This is even more important if large injector volumes (>100 μL) are needed. System suitability tests may evaluate peak shape; separation of the main peak from solvent disturbance and from closely eluting peaks; and injection precision. At a minimum, the precision is critical.

Ideally, the standard solutions should be diluted with the dissolution media at a concentration within the linear range of the method, e.g., 100%, or the selected Q value of the dosage strength. However, organic solvent may be used in the preparation of the standard, provided that the accuracy criteria can be met during validation. In some cases, the sample may be diluted with mobile phase to improve the peak shape. The standard and sample solutions should both be prepared in the linear concentration range and measured at the same wavelength.

4. AUTOMATION

Automated dissolution systems may be configured in various ways and degrees. The elements of test preparation, initiation, sampling and timing, and cleaning all can be automated. Fully automated systems are available, as are systems where individual steps, such as media preparation or sampling, are automated. This section will discuss operational steps that can be automated. The level of complexity for automation depends on whether the instrument configuration is open or closed loop and also whether the analytical device is coupled online or offline. Online analysis returns the sample aliquot to the test system, as in the case of spectrophotometry with flow-through cuvettes. Offline analysis removes the sample aliquot from the dissolution medium for subsequent analysis, typically by HPLC, where the analysis consumes the sample. The decision on the configuration usually depends on the number of samples to be processed and the time required for their analysis.

Automation may require deviations from the pharmacopeial specifications of the instruments, such as incorporation of an integrated outlet on the bottom of the vessel for cleaning and replacement of medium.

Operational steps that are not part of the compendial procedure should be validated. Deviations from the standard procedure described in ⟨711⟩, such as use of sampling probes or fiber-optic probes, should be validated against the standard procedure.

4.1 Medium Preparation

Automated media preparation generally is accomplished by diluting concentrates. Automated media preparation systems typically dispense the volume of medium into the vessel by monitoring either the weight or volume. Chemical and physical stability of the concentrates as well as homogeneity of the dilutions over the intended period of use are important issues and should be understood. Concentrates of buffer solutions and surfactants may have stability issues, such as chemical degradation and pH change. Physical instability may manifest as precipitation, re-crystallization, or phase separation and should be prevented.

If deaeration of the medium is required, the level of deaeration should be specified.

The concentration of the dissolved oxygen can be used to evaluate the efficiency of deaeration procedures discussed in section *2.1 Deaeration*.

4.2 Sample Introduction and Timing

   Samples should be inserted in the vessel in a reproducible way. Automated sample introduction and aliquot withdrawal provide an advantage over manual sampling because the automated techniques can reduce the variability in the vessel-to-vessel timing of the test intervals. However, automated sample handling may impose timing limitations that need to be considered. The pharmacopeial tolerance of ±2% of the specified dissolution test time may be difficult to meet for early time points.

4.3 Sampling and Filtration

   Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. The transfer and filtration of sample solutions from the dissolution instrument to the analytical unit may be undertaken via tube connections or via robotic devices operated in a stepwise procedure. Sample volumes may be removed from the dissolution medium and not returned (consumptive sampling), or the sample volume may be returned to the dissolution medium (recirculated sampling).
   There are many brands of autosamplers, including semi-automated and fully automated systems. Routine performance checks, cleaning, and maintenance, as described in the pertinent standard operating procedures or metrology documents, help to ensure reliable operation of these devices.
   Sampling probes may or may not remain in the vessel throughout the entire run. Sampling probes or fiber-optic probes can disturb the hydrodynamics of the vessel; therefore, adequate validation should be performed to ensure that the probes are not causing a significant change in the dissolution rate. If filters are used that are different from those used for manual sampling, then these different filters should also be evaluated separately. The position of the pharmacopeial sampling zone for Apparatus 1 and Apparatus 2 is midway from the top of the stirring element to the medium surface and depends on the medium volume. Sampling probes should pull the sample from the sampling zone. Instruments for which the sampling occurs through the hollow shaft should be designed with a means to adjust the depth of the inlet aperture to allow conformance with this requirement. The programmed sampling volume depends on the dead volume of the tubing, cuvettes, and other devices and has to be adjusted accordingly.
   A recirculated sampling alignment can be operated either by discharging the tubing contents into the vessel after each sampling or by allowing the tubing to remain filled with solution in the intervals between sampling points. In the latter case, the dead volume and carryover effects are important considerations.
   The need for sample volume replacement should be considered. In consumptive sampling with multiple sampling time points, the withdrawn volume may be replaced with an equal volume of fresh medium. The sampling volume may be critical if, in total, it exceeds 1% of the stated volume of dissolution medium required by the procedure. If it can be shown that replacement of the medium is not necessary, the volume change must be part of the calculation of results. See section *2.5 Data Handling*.
   Carryover may occur when subsequent samples are affected by residues or conditions of previous samples; the effect of the first sample or condition "carries over" to the second. In liquid handling, residues of liquids previously in the sample solution may contaminate subsequent sample solutions. Dissolution media containing surfactants or lipids may present problems. Carryover may occur for successive samples taken over a multiple time-point test, as well as at the beginning of a new test due to the cleaning solution. This topic is discussed in section *4.4 Cleaning*.
   Interaction of dissolved drug substance with the sampling and transfer devices is an important consideration. When adsorption of the dissolved drug substance occurs, it most often involves surfaces of the dissolution apparatus or sampling filters and tubing. Adsorption may be pH dependent in the case of charged, dissolved drug substance. Adsorption of the dissolved drug to the parts of the sampling device should be assessed using a typical sample solution (dissolution sample from the product or drug substance with formulation matrix) with known concentration. The typical design is a cross-validation with aliquots of the same sample solution passing and bypassing the sampling device (including the sampling probe, filter, tubing, valves, and pump). There is no general recommendation that may give preference to any kind of material or equipment construction (e.g., glass or specific polymers). See section *5.7 Considerations for Automation* for more information.
   In addition to the information in section *2.4.3 Sampling*, connections of pumps and tubing may be sources of contamination in automated systems. Interferences with the spectroscopic analytical procedures, which are commonly used for dissolution testing, are less of a concern. However, interferences must be evaluated if the product under investigation contains low-dose metal salts, as do some dietary supplements.
   Liquid transfer usually is undertaken via polymeric tubing. Inert materials such as polytetrafluoroethylene (PTFE) sometimes cannot be used because of their mechanical properties. Where flexible tubes are required, for example in peristaltic pumps or for coiling in a small radius, polypropylene (PP) or high-density polyethylene (HDPE) may be the preferred materials. Depending on the type of polymer and its crystallinity and density, leaching of constituents, mainly plasticizers, may occur. Leachables can interfere with the analytical procedure. The concentration leached to the sample solution usually depends on the surface, the temperature, the exposure time, the hydrodynamic conditions, and the composition of the media.

4.4 Cleaning

In addition to the information in section *2.4.4 Cleaning*, automated systems have specific cleaning issues. For example, evaluation of the effectiveness of purging and rinsing between sampling times and within-run condition of the tubing is recommended. Also it is important to evaluate the cleaning process between tests.

4.5 Operating Software and Computation of Results

The software systems for data evaluation and instrument operation must be validated as per 21 CFR 11 *(17)*.

4.6 Common Deviations from the Compendial Procedures That May Require Validation

Some common areas of deviation from compendial procedures include the following:
• Sample introduction relative to start of spindle rotation
• Residence time and positioning of sampling probes
• Recirculated versus consumptive sampling
• Sample volume replacement in consumptive sampling.

5. VALIDATION

The validation topics described in this section are typical but not all-inclusive and can be viewed in the context of *Validation of Compendial Procedures* ⟨1225⟩, as well as the International Conference on Harmonization (ICH) document, *Validation of Analytical Procedures (18)*. Validation for both parts of the dissolution procedure, the analytical finish and the dissolution step, will be discussed in this section. The dissolution step is the release of the drug in the dissolution medium and sampling. The analytical finish is defined in section *3. Analytical Finish*. Validation of the analytical finish will evaluate the attributes, linearity and range, precision, specificity, accuracy/recovery, robustness, and stability of the sample and standard solutions. Validation of the dissolution step will include evaluation of precision and robustness of the dissolution sample preparation. Validation of the analytical finish is performed either using a standard solution or spiked placebo or by the method of standard addition (spiked drug product as described in *Accuracy* in ⟨1225⟩), as specified in the sections below. Validation of the dissolution step requires the use of a well-characterized dosage form (e.g., having tight content uniformity and uniform performance). Depending on the parameter of interest, validation of the sample handling and analytical procedure can be performed in situ, e.g., within the dissolution vessel. The validation parameters addressed and the extent of the validation may vary, depending on the phase of development or the intended use for the data.

The acceptance criteria are presented as guidelines only, and may differ for some products. Manufacturers should document the appropriate acceptance criteria for their products in pertinent Standard Operating Procedures (SOPs) or in validation protocols. Other considerations may be important for special dosage forms. Validation studies should be performed across the range of profile time points. For products containing more than a single active ingredient, the dissolution procedure needs to be validated for each active ingredient. It is expected that investigations into filter suitability and the potential for glass adsorption will have been undertaken already (see *1.1 Performing Filter Compatibility*). Validation of these assessments may occur during spiked recovery experiments.

5.1 Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradants. The placebo consists of all the excipients and coatings, with inks and capsule shells included if appropriate, without the active ingredient. Placebo interference can be evaluated by using a spiked placebo that is prepared by weighing samples of the placebo blend, dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing, and adding a known amount of the drug in solution. It may be preferable to perform this experiment at 37°, comparing the solution to a standard solution at the concentration expected to be encountered during testing, by using the formula:

$$\text{Result} = (A_P/A_S) \times C_S \times (V/L) \times 100$$

A_P = absorbance of the placebo
A_S = absorbance of the standard
C_S = concentration of the standard (mg/mL)
V = volume of the medium (mL)
L = label claim (mg)

The interference should not exceed 2%. Note that for extended-release products, a placebo version of the finished dosage form may be more appropriate than blends because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate

potential interference at multiple sampling points in the release profile, with worst-case interference expected at the later sampling points.

The blank is the dissolution medium without dissolved sample, and it is treated in the same manner as the sample. The effect of the absorbance of the blank at the analytical wavelength should be evaluated. In most cases, the absorbance of the dissolution medium blank may not exceed 1% of the standard solution at the concentration used for analysis. Values >1% should be evaluated on a case-by-case basis.

If the placebo interference exceeds 2%, modification of the method may be necessary. Possible modifications include choosing another wavelength, subtracting baseline using a longer wavelength, transforming absorbance values (e.g., first derivative), and using an alternative analytical technique such as HPLC. Other means for minimizing the placebo interference would be acceptable with appropriate justification. When other active drug substances or significant levels of degradants are present, it is necessary to show that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug substance or degradant: any interference should not exceed 2%. Similar approaches may be used if other techniques are used for the analytical finish.

5.2 Linearity and Range

Linearity is typically established by preparing solutions of the drug substance, ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. The solutions may be prepared either using either a standard solution or spiked solution or by the method of standard addition. A minimum of five concentrations is normally used (see ⟨1225⟩). Typically, solutions are made from a common stock if possible. The concentration range may not exceed the linearity limits of the method, including the instrumentation. Organic solvents may be used to enhance drug solubility for the preparation of the linearity standard solutions. However, no more than 5% (v/v) of organic solvent should be present in the final solution unless validated. Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the *y*-intercept must not be importantly different from zero.

The range of the procedure is the interval between the upper and lower concentrations of the drug substance (including these levels) that has been demonstrated to have a suitable level of precision, accuracy, and linearity using the procedure as written.

5.3 Accuracy/Recovery

Accuracy/recovery is typically established by preparing multiple samples containing the drug substance and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from less than the lowest expected concentration to more than the highest concentration during release. Accuracy/recovery may be done in conjunction with linearity determination. The method of standard addition can also be used. Before this activity, it is expected that filter assessment will already have been performed, and adsorption of drug onto the glass has also been investigated and ruled out.

Individual solutions may be directly prepared in the dissolution medium. Alternatively, to enhance drug solubility it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5% organic solvent in the final dissolution media) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be used instead of the drug substance powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts.

The measured recovery is typically 95%–105% of the amount added. Bracketing or matrixing of multiple strengths may be useful. A special case for validation is the *Acid Stage* procedure described in ⟨711⟩, *Delayed-Release Dosage Forms*. The limit of NMT 10% needs to be validated. Recovery experiments for drugs that have low solubility in acidic media may be challenging or impossible to perform and may need to be addressed on a case-by-case basis. If the compound degrades in acid, the validation experiment must address this fact.

5.4 Precision

5.4.1 REPEATABILITY OF ANALYSIS

For the analytical finish, repeatability is evaluated by obtaining replicate measurements of standard and/or spiked placebo/standard addition solutions. It can be determined by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or by using the accuracy or linearity data. ICH guidance, *Validation of Analytical Procedures: Methodology*, recommends that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration. A typical acceptance criterion is an RSD of <2%. The demonstration of the

repeatability for the dissolution step is conducted by performing the dissolution step on separate units of a well-characterized dosage form or equivalent composite.

5.4.2 INTERMEDIATE PRECISION/RUGGEDNESS

Assuming that the major contributor to the variance is from the dissolution step, intermediate precision may be evaluated to determine the effects of random events on the precision of the dissolution procedure. This evaluation is typically done later in the development of the drug product and is required for full method validation. For many analytical procedures intermediate precision is typically assessed by determination of contributions to variance and, possibly, by a comparison of means. The use of an experimental matrix design is encouraged for evaluation of intermediate precision because interaction effects may be observed more clearly relative to a single variable experiment. In dissolution testing, a ruggedness approach that compares means alone is often taken to investigate the factors that contribute to intermediate precision. The ruggedness can be evaluated across the range of product strengths. Typical variations to be studied include different days, analysts, and equipment. If possible, ruggedness can be evaluated using a drug product lot if well characterized, for example, by having tight content uniformity and uniform performance, but if this type of lot is not available, a premeasured placebo with active ingredients may be used to investigate the intermediate precision. The use of such a spiked placebo would additionally support the assessment of the contribution of the analytical finish to the observed variability of results.

The dissolution procedure on the same lot of well-characterized dosage form may be run by at least two different analysts from the same laboratory, with each analyst preparing the standard solutions and the medium and following the defined extraction/quantification procedure. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers, and they perform the test on different days. Full profiles are assessed where relevant to the product. This procedure may not be necessary at each strength; instead, bracketing with high and low strengths may be acceptable.

Acceptance criteria for intermediate precision or for ruggedness are predetermined. A typical acceptance criterion for ruggedness is that the difference in the mean value for dissolution results between any two conditions, using the same strength, does not exceed an absolute 10% at time points with <85% dissolved and does not exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

5.4.3 REPRODUCIBILITY

Reproducibility follows the general concepts of intermediate precision, but is performed by two different analysts at different labs.

5.5 Robustness

Evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in development of the drug product and is a requirement for full method validation. It is performed using a well-characterized lot of drug product, for example having tight content uniformity and uniform performance. The number of replicates (typically 3 or 6) is dependent on the intermediate precision. All profile points should be evaluated.

Selection of parameters to be varied depends on the dissolution procedure and analysis type. The parameters may include medium composition (e.g., buffer or surfactant concentration, pH, deaeration), volume, agitation rate, sampling time, and temperature. Statistical analysis of the data generated will help determine the extent to which the parameters must be controlled in the method. The robustness assessment is well suited to Design of Experiments (DoE) methodologies to efficiently investigate the impact of the individual parameters and/or their interaction.

Robustness of analytical finish is referenced in ⟨1225⟩. HPLC analysis parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

5.6 Stability of Standard and Sample Solutions

The standard solution is stored under conditions that ensure stability. The stability of the standard solution is analyzed over a specified period of time (for at least the time of the entire dissolution procedure), using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is influenced by the concentration and is typically between 98% and 102% at the expected final concentration.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time, using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102%, compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider include temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

5.7 Considerations for Automation

Automated methods offer opportunities for increased precision and reproducibility; however, bias may be introduced. In particular, the sampling probe and the sample lines warrant attention as places where inaccuracies may occur. Deviations from the procedure described in ⟨711⟩, such as resident sampling probes, sampling through the stirring element shaft (hollow-shaft sampling), or fiber-optic probes, should be validated. Other aspects of automation validation may include carryover of residual drug, effect of an in-residence probe, adsorption of drug, and cleaning and/or rinse cycles. Validation is performed using the automated dissolution system including materials. Therefore, any change in materials will require demonstration of suitability based on the validation attributes that are impacted by the change.

Manual and automated procedures should be compared to evaluate the interchangeability of the procedures. This is done by performing two automated runs at each dosage concentration, using all sampling points, compared to manually sampled runs of the same samples. The effect of the in-resident probe cannot be determined by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision if the procedures are to be considered interchangeable. The difference in the mean value for dissolution results between any two conditions using the same strength should not exceed an absolute 10% at time points with <85% dissolved nor exceed 5% for time points >85%. Acceptance criteria may be product specific, and other statistical tests and limits may be used.

Revalidation may be necessary when the automated system is used with different formulations because of the interaction with excipients. Dissolution media containing surfactants or lipids may require additional validation efforts.

6. ACCEPTANCE CRITERIA

The acceptance criteria should be consistent with historical release or stability data. There is an expectation that acceptable batches will have results that fall within the acceptance criteria and that all manufactured batches should have similar dissolution behavior, thus highlighting the importance of having a method that is not highly variable. The acceptance criteria and time point(s), therefore, should discriminate between an acceptable and an unacceptable batch. In addition, the dissolution test results are viewed as a link to the pivotal clinical trial batches. When changes in dissolution rate have been shown to affect bioavailability significantly, the dissolution test and acceptance criterion should distinguish batches with unacceptable bioavailability *(19)*. Likewise, when changes in the formulation and manufacturing process significantly affect dissolution and such changes are not controlled by another aspect of the specification, the dissolution test and criteria should distinguish these changes.

6.1 Immediate-Release Dosage Forms

Although release and stability data are collected during dosage form development, it is common to record the entire dissolution profile or the amount of drug dissolved at specified intervals, such as 10, 20, 30, 40, 50, and 60 min or 15, 30, 45, and 60 min. At registration, dissolution for an immediate-release tablet usually becomes a single-point test. The acceptance criterion and test time are established by evaluating the dissolution profile data. The acceptance criterion for a dissolution test is a function of Q, which is expressed as a percentage of label claim of drug dissolved at a specified time. Typical Q values are in the range of 75%–80% dissolved. Q values in excess of 80% are not generally used because allowance needs to be made for assay and content uniformity ranges.

6.2 Delayed-Release Dosage Forms

The discussion about dissolution of delayed-release dosage forms in ⟨711⟩ focuses on enteric-coated dosage forms, which is the most common delayed-release dosage form. A dissolution test for a delayed-release tablet or capsule is a two-part test, and each part has acceptance criteria. First, the dosage forms are exposed to an acid medium, followed by exposure to a buffer medium. To ensure that the enteric coating performs properly, a "NMT" acceptance criterion is indicated in ⟨711⟩ for the acid stage. The medium used for an acid stage is usually 0.1 N HCl, and the duration of this stage is typically 2 h. The dosage forms are then exposed to a buffer medium, usually 0.05 M phosphate buffer at pH 6.8, but other buffers and pH targets may be used if justified. The duration of the buffer stage is usually 45 min for compendial tests, but this duration may vary, depending on the drug product. As with immediate-release dosage forms, a Q value and time point are determined by evaluating the entire dissolution profile.

6.3 Extended-Release Dosage Forms

A dissolution test for an extended-release dosage form is generally similar to that used for an immediate- or delayed-release drug product, except that the duration of the test is longer, and at least three time points are specified for pharmacopeial purposes *(20)*. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a final time point is chosen to show essentially complete release of the drug *(20)*. The time points for

the test should be determined by evaluating the dissolution profile across the desired test duration. Often, additional time points are obtained during dosage form development to aid with selecting the appropriate time points for the specification or monograph.

As with an immediate- or delayed-release drug product, the acceptance criteria and time points for an extended-release drug product should discriminate between an acceptable and an unacceptable batch. The acceptance criteria for the first stage of testing (L₁) should be established on the basis of available batch data *(19,20)*. If human bioavailability data are available for formulations exhibiting different release rates, then an in vitro/in vivo relationship may be used to establish acceptance criteria *(19,20)*. Acceptance criteria for the second (L₂) and third (L₃) stages are derived from the L₁ criteria using *Acceptance Table 2* in ⟨711⟩.

6.4 Multiple Dissolution Tests

Typically, monographs for extended-release dosage forms contain multiple dissolution tests representing specific products. In accordance with *General Notices and Requirements 4.10.10*, the appropriate test, if not *Test 1*, is indicated on the product labeling. For example, the *USP* monograph for *Oxycodone Hydrochloride Extended-Release Tablets (21)* lists two dissolution tests, each of which has either three or four time points. If the Tablets are analyzed using *Test 2* and the dissolution results comply with the criteria provided in the monograph, the labeling for Tablets can indicate that the Tablets meet USP *Dissolution Test 2*. Multiple dissolution tests also can be found in monographs for immediate- and delayed-release dosage forms. For example, the *USP* monographs for *Levothyroxine Sodium Tablets* and *Pantoprazole Sodium Delayed-Release Tablets* provide four dissolution tests *(22,23)*.

6.5 Interpretation of Dissolution Results

The *Interpretation* section of ⟨711⟩ discusses immediate-, delayed-, and extended-release dosage forms. The discussion for each of these release patterns is expanded here with examples to assist with applying the criteria during the various stages of testing. Understanding how these criteria are applied will assist in setting appropriate acceptance criteria.

6.5.1 IMMEDIATE-RELEASE DOSAGE FORMS

Once the *Q* value is established, the dissolution test is a staged test of three levels. In the first level of testing called S₁, six dosage forms are tested. Each dosage form must be *Q* + 5% (absolute percentage points) dissolved at a specified time. For example, the time and tolerances in a monograph would be:

Time: 30 min
Tolerances: NLT 80% (*Q*) of the labeled amount of "drug substance" is dissolved.

If the *Q* value for a 200-mg label claim (LC) immediate-release tablet is specified as 80% and the time point is 30 min, then NLT 85% LC (170 mg) of the drug substance in each tablet must be dissolved at 30 min.

If this criterion is not met, then 6 additional tablets are tested at level 2 (S₂). To pass the S₂ acceptance criteria, the average of all 12 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example), and no tablet has less than *Q* – 15% (65% LC; 130 mg in the above example).

If these criteria are not met, then level 3 or S₃ testing must be performed by testing 12 additional tablets. To pass S₃, the average of all 24 tablets must be equal to or greater than *Q* (80% LC; 160 mg in the above example). Two additional criteria must be met as well: 1) no more than 2 tablets are less than *Q* – 15% (65% LC; 130 mg in the above example), and 2) no tablet is less than *Q* – 25% dissolved (55% LC; 110 mg in the above example.)

6.5.2 DELAYED-RELEASE DOSAGE FORMS

An aliquot of the acid medium from each vessel is analyzed at the end of the acid stage. For the acid stage, the acceptance criteria have three levels. Level 1 (A₁) testing is passed if no individual value exceeds 10% dissolved. If the A₁ criteria are not met, then the dissolution test is performed on 6 additional dosage forms for level 2 (A₂) testing. Level A₂ criteria are passed if the average of all 12 dosage forms in the acid stage is NMT 10% dissolved and if no individual dosage form is more than 25% dissolved. Level 3 testing is performed if the A₂ criteria are not met. The A₃ criteria are passed if the average of all 24 dosage forms in the acid stage is NMT 10% dissolved and if no individual tablet is more than 25% dissolved. For the special case in which the solubility of the drug in an acidic medium because of conversion to the free acid is too low to support an acceptance criterion of not more than 10% the drug product should be exposed to the acid stage for the defined duration and then exposed to the buffered medium. Alternate acceptance criteria for the acid stage based on drug solubility may be justified.

For delayed-release dosage forms, the total percentage dissolved is determined by adding the measured amounts in the acid and buffer phases for each individual dosage form. These calculated values are then compared to staged acceptance criteria (B₁, B₂, and B₃) that are based on a *Q* value. The B₁, B₂, and B₃ criteria are identical to those for the immediate release S₁, S₂, and S₃ criteria.

6.5.3 EXTENDED-RELEASE DOSAGE FORMS

In the following hypothetical example, which is used to describe the criteria for an extended-release dosage form, the time points are 1, 4, and 8 h. The acceptance range for each time point is as follows:
- Between 24% and 44% LC drug substance dissolved at 1 h
- Between 56% and 76% LC drug substance dissolved at 4 h
- NLT 85% LC drug substance dissolved at 8 h.

Acceptance ranges are often expressed in tabular form in the *USP–NF* (see *Table 3*):

Table 3. L_1 Criteria

Time (h)	Amount Dissolved
1	24%–44%
4	56%–76%
8	NLT 85%

Six tablets are analyzed at Level 1 (L_1); acceptance criteria are met if no individual value lies outside each of the stated ranges, and no individual value is less than the percentage specified for the final time point. If the L_1 criteria are not met, then 6 additional tablets are analyzed at level 2 (L_2). The L_2 criteria are met if these three conditions are met:
1. The average value of the 12 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. None of the 12 tablets is >10% of the labeled content outside each of the stated ranges.
3. None of the 12 tablets is >10% of the labeled content below the stated amount at the final test time.

For the above example, the L_2 acceptance criteria for the 12 tablets (see *Table 4*) are as follows:

Table 4. L_2 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%
Individual tablets	14%–54%	46%–86%	NLT 75%

If the L_2 criteria are not met, then 12 additional tablets are tested at level 3 (L_3). The L_3 criteria are met if these five conditions are met:
1. The average value of the 24 tablets lies within each of the stated ranges and is NLT the stated range of the final time point.
2. NMT 2 of the 24 tablets are >10% of labeled content outside each of the stated ranges.
3. NMT 2 of the 24 tablets are >10% of the labeled content below the stated amount at the final test time.
4. None of the 24 tablets is >20% of the labeled content outside each of the stated ranges.
5. None of the 24 tablets is >20% of the labeled content below the stated amount at the final test time.

The L_3 acceptance criteria for the 24 tablets in the above example are summarized in *Table 5*:

Table 5. L_3 Criteria

	1 h	4 h	8 h
Average	24%–44%	56%–76%	NLT 85%
Individual Tablets	NMT 2 tablets are outside the range of 14%–54%, and no individual tablet is outside the range of 4%–64%	NMT 2 tablets are outside the range of 46%–86%, and no individual tablet is outside the range of 36%–96%	NMT 2 tablets release <75% and no individual tablet releases <65%

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