Key to interpreting proposals

Proposed revisions are shown with language proposed for deletion or replacement crossed off. New text (if any) follows, and is enclosed in symbols as shown in the examples below:

▲new text▲FCC 7
if slated for FCC 7; and
▼new text▼1S (FCC 7)
if slated for a Supplement to FCC. The same symbols not set off by an extra paragraph break and enclosing text with no increase in type size indicate recent revisions that are already official. Where the symbols appear together with no enclosed text, such as ▼▼ or ▲▲, it means that text has been deleted and no new text was proposed to replace it. In all revisions, the closing symbol is accompanied by an identifier that indicates the particular Supplement or indicates the FCC as the publication where the revision will appear if approved. For example, ▼1S (FCC 7) indicates that the proposed revision is slated for the First Supplement to FCC 7, and ▲FCC 7 indicates that the revisions are proposed for FCC 7.
BRIEFING

Alitame, FCC 7 page 30. On the basis of comments and data received, the following revisions are proposed:

1. Clarify the subject of this monograph as the 2.5 hydrate material only by removing the chemical structure, chemical formula, formula weight, and CAS number specific for the anhydrous material, and revise the chemical synonym to be clear that it is the hydrated material. Data and comments received indicate that the 2.5 hydrate material was the commercially-available product on which the monograph was based.

2. On the basis of efforts to modernize the Identification test methods, it is proposed to change the existing infrared method under Identification test A to one that utilizes a USP Reference Standard instead of comparison to a printed spectrum. Although the printed spectrum will no longer be used in the Acceptance criteria for this test, it will be moved to the General Information section of FCC and available for informational purposes.

3. On the basis of efforts to modernize the procedures that utilize reference materials but do not specify the quality of the material to be used, a revision is proposed to replace the generic alitame reference standard material in the Assay with a USP Reference Standard.

4. Specify that the sample used in the test for Optical (Specific) Rotation should be the undried 2.5 hydrate material because further drying of the material will yield results outside of the specified range.

(FIEC: K. Laurvick) C96176

Add the following:

▲Alitame

Change to read:

\[ \text{L-\alpha-Aspartyl-N(2,2,4,4-tetramethyl-3-thietanyl)-d-alaninamide, \ hydrated\ 3S (FCC7)} \]

\[ \text{C}_{14}\text{H}_{25}\text{N}_{3}\text{O}_{4}\text{S} \]

\[ \text{C}_{14}\text{H}_{25}\text{N}_{3}\text{O}_{4}\text{S} \cdot 2.5 \text{H}_{2}\text{O} \]
DESCRIPTION

Alitame occurs as a white, odorless, crystalline powder having an intensely sweet taste. One method of production is through a multi-step synthesis involving the reaction between two intermediates, (S)-[2,5-dioxo-(4-thiazolidine)] acetic acid and (R)-2-amino-N-(2,2,4,4-tetramethyl-3-thietanyl)propanamide. The final product is isolated and purified through crystallization of an alitame/4-methylbenzenesulfonic acid adduct followed by additional purification steps, and finally recrystallization from water as the 2.5 hydrate. It is freely soluble in water and alcohol, and the pH of a 5% solution is between 5.0 and 6.0.

Function: Sweetener; flavor enhancer

Packaging and Storage: Store in tight containers in a cool place.

IDENTIFICATION

Change to read:

A. Infrared Spectra—Spectrophotometric Identification Tests, Appendix IIIC

 acceptance criteria: The spectrum of a potassium bromide dispersion of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

Reference standard: USP Alitame RS

Sample and standard preparation: K

Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

B. Procedure

Sample: 10 mg

Analysis: To 5 mL of a solution containing 300 mg of ninhydrin in 100 mL of n-butanol and 2 mL of glacial acetic acid, add the Sample and heat to gentle reflux.

Acceptance criteria: An intense blue-violet color is formed.

C. Procedure

Sample: 10 mg

Analysis: To 5 mL of a freshly prepared 0.001 M potassium permanganate solution, add the Sample and mix thoroughly.

Acceptance criteria: The purple solution changes to brown.
ASSAY

Change to read:

• Procedure

[Note—In this procedure, alitame and its impurities, alanine amide (N(2,2,4,4-tetramethyl-3-thietanyl)-d-alaninamide) and beta-isomer (L-asparyl-N(2,2,4,4-tetramethyl-3-thietanyl)-d-alaninamide hydrate) [2:5]), are measured by reverse-phase ion-pair high performance liquid chromatography.]

Solution A: Dissolve 0.69 g of sodium phosphate monobasic monohydrate and 4.32 g of 1-octanesulfonate, sodium in 200 mL of water. Adjust with 85% phosphoric acid to a pH of 2.5, then dilute with water to 1000 mL. Pass through a 0.22-µm Millipore filter, or equivalent.

Mobile phase: Acetonitrile and Solution A (1:4). [Note—Degas by sonication under aspirator vacuum for 2 min.]

Standard solution A: Transfer 25 mg each of a suitable alanine amide reference standard and a suitable beta-isomer reference standard to a 500-mL volumetric flask using 50 mL of methanol to aid in dissolution. Dilute with water to volume. [Note—Store in a refrigerator.]

Dilute standard solution A: Transfer 15.0 mL of Standard solution A to a 50-mL volumetric flask and dilute with water to volume.

Standard solution B: Transfer 50 mg of a suitable alitame reference standard USP Alitame RS 3S (FCC7) to a 10-mL volumetric flask. Add 3 mL of water to dissolve, the alitame 3S (FCC7) then add 5 mL of Dilute standard solution A, and dilute with water to volume.

Dilute standard solution B: Transfer 5 mL of Standard solution B to a 50-mL volumetric flask, and dilute with water to volume.

Sample solution: 5 mg/mL

Dilute sample solution: 0.5 mg/mL, from the Sample solution

Chromatographic system, Appendix IIA

Mode: High-performance liquid chromatography
Detector: UV 217 nm
Column: 15-cm × 0.39-cm NovaPak C18 reverse phase ion-pair (Waters, or equivalent)
Flow rate: 1.0 mL/min. [Note—Maintain the Mobile phase at a pressure and flow rate capable of giving the elution times listed under System suitability.]
Injection size: 100 µL

System suitability

Sample: Dilute standard solution B (three replicates)
Suitability requirement: The relative standard deviation is NMT 2% for the alitame peak area.

Analysis: [Note—All injections should be done in triplicate. The retention times for the beta-isomer, alitame, and alanine amide should be approximately 6, 10, and 15 min, respectively. If a column of a different make or length is used, the retention times may vary proportionally to the times listed.] Equilibrate the column by pumping Mobile phase through it until a drift-free baseline is obtained. Inject the Dilute sample solution and Dilute standard solution B into the chromatograph and record the chromatograms. Calculate the average peak areas for alitame from both chromatograms.

Calculate the weight percent for alitame in the sample taken:

Result = \left( \frac{r_{DU}}{r_{DS}} \right) \times \left( \frac{C_{DS}}{C_{DU}} \right) \times 100
\[ r_{DU} = \text{peak response for alitame from the Dilute sample solution} \]
\[ r_{DS} = \text{peak response for alitame from Dilute standard solution B} \]
\[ C_{DS} = \text{concentration of alitame in Dilute standard solution B, corrected for water content and purity (mg/mL)} \]
\[ C_{DU} = \text{concentration of the Dilute sample solution, corrected for water (mg/mL)} \]

**Acceptance criteria:** 98.0%–101.0% of alitame, calculated on the anhydrous basis

**IMPURITIES**

**Inorganic Impurities**

- **LEAD, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Appendix IIIb**
  - Sample: 5 g
  - Acceptance criteria: NMT 1 mg/kg

**Organic Impurities**

- **ALANINE AMIDE AND BETA-ISOMER**
  - **Solution A, Mobile phase, Standard solution A, Dilute standard solution A, Standard solution B, Dilute standard solution B, Sample solution, Dilute sample solution, and Chromatographic system:** Proceed as directed in the Assay.
  - **Analysis:** Proceed as directed in the Assay. Inject the Sample solution and Standard solution B into the chromatograph and record the chromatograms. Calculate the average peak areas for beta-isomer and alanine amide from both chromatograms.
  - Calculate the weight percent of alanine amide and beta-isomer in the sample taken:
    
    \[
    \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
    \]

    \[ r_U = \text{peak response for the analyte from the Sample solution} \]
    \[ r_S = \text{peak response for the analyte from Standard solution B} \]
    \[ C_S = \text{concentration of the analyte in Standard solution B, corrected for water content and purity (mg/mL)} \]
    \[ C_U = \text{concentration of the Sample solution, corrected for water (mg/mL)} \]

    **Acceptance criteria**
    - Alanine amide: NMT 0.2%, calculated on the anhydrous basis
    - Beta-isomer: NMT 0.3%, calculated on the anhydrous basis

**SPECIFIC TESTS**

- **Residue on Ignition (Sulfated Ash), Appendix IIc**
  - Sample: 1 g
  - Acceptance criteria: NMT 1.0%

**Change to read:**

- **Optical (Specific) Rotation, Appendix IIb**
  - Sample solution: 10 mg/mL, on the as-is (undried) basis \(3S\) (FCC7)
    - Acceptance criteria: \([\alpha]_D^{25}\) between +40° and +50°, on the as-is basis \(3S\) (FCC7)

- **WATER, Water Determination, Appendix IIb**
  - Acceptance criteria: Between 11% and 13%

\(^{\text{FCC7}}\)
**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>
ARA from Fungal (Mortierella alpina) Oil. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received.

1. The tests proposed for Arsenic, Lead, and Mercury under Inorganic Impurities are consistent with tests used for similar oils in FCC.
2. The sponsor of the monograph proposed a specification for Mercury of 0.05 mg/kg; however, it is not clear that the methods referenced can accurately measure to that limit. Comments and/or data supporting a lower limit of 0.05 mg/kg are encouraged.
3. The instructions given in the Labeling section are consistent with newer FCC monographs for similar oil products, DHA from Algal (Cryptochromen) Oil and DHA from Algal (Schizochytrium) Oil.
4. The tests for Lovibond Color and Unsaponifiable Matter under Specific Tests are based on revisions being proposed to Appendix VII, appearing elsewhere in this Forum. See the Briefing under Appendix VII: Fats and Related Substances.

Add the following:

- ARA from Fungal (Mortierella alpina) Oil
- Arachidonic Acid-Rich Oil
- ARA Fungal Oil
- ARA-Rich Oil
- Mortierella alpina Oil
- Refined Arachidonic Acid-Rich Oil (RAO)

DESCRIPTION
ARA from Fungal (Mortierella alpina) Oil occurs as a clear, yellow-colored oil providing a source of arachidonic acid (ARA, \(\text{C}_20\text{H}_{32}\text{O}_2\)) (C20:4 n-6), an omega-6 long-chain polyunsaturated fatty acid. It is obtained from fermentation of the species of fungus Mortierella alpina followed by solvent extraction. The oil may be winterized, bleached, and deodorized to substantially remove free fatty acids, phospholipids, odor and flavor components, and other material. Arachidonic acid is the main polyunsaturated fatty acid present; ARA content may be standardized with other oils. Suitable antioxidants may be added.

Function: Source of ARA

Packaging and Storage: Store in tight, light-resistant containers. Avoid exposure to excessive heat.

IDENTIFICATION
- **Fatty Acid Composition**, Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids, Appendix VII

Acceptance criteria: The retention times of the peaks of the arachidonic acid methyl ester from the Sample Preparation correspond to those from the Standard Solution. The percent of the fatty acids (calculated
using the results from the corresponding methyl esters) from the Sample Preparation, determined as the percent of total fat, meet the requirements for each fatty acid indicated in the table below.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Shorthand Notation</th>
<th>Lower Limit (Area %)</th>
<th>Upper Limit (Area %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>14:0</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>16:0</td>
<td>4.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>16:1 n-9</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>18:0</td>
<td>3.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1 n-9</td>
<td>2.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18:2 n-6</td>
<td>5.6</td>
<td>10.1</td>
</tr>
<tr>
<td>gamma-Linolenic acid</td>
<td>18:3 n-6</td>
<td>1.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Arachidic acid</td>
<td>20:0</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Dihomo-gamma-linolenic acid</td>
<td>20:3 n-6</td>
<td>3.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20:4 n-6</td>
<td>40.0</td>
<td>48.5</td>
</tr>
<tr>
<td>Behenic acid</td>
<td>22:0</td>
<td>2.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Lignoceric acid</td>
<td>24:0</td>
<td>9.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>

**ASSAY**

- **ARA, Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids, Appendix VII**

  **Analysis:** Proceed as directed. Calculate the percent ARA (w/w, as a percent of total fat) in the sample:

  \[
  \text{Result} = \left( W_{FAMEx} \times F_{FAX} \right) / \sum W_{TAG}
  \]

  [Note—Use the definitions provided for \( W_{FAMEx} \), \( F_{FAX} \), and \( \sum W_{TAG} \) in the method referenced, where \( x \) is arachidonic acid.]

  **Acceptance criteria:** NLT 40.0% arachidonic acid (ARA)

**IMPURITIES**

**Inorganic Impurities**

- **Arsenic, Elemental Impurities by ICP, Appendix IIIC**

  [Note—Alternatively, the arsenic content may be determined by the following method.]

  **Apparatus**

  **Sample digestion:** Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

  **Sample analysis:** Use a suitable graphite furnace atomic absorption spectrophotometer (GFAAS) equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. An electrodeless discharge lamp serves as the source, argon as the purge gas, and air as the alternate gas. Set up the instrument according to the manufacturer’s specifications, with consideration of current good GFAAS practices. The instrument parameters are as follows:

  **Wavelength:** 193.7 nm

  **Lamp current:** 300 (EDL) modulated
Pyrolysis: 1000°
Atomization: 2400°
Slit: 0.7

Characteristic mass: 15 pg

Glassware: Acid wash all glass, Teflon, and plastic vessels by soaking them in a nitric acid bath containing a 4:1 solution of water:nitric acid. [CAUTION—Wear a full face shield, protective clothing, and gloves at all times when working with acid baths.] After acid soaking, rinse the acid-washed items in deionized water, dry them, and store them in clean, covered cabinets.

Calibration standard stock solution: 100 µg/L. Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

Calibration standard solutions: 2.0, 5.0, 10.0, 25.0, and 50.0 µg/L in 2% nitric acid, from the Calibration standard stock solution

1% Palladium stock solution: Mix 1 g of ultrapure palladium metal with 20 mL of water and 10 mL of nitric acid in a Teflon beaker, and warm the solution on a hot plate to dissolve the palladium. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Mix 1 g of ultrapure magnesium nitrate with 40 mL of water and 1 mL of nitric acid in a Teflon beaker, and warm the solution on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

[Note—Because of the difficulty in preparing matrix modifier stock solutions with the required purity, purchasing modifier stock solutions and using them to prepare modifier working solutions is recommended.]

Modifier working solution: Transfer 3 mL of 1% Palladium stock solution and 2 mL of 1% Magnesium nitrate stock solution to a 10-mL volumetric flask and dilute with 2% nitric acid to volume. A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Sample solution: [CAUTION—Wear proper eye protection, protective clothing, and gloves during sample preparation. Closely follow the manufacturer’s safety instructions for use of the microwave digestion apparatus.] Transfer 500 mg of sample into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressuresensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature (a cool water bath may be used to speed the cooling process). Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with deionized water to volume.

Analysis: The graphite furnace program is as follows:

1 Dry at 115° using a 1-s ramp, a 65-s hold, and a 300-mL/min argon flow.
2 Char the sample at 1000° using a 1-s ramp, a 20-s hold, and a 300-mL/min air flow.
3 Cool down and purge the air from the furnace for 10 s using a 20° set temperature and a 300-
mL/min argon flow.

4Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped.

5Clean out at 2600° with a 1-s ramp and a 5-s hold.

Use the autosampler to inject 20-µL aliquots of blanks, Calibration standard solutions, Sample solution, and 5 µL of Modifier working solution. Inject each solution in duplicate, and average the results. Use peak area measurement for all quantitations. After ensuring that the furnace is clean by running a 5% nitric acid blank, check the instrument’s sensitivity by running a 20-µL aliquot of the 25.0-µg Calibration standard solution. Compare the results obtained with the expected results for the equipment used, and take the necessary steps to correct any problems.

Calculate the characteristic mass. Record and track the integrated absorbance and characteristic mass for reference and quality assurance.

Inject each Calibration standard solution in duplicate. Use the algorithms provided in the instrument software to establish calibration curves. Recheck calibration periodically, and recalibrate if the recheck differs from the original calibration by more than 10%.

Inject the Sample solution in duplicate, and record the integrated absorbance. If the instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample's response into the working range, and note the dilution factor (DF). All sample analyses should be blank corrected using a sample solution blank.

If a computer-based instrument is used, the data output is reported as µg/L. Calculate the concentration of arsenic, in µg/g (equivalent to mg/kg), in the original sample taken:

\[
\text{Result} = \frac{C \times DF \times V}{W}
\]

\[
C = \text{concentration of arsenic in the sample aliquot injected (µg/L)}
\]

\[
DF = \text{dilution factor of the Sample solution}
\]

\[
V = \text{final volume of the Sample solution (L)}
\]

\[
W = \text{weight of the sample taken to prepare the Sample solution (g)}
\]

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

Acceptance criteria: NMT 0.1 mg/kg

- Cadium, Elemental Impurities by ICP, Appendix IIIC
  Acceptance criteria: NMT 0.1 mg/kg

- Lead, Elemental Impurities by ICP, Appendix IIIC
  [Note—Alternatively, the lead content may be determined by the following method.]

Apparatus

Sample digestion: Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

Sample analysis: See Apparatus in Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB

Calibration standard stock solution: 100 µg/L. Prepare from a suitable standard, which may be purchased (accuracy certified against NIST spectrometric standard solutions).

Calibration standard solutions: 2.0, 5.0, 10.0, 25.0, and 50.0 µg/L in 2% nitric acid, from the Calibration standard stock solution.
10% Ammonium dihydrogen phosphate stock solution: Mix 10 g of ultrapure ammonium dihydrogen phosphate with 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate stock solution: Mix 1 g of ultrapure magnesium nitrate with 40 mL of water and 1 mL of nitric acid in a Teflon beaker, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

[Note—Because of the difficulty in preparing matrix modifier stock solutions with the required purity, purchasing modifier stock solutions and using them to prepare modifier working solutions is recommended.]

Modifier working solution: Transfer 4 mL of 10% Ammonium dihydrogen phosphate stock solution and 2 mL of 1% Magnesium nitrate stock solution to a 10-mL volumetric flask, and dilute with 2% nitric acid to volume. A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Sample solution: Prepare as directed for Sample solution in the test for Arsenic.

[CAUTION—Wear proper eye protection, protective clothing, and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus.]

Analysis: The graphite furnace program is as follows:

1. Dry at 120°C using a 1-s ramp, a 55-s hold, and a 300-mL/min argon flow.
2. Char the sample at 850°C using a 1-s ramp, a 30-s hold, and a 300-mL/min air flow.
3. Cool down and purge the air from the furnace for 10 s using a 20°C set temperature and a 300-mL/min argon flow.
4. Atomize at 2100°C using a 0-s ramp and a 5-s hold with the argon flow stopped.
5. Clean out at 2600°C with a 1-s ramp and a 5-s hold.

Use the autosampler to inject 20-µL aliquots of blanks, Calibration standard solutions, Sample solution, and 5 µL of Modifier working solution. Inject each solution in duplicate, and average the results. Use peak-area measurement for all quantitation. After ensuring that the furnace is clean by running a 5% nitric acid blank, check instrument sensitivity by running an aliquot of the 25.0-µg Calibration standard solution. Compare the results obtained with the expected results for the equipment used, and take the necessary steps to correct any problems.

Calculate the characteristic mass, and record and track the integrated absorbance and characteristic mass for reference and quality assurance.

Inject each Calibration standard solution in duplicate. Use the algorithms provided in the instrument software to establish calibration curves. Recheck the calibration periodically, and recalibrate if recheck differs from the original calibration by more than 10%.

Inject the Sample solution in duplicate, and record the integrated absorbance. If the instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into the working range, and note the dilution factor (DF). All sample analyses should be blank corrected using a sample solution blank.

If a computer-based instrument is used, the data output is reported as µg/L. Calculate the concentration, in µg/g (equivalent to mg/kg), of lead in the original sample:

\[
\text{Result} = \frac{C \times DF \times V}{W}
\]
C = concentration of lead in the sample aliquot injected (µg/L)
DF = dilution factor of the Sample solution
V = final volume of the Sample solution (L)
W = weight of the sample taken to prepare the Sample solution (g)

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

Acceptance criteria: NMT 0.1 mg/kg

Mercury

Apparatus

Sample digestion: Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

Sample analysis: Use a suitable atomic absorption spectrophotometer equipped with an atomic vapor assembly. An electrodeless discharge lamp serves as the source, with an inert gas such as argon or nitrogen as the purge gas. Set up the instrument according to the manufacturer's specifications.

Instrument parameters are as follows:
Wavelength: 253.6 nm
Slit: 0.7
Reagent setting: 5
Gas flow: 5 to 6 L/min
Reaction time: 0.5 min

Glassware: Acid wash all glass, Teflon, and plastic vessels by soaking them in a nitric acid bath containing a 4:1 solution of water:nitric acid. [CAUTION—Wear a full face shield, protective clothing, and gloves at all times when working with acid baths. ] After acid soaking, rinse the acid-washed items in deionized water, dry, and store them in clean, covered cabinets.

Calibration standard stock solution: 200 ng/g of mercury. Prepare from a suitable standard, which may be purchased (accuracy certified against NIST spectrometric standard solutions).

Calibration standard solutions: 20, 60, 100, 200, and 400 ng of mercury in 1 N hydrochloric acid from the Calibration standard stock solution

Reducing reagent: 5% Stannous chloride in 25% hydrochloric acid (trace-metal grade). [Note—Prepare daily.]

Sample solution: Prepare as directed for the Sample solution in the test for Arsenic. [CAUTION—Wear proper eye protection, protective clothing, and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus. ]

Analysis: Optimize the instrument settings for the spectrophotometer as described in the instrument manual. The instrument parameters for cold vapor generation are as follows:
Wavelength: 253.6 nm
Slit: 0.70 nm
Reagent setting: 5
Gas flow: 5 to 6 L/min
Reaction time: 0.5 min

Use a peak height integration method with a 40-s integration time and a 20-s read delay in an unheated absorption cell. Zero the instrument as follows: Place a Fleaker containing 50 mL of 1 N hydrochloric acid in the sample well of the hydride generator. Press "start" on the vapor generator and "read" on the atomic absorption spectrophotometer. The instrument will automatically flush the
sample container with nitrogen, dispense the designated amount of reagent, stir the sample for a
designated reaction time, and purge the head volume again with nitrogen, sweeping any vapor into
the quartz cell for determination of absorption. The atomic absorption spectrophotometer will
automatically zero on this sample when “autozero” is selected from the calibration menu.

Generate a standard curve of concentration versus absorption by analyzing the five Calibration
standard solutions prepared as described for daily standards under Calibration standard solutions.
Analyze each solution in duplicate, generate the calibration curve, and store, using procedures
specific for the instrumentation.

Transfer an appropriate aliquot of Sample solution (usually 2 mL) in a Fleaker containing 50 mL of 1 N
hydrochloric acid. Analyze solutions in duplicate using the procedure specified in the instrument
manual. Using the calibration algorithm provided in the instrument software, calculate and report the
mercury concentration in ng of mercury in the aliquot analyzed.

Calculate the level of mercury, as µg/g (equivalent to mg/kg), in the original sample:

\[ \text{Result} = \frac{A \times DF}{W \times 1000} \]

\[ A = \text{amount of mercury in the aliquot analyzed (ng)} \]
\[ DF = \text{dilution factor (final volume of Sample solution/volume taken for analysis)} \]
\[ W = \text{weight of the sample taken to prepare the Sample solution (g)} \]

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze
blanks, spiked blanks, and a spiked oil with each digestion set.]

**Acceptance criteria:** NMT 0.1 mg/kg

**Organic Impurities**

- **Hexane Residues**
  - **Vegetable oil:** Use solvent-free vegetable oil that is similar in nature to the sample. Deodorization of the oil
    in the laboratory may be used to reduce the amount of extraction solvent present in the oil.
  - **Internal standard:** n-Heptane

**Calibration solutions:** Prepare a series of solutions by adding 0, 20, 40, 60, 80, and 100 µL of n-hexane,
separately, to a series of vials, each containing 25 g of Vegetable oil. Close the vials, then mechanically
shake them vigorously for 1 h at room temperature. After shaking the vials, add 5 µL of the Internal
standard to each vial, using a syringe. [Note—The vial with 0 µL of n-hexane added is the blank.]

**Sample solution:** Weigh 25.00 g of the sample into a septum vial. Close the vial and, using a syringe, add
25 µL of the Internal standard to the sample. Shake the vial vigorously for about 1 min before proceeding
with the Analysis.

**Chromatographic system,** Appendix IIA

- **Mode:** Headspace gas chromatography
- **Detector:** Flame ionization
- **Column:** 30-m × 0.3-mm fused silica or glass capillary column coated with methyl polysiloxane (0.2-µm
  thickness)\(^7\)
- **Temperature**
  - **Oven:** 40°
  - **Injection port:** 120°
  - **Detector:** 120°
- **Headspace sampling conditions**
  - **Sample heating temperature:** 60°
**Sample heating time:** 30 min

**Syringe temperature:** 60°

**Carrier gas:** Helium

**Flow rate:** Optimize accordingly

**Injection volume:** 1000 µL

**Determination of calibration factors:** Warm a 1000-µL gas-tight syringe to 60°. Temper each *Calibration solution* in a water bath maintained at 60° for exactly 30 min, then, without removing the vial from the bath, use the gas-tight syringe and withdraw 1000 µL of the headspace above the oil. Inject immediately into the gas chromatograph, record the chromatograms from each *Calibration solution*, and determine the peak areas.

For each of the *Calibration solutions* containing \(n\)-hexane (not including the blank solution) calculate the calibration factor, \(F\):

\[
F = \frac{C_S \times A_I}{(A_H - A_B - A_I) \times C_I}
\]

- \(C_S\) = concentration of \(n\)-hexane in the *Calibration solution* of interest (mg/kg)
- \(A_I\) = peak area corresponding to the *Internal standard* in the chromatogram of the *Calibration solution*
- \(A_H\) = total peak area of solvent hydrocarbons in the chromatogram of the *Calibration solution*, including the area of the *Internal standard*, not including peaks due to the oxidation products
- \(A_B\) = peak area of the solvent hydrocarbons present in the blank solution, minus the peak area of the *Internal standard*
- \(C_I\) = quantity of the *Internal standard* added to the *Calibration solution*, in mg/kg of oil (680 mg)

[Note—Calculate calibration factors to three decimal points. The mean calibration factor will be used in the Analysis.]

**Analysis:** Warm a 1000-µL gas-tight syringe to 60°. Temper the *Sample solution* in a water bath maintained at 60° for exactly 30 min, then, without removing the vial from the bath, use the gas-tight syringe and withdraw 1000 µL of the headspace above the oil. Inject immediately into the gas chromatograph, record the chromatogram, and determine the peak areas.

Determine the residual solvent content, in mg/kg of hexane:

\[
\text{Result} = (A_H - A_I) \times F \times C_I \times (1/A_I)
\]

- \(A_H\) = total peak area of solvent hydrocarbons in the chromatogram of the *Sample solution*, including the area of the *Internal standard* (do not include peaks due to oxidation products)
- \(A_I\) = peak area corresponding to the *Internal standard* in the chromatogram of the *Sample solution*
- \(F\) = mean calibration factor determined above
- \(C_I\) = quantity of the *Internal standard* added to the *Sample solution*, in mg/kg of sample (680 mg)

**Acceptance criteria:** NMT 1.0 mg/kg
SPECIFIC TESTS

- **Acid Value**, Appendix VII
  - Acceptance criteria: NMT 1.0

- **Anisidine Value**, Appendix VII
  - Acceptance criteria: NMT 20

- **Free Fatty Acids (as Oleic Acid)**, Appendix VII
  - Analysis: Use 28.2 for the equivalence factor (e) in the formula given in the procedure.
  - Acceptance criteria: NMT 0.2%

- **Lovibond Color**, Appendix VII
  - Analysis: Use a 25.4-mm cell.
  - Acceptance criteria
    - Yellow: NMT 50
    - Red: NMT 5

- **Peroxide Value**, Appendix VII
  - Acceptance criteria: NMT 2.0 mEq/kg

- **Unsaponifiable Matter, Method II**, Appendix VII
  - Acceptance criteria: NMT 3.0%

OTHER REQUIREMENTS

- **Labeling**: Label to indicate the content of arachidonic acid in mg/g (%). Indicate the name of any added antioxidant and the presence of any other oil(s) used to standardize the arachidonic acid content.

---

1 CEM Model MDS-2100, or equivalent.

2 This method was developed using a Perkin-Elmer Model 5100, HGA-600 furnace, and an AS-60 autosampler with Zeeman effect background correction.

3 A palladium (0.3%) and magnesium nitrate (0.2%) solution may be purchased from High Purity Standards, or equivalent.

4 An ammonium dihydrogen phosphate (4%) and magnesium nitrate (0.2%) solution may be purchased from High Purity Standards, or equivalent.

5 This method was developed using a Perkin-Elmer Model 5100 and IL 440 Thermo Jarrell Ash atomic vapor assembly.

6 Adapted from AOCS Method Ca 3b-87 (1997). The original method is available from the American Oil Chemists’ Society (AOCS) at www.aocs.org.

7 HP-1 (Agilent Technologies), or equivalent.

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>
**BRIEFING**

**Astaxanthin Esters from *Haematococcus pluvialis*.** Because there is no existing *FCC* monograph for this food ingredient, a new monograph is proposed based on comments and data received.

1. In an effort to provide the ability to differentiate between astaxanthin esters from *Haematococcus pluvialis* and from krill oil, the *EPA Content* test under *Identification* is proposed. Data gathered indicates that krill oil has a comparatively significant content of EPA (greater than 10%), while products from *Haematococcus pluvialis* do not. Comments and additional data are encouraged.

2. The *Assay for Astaxanthin* was submitted without system suitability data and criteria. Comments including appropriate system suitability criteria with data are encouraged. It should be noted that this test is for total astaxanthin content. The sponsor of this monograph indicated that they have not seen a suitable validated method for separating free, mono-, and diesterified astaxanthin. Comments including suggestions for alternate methods and supporting data are encouraged as well.

3. The sponsor of the monograph originally proposed microbiological specifications for *Salmonella* sp., *E. coli, Staphylococcus aureus*, combined yeasts and molds, and total aerobic count. These specifications are not included because of *FCC*’s policy on microbiological limits (from *FCC* 5 page 3), which includes the following statement: “The General Policy for microbiological safety and quality of *FCC* substances is such that substances be produced, handled, and used in food processing following GMPs and applicable food safety systems. Therefore, the *FCC* does not list specific microbiological criteria for *FCC* substances other than those for which scientifically valid data are available to the committee that support the need for such criteria.” Comments regarding the omission of microbial specifications are encouraged.

4. The test for *Phaeophorbide Content* is a method from literature used by the sponsor and supported with batch data, however, a chromatographic technique may be preferred. Comments are encouraged.

5. The *Thin-Layer Chromatography* test under *Identification* is intended to differentiate between astaxanthin esters from *Haematococcus pluvialis* versus other sources, such as krill oil and *Xanthophyllomyces dendrorhous* (formerly *Phaffia* yeast). This test also allows differentiation between free astaxanthin from synthetic sources and esterified astaxanthin from *Haematococcus pluvialis*.

(FIEC: K. Laurvick)  C93993

---

**Add the following:**

- **Astaxanthin Esters from *Haematococcus pluvialis***

Astaxanthin

Astaxanthin Esters

Astaxanthin Fatty Acid Esters

*(3S,3'S)-3,3'-dihydroxy-β,β-carotene-4,4'-dione*

**DESCRIPTION**

Astaxanthin Esters from *Haematococcus pluvialis* occurs as a dark red, viscous oil. It is the product of the fermentation of *Haematococcus pluvialis*, extracted with either super critical CO2 or acetone. It is a complex
mixture, primarily composed of lipids, with astaxanthin esterified with common edible fatty acids to form both mono- and diesters. Esterified astaxanthin is the primary carotenoid present and the approximate astaxanthin composition is: 75% monoester, 20% diester, and 5% free form astaxanthin. Astaxanthin Esters from Haematococcus pluvialis is soluble in n-hexane, acetone, and ether; partially soluble in alcohol; practically insoluble in water and hot water. Suitable antioxidants may be added.

**Function:** Source of astaxanthin

**Packaging and Storage:** Store in tight, light-resistant containers in a cool place.

### IDENTIFICATION

- **EPA CONTENT, Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids,** Appendix VII
  
  **Analysis:** Proceed as directed, then calculate the amount of EPA (eicosapentaenoic acid; C20:5 n-3) present as the percent of total fatty acids.
  
  **Acceptance criteria:** NMT 1.0%

- **Thin-Layer Chromatography,** Appendix IIA
  
  **Sample solution:** 10 mg/mL in acetone
  
  **Standard solution:** 10 mg/mL of USP Astaxanthin Esters from Haematococcus pluvialis RS in acetone
  
  **Adsorbent:** 0.25-mm layer of chromatographic silica gel. [Note—Dry silica gel at 110°C for 1 h before use.]
  
  **Developing solvent system:** Hexane and acetone [70:30]
  
  **Application volume:** 5 µL
  
  **Analysis:** Develop the chromatogram in the Developing solvent system until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber and dry in a current of air.
  
  **Acceptance criteria:** The principal spots obtained from the Sample solution correspond in color, size, and Rf value to those obtained from the Standard solution.

### ASSAY

- **Astaxanthin (Total)** [Note—Astaxanthin measured by this method is total astaxanthin, including free astaxanthin and both mono- and diesters.]
  
  **Buffer solution:** Dissolve 6.06 g of tris(hydroxymethyl)aminomethane in 750 mL of water, adjust with 1 M hydrochloric acid to a pH of 7.0, and dilute with water to 1000 mL.
  
  **Solution A:** 4 U/mL of cholesterol esterase in Buffer solution. [Note—Prepare fresh daily.]
  
  **Internal standard solution:** 37.5 µg/mL of USP Apocarotenal RS in acetone
  
  **Standard stock solution:** Transfer 30 mg of USP Astaxanthin Esters from Haematococcus pluvialis RS to a 100-mL volumetric flask. Dissolve in 30 mL of acetone, shake by mechanical means, and dilute with acetone to volume.
  
  **Standard solution:** Combine 2.0 mL of the Standard stock solution and 1.0 mL of the Internal standard solution in a glass centrifuge tube. Add 3.0 mL of Solution A to the tube and mix gently by inversion. Place the tube in a block heater set to 37°C and allow the reaction to continue for 45 min, gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of sodium sulfate decahydrate and 2 mL of petroleum ether to the tube. Vortex the tube for 30 s, then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of sodium sulfate anhydrate. Be careful to avoid pipetting the intermediate emulsive layer. Evaporate the petroleum ether layer using a vacuum or a stream of inert gas at room temperature, add 3 mL of acetone, sonicate, and filter the mixture. The filtered solution is the Standard solution.
  
  **Sample stock solution:** Warm a quantity of the sample in a water bath at 50°–60° for 30 min. Shake the
sample well at 10-min intervals. After 30 min, transfer 30 mg of the sample to a 100-mL volumetric flask. Dissolve in 30 mL of acetone, shake by mechanical means, and dilute with acetone to volume. [Note—Prepare in triplicate.]

**Sample solution:** Combine 2.0 mL of the Sample stock solution and 1.0 mL of the Internal standard solution in a glass centrifuge tube. Add 3.0 mL of Solution A to the tube and mix gently by inversion. Place the tube in a block heater set to 37°C and allow the reaction to continue for 45 min, gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of sodium sulfate decahydrate and 2 mL of petroleum ether to the tube. Vortex the tube for 30 s, then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of sodium sulfate anhydrate. Be careful to avoid pipetting the intermediate emulsive layer. Evaporate the petroleum ether layer using a vacuum or a stream of inert gas at room temperature, add 3 mL of acetone, sonicate, and filter the mixture. The filtered solution is the Sample solution.

**Chromatographic system,** Appendix IIA

**Mode:** High-performance liquid chromatography

**Detector:** 474 nm

**Column:** 4.6-mm × 250-mm column with a C30 silane bonded stationary phase on fully porous spherical silica, 5-µm in diameter

**Column temperature:** 25°C

**Flow rate:** 1.0 mL/min

**Injection size:** 20 µL

**Mobile phase:** See the gradient table below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Methanol (%)</th>
<th>t-Butylmethyl ether (%)</th>
<th>Phosphoric acid, 1% aqueous (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>81</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>66</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>23</td>
<td>16</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>27</td>
<td>16</td>
<td>80</td>
<td>4</td>
</tr>
<tr>
<td>27.1</td>
<td>81</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>35</td>
<td>81</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

**Analysis:** Separately inject the Standard solution and the Sample solution into the chromatograph. Record the chromatograms, and identify the peaks by comparison to the Reference Chromatograms supplied with the USP Apocarotenal RS (internal standard) and with the USP Astaxanthin Esters from Haematococcus pluvialis RS. [Note—The approximate retention times for 13-cis-astaxanthin, trans-astaxanthin, 9-cis-astaxanthin, and the internal standard apocarotenol (trans-beta-apo-8'carotenal) are 9, 10, 14, and 17 min, respectively.]

For the Standard solution and the Sample solution, separately calculate the ratios of the peak responses of total astaxanthin to the internal standard obtained from the individual analysis:

\[
\text{Result} = \frac{(F_1 P_{13\text{-cis}} + P_{\text{trans}} + F_2 P_{9\text{-cis}})}{P_{\text{IS}}}
\]
Relative response coefficients of 13-cis- and 9-cis-astaxanthin to trans-astaxanthin are given by

\[ F_1 = \text{relative response coefficient of 13-cis-astaxanthin to trans-astaxanthin (1.3)} \]

\[ P_{13\text{-cis}} = \text{peak response for 13-cis-astaxanthin obtained from the chromatogram} \]

\[ P_{\text{trans}} = \text{peak response for trans-astaxanthin obtained from the chromatogram} \]

\[ F_2 = \text{relative response coefficient of 9-cis-astaxanthin to trans-astaxanthin (1.1)} \]

\[ P_{9\text{-cis}} = \text{peak response for 9-cis-astaxanthin obtained from the chromatogram} \]

\[ P_{\text{IS}} = \text{peak response for the internal standard, apocarotenal} \]

Calculate the percentage of astaxanthin (w/w) in the sample taken:

\[ \text{Result} = \left( \frac{R_U}{R_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100 \]

\[ R_U = \text{ratio of peak responses of total astaxanthin to the internal standard obtained from the Sample solution} \]

\[ R_S = \text{ratio of peak responses of total astaxanthin to the internal standard obtained from the Standard solution} \]

\[ C_S = \text{concentration of astaxanthin in the Standard solution (mg/mL)} \]

\[ C_U = \text{concentration of the Sample solution (mg/mL)} \]

Acceptance criteria: 5.0%–15.0%

**IMPURITIES**

Inorganic Impurities

- **As** [Note—Alternatively, the arsenic content may be determined by the following method.]

**Apparatus**

**Sample digestion:** Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

**Sample analysis:** Use a suitable graphite furnace atomic absorption spectrophotometer (GFAAS) equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. An electrodeless discharge lamp serves as the source, argon as the purge gas, and air as the alternate gas. Set up the instrument according to manufacturer's specifications, with consideration of current good GFAAS practices. The instrument parameters are as follows:

**Wavelength:** 193.7 nm

**Lamp current:** 300 (EDL) modulated

**Pyrolysis:** 1000°C

**Atomization:** 2400°C

**Slit:** 0.7

**Characteristic mass:** 15 pg

**Glassware:** Acid wash all glass, Teflon, and plastic vessels by soaking them in a nitric acid bath containing a 4:1 solution of water and nitric acid. **CAUTION**—Wear a full face shield and protective clothing and
gloves at all times when working with acid baths. After acid soaking, rinse acid-washed items in deionized water, dry them, and store them in clean, covered cabinets.

**Calibration standard stock solution:** 100 µg/L

Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

**Calibration standard solutions:** 2.0, 5.0, 10.0, 25.0, and 50.0 µg/L in 2% nitric acid, from the Calibration standard stock solution

1% Palladium stock solution: Mix 1 g of ultrapure palladium metal, with 20 mL of water and 10 mL of nitric acid in a Teflon beaker, and warm the solution on a hot plate to dissolve the palladium. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Mix 1 g of ultrapure magnesium nitrate, with 40 mL of water and 1 mL of nitric acid in a Teflon beaker, and warm the solution on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

[Note—Because of the difficulty in preparing matrix modifier stock solutions with the required purity, purchasing modifier stock solutions and using them to prepare working modifier solutions is recommended. A palladium (0.3%) and magnesium nitrate (0.2%) solution may be purchased from High Purity Standards, or equivalent.]

**Modifier working solution:** Transfer 3 mL of 1% Palladium stock solution and 2 mL of 1% Magnesium nitrate stock solution to a 10-mL volumetric flask and dilute with 2% nitric acid to volume. A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

**Sample solution:** [CAUTION—Wear proper eye protection and protective clothing and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus.]

Transfer 500 mg of the sample into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature (a cool water bath may be used to speed the cooling process). Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with deionized water to volume.

**Analysis:** The graphite furnace program is as follows:

1. Dry at 115° using a 1-s ramp, a 65-s hold, and a 300-mL/min argon flow.
2. Char the sample at 1000° using a 1-s ramp, a 20-s hold, and a 300-mL/min air flow.
3. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and a 300-mL/min argon flow.
4. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped.
5. Clean out at 2600° with a 1-s ramp and a 5-s hold.
Use the autosampler to inject 20-µL aliquots of blanks, Calibration standard solutions, and Sample solutions and 5 µL of Modifier working solution. Inject each solution in duplicate, and average the results. Use peak area measurement for all quantitations. After ensuring that the furnace is clean by running a 5% nitric acid blank, check the instrument's sensitivity by running a 20-µL aliquot of the 25-µg Calibration standard solution. Compare the results obtained with the expected results for the equipment used, and take the necessary steps to correct any problems. Calculate the characteristic mass. Record and track the integrated absorbance and characteristic mass for reference and quality assurance.

Inject each Calibration standard solution in duplicate. Use the algorithms provided in the instrument software to establish calibration curves. Recheck calibration periodically, and recalibrate if the recheck differs from the original calibration by more than 10%.

Inject the Sample solution in duplicate, and record the integrated absorbance. If the instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample's response into the working range, and note the dilution factor (DF). All sample analyses should be blank corrected using a sample solution blank.

If a computer-based instrument is used, the data output is reported as µg/L. Calculate the concentration of arsenic, in µg/g (equivalent to mg/kg), in the original sample taken:

\[
\text{Result} = \left( C \times \text{DF} \times V \right) / W
\]

\[
\begin{align*}
C & = \text{concentration of arsenic in the sample aliquot injected (µg/L)} \\
\text{DF} & = \text{dilution factor of the Sample solution} \\
V & = \text{final volume of the Sample solution (L)} \\
W & = \text{weight of the sample taken to prepare the Sample solution (g)}
\end{align*}
\]

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

**Acceptance criteria:** NMT 2.0 mg/kg

- **Cadmium, Elemental Impurities by ICP, Appendix IIIC**

**Acceptance criteria:** NMT 1.0 mg/kg

- **Lead, Elemental Impurities by ICP, Appendix IIIC**

[Note—Alternatively, the lead content may be determined by the following method.]

**Apparatus**

**Sample digestion:** Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

**Sample analysis:** See Apparatus in Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB

**Calibration standard stock solution:** 100 µg/L

Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

**Calibration standard solutions:** 2.0, 5.0, 10.0, 25.0, and 50.0 µg/L in 2% nitric acid, from the Calibration standard stock solution

**10% Ammonium dihydrogen phosphate stock solution:** Mix 10 g of ultrapure ammonium dihydrogen phosphate, with 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.
1% Magnesium nitrate stock solution: Mix 1 g of ultrapure magnesium nitrate, with 40 mL of water and 1 mL of nitric acid in a Teflon beaker, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

[Note—Because of the difficulty in preparing matrix modifier stock solutions with the required purity, purchasing modifier stock solutions and using them to prepare working solutions is recommended. An ammonium dihydrogen phosphate (4%) and magnesium nitrate (0.2%) solution may be purchased from High Purity Standards, or equivalent.]

Modifier working solution: Transfer 4 mL of 10% Ammonium dihydrogen phosphate stock solution and 2 mL of 1% Magnesium nitrate stock solution to a 10-mL volumetric flask and dilute with 2% nitric acid to volume. A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Sample solution: Prepare as directed for the Sample solution in the Arsenic test.

[CAUTION—Wear proper eye protection and protective clothing and gloves during sample preparation. Closely follow the manufacturer’s safety instructions for use of the microwave digestion apparatus.]

Analysis: The graphite furnace program is as follows:

1. Dry at 120° using a 1-s ramp, a 55-s hold, and a 300-mL/min argon flow.
2. Char the sample at 850° using a 1-s ramp, a 30-s hold, and a 300-mL/min air flow.
3. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and a 300-mL/min argon flow.
4. Atomize at 2100° using a 0-s ramp and a 5-s hold with the argon flow stopped.
5. Clean out at 2600° with a 1-s ramp and a 5-s hold.

Use the autosampler to inject 20-µL aliquots of blanks, Calibration standard solutions, Sample solutions, and 5 µL of Modifier working solution. Inject each solution in duplicate, and average the results. Use peak-area measurement for all quantitation. After ensuring that the furnace is clean by running a 5% nitric acid blank, check instrument sensitivity by running an aliquot of the 25-µg Calibration standard solution. Compare the results obtained with the expected results for the equipment used, and take the necessary steps to correct any problems.

Calculate the characteristic mass, and record and track the integrated absorbance and characteristic mass for reference and quality assurance.

Inject each Calibration standard solution in duplicate. Use the algorithms provided in the instrument software to establish calibration curves. Recheck the calibration periodically and recalibrate if recheck differs from the original calibration by more than 10%.

Inject the Sample solution in duplicate, and record the integrated absorbance. If the instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into the working range, and note the dilution factor (DF). All sample analyses should be blank corrected using a sample solution blank.

If a computer-based instrument is used, the data output is reported as micrograms per liter. Calculate the concentration, in µg/g (equivalent to mg/kg), of lead in the original sample taken:

\[ \text{Result} = \left( \frac{C \times DF \times V}{W} \right) \]
\[ C = \text{concentration of lead in the sample aliquot injected (µg/L)} \]

\[ DF = \text{dilution factor of the Sample solution} \]

\[ V = \text{final volume of the Sample solution (L)} \]

\[ W = \text{weight of the sample taken to prepare the Sample solution (g)} \]

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

**Acceptance criteria:** NMT 1.0 mg/kg

- **Mercury**

**Apparatus**

**Sample digestion:** Use a microwave oven (CEM Model MDS-2100, or equivalent) equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

**Sample analysis:** Use a suitable atomic absorption spectrophotometer equipped with an atomic vapor assembly. This method was developed using a Perkin-Elmer Model 5100 and IL 440 Thermo Jarrell Ash atomic vapor assembly. An electrodeless discharge lamp serves as the source, with an inert gas such as argon or nitrogen as the purge gas. Set up the instrument according to manufacturer's specifications. Instrument parameters are as follows:

- **Wavelength:** 253.6 nm
- **Slit:** 0.7
- **Reagent setting:** 5
- **Gas flow:** 5 to 6 L/min
- **Reaction time:** 0.5 min

**Glassware:** Acid wash all glass, Teflon, and plastic vessels by soaking them in a nitric acid bath containing a 4:1 solution of water and nitric acid. [\textbf{CAUTION—}Wear a full face shield and protective clothing and gloves at all times when working with acid baths. ] After acid soaking, rinse acid-washed items in deionized water, dry, and store them in clean, covered cabinets.

**Calibration standard stock solution:** 200 ng/g of mercury. Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

**Calibration standard solutions:** 20, 60, 100, 200, and 400 ng of mercury in 1 N hydrochloric acid from the **Calibration standard stock solution**

**Reducing reagent:** 5% stannous chloride in 25% hydrochloric acid (trace-metal grade) [Note—Prepare daily.]

**Sample solution:** Prepare as directed for the Sample solution in the Arsenic test. [\textbf{CAUTION—}Wear proper eye protection and protective clothing and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus. ]

**Analysis:** Optimize the instrument settings for the spectrophotometer as described in the instrument manual. The instrument parameters for cold vapor generation are as follows:

- **Wavelength:** 253.6 nm
- **Slit:** 0.70 nm
- **Reagent setting:** 5
- **Gas flow:** 5 to 6 L/min
- **Reaction time:** 0.5 min

Use a peak height integration method with a 40-s integration time and a 20-s read delay in an unheated absorption cell. Zero the instrument as follows: Place a Fleaker containing 50 mL of 1 N
hydrochloric acid in the sample well of the hydride generator. Press “start” on the vapor generator and “read” on the atomic absorption spectrophotometer. The instrument will automatically flush the sample container with nitrogen, dispense the designated amount of reagent, stir the sample for a designated reaction time, and purge the head volume again with nitrogen, sweeping any vapor into the quartz cell for determination of absorption. The atomic absorption spectrophotometer will automatically zero on this sample when “autozero” is selected from the calibration menu.

Generate a standard curve of concentration versus absorption by analyzing the five Calibration standard solutions prepared as described for daily standards under Calibration standard solutions. Analyze each solution in duplicate, generate the calibration curve, and store, using procedures specific for the instrumentation.

Transfer an appropriate aliquot of the Sample solution (usually 2 mL) in a Fleake containing 50 mL of 1 N hydrochloric acid. Analyze solutions in duplicate using the procedure specified in the instrument manual. Using the calibration algorithm provided in the instrument software, calculate and report the mercury concentration in nanograms of mercury in the aliquot analyzed.

Calculate the level of mercury as µg/g (equivalent to mg/kg), in the original sample taken:

\[
\text{Result} = \frac{A \times \text{DF}}{W \times 1000}
\]

A = amount of mercury in the aliquot analyzed (ng)
DF = dilution factor (final volume of Sample solution/volume taken for analysis)
W = weight of the sample taken to prepare the Sample solution (g)

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

Acceptance criteria: NMT 1.0 mg/kg

Organic Impurities

- **Pheophorbide Content**

  **Solution A**: 50 mg/mL of sodium sulfate

  **Solution B**: Saturated solution of sodium sulfate

  **Sample stock solution**: Transfer 100 mg of the sample to a 10-mL test tube, add 10 mL of acetone, and dissolve with sonication. Quantitatively transfer this solution to a separatory funnel, rinsing the test tube three times with 10-mL portions of acetone and adding the rinsings to the funnel. Add 30 mL of ethyl ether to the separatory funnel, followed by 50 mL of Solution A. Mix the contents of the separatory funnel by shaking gently, then draw off and discard the lower layer. Repeat washing with Solution A three times. Dehydrate the remaining extract with sodium sulfate anhydrate, then transfer the extract to a 50-mL volumetric flask. Dilute with ethyl ether to volume.

  **Sample solution**: Transfer 20 mL of the Sample stock solution to a small beaker. Add 20 mL of 17% hydrochloric acid and mix the solution vigorously. Transfer the hydrochloric acid layer to a separatory funnel and repeat the extraction with a second 10-mL portion of 17% hydrochloric acid, adding the hydrochloric acid layer to the separatory funnel. Add 150 mL of Solution B, 20 mL of ethyl ether, and mix the contents of the separatory funnel by shaking. Transfer the ethyl ether layer to a 20-mL volumetric flask and dilute with ethyl ether to volume.

  **Analysis**: Using a suitable spectrophotometer, determine the absorbance of the Sample solution at 667 nm in a 1-cm cuvette, using ethyl ether as the blank. If necessary, the Sample solution may be further diluted with ethyl ether to obtain an absorbance within the linear operating range of the instrument.

  Calculate the percentage of pheophorbide in the sample taken:

  \[
  \text{Result} = \frac{A_U (C_U \times E )}{100}
  \]
\[ A_U = \text{absorbance of the Sample solution} \]
\[ C_U = \text{concentration of the Sample solution (mg/mL)} \]
\[ E = \text{absorption constant for 1 mg/mL pheophorbide in ethyl ether at 667 nm in a 1-cm cuvette (70.2 mL/mg^{-1}cm^{-1})} \]

**Acceptance criteria:** NMT 0.02%

**SPECIFIC TESTS**
- **Water**, *Water Determination*, Appendix IIB
  - **Acceptance criteria:** NMT 1.0%

**OTHER REQUIREMENTS**
- **Labeling** Label to indicate the name of any added antioxidant.
- **3S (FCC7)**

---

1. Commercial products marketed as astaxanthin are actually often mixtures of free and esterified astaxanthin or primarily esterified astaxanthin.


3. YMC-Carotenoid™ S 5-µ column, available at www.ymc.co.jp/en, or equivalent.

4. CEM Model MDS-2100, or equivalent.

5. This method was developed using a Perkin-Elmer Model 5100, HGA-600 furnace, and an AS-60 autosampler with Zeeman effect background correction.

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick Scientific Liaison 1-301-816-8356</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

Beta Glucan from Baker’s Yeast (*Saccharomyces cerevisiae*). Because there is no existing FCC monograph for this food ingredient, a new monograph for Beta Glucan from Baker's Yeast is proposed based on comments and data received.

1. The method given for Identification references a new Nuclear Magnetic Resonance Spectroscopy test in Appendix II. The Nuclear Magnetic Resonance Spectroscopy section is based on General Chapter 761 of the *United States Pharmacopeia and National Formulary* (USP–NF) as published in USP 32–NF 27. Data submitted to support this test in this new monograph proposal was generated using nuclear magnetic resonance spectroscopy. See the Briefing under Appendix II: Physical Tests and Determinations.

2. The specifications and method proposed in the tests for Inorganic Impurities are adapted from AOAC Official Method 984.27, Inductively Coupled Plasma Emission Spectroscopic Method for Calcium, Copper, Iron, Magnesium, Manganese, Phosphorus, Potassium, Sodium, and Zinc Detection in Infant Formula.

Interested parties are encouraged to submit comments.

(FIEC: C. Mejia) C94589

---

Add the following:

**Beta Glucan from Baker’s Yeast (*Saccharomyces cerevisiae*)**

Baker's Yeast Beta Glucan

(1-3), (1-6)-β-d-glucan, Poly-(1-6)-β-d-glucopyranosyl-(1,3)-β-d-glucopyranose

**DESCRIPTION**

Beta Glucan from Baker's Yeast (*Saccharomyces cerevisiae*) is a light beige to tan fine powder. This ingredient is the result of the fermentation of food-grade baker's yeast (*Saccharomyces cerevisiae*) and later lysis through a thermal process. The cell wall component is separated from the yeast extract using centrifugation. Then, the cell wall isolate undergoes a caustic treatment to strip the mannosylated cell wall proteins that are linked to the cell wall and to remove the residual cellular lipids. After that, the isolate undergoes an acid treatment, which results in the removal of most of the chitin. Lastly, the yeast wall slurry undergoes flash sterilization, followed by pH adjustment steps, which results in the final dry product. It is comprised mainly of β-(1,3)/(1,6) branched glucan polymers, and trace amounts of protein and lipid. Small amounts of β-(1,6)-glucan and chitin are also expected to be present in the final product.

**Function:** Nutrient

**Packaging and Storage:** Store in closed, sealed packages in a dry controlled environment (21° and 50% RH).

**IDENTIFICATION**

- **$^1$H NMR Spectroscopy, Nuclear Magnetic Resonance Spectroscopy,** Appendix IIC

  **Reference standard solution:** Dissolve 10 mg of USP Beta Glucan RS in 0.6 mL of dimethyl sulfoxide–$d_6$ at 100° for 1 h. After incubation time, add 0.1 mL of D$_2$O, mix the solution, and transfer to an NMR tube.
**Sample solution:** Dissolve 10 mg of sample in 0.6 mL of dimethyl sulfoxide–d$_6$ at 100° for 1 h. After incubation time, add 0.1 mL of D$_2$O, mix the solution, and transfer to an NMR tube.

**Analysis:** Collect $^1$HNMR spectra at 80°, and compare individual resonances from the Sample solution to those from the Reference standard solution. The major signals associated with this ingredient are shown in the table below. The relative area of the resonance corresponding to the $^1$H signal of (1,6) linked beta glucan with respect to the $^1$H signal of (1,3) linked beta glucan is also measured and compared to the range shown in the table.

<table>
<thead>
<tr>
<th>$^1$HNMR Major Signals</th>
<th>USP Beta Glucan RS</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (1,3-glucan)</td>
<td>4.52, d, J = 7.5 Hz, 1H</td>
</tr>
<tr>
<td>H-2, 4, and 5 (1,3-)</td>
<td>3.27–3.33, m, 3H</td>
</tr>
<tr>
<td>H-3 and 6b (1,3-)</td>
<td>3.45–3.48, m, 2H</td>
</tr>
<tr>
<td>H-6a (1,3-)</td>
<td>3.71, d, J = 11 Hz, 1H</td>
</tr>
<tr>
<td>H-1 (1,6-glucan)</td>
<td>4.27, d, J = 7.7 Hz, 1H</td>
</tr>
<tr>
<td>Relative % of 1,6</td>
<td>10%–18%</td>
</tr>
</tbody>
</table>

Integrate the area under the peaks five times for each sample and average. Integration values are then used in the equation below for the peaks at 4.52 and 4.27 ppm to determine the relative percentage of (1,6) linked glucan in the sample.

$$\text{Result} = \frac{\text{A}}{(\text{A} + \text{B})} \times 100$$

A = integration values of H-1 from (1,6) glucan

B = integration values of H-1 from (1,3) glucan

**Acceptance criteria:** The spectrum obtained for the Sample solution exhibits a chemical shift pattern with signal locations and intensities that match those obtained from the preparation of the Reference standard solution. Also, the relative percentage of (1,6) linked glucan is 10%–18% of the total linkages.

**ASSAY**

**• Procedure**

**Buffer solution A (sodium acetate buffer, pH 5, 200 mM):** Add 11.6 mL of glacial acetic acid to approximately 900 mL of water with stirring. Adjust to pH 5 using sodium hydroxide solution (4 M). Transfer to a 1-L volumetric flask and adjust to volume.

**Buffer solution B (sodium acetate buffer, pH 3.8, 1.2 M):** Add 69.6 mL of glacial acetic acid to approximately 800 mL of water with stirring. Adjust to pH 3.8 using sodium hydroxide solution (4 M). Transfer to a 1-L volumetric flask and adjust to volume.

**10 X TES buffer (10 X (hydroxymethyl)aminomethane (TRIS)/EDTA/Saline):** Dissolve 12.12 g TRIS, 11.69 g NaCl and 4.16 g EDTA tetrasodium dihydrate salt in approximately 900 mL of purified water with stirring. Adjust the pH to 7.5 with concentrated HCl or 4 M NaOH. Transfer the solution to a 1-L volumetric flask, and dilute with water to volume. [Note—Buffer can be stored for 1 year at 2°–8°]

**Lyticase solution (10 U/µL in 1 X TES buffer):** Prepare the required volume of lyticase from *Arthrobacter luteus*¹ at a concentration of 10 U/µL by dissolving the quantity stated by the manufacturer (U/mg) in a solution containing 10% 10 X TES buffer (w/v). [Note—Unused solution can be stored at NMT -15° with an expiration date of 1 year. Every time a different lot of lyticase is used, the concentration of lyticase solution required needs to be qualified.]

**(1,6)-glucanase solution:** Dissolve lyophilized (1,6)-glucanase² in Buffer solution A in amounts that yield
1U/300 µL solution. [Note—Solids may not fully dissolve. So, this solution should be handled as a homogeneous suspension. Solution is stable for at least 60 days at NMT -15°.]

**Polishing enzyme mix:** For 100 mL total volume, mix 2000 U of exo-beta-glucanase and 400 U of beta-glucosidase with *Buffer solution A* in a 100-mL volumetric flask. A pre-mix of the enzymes may be used as an alternative. Mix well by inverting at least 10 times. [Note—Store on ice during the procedure, and for use in a same-day assay. Unused *Polishing enzyme mix* can be refrozen once at NMT -15° with an expiration date of 2 years.]

**Glucose oxidase/peroxidase buffer** $^6$: In a 1-L volumetric flask, add 45.287 g of potassium phosphate dibasic, 30.382 g of $p$-hydroxybenzoic acid, and 4 g of sodium azide. Carefully add 800 mL of water. Mix with a stir bar and mild heat until fully dissolved. Transfer the contents to a large beaker. Adjust to pH 7.4 with 2 M KOH solution. Transfer solution back to the 1-L volumetric flask, and fill with water to volume. Mix by inverting at least six times. [Note—Store buffer in an amber bottle with an expiration date of 3 years at 4°.

**Glucose oxidase/peroxidase reagent:** Dissolve 50 mL of *Glucose oxidase/peroxidase buffer* in water to a total volume of 1 L. In this entire volume, dissolve the contents of the Glucose Determination Reagent$^7$. [Note—Store reagent in an amber bottle and label with an expiration date of 3 months at a temperature between 2° and 8° or 1 year at NMT -17°. Minimize time spent at room temperature.]

**Sample solution:** Accurately weigh 15–20 mg of sample into a 16 × 100 mm glass vial. Place vial in an ice bath. Add 0.4 mL of cold KOH solution (2 M) all in one aliquot while vortexing to disperse the powder. Return vial to the ice bath. Continue cycling through vortexing and placing vials in the ice bath as much as possible for 20 min. The mixture should turn into a homogenous, translucent dispersion. [Note—Prepare *Sample solution* and *Standard solution* in triplicate. It is critical for the success of the assay that the sample is well dispersed.]

**Lyticase digestion:** Upon removal of all vials containing *Sample* or *Standard solution* from the ice bath, add 1.6 mL *Buffer solution B* and 600 µL of *Lyticase solution* to each vial. Incubate the mixture at 50° for 12–18 h, and cool to room temperature. (1,6)-glucanase digestion: After cooling of all vials, remove a 130 µL aliquot of each vial and digest further by adding 25 µL of KOH solution (2 M) and 300 µL of (1,6)-glucanase solution. Incubate vials at 80° for 15 min, and cool to room temperature.

**Beta glucanase/glucosidase digestion:** After cooling of all vials, add 390 µL of the *Polishing enzyme mix* to each vial and incubate the vials at 40° for 1 h. Cool them to room temperature, centrifuge, and transfer 50 µL aliquots (in duplicate) to new vials. [Note—Store enzyme blanks in an amber bottle and label with an expiration date of 3 months at a temperature between 2° and 8° or 1 year at NMT -17°. Minimize time spent at room temperature.]

**Enzyme blank solution:** Prepare enzyme blanks in triplicate by combining all the reagents used during the digestion steps except the Sample or Standard solution.

**Analysis:** Dilute the 50 µL aliquots obtained after the Beta glucanase/glucosidase digestion with 50 µL of water and then add 3 mL of *Glucose oxidase/peroxidase reagent*. Incubate vials for 20 min at 40°. Using a suitable spectrophotometer, determine the absorbance of each vial with Sample or Standard solution at 510 nm against the Enzyme blank solution. Prepare a standard curve using the absorbance of similarly treated series of glucose standards (0, 0.1, 0.25, 0.5, and 1.0 mg/mL). From the slope of the standard curve and the absorbance of the digested Sample and Standard solutions, determine the concentration of liberated glucose in the cuvette (C), in mg/mL:

\[
\text{Result} = \frac{(\text{Abs}_S - \text{Abs}_B) / \text{slope}}{50} \times 510 \times 3 \times \frac{1}{5000} \times 1000
\]

Result = (Abs$_S$ - Abs$_B$)/slope
Abs_S = average absorbance of sample or USP Beta Glucan RS
Abs_B = average absorbance of Enzyme blank solution

Calculate the percent of beta glucan as glucose in the sample:

\[
\text{Result} = 100 \times \frac{C}{(W_{TS}/F_1) \times (F_2/F_3)/2}
\]

\(W_{TS}\) = original weight of the sample or USP Beta Glucan RS (mg)
\(F_1\) = total volume in the vial during Lyticase digestion, 2.6 (mL)
\(F_2\) = volume of the sample or USP Beta Glucan RS transferred to a new vial during (1,6)-glucanase digestion, 0.130 (mL)
\(F_3\) = total volume during Beta glucanase/glucosidase digestion, 0.845 (mL)

Acceptance criteria: NLT 70% beta glucan as glucose, calculated on the dried basis

IMPURITIES

Inorganic Impurities

- **Arsenic**, Elemental Impurities by ICP, Method I: ICP-OES, Appendix IIIC
  - Acceptance criteria: NMT 0.5 ppm
- **Cadmium**, Elemental Impurities by ICP, Method I: ICP-OES, Appendix IIIC
  - Acceptance criteria: NMT 0.5 ppm
- **Lead**, Elemental Impurities by ICP, Method I: ICP-OES, Appendix IIIC
  - Acceptance criteria: NMT 0.5 ppm
- **Mercury**, Elemental Impurities by ICP, Method I: ICP-OES, Appendix IIIC
  - Acceptance criteria: NMT 0.1 ppm

Organic Impurities

- **Glycogen**

  Amyloglucosidase/invertase solution\(^8\): Dissolve amyloglucosidase and invertase in 20 mL of glycerol solution (50% v/v) to obtain a solution containing 1630 U/mL of amyloglucosidase and 500 U/mL of invertase.

  **Procedure**: Weigh 100 mg of sample, in triplicate, into individual 16- × 150-mm glass screw cap vials. Place vials in an ice bath and add to each vial 2 mL of cold 2 M KOH (in one aliquot) while vortexing to disperse the powder. Return the vial to the ice bath. Continue cycling through vortexing and placing vials in the ice bath as much as possible for 20 min. The mixture should turn into a homogenous, translucent dispersion. Add 8 mL of **Buffer solution B**. Vortex thoroughly, and immediately add 200 µL of Amyloglucosidase/invertase solution and vortex again. Incubate the mixture at 40° for 30–35 min. Cool to room temperature. Vortex again, transfer to a suitable centrifuge tube, and centrifuge until a clear supernatant is obtained. Transfer duplicate 50 µL aliquots of supernatant into new vials and proceed with the Analysis as outlined in the Assay section above.

  Acceptance criteria: NMT 1.0%

- **Mannose**

  **Mobile Phase A**: 100% Purified Water
  **Mobile Phase B**: 956 mM NaOH

  **Internal standard solution**: Dissolve USP Inositol RS, or equivalent, in water (0.8 mg/mL).
  **Standard solutions**: Dissolve USP Dextrose RS, or equivalent, and USP Mannose RS, or equivalent, in water, aliquot them, in duplicate, as shown in the table below and freeze dry them. Continue preparation as directed under **Sample solution**, beginning with “Add 500 µL of pure trifluoroacetic acid”.
Sample solution: Weigh 2.0–4.0 mg of sample, in duplicate, into vials with stir bars. Add 500 µL of pure trifluoroacetic acid (TFA), and allow the mixture to form a uniform dispersion by stirring for 1 h at room temperature. Incubate in an 80 °C water bath for 2 h with stirring, and then cool to room temperature. Add 100 µL of *Internal standard solution* to each vial, and incubate with stirring in a boiling water bath for 15 min. Cool again to room temperature, then add 1.07 mL of water to each vial, and incubate with stirring in a boiling water bath for 1 h. Cool the solutions to room temperature, and dry overnight on a SpeedVac, or equivalent, at low heat with the cryopumping system off. Dissolve the dried preparation in 2.5 mL of deionized water, and filter through a 0.2-µm PTFE syringe filter. Dilute with an equal volume of water before injection.

**Chromatographic system,** Appendix IIA

**Mode:** High-performance liquid chromatography

**Column:** Strong anion-exchange column 4.0- × 250-mm (CarboPac MA-1, with CarboPac MA-1 4.0- × 50-mm guard column, Dionex, or equivalent)

**Column temperature:** 30 °C

**Flow rate:** 0.4 mL/min

**Injection volume:** 10 µL

**Gradient program:** See the gradient table below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase A (%)</th>
<th>Mobile phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>36.0</td>
<td>64.0</td>
</tr>
<tr>
<td>15.0</td>
<td>36.0</td>
<td>64.0</td>
</tr>
<tr>
<td>35.0 (sample injection)</td>
<td>59.4</td>
<td>40.6</td>
</tr>
<tr>
<td>80.0</td>
<td>59.4</td>
<td>40.6</td>
</tr>
</tbody>
</table>

[Note—Run time typically required is 80 min.]

**Detector mode:** Integrated amperometry

**Detector range:** 3000 µC (may be modified if needed)

**Working electrode:** Gold

**Reference electrode:** pH, Ag/AgCl

**Electrochemical waveform:** See the table below.
<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Potential (V)</th>
<th>Integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.10</td>
<td>Start</td>
</tr>
<tr>
<td>0.40</td>
<td>0.10</td>
<td>End</td>
</tr>
<tr>
<td>0.41</td>
<td>-2.00</td>
<td></td>
</tr>
<tr>
<td>0.42</td>
<td>-2.00</td>
<td></td>
</tr>
<tr>
<td>0.43</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td>-0.10</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>-0.10</td>
<td></td>
</tr>
</tbody>
</table>

**Analysis:** Separately inject equal volumes of the *Standard solutions* and *Sample solution* into the chromatograph, and measure the responses for the major peaks on the resulting chromatograms. For each injection, calculate the ratios of the area of the glucose and mannose peaks to the area of the internal standard inositol peak. Make two standard curves of the peak area ratio versus the concentration for the glucose and mannose standards, and calculate their linear regression. The glucose and mannose concentration of the sample is then calculated from their peak area ratio using the respective slope and intercept of the standards regression lines. Calculate the percentage of mannose present in the sample:

\[ \text{Result} = C_M(C_M + C_G) \times 100 \]

- \( C_M \) = mannose concentration in the sample (µL/mL)
- \( C_G \) = glucose concentration in the sample (µL/mL)

**Acceptance criteria:** NMT 1.0% mannose, as a function of total hexose recovered (glucose and mannose)

**SPECIFIC TESTS**

- **Loss on Drying,** Appendix IIIC: 105°, 3 h
  - Sample: 0.9–1.2 g
  - **Acceptance criteria:** NMT 8.0%
- **Residue on Ignition (Sulfated Ash),** Appendix IIIC
  - Sample: 2.0 g
  - **Acceptance criteria:** NMT 2.5%

---

1. Lyticase from *Arthrobacter luteus*, Sigma L4025, or equivalent.
2. Commercially available as Rustulanase, Cef136, Prokzyme, or equivalent.
3. E-EXBGL 200 U/ml, 200 U/bottle, Megazyme, or equivalent.
4. 200 U/bottle, Megazyme, or equivalent.
5. E-EXBGOS, Megazyme, or equivalent.
6. This buffer is also available as Bottle #3 of the K-YBGL kit (Megazyme), or Bottle #1 of the GQPOD kit (Megazyme).
7. Bottle #4 of K-YBGL kit, or Bottle #2 of GQPOD kit, Megazyme, or equivalent.
8. Alternatively, Bottle #2 of K-YBGL kit (Megazyme, or equivalent) could be used directly.

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.
<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

**Balsam Peru Oil, FCC 7 page 79.** On the basis of comments and data received, it is proposed to revise the
Acceptance criteria range in the test for Specific Gravity to correspond with current food-grade materials in commerce. The proposed range is higher and tighter than the current range.
(FIEC: J. Moore)     C97209

---

**Balsam Peru Oil**

CAS: [8007-00-9]

**DESCRIPTION**

Balsam Peru Oil occurs as a yellow to pale brown, slightly viscous liquid having a sweet, balsamic odor. It is obtained by extraction or distillation of Peruvian Balsam obtained from *Myroxylon pereirae Royle Klotzsche* (Fam. Leguminosae). Occasionally, crystals may occur within the liquid. It is soluble in most fixed oils, and is soluble, with turbidity, in mineral oil. It is partly soluble in propylene glycol, but it is practically insoluble in glycerin.

**Function:** Flavoring agent

**Packaging and Storage:** Store in a cool place protected from light in full, tight containers that are made from steel or aluminum and that are suitably lined.

**IDENTIFICATION**

- **Infrared Spectra, Spectrophotometric Identification Tests, Appendix IIIC**
  - **Acceptance criteria:** The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

- **Specific Tests**
  - **Acid Value, Appendix VI**
    - **Acceptance criteria:** Between 30 and 60
  - **Angular Rotation, Optical (Specific) Rotation, Appendix IIB:** Use a 100-mm tube.
    - **Acceptance criteria:** Between -1° and +2°
  - **Esters, Ester Value, Appendix VI**
    - **Sample:** 1 g
    - **Acceptance criteria:** Between 200 and 225
  - **Refractive Index, Appendix IIB**
    - [Note—Use an Abbé or other refractometer of equal or greater accuracy.]
    - **Acceptance criteria:** Between 1.567 and 1.579 at 20°
  - **Solubility in Alcohol, Appendix VI**
    - **Acceptance criteria:** One mL of the sample dissolves in 0.5 mL of 90% alcohol, and remains in solution on
further dilution to 10 mL.

**Change to read:**

- **SPECIFIC GRAVITY:** Determine by any reliable method (see General Provisions).
  
  **Acceptance criteria:** Between 1.095 and 1.110, between 1.110 and 1.120. \(3S\) (FCC7)

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
</table>
| Monograph      | Kristie Laurvick  
Scientific Liaison  
1-301-816-8356 | (FI2010) Monographs - Food Ingredients |

*FCC Seventh Edition Page 79*
BRIEFING

Bentonite, FCC 7 page 85. On the basis of comments and data received, a revision is proposed to remove the pH requirement from Specific Tests and to include a typical pH range in the Description instead of as a monograph requirement. Information received indicates that the pH of a bentonite solution is not necessarily a characteristic of the quality of the ingredient because the pH of bentonite from different geographical locations naturally varies widely.

(FIEC: K. Laurvick) C96669

Bentonite

Smectite

Aluminum Silicate

INS: 558

CAS: [1302-78-9]

DESCRIPTION

Change to read:

Bentonite occurs commercially as powders ranging in colors and tints from off white to pale brown to gray depending on the cations present in natural deposits. It comprises natural smectite clays consisting primarily of colloidal hydrated aluminum silicates of the montmorillonite or hectorite type of minerals with varying quantities of alkalies, alkaline earths, and iron. It is insoluble in water, in alcohol, in dilute acids, and in alkalies.

The pH of a 2% suspension of Bentonite is typically in the range of 4.5–10.5.

Function: Clarifying, filter agent

Packaging and Storage: Store in tight containers.

IDENTIFICATION

• A. X-RAY DIFFRACTION

Sample preparation A: With intense agitation, add 2 g of sample, in small portions, to 100 mL of water. Allow the mixture to stand for 12 h to ensure complete hydration. Place 2 mL of the mixture so obtained on a suitable glass slide, and allow it to air dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that ethylene glycol saturates the desiccator chamber. Allow the slide to stand for 12 h.

Sample preparation B: Prepare a random powder specimen of the sample.

Analysis: Record the X-ray diffraction pattern using a copper source, and calculate the d values. [Note —For Sample preparation B, determine the d value between the range of 1.48 and 1.54 Å.] Acceptance criteria: For Sample preparation A, the largest peak corresponds to a d value between 15.0 and 17.2 Å. For Sample preparation B, the peak is between 1.492 and 1.504 Å or between 1.510 and 1.540 Å.

• B. PROCEDURE

Sample: 0.5 g

Analysis: Add 1 g of potassium nitrate and 3 g of anhydrous sodium carbonate to the Sample contained in a metal crucible, heat until the mixture has melted, and allow it to cool. Add 20 mL of boiling water to the
residue, mix, filter, and wash the residue with 50 mL of water. Add 1 mL of hydrochloric acid and 5 mL of water to the residue and filter. Add 1 mL of 10 N sodium hydroxide to the filtrate, filter, and add 3 mL of 2 M ammonium chloride.

**Acceptance criteria:** A gelatinous, white precipitate forms.

## IMPURITIES

### Inorganic Impurities

- **Arsenic, Arsenic Limit Test, Appendix IIIB**
  
  **Sample solution:** Transfer 8.0 g of dried sample into a 250-mL beaker containing 100 mL of 1:25 hydrochloric acid, mix, cover with a watch glass, and boil gently, stirring occasionally, for 15 min without allowing excessive foaming. Filter the hot supernatant liquid through a rapid-flow filter paper into a 200-mL volumetric flask, and wash the filter with four 25-mL portions of hot, 4% hydrochloric acid, collecting the washings in the volumetric flask. Cool the combined filtrates to room temperature, add 4% hydrochloric acid to volume, and mix.

  **Control:** 5 µg As (5 mL of Standard Arsenic Solution)

  **Analysis:** Proceed as directed using a 25-mL aliquot of the Sample solution.

  **Acceptance criteria:** NMT 5 mg/kg

- **Lead**

  [Note—The Standard solution and the Sample solution may be modified, if necessary, to obtain solutions of suitable concentrations, adaptable to the linear or working range of the spectrophotometer used.]

  **Sample preparation:** Transfer 3.75 g of dried sample into a 250-mL beaker containing 100 mL of 1:25 hydrochloric acid, stir, cover with a watch glass, and boil for 15 min. Cool to room temperature, and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400-mL beaker. Wash the filter with four 25-mL portions of hot water, collecting the filtrate in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate forms, add 2 to 3 drops of nitric acid, heat to boiling, and cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume, and mix.

  **Standard solution:** 3 µg/mL Pb: from Lead Nitrate Stock Solution, Lead Limit Test, Flame Atomic Absorption Method, Appendix IIIB.  [Note—Prepare this solution on the day of use.]

  **Analysis:** Using a suitable atomic absorption spectrophotometer equipped with a lead hollow-cathode lamp, deuterium arc background correction, a single-slot burner, and using an oxidizing air–acetylene flame, determine the absorbances of the Sample preparation and the Standard solution at 284 nm.

  **Acceptance criteria:** The absorbance of the Sample preparation is not greater than that of the Standard solution (NMT 4 mg/kg).

## SPECIFIC TESTS

- **Coarse Particles**

  **Sample preparation:** 20 g in 100 mL of water, mixed for 15 min at NLT 5000 rpm

  **Analysis:** Transfer the Sample preparation to a wet sieve of nominal mesh aperture (75 µm), previously dried at 100° to 105° and weighed, and wash with three 500-mL volumes of water, ensuring that any agglomerates are dispersed. Dry at 100° to 105°, and weigh. The difference in weight corresponds to the measure of coarse particles.

  **Acceptance criteria:** NMT 0.5% of sample is retained on a 75-µm sieve

- **Gel Formation**

  **Sample:** 6 g

  **Analysis:** Mix the Sample with 300 mg of magnesium oxide. Add the mixture, in several divided portions, to
200 mL of water contained in a blender jar with an approximately 500-mL capacity. Blend thoroughly for 5 min at high speed, transfer 100 mL of the mixture into a 100-mL graduated cylinder, and leave undisturbed for 24 h.

**Acceptance criteria:** NMT 2 mL of supernatant appears on the surface

**Change to read:**

- **Loss on Drying,** Appendix IIC (105° for 2 h)
  
  **Acceptance criteria:** ▲NMT 12.0%▲

- **Microbial Limits**

  [Note—Current methods for the following tests may be found by accessing the Food and Drug Administration's Bacteriological Analytical Manual (BAM) online at www.cfsan.fda.gov.]

  **Acceptance criteria**

  - **Aerobic plate count:** NMT 1000 cfu/g
  - **E. coli:** Negative in 25 g

**Delete the following:**

- **pH, pH Determination,** Appendix IIB

  **Sample preparation:** Disperse 4.0 g of sample in 200 mL of water, mixing vigorously to facilitate wetting.

  **Acceptance criteria:** 8.5–10.5

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick, Scientific Liaison 1-301-816-8356</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 85*
BRIEFING

**Brown HT.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Brown HT monograph from the 59th session (2002) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and specifications published in the European Union (EU) Commission Directive 2008/128/EC for this color additive. It should be noted that this color additive is not approved for use in the US, but is approved in other countries.

1. The specification for *Visible Absorption Spectrum* under *Identification* is based on that from the EU. Stakeholders are encouraged to submit additional, more modern test procedures to aid in identifying and distinguishing this ingredient from other synthetic color additives.

2. In the test for *Lead* under *Inorganic Impurities*, it is proposed to use the specifications for lead from the JECFA monograph and test procedures already in FCC for this impurity in Appendix III. Stakeholders are encouraged to submit a more modern lead procedure validated for this ingredient.

3. A test procedure and specification for *4-Aminonaphthalene-1-sulfonic Acid* is proposed based on that in JECFA. Stakeholders are encouraged to submit information on a supplier and appropriate concentration for reference standards used in this test procedure.

4. Specifications in the tests for *Loss on Drying*, *Chloride*, and *Sulfates* in the *Combined Tests* section are proposed based on those in JECFA, but using different test procedures—ones already existing in FCC Appendix IIIIC for the analysis of colors.

5. Specifications in the test for *Ether Extracts* and the *Assay* are proposed based on those in JECFA using FCC test procedures in FCC Appendix IIIIC, which are equivalent to those used in JECFA.

6. The specifications and test procedures for *Subsidiary Coloring Matters* and *Unsulfonated Primary Aromatic Amines* are based on those in JECFA. Stakeholders are encouraged to submit more modern test procedures, especially for *Subsidiary Coloring Matters*, for consideration in this monograph.

7. The specification for *Water-Insoluble Matter* is based on that in JECFA using a similar test procedure already in FCC Appendix IIIIC.

(FIEC: J. Moore)  C94721

---

**Add the following:**

- **Brown HT**
  - Chocolate Brown HT

CI Food Brown 3

CI No. 20285

Class: Bis-azo

Disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylene-bisazo) di-1-naphthalene-sulfonate
C₂₇H₁₈N₄Na₂O₆S₂

Formula wt 652.57

INS: 155

CAS: [4553-89-3]

DESCRIPTION
Brown HT occurs as a brown powder or granules. It is principally the disodium salt of 4,4’-(2,4-dihydroxy-5-
hydroxymethyl-1,3-phenylene-bisazo) di-1-naphthalene-sulfonate and subsidiary coloring matters with sodium
chloride and/or sodium sulfate as the principal uncolored components. It is soluble in water and insoluble in
ethanol.

Function: Color
Packaging and Storage: Store in well-closed containers.

IDENTIFICATION
• **Visible Absorption Spectrum**
  Sample solution: Dissolve a sample in water and adjust the pH to 7.
  Analysis: Measure the absorption spectrum of the Sample solution using a suitable UV-visible
  spectrophotometer.
  Acceptance criteria: The Sample solution exhibits a wavelength maximum at 460 nm.

ASSAY
• **Total Color, Colors, Method I, Appendix IIIC**
  Diluent: Phosphate buffer, pH 7, prepared by combining 50 mL of 0.2 M potassium dihydrogen phosphate
  and 29.54 mL of 0.2 M sodium hydroxide, and diluting to 200 mL with water.
  Sample stock solution: 250 mg/L in diluent
  Sample solution: 10 mg/L prepared by diluting the Sample stock solution with diluent
  Analysis: Determine the absorbance of the Sample solution (instead of the directed sample preparation) at
  460 nm. Calculate the total color as directed using 0.0403 L/(mg·cm) for the absorptivity (a) for Brown HT.
  Acceptance criteria: NLT 70.0% total coloring matters

IMPURITIES
Inorganic Impurities
• **Lead, Lead Limit Test, Appendix IIIB**
  Sample solution: Prepare as directed for organic compounds.
  Control: 2 μg Pb (2 mL of Diluted Standard Lead Solution)
  Acceptance criteria: NMT 2 mg/kg

Organic Impurities
• **4-Aminonaphthalene-1-sulfonic Acid**
  Solution A: 0.2 N ammonium acetate
  Solution B: Methanol
  Mobile phase: Exponential gradient program from (99% A and 1% B) to (0% A and 100% B) at a rate of 2%
  per min
**Sample solution:** 5 mg/mL in 0.02 M ammonium acetate

**Standard solution:** 35 µg/mL of 4-aminonaphthalene-1-sulfonic acid

**Chromatographic system,** Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** UV
- **Column:** 25-cm × 4.6-mm C18 analytical column (5-µm), with a 15-mm × 4.6-mm C18 guard column (5-µm)
- **Column temperature:** Ambient
- **Flow rate:** 1.0 mL/min
- **Injection volume:** 20 µL

**Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph, and measure the responses for the major peaks on the resulting chromatograms.

Calculate the percent of 4-aminonaphthalene-1-sulfonic acid in the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 1000 \times 100
\]

- \(r_U\) = peak area for 4-aminonaphthalene-1-sulfonic acid in the Sample solution
- \(r_S\) = peak area for 4-aminonaphthalene-1-sulfonic acid in the Standard solution
- \(C_S\) = concentration of 4-aminonaphthalene-1-sulfonic acid in the Standard solution (µg/mL)
- \(C_U\) = concentration of sample in the Sample solution (mg/mL)
- 1000 = mg-to-µg conversion factor

**Acceptance criteria:** NMT 0.7%

**SPECIFIC TESTS**

- **Combined Tests**
  - **Tests**
    - Loss on Drying (Volatile Matter), Colors, Appendix IIIC
    - Chloride, Sodium Chloride, Colors, Appendix IIIC
    - Sulfates (as sodium salts), Sodium Sulfate, Colors, Appendix IIIC
  - **Acceptance criteria:** NMT 30%, combined as the sum of all three tests

- **Ether Extracts,** Colors, Appendix IIIC
  - **Acceptance criteria:** NMT 0.2%

- **Subsidiary Coloring Matters**
  - [Note—In this method, subsidiary coloring matters are separated from the main coloring matter of Brown HT by ascending paper chromatography (see Paper Chromatography, Appendix IIA), and extracted separately from the chromatographic paper. The absorbance of each extract is measured at the wavelength of maximum absorption for Brown HT (460 nm) by visible spectrophotometry. Because it is impractical to identify each subsidiary coloring matter using this procedure, and because the subsidiary coloring matters are usually minor components of food colors, the method assumes that the maximum absorbance of each subsidiary coloring matter is the same as that of the total coloring matters. The subsidiary coloring matters content is calculated by adding together the absorbances of the extracts in conjunction with the total coloring matters content of the sample.]

**Chromatographic apparatus:** The chromatography tank (Figures 1 and 2) is comprised of a glass tank (A) and cover (B); frame to support chromatography paper (C); solvent tray (D); secondary frame (E) for supporting “drapes” of the filter paper; and 20-cm × 20-cm chromatography grade paper². Mark out the
chromatography paper as shown in Figure 3.

Figure 1. Assembly of the Chromatographic Apparatus.

Figure 2. Components of the Chromatographic Apparatus.

Figure 3. Method for Marking the Chromatographic Paper.

Chromatographic solvent: Prepare of mixture of n-butanol, glacial acetic acid, and water (4:1:5). Shake for 2 min, allow the layers to separate, and use the upper layer as the chromatographic solvent.

Sample solution: 10 mg/mL sample

Standard solution: Dilute 1.0 mL of the Sample solution to 100 mL with water and mix. Transfer 0.1 mL of this solution to a test tube; add 5.0 mL of a water and acetone mixture (1:1 v/v), and then add 14.9 mL of 0.05 N sodium carbonate solution and shake the tube to ensure mixing.

Application volume: 0.10 mL

Analysis: No less than 2 h before analysis, arrange the filter-paper drapes in the glass tank and pour a sufficient amount of the Chromatographic solvent over the drapes and into the bottom of the tank to cover the bottom of the tank to a depth of 1 cm. Place the solvent tray in position and fit the cover to the tank. Using a microsyringe capable of delivering 0.1 mL with a tolerance of ±0.002 mL, apply a 0.1 mL aliquot of
the *Sample solution* to a chromatograph sheet as uniformly as possible within the confines of the 18-cm × 7-mm rectangle, holding the nozzle of the microsyringe steadily in contact with the paper. Allow the papers to dry at room temperature for 1–2 h or at 50° in a drying cabinet for 5 min followed by 15 min at room temperature. Mount the dried sheet together with a plain sheet to act as a blank on the supporting frame. [Note—If required, several dried sheets may be developed simultaneously.] Pour a sufficient amount of the *Chromatographic solvent* into the solvent tray to bring the surface of the solvent about 1 cm below the base line of the chromatography sheets. The volume necessary will depend on the dimensions of the apparatus and should be predetermined. Put the supporting frame into position and replace the cover. Allow the system to develop for approximately 14 h, then remove the supporting frame and transfer it to a drying cabinet at 50–60° for 10–15 min. Remove the sheets from the frame. Cut each subsidiary band from each chromatogram sheet as a strip, and cut an equivalent strip from the corresponding position of the plain (blank) sheet. Add 5.0 mL of water and acetone (1:1 by vol) to each test tube, swirl for 2–3 min, add 15.0 mL of 0.05 N sodium hydrogen carbonate solution, and shake the tube to ensure mixing. Filter the colored extracts and blanks through 9-cm coarse porosity filter papers into clean test tubes and determine the absorbances of the colored extracts at 460 nm using a suitable spectrophotometer with 40-mm closed cells against a filtered mixture of 5.0 mL of water and acetone (1:1 by vol) and 15.0 mL of the 0.05 N sodium hydrogen carbonate solution. Measure the absorbances of the extracts of the blank strips at 460 nm and correct the absorbances of the colored extracts with the blank values.

Measure the absorbance of the *Standard solution* at 460 nm using a suitable spectrophotometer with 40-mm closed cells against a filtered mixture of 5.0 mL of water and acetone (1:1 by vol), 14.9 mL of the 0.05 N sodium hydrogen carbonate solution, and 0.1 mL of water. Calculate the percent subsidiary coloring matter in the portion of the sample taken:

\[
\text{Result} = 0.01 \times D \times \left[ \frac{A_A + A_B + A_C \ldots + A_N}{A_S} \right] \times 100
\]

0.01 = dilution factor for the *Standard solution*

\(D\) = total coloring matter content of the sample, determined from the *Total Color* test above, expressed as a decimal

\(A_S\) = the absorbance from the *Standard solution* corrected for the blank

\(A_A + A_B + A_C \ldots + A_N\) = the sum of the absorbances of the subsidiary coloring matters from the *Sample solution*, corrected for the blank values

**Acceptance criteria:** NMT 10%

**• Unsulfonated Primary Aromatic Amines**

[Note—Under the conditions of this test, unsulfonated primary aromatic amines are extracted into toluene from an alkaline solution of the sample, re-extracted into acid, and then determined spectrophotometrically after diazotization and coupling.]

**R salt solution:** 0.05 N 2-naphthol-3,6-disulfonic acid, disodium salt

**Sodium carbonate solution:** 2 N sodium carbonate

**Standard stock solution:** Weigh 0.100 g of redistilled aniline into a small beaker and transfer to a 100-mL volumetric flask, rinsing the beaker several times with water. Add 30 mL of 3 N hydrochloric acid and dilute to the mark with water at room temperature. Dilute 10.0 mL of this solution to 100 mL with water and mix well; 1 mL of this solution is equivalent to 0.0001 g of aniline. [Note—Prepare the *Standard stock solution* fresh.]

**Standard solutions:** Separately dilute 5-, 10-, 15-, 20-, and 25-mL aliquots of the *Standard stock solution* to
100 mL with 1 N hydrochloric acid.

**Standard blank solution:** In a 25-mL volumetric flask mix 10.0 mL of 1 N hydrochloric acid, 10.0 mL of Sodium carbonate solution, 2.0 mL of R salt solution, and dilute with water to volume.

**Sample solution:** Add 2.0 g of the sample into a separatory funnel containing 100 mL of water, rinse down the sides of the funnel with 50 mL of water, swirling to dissolve the sample, and add 5 mL of 1 N sodium hydroxide. Extract with two 50-mL portions of toluene and wash the combined toluene extracts with 10-mL portions of 0.1 N sodium hydroxide to remove traces of color. Extract the washed toluene with three 10-mL portions of 3 N hydrochloric acid, and dilute the combined extract with water to 100 mL.

**Sample blank solution:** In a 25-mL volumetric flask mix 10.0 mL of the Sample solution, 10 mL of Sodium carbonate solution, and 2.0 mL of R salt solution, and dilute with water to volume.

**Analysis:** Pipet 10-mL aliquots of the Sample solution and each of the Standard solutions into separate, clean dry test tubes. Cool the tubes for 10 min by immersion in a beaker of ice water, add 1 mL of 50% potassium bromide solution and 0.05 mL of 0.5 N sodium nitrite solution. Mix and allow the tubes to stand for 10 min in the ice water bath while the aniline is diazotized. Into each of five 25-mL volumetric flasks, measure 1 mL of R salt solution and 10 mL of Sodium carbonate solution. Separately pour each diazotized aniline solution into a 25-mL volumetric flask containing R salt solution and Sodium carbonate solution, rinse each test tube with a small volume of water to allow for a quantitative transfer. Dilute to the mark with water, stopper the flasks, mix the contents well and allow them to stand for 15 min in the dark. Measure the absorbance of each of the solutions containing the coupled Standard solutions at 510 nm using a suitable spectrophotometer with 40-mm cells against the Standard blank solution. Plot a standard curve relating absorbance to weight (g) of aniline in each 100 mL of the Standard solutions. Measure the absorbance of the solutions containing the coupled Sample solution at 510 nm using a suitable spectrophotometer with 40-mm cells against the Sample blank solution. From the standard curve, determine the weight (g) of aniline in each 100 mL of the Sample solution.

Calculate the percent unsulfonated primary aromatic amine (as aniline) in the portion of the sample taken:

\[ \text{Result} = \frac{W_A}{W} \times 100 \]

\[ W_A = \text{weight of aniline in the Sample solution calculated from the standard curve (g/100 mL)} \]

\[ W = \text{weight of sample used to prepare the Sample solution (g)} \]

**Acceptance criteria:** NMT 0.01%, calculated as aniline

- **Water-insoluble Matter, Colors, Appendix IIIC**
  - **Acceptance criteria:** NMT 0.2%

---

1 Brown HT is approved for use in some countries but banned in others, such as the United States.

2 Whatman No 1, or equivalent.

**Auxiliary Information—** Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
</table>
| Monograph      | Jeffrey Moore, Ph.D.  
Scientific Liaison  
1-301-816-8288 | (FI2010) Monographs - Food Ingredients |
BRIEFING

Calcium Cyclamate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Calcium Cyclamate monograph prepared at the 63rd Session (2004) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and on the basis of comments received. The analysis for Organic Impurities is based on the analysis for organic impurities in the Sodium Cyclamate monograph of the British Pharmacopoeia (Vol. I, II, 2010) and European Pharmacopoeia (6th Ed.). Interested parties are encouraged to submit comments.

(FIEC: C. Mejia) C93555

Add the following:

- **Calcium Cyclamate**
- Calcium Cyclohexanesulfamate
- Calcium Cyclohexylsulfamate

![Chemical structure of Calcium Cyclamate]

C₁₂H₂₄CaN₂O₆S₂·2H₂O

Formula wt, anhydrous 396.53
Formula wt, dihydrate 432.57

INS: 952(ii)

CAS: anhydrous [139-06-0]
dihydrate [5897-16-5]

DESCRIPTION

Calcium Cyclamate occurs as colorless to white crystals or crystalline powder. It is soluble in water and sparingly soluble in ethanol.

**Function:** Sweetener

**Packaging and Storage:** Store in tight containers in a cool, dry place.

IDENTIFICATION

- **Calcium**, Appendix IIIA
  
  Sample solution: 50 mg/mL
  
  Acceptance criteria: Passes test

- **Infrared Absorption**, Spectrophotometric Identification Tests, Appendix IIIC
  
  Reference standard: USP Calcium Cyclamate RS
  
  Sample and standard preparation: K
  
  Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

ASSAY

- **Procedure**
Sample: 0.4 g

Analysis: Dissolve the Sample in a mixture of 50 mL of water and 5 mL of hydrochloric acid TS, diluted. Titrate the solution with 0.1 M sodium nitrite. Add the last mL of titrant dropwise until a blue color is produced immediately when a glass rod dipped into the titrated solution is streaked on a piece of starch iodide test paper. Alternatively, the endpoint may be determined potentiometrically. When the titration is complete, the endpoint is reproducible after the mixture has been allowed to stand for 1 min. Each mL of 0.1 M sodium nitrite is equivalent to 19.83 mg of \( \text{C}_{12}\text{H}_{24}\text{CaN}_{2}\text{O}_{6}\text{S}_{2} \).

Acceptance criteria: 98.0%–102.0%, calculated on the anhydrous basis

IMPURITIES

Inorganic Impurities

- Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB
  
  Sample: 5 g
  
  Acceptance criteria: NMT 1.0 mg/kg

Organic Impurities

- Cyclohexanamine, Aniline, and N-Cyclohexylcyclohexanamine

  Internal standard solution: Dissolve 0.02 µL/mL of tetradecane in methylene chloride.
  
  Solution A: Dissolve 10 mg of cyclohexanamine, 1 mg of N-cyclohexylamine, and 1 mg of aniline in water, then dilute with the same solvent to 1000 mL. Dilute 10 mL of this solution with water to 100 mL.
  
  Solution B: 42% w/v sodium hydroxide solution
  
  Standard solution: To 20 mL of Solution A, add 0.5 mL of Solution B, and extract with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of water and an acetic acid solution (12% w/v). Separate the lower layer, add 0.5 mL of Solution B and 0.5 mL of the Internal standard solution, and shake. Use the lower layer immediately after separation.
  
  Sample solution: Dissolve 2 g of sample in 20 mL of water, add 0.5 mL of Solution B, and shake with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of an acetic acid solution (12% w/v) and water. Separate the lower layer, add 0.5 mL of Solution B and 0.5 mL of the Internal standard solution, and shake. Use the lower layer immediately after separation.

Chromatographic system, Appendix IIA

Mode: Gas chromatography

Detector: Flame ionization

Column: 25-m × 0.32-mm (i.d.) fused-silica column with poly(dimethyl)(diphenyl)siloxane containing 95% of methyl groups and 5% of phenyl groups (DB-5, SE52) as stationary phase (film thickness 0.51 µm)

Carrier gas: Helium

Flow rate: 1.8 mL/min

Temperature

- Injection port: 250°
- Detector: 270°

Column: See the temperature program in the table below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>85</td>
</tr>
<tr>
<td>1–9</td>
<td>85–150</td>
</tr>
<tr>
<td>9–13</td>
<td>150</td>
</tr>
</tbody>
</table>

Injection volume: 1.5 µL. Use a split vent at a flow rate of 20 mL/min.

Analysis: Separately inject equal volumes of the Standard solution and Sample solution into the
chromatograph, record the chromatograms, and measure the responses. [Note—The approximate retention times (relative to cyclohexanamine, which has a retention time of about 2.3 min) for aniline, tetradecane, and N-cyclohexylcyclohexanamine are about 1.4, 4.3, and 4.5, respectively.]

Acceptance criteria
- Cyclohexanamine: NMT 10.0 mg/kg
- Aniline: NMT 1.0 mg/kg
- N-Cyclohexylcyclohexanamine: NMT 1.0 mg/kg

SPECIFIC TESTS
- Loss on Drying, Appendix II C: 140° for 2 h
  Acceptance criteria: 6.0%–9.0%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

**Cholic Acid, FCC 7 page 211.** In an effort to modernize Identification methods in FCC, it is proposed to add the more specific infrared comparison to a USP Reference Standard. Comments from interested parties are encouraged.

(FIEC: C. Mejia) C95345

**Cholic Acid**

Cholalic Acid

3,7,12-Trihydroxycholanic Acid

![Chemical Structure](image)

\[ C_{24}H_{40}O_5 \]

Formula wt 408.58

INS: 1000

CAS: [81-25-4]

**DESCRIPTION**

Cholic Acid occurs as colorless plates or as a white, crystalline powder. One g dissolves in about 30 mL of alcohol or acetone and in about 7 mL of glacial acetic acid. It is very slightly soluble in water.

**Function:** Emulsifier

**Packaging and Storage:** Store in tight containers.

**IDENTIFICATION**

**Add the following:**

- **A. IR\text{RED} ABSORPTION, Spectrophotometric Identification Tests, Appendix IIIC**
  - **Reference standard:** USP Cholic Acid RS
  - **Sample and standard preparation:** K
  - **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.\textit{3S (FCC7)}

**Change to read:**

- **B. 3S (FCC7) PROCEDURE**
  - **Sample solution:** 0.2 mg/mL in 50% acetic acid
  - **Analysis:** To 1 mL of the \textit{Sample solution} add 1 mL of a 1:100 furfural solution. Cool in an ice bath for 5 min, add 15 mL of 1:2 sulfuric acid, mix, and warm in a water bath at 70° for 10 min. Immediately cool in an ice bath, and stir for 2 min.
Acceptance criteria: A blue color appears.

ASSAY

- **Procedure**
  - **Sample**: 400 mg
  - **Analysis**: Transfer the Sample into a 250-mL Erlenmeyer flask, add 20 mL of water and 40 mL of alcohol, cover with a watch glass, heat gently on a steam bath until dissolved, and cool. Add 5 drops of phenolphthalein TS and, using a 10-mL microburet, titrate with 0.1 N sodium hydroxide to the first pink color that persists for 15 s. Perform a blank determination (see General Provisions), and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 40.86 mg of C\textsubscript{24}H\textsubscript{40}O\textsubscript{5}.

Acceptance criteria: NLT 98.0% of C\textsubscript{24}H\textsubscript{40}O\textsubscript{5}, calculated on the dried basis

IMPURITIES

Inorganic Impurities

- **Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**
  - **Sample**: 10 g
  - **Acceptance criteria**: NMT 4 mg/kg

SPECIFIC TESTS

- **Loss on Drying, Appendix IIC**: 140° under a vacuum of NMT 5 mm Hg, for 4 h
  - **Acceptance criteria**: NMT 0.5%
- **Melting Range or Temperature, Appendix IIB**
  - **Acceptance criteria**: Between 197° and 202°
- **Optical (Specific) Rotation, Appendix IIB**
  - **Sample solution**: 20 mg/mL in alcohol
  - **Acceptance criteria**: [\alpha]D\textsubscript{25} ^\circ NLT +37°, calculated on the dried basis
- **Residue on Ignition (Sulfated Ash), Appendix IIC**
  - **Sample**: 2 g
  - **Acceptance criteria**: NMT 0.1%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(Fl2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 211
BRIEFING

Citric and Fatty Acid Esters of Glycerol. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Citric and Fatty Acid Esters of Glycerol monograph prepared at the 35th Session (1989) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and on the basis of comments supplied. Interested parties are encouraged to submit additional comments. The tests in the Assay section are proposed to be consistent with JECFA. However, those tests are unable to differentiate this food ingredient from monoglyceride citrate and stearyl monoglyceridyl citrate. Comments and data pertaining to methods of analysis that are able to differentiate these materials are especially requested.

(FIEC: C. Mejia) C89032

Add the following:

Citric and Fatty Acid Esters of Glycerol

CITREM

Citric Acid Esters of Mono- and Diglycerides

Citroglycerides

INS: 472c

DESCRIPTION

Citric and Fatty Acid Esters of Glycerol occurs as a yellowish to light brown liquid of variable viscosity. It consists of mixed esters of citric acid and edible fatty acids with glycerol. It may contain minor amounts of free fatty acids, free glycerol, free citric acid, and mono- and diglycerides, and may be fully or partially neutralized with substances suitable for the purpose (as declared on the label). It is obtained by esterification of glycerol with citric acid and edible fatty acids or by reaction of a mixture of mono- and diglycerides of edible fatty acid with citric acid. Citroglycerides can be differentiated from stearyl citrate by the distinctive amount of stearyl alcohol in the latter. Because the mono- or diglycerides in citroglycerides may include either one or two fatty acids, and because there is a variety of edible fatty acids with chain lengths ranging most commonly from 12 to 18, there is no single molecular or structural formula. It forms a dispersion in hot water; is soluble in oils and fats; insoluble in cold water and in cold ethanol.

Function: Stabilizer, emulsifier, dough conditioner, antioxidant synergist

Packaging and Storage: Store in well-closed containers.

ASSAY

• Total Glycerin

  Solution A: Mix exactly 99 mL (from a buret) of chloroform and 25 mL of glacial acetic acid in a 1-L volumetric flask.

  Sample solution: Weigh accurately 2 g of sample into a saponification flask, add 50 mL of ethanolic potassium hydroxide solution (0.5 M), and gently boil for 30 min. Quantitatively transfer the content of the saponification flask to the 1-L volumetric flask with Solution A, using three 25-mL portions of water. Add about 500 mL of water, and shake vigorously for about 1 min. Dilute with water to volume, mix thoroughly, and set aside for separation of layers. The aqueous layer result is the Sample solution.

  Analysis: Pipet 50 mL of acetic periodic acid TS into a series of 400-mL beakers. Prepare two blanks by
adding 50 mL of water to each. Pipet 50 mL of the Sample solution into one of the beakers containing 50 mL of acetic periodic acid TS, shake gently to mix, cover with a watch glass, and allow to stand 30 min but NMT 90 min. Add 20 mL of 15% ethanolic potassium iodide solution, shake gently to mix, and allow to stand at least 1 min but NMT 5 min. Do not allow to stand in bright or direct sunlight. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS. Use a variable speed electric stirrer to keep the solution thoroughly mixed. Continue the titration until the brown iodine color dissappears from the aqueous layer. Add 2 mL of starch TS, and continue the titration until the blue iodine-starch color dissapears from both the thin chloroform layer (separated during titration) and the aqueous layer.

Calculate the percent total glycerol in the sample taken:

\[ \text{Result} = \frac{(B - S) \times N \times K}{W} \]

- \( B \) = volume of titrant consumed by the blank containing 50 mL of water (mL)
- \( S \) = volume of titrant consumed by the Sample solution (mL)
- \( N \) = exact normality of 0.1 N thiosulfate
- \( K \) = molecular weight of glycerin divided by 40, 2.302
- \( W \) = weight of the original sample taken (g)

**Acceptance criteria:** Between 8% and 33%

### Total Fatty Acid

**Analysis:** Transfer 5 g of sample into a 250-mL round-bottomed flask, add 50 mL of 1 N ethanolic potassium hydroxide, and reflux for 1 h on a water bath. Quantitatively transfer the contents of the saponification flask to a 1000-mL separating funnel, using three 25-mL portions of water, and add 5 drops of methyl orange TS. Cautiously add concentrated hydrochloric acid until the solution color changes clearly to red, and shake well to separate fatty acids. Extract the separated fatty acids with three 100-mL portions of diethyl ether. Combine the extracts, and wash with 50-mL portions of 10% sodium chloride solution until the washed sodium chloride solution becomes neutral. Dry the ether solution with anhydrous sodium sulfate. Then evaporate off ether on a steam bath, leave an additional 10 min on the steam bath, and weigh the residue.

**Acceptance criteria:** Between 37% and 81%

### Total Citric Acid

[Note—In this test, the sample is saponified with an alcoholic potassium hydroxide solution and the fatty acids are removed by extraction. The citric acid present is converted to trimethylsilyl derivatives and analyzed by gas liquid chromatography.]

**Internal standard solution:** 1 mg/mL of tartaric acid solution

**Standard stock solution:** 3 mg/mL of USP Citric Acid RS solution

**Sample solution:** Weigh accurately 1 g of sample into a round-bottomed flask, add 25 mL of 0.5 M ethanolic potassium hydroxide, and reflux for 30 min. Acidify the mixture with hydrochloric acid, and evaporate in a rotary evaporator or by another suitable method. Quantitatively transfer the contents of the flask to a separator, using NMT 50 mL of water, and extract with three 50-mL portions of heptane, discarding the extracts. Transfer the aqueous layer to a 100-mL volumetric flask, neutralize, dilute with water to volume, and mix. Transfer 1 mL of this mixture and 1 mL of the Internal standard solution into a 10-mL round-bottom flask, and evaporate to dryness. Add to the flask 1.0 mL of pyridine, 0.2 mL of trimethyl-chlorosilane, 0.4 mL of hexamethyl-disilazane, and 0.1 mL of N-methyl-N-trimethylsilyl-trifluoroacetamide. Cap the flask tightly, and swirl carefully to dissolve completely. Heat the flask in an oven at 60° for 1 h.

**Standard solution:** Transfer 1 mL of the Standard stock solution and 1 mL of the Internal standard solution into a 10-mL round-bottom flask, and evaporate to dryness. Add to the flask 1.0 mL of pyridine, 0.2 mL of trimethyl-chlorosilane, 0.4 mL of hexamethyl-disilazane, and 0.1 mL of N-methyl-N-trimethylsilyl-tri-
fluoroacetamide. Cap the flask tightly, and swirl carefully to dissolve completely. Heat the flask in an oven at 60° for 1 h.

**Chromatographic system**, Appendix IIA  
**Mode**: Gas chromatography  
**Detector**: Flame ionization  
**Column**: 1.8-m × 2.0-mm (id) glass column packed with 10% DC-200 on 80- to 100-mesh, chromosorb Q, or equivalent  
**Temperature**  
- **Oven**: 165°  
- **Injection block**: 240°  
- **Detector block**: 240°  
**Carrier gas**: Nitrogen  
**Flow rate**: 24 mL/min  
**Injection volume**: 5 µL  
**Analysis**: Separately inject derivatized *Sample solution* and *Standard solution* into the chromatograph. Measure each peak area by a suitable method, and calculate the percentage of citric acid in the sample taken:  
[Note—The retention times for tartaric acid and citric acid/tartaric acid are about 12 min and 2.3 min, respectively.]

\[
\text{Result} = R_S \times 100 \times R_O \times 100 \times \left(\frac{W_O}{W_S}\right)
\]

- \(R_S\) = peak area ratio of citric acid and tartaric acid from the *Sample solution*  
- \(R_O\) = peak area ratio of tartaric acid and citric acid from the *Standard solution*  
- \(W_O\) = weight of USP Citric Acid RS used in the *Standard solution*  
- \(W_S\) = sample weight (g)  

**Acceptance criteria**: Between 13% and 50%

**IMPURITIES**  
**Inorganic Impurities**  
- **Lead**, *Lead Limit Test*, *Flame Atomic Absorption Spectrophotometric Method*, Appendix IIIB  
  - **Sample**: 10 g  
  - **Acceptance criteria**: NMT 2 mg/kg

**SPECIFIC TESTS**  
- **Residue on Ignition (Sulfated Ash)**, *Method 1*, Appendix IIIC  
  - **Sample**: 2 g  
  - **Acceptance criteria**: NMT 0.5% for non-neutralized products, NMT 10% for partially or wholly neutralized products  
- **Free Glycerin**, *Free Glycerin or Propylene Glycol*, Appendix VII  
  - **Acceptance criteria**: NMT 4%

**OTHER REQUIREMENTS**  
- **Labeling**: Indicate substances used to neutralize material.
**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

(+)-Dihydrocarvone, page 1461 of the First Supplement to FCC 7. On the basis of comments and data received, the food-grade materials of commerce for this ingredient have a different Angular Rotation range than the current requirement in this monograph. According to comments received, this is due to the higher enantiomeric purity of the material currently in commerce. It is therefore proposed to change the Acceptance criteria in the test for Angular Rotation to a range that reflects materials currently available. Comments of interested parties are encouraged.

(FIEC: C. Mejia) C97213

Change to read:

1. (+)-Dihydrocarvone 1S (FCC7)

Change to read:

• d-Dihydrocarvone

1. 1S (FCC7)

d-2-Methyl-5-(1-methylethenyl)-cyclohexanone

C\text{10}H_{16}O

Formula wt 154.24
FEMA: 3565

DESCRIPTION

Characteristics:

Chemical
Change to read:

(+)-Dihydrocarvone\textsubscript{1S} (FCC7) occurs as an almost colorless liquid.

**Odor:** Herbaceous, spearmint

**Solubility:** Soluble in alcohol, most fixed oils; insoluble or practically insoluble in water

**Boiling Point:** $\sim 222^\circ$

**Solubility in Alcohol,** Appendix VI: One mL dissolves in 1 mL of 95% alcohol.

**Function:** Flavoring agent

### IDENTIFICATION

**Change to read:**

- **Infrared Spectra**, *Spectrophotometric Identification Tests*, Appendix IIIC

**Acceptance criteria:** The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

![Infrared Spectrum](image)

### ASSAY

**Procedure:** Proceed as directed under M-1a, Appendix XI

**Acceptance criteria:** NLT 92.0% of $\text{C}_{10}\text{H}_{16}\text{O}$ (sum of two isomers)

### SPECIFIC TESTS

- **Refractive Index,** Appendix II: At $20^\circ$

  **Acceptance criteria:** Between 1.470 and 1.474

- **Specific Gravity:** Determine at $25^\circ$ by any reliable method (see *General Provisions*).

  **Acceptance criteria:** Between 0.923 and 0.928

### OTHER REQUIREMENTS

**Change to read:**

- **Angular Rotation,** *Optical (Specific) Rotation*, Appendix IIB

  **Acceptance criteria:** Between $+14^\circ$ and $+22^\circ$ Between $+14^\circ$ and $+25^\circ$ \textsubscript{3S} (FCC7)

### Auxiliary Information— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 293*

*FCC Seventh Edition Supplement 1 Page 1461*
BRIEFING

**Ethyl Cellulose, FCC 7 page 350.** On the basis of comments received, it is proposed to revise the Acceptance criteria in the test for Viscosity to be consistent with the Ethylcellulose monograph published in *USP 32–NF 27.*

(FIEC: J. Moore) C95023

**Ethyl Cellulose**

Modified Cellulose, EC

INS: 462

CAS: [9004-57-3]

**DESCRIPTION**

Ethyl Cellulose occurs as a free-flowing, white to light tan powder. It is heat-labile, and exposure to high temperatures (240°) causes color degradation and loss of properties. It is practically insoluble in water, in glycerin, and in propylene glycol, but is soluble in varying proportions in certain organic solvents, depending on the ethoxyl content. Ethyl Cellulose containing less than 46% to 48% of ethoxyl groups is freely soluble in tetrahydrofuran, in methyl acetate, in chloroform, and in aromatic hydrocarbon–alcohol mixtures. Ethyl Cellulose containing 46% to 48% or more of ethoxyl groups is freely soluble in alcohol, in methanol, in toluene, in chloroform, and in ethyl acetate. A 1:20 aqueous suspension is neutral to litmus.

**Function:** Protective coating; binder; filler

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

- **Procedure**
  - **Sample solution:** Dissolve 5 g of the sample in 95 g of an 80:20 (w/w) mixture of toluene–ethanol.
  - **Analysis:** Pour a few mL of the *Sample solution* onto a glass plate, and allow the solvent to evaporate.
  - **Acceptance criteria:** The *Sample solution* is clear, stable, and slightly yellow; and following the *Analysis*, a thick, tough, clear, flammable film remains.

**ASSAY**

- **Procedure**
  - **Sample:** Place about 50 mg of the sample, previously dried, in a tared gelatin capsule.
  - **Analysis:** Transfer the capsule and its contents into the boiling flask of a methoxyl determination apparatus, and proceed as directed under *Methoxyl Determination*, Appendix IIIC. Each mL of 0.1 N sodium thiosulfate is equivalent to 751 mg of ethoxyl groups (–OC₂H₅).
  - **Acceptance criteria:** NLT 44.0% and NMT 50.0% of ethoxyl groups (–OC₂H₅), on the dried basis (equivalent to NMT 2.6 ethoxyl groups per anhydroglucose unit)

**IMPURITIES**

**Inorganic Impurities**

- **Lead, Lead Limit Test, Appendix IIIB**
  - **Sample solution:** Prepare as directed for organic compounds, using a 2-g sample.
  - **Control:** 6 µg of Pb (6 mL of *Diluted Standard Lead Solution*)
  - [Note—Alternatively, determine as directed for *Flame Atomic Absorption Spectrophotometric Method* under]
Lead Limit Test, Appendix IIIB, using a 10-g sample.

Acceptance criteria: NMT 3 mg/kg

SPECIFIC TESTS

- Loss on Drying, Appendix IIC: 105°C for 2 h
  Acceptance criteria: NMT 3.0%

- Residue on Ignition (Sulfated Ash), Appendix IIC
  Sample: 1 g
  Analysis: Proceed as directed, but igniting at 600°C ± 50°C.
  Acceptance criteria: NMT 0.5%

Change to read:

- Viscosity, Viscosity of Methylcellulose, Appendix IIB

  Solvent systems: For Ethyl Cellulose containing less than 46% to 48% of ethoxyl groups, prepare a solvent consisting of a 60:40 (w/w) mixture of toluene–alcohol; for Ethyl Cellulose containing 46% to 48% or more of ethoxyl groups, prepare a solvent consisting of an 80:20 (w/w) mixture of toluene–alcohol.

  Sample solution: Transfer 5.0 g of the previously dried sample into a bottle containing 95 ± 0.5 g of the appropriate solvent system. Shake or tumble the bottle until the sample is completely dissolved, and then adjust the temperature of the solution to 25 ± 0.1°C.

  Analysis: Determine the viscosity of the Sample solution as directed, but make all determinations at 25°C instead of at 20°C.

  Acceptance criteria

  Ethyl Cellulose labeled as 10 centipoises or more: NLT 90% and NMT 110% of the viscosity stated on the label

  Ethyl Cellulose labeled as 10 centipoises or less: NLT 80% and NMT 120% of the viscosity stated on the label

  Ethyl Cellulose labeled as more than 6 centipoises (mPa·s): NLT 80% and NMT 120% of the viscosity stated on the label

  Ethyl Cellulose labeled as 6 centipoises (mPa·s) or less: NLT 75% and NMT 140% of the viscosity stated on the label

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 350
**BRIEFING**

**Ethyl Lauroyl Arginate.** A proposed monograph for this ingredient appeared in the *FCC Forum* [December 2009], but did not reach the ballot due to comments received indicating that some of the HPLC procedures for the *Assay* and *Organic Impurities* were unreliable. This revised proposal replaces the five originally proposed HPLC procedures with an HPLC procedure capable of separating and quantifying Ethyl Lauroyl Arginate and its five related compounds. This revised method is based on data and comments received. The acceptance criteria for the *Assay* and *Related Compounds* were also revised and clarified to be on the as-is basis. Interested parties are encouraged to submit comments to Jeff Moore, Ph.D., email jm@usp.org.

(FIEC: J. Moore)  C91748

---

**Add the following:**

- **Ethyl Lauroyl Arginate**

  Ethyl-$\text{N}^\alpha$-Lauroyl-$l$-Arginate · HCl

  Ethyl-$\text{N}^\alpha$-Dodecanoyl-$l$-Arginate · HCl

  Lauric Arginate Ethyl Ester

  Lauramide Arginine Ethyl Ester

  **LAE**

  ![Chemical Structure](image)

  $\text{C}_{20}\text{H}_{41}\text{N}_4\text{O}_3\text{Cl}$

  Formula wt 421.02

  INS: 243

  CAS: [60372-77-2]

**DESCRIPTION**

Ethyl Lauroyl Arginate occurs as a white powder. It is synthesized by esterifying arginine with ethanol, followed by reacting the ester with lauroyl chloride. The resultant ethyl lauroyl arginate is recovered as hydrochloride salt which is filtered off and dried. It is freely soluble in water, ethanol, propylene glycol, and glycerol.

**Function:** Preservative

**Packaging and Storage:** Store in tight containers in a dry place.

**IDENTIFICATION**

- **Procedure**

  **Acceptance criteria:** The retention time of the major peak (excluding the solvent peak) in the chromatogram of the *Sample solution* corresponds to that of the *Standard solution* in the *Assay* (below).
ASSAY

- **PROCEDURE**

  **Mobile phase:** Acetonitrile and water (50:50, v/v) containing 0.1% trifluoroacetic acid

  **Standard stock solution:** 3000 µg/mL USP Ethyl Lauroyl Arginate Hydrochloride RS¹ and 100 µg/mL USP Lauroyl Arginine RS² in Mobile phase

  **Standard solutions:** Separately dilute 2-, 4-, 6-, 8-, and 10-mL aliquots of Standard stock solution with Mobile phase to 25 mL.

  **Sample solution:** 1000 µg/mL in Mobile phase

  **Chromatographic system,** Appendix II.A.

  - **Mode:** High-performance liquid chromatography
  - **Detector:** UV 215 nm
  - **Column:** 15-cm × 3.9-mm, packed with octadecylsilane chemically bonded to porous silica or ceramic micro-particles (5-µm particle diameter)³
  - **Column temperature:** Ambient
  - **Flow rate:** 1 mL/min
  - **Injection volume:** 10 µL

  **System suitability**

  - **Sample:** Standard solutions

  - **Suitability requirement:** The relative standard deviation is NMT 2.0% for the slope of the ethyl-N⁷-lauroyl-l-arginate · HCl standard curve.

  **Analysis:** [Note—Equilibrate the column by pumping Mobile phase through it for 30 min. All injections should be done in triplicate.] Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. [Note—The retention times for ethyl-N⁷-lauroyl-l-arginate · HCl and N⁷-lauroyl-l-arginine are approximately 4.3 and 2.2 min, respectively.]

  Prepare a standard curve for ethyl-N⁷-lauroyl-l-arginate · HCl by plotting peak areas versus concentrations in µg/mL of the Standard solutions. Calculate the percentage of ethyl-N⁷-lauroyl-l-arginate · HCl in the portion of the sample taken:

  \[
  \text{Result} = \frac{C_U}{C_{SMP}} \times 100
  \]

  \(C_U\) = concentration of ethyl-N⁷-lauroyl-l-arginate · HCl in the Sample solution determined from the standard curve (µg/mL)

  \(C_{SMP}\) = concentration of sample in the Sample solution (µg/mL)

  **Mobile phase:** 0.20% sulfuric acid and acetonitrile (1:1, v/v)

  **Diluent:** Water and acetonitrile (1:1, v/v)

  **Standard solution:** 100 µg/mL of USP Ethyl Lauroyl Arginate RS in Diluent. [Note—Sonication may be necessary to aid in dissolution.]

  **Sample solution:** 100 µg/mL of sample in Diluent. [Note—Sonication may be necessary to aid in dissolution.]

  **Chromatographic system,** Appendix II.A.

  - **Mode:** High-performance liquid chromatography
  - **Detector:** UV 209 nm
  - **Column:** 2.1- × 150-mm, mixed mode column (reverse phase with embedded acidic ion-pairing groups), 5-
µm particle diameter

Column temperature: 30°C
Flow rate: 0.6 mL/min
Injection volume: 50 µL

System suitability
Sample: Standard solution
Suitability requirement 1: The relative standard deviation for peak area is NMT 0.5% for replicate injections.
Suitability requirement 2: The peak tailing factor is NMT 2.0%.

Analysis: Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. [Note —The retention times for ethyl lauroyl arginate is approximately 12.8 min.] Calculate the percent ethyl lauryl arginate in the sample taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100 \]

\[ r_U = \text{peak area response for ethyl lauryl arginate in the Sample solution} \]
\[ r_S = \text{peak area response for ethyl lauryl arginate in the Standard solution} \]
\[ C_S = \text{concentration of USP Ethyl Lauryl Arginate RS in the Standard solution, corrected for purity based on the label claim (µg/mL)} \]
\[ C_U = \text{concentration of sample in the Sample solution (µg/mL)} \]

Acceptance criteria: 85%–95% NLT 85% on the as-is basis

IMPURITIES
Inorganic Impurities
• Lead, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB
  Acceptance criteria: NMT 1 mg/kg

Organic Impurities
• L-Arginine Hydrochloride Related Compounds
  Mobile phase: Methanol, 15 mM sodium heptanesulfonate, 27 mM phosphoric acid solution, and 3 mM sodium di-hydrogen phosphate solution (1.5:1:1:1) (v/v/v/v)
  Standard stock solution: 400 µg/mL USP Arginine Hydrochloride RS
  Standard solutions: 20, 40, and 60 µg/mL of USP Arginine Hydrochloride RS, made from Standard stock solution
  Sample solution: 4000 µg/mL
  Derivatizing solution: Mix 1 L of 0.2 M borate buffer solution (pH 9.4) with 0.8 g of o-phthalaldehyde dissolved in 5 mL of methanol and 2 mL of 2-mercaptoethanol. [Note —This solution is stable for 48 h at room temperature and without additional preventative measure but it is advisable to keep the solution under nitrogen and to prepare it freshly every 24–48 h.]

Chromatographic system, Appendix IIA.
Mode: High performance liquid chromatography (equipped with post-column derivatization)
Detector: UV 340 nm
Column: 30 cm × 3.9 mm, packed with octadecylsilane chemically bonded to porous silica or ceramic microparticles (10 µm particle diameter)
**Column temperature:** 65°C
**Flow rate:** 0.8 mL/min
**Injection size:** 10 µL

**Derivatization:** Post-column derivatization is produced employing the Derivatizing solution at 65°C in a teflon tubular reactor (650–800 × 0.3 mm)

**System suitability:**

**Sample:** Standard solutions

**Suitability requirement:** The relative standard deviation is NMT 5.0% for the slope of the \( l \)-arginine·HCl standard curve.

**Analysis:** [Note—Equilibrate the column by pumping Mobile phase through it for 30 min. All injections should be done in triplicate.] Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. [Note—The retention time for \( l \)-arginine·HCl is approximately 5.03 min.]

Prepare standard curves for \( l \)-arginine·HCl by plotting peak areas versus concentrations in µg/mL of the Standard solutions. Calculate the percentage of \( l \)-arginine·HCl in the portion of the sample taken:

\[
\text{Result} = \frac{C_U}{C_{SMP}} \times 100
\]

- \( C_U \) = concentration of \( l \)-arginine·HCl in the Sample solution determined from the standard curve (µg/mL)
- \( C_{SMP} \) = concentration of sample in the Sample solution (µg/mL)

**Mobile phase, Diluent, and Chromatographic System:** Proceed as directed under Assay.

**Standard solution:** 1000 µg/mL of USP Ethyl Lauroyl Arginate RS, 10 µg/mL of USP Arginine Hydrochloride RS, 10 µg/mL of USP Arginine Ethyl Ester Dihydrochloride RS, 50 µg/mL of USP Lauric Acid RS, 30 µg/mL of USP Ethyl Laurate RS, and 30 µg/mL of USP Lauroyl Arginine RS in Diluent. [Note—Sonication may be necessary to aid in dissolution.]

**Sample solution:** 1000 µg/mL of sample in Diluent. [Note—Sonication may be necessary to aid in dissolution.]

**System suitability**

**Sample:** Standard solution

**Suitability requirement 1:** The relative standard deviation for peak area is NMT 2.0% for any of the five measured related compounds, for replicate injections.

**Suitability requirement 2:** The resolution is NLT 1.5 between any of the five related compound peaks, and NLT 1.0 between the ethyl laurate and ethyl lauroyl arginate peaks.

**Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. [Note—The relative retention times for arginine hydrochloride, lauric acid, arginine ethyl ester dihydrochloride, lauroyl arginine, ethyl laurate, and ethyl lauroyl arginate RS are 0.2, 0.3, 0.4, 0.5, 0.8, and 1, respectively.] Calculate the percents of the five related compounds (arginine hydrochloride, lauric acid, arginine ethyl ester dihydrochloride, lauroyl arginine, and ethyl laurate) in the sample taken:

\[
\text{Result} = \left( \frac{n_U}{n_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]
\( r_U \) = peak area response for analyte in the Sample solution
\( r_S \) = peak area response for analyte in the Standard solution
\( C_S \) = concentration of corresponding analyte USP RS in the Standard solution, corrected for purity based on the label claim if applicable (\( \mu g/mL \))
\( C_U \) = concentration of sample in the Sample solution (\( \mu g/mL \))

Acceptance criteria NMT 1%

Arginine hydrochloride: NMT 1.0% on the as-is basis
Lauric acid: NMT 5.0% on the as-is basis
Arginine ethyl ester dihydrochloride: NMT 1.0% on the as-is basis
Lauroyl arginine: NMT 3.0% on the as-is basis
Ethyl laurate: NMT 3.0% on the as-is basis

\[ \text{L-Arginine Ethyl Ester Dihydrochloride} \]

Mobile phase, Derivatizing solution, and Chromatographic system: Prepare as directed in the test procedure for L-Arginine Hydrochloride (above):

Standard stock solution: 8000 \( \mu g/mL \) USP Arginine Ethyl Ester Dihydrochloride RS

Standard solutions: 400, 800, and 1200 \( \mu g/mL \) of USP Arginine Ethyl Ester Dihydrochloride RS, made from Standard stock solution

Sample solution: 80 mg/mL

System suitability-
Sample: Standard solutions
Suitability requirement: The relative standard deviation is NMT 5.0% for the slope of the L-arginine ethyl ester \( \cdot 2HCl \) standard curve.

Analysis: [Note—Equilibrate the column by pumping Mobile phase through it for 30 min. All injections should be done in triplicate.] Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. [Note—The retention time for L-arginine ethyl ester \( \cdot 2HCl \) is approximately 6.70 min.]

Prepare a standard curve for L-arginine ethyl ester \( \cdot 2HCl \) by plotting peak areas versus concentrations in \( \mu g/mL \) of the Standard solutions. Calculate the percentage of L-arginine ethyl ester \( \cdot 2HCl \) in the portion of the sample taken:

\[
\text{Result} = \frac{C_U}{C_{SMP}} \times 100
\]

\( C_U \) = concentration of analyte in the Sample solution determined from the standard curve (\( \mu g/mL \))
\( C_{SMP} \) = concentration of sample in the Sample solution (\( \mu g/mL \))

Acceptance criteria: NMT 1%

\[ \text{Ethyl Laurate and Lauric Acid} \]

Mobile phase: Acetonitrile and water (85:15) (v/v), containing 0.1% trifluoroacetic acid

Standard stock solution: 2500 \( \mu g/mL \) of USP Lauric Acid RS and 1500 \( \mu g/mL \) of USP Ethyl Laurate RS in Mobile phase

Standard solutions: Separately dilute 5-, 10-, and 15-mL aliquots of Standard stock solution with Mobile phase to 50 mL.

Sample solution: 10 mg/mL

Chromatographic system, Appendix II A:

Mode: High-performance liquid chromatography
Detector: UV 242 nm
Column: 45 cm × 3.9-mm, packed with octadecylsilane chemically bonded to porous silica or ceramic micro-particles (5-µm particle diameter)

Column temperature: Ambient
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability:
Sample: Standard solutions
Suitability requirement: The relative standard deviation is NMT 5.0% for the slope of the lauric acid and ethyl laurate standard curves.

Analysis: [Note—Equilibrate the column by pumping Mobile phase through it for 30 min. All injections should be done in triplicate.]—Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms.—[Note—The retention times for lauric acid and ethyl laurate are approximately 3.65 and 11.2 min, respectively.]
Prepare standard curves for ethyl laurate and lauric acid by plotting their peak areas versus concentrations in µg/mL of the Standard solutions, corrected for purity. Separately calculate the percentages of ethyl laurate and lauric acid in the portion of the sample taken:

Result = C_U / C_SMP × 100

C_U = concentration of analyte in the Sample solution determined from the standard curve (µg/mL)
C_SMP = concentration of sample in the Sample solution (µg/mL)

Acceptance criteria—
Ethyl laurate: NMT 3%
Lauric acid: NMT 5%

Lauroyl arginine
Mobile phase, Standard stock solution, Standard solutions, Sample solution, and Chromatographic system: Proceed as directed under Assay.
System suitability—
Sample: Standard solutions
Suitability requirement: The relative standard deviation is NMT 5.0% for the slope of the N-lauroyl-L-arginine standard curve.

Analysis: Proceed as directed under Assay. Prepare a standard curve for lauroyl arginine by plotting N-lauroyl-L-arginine peak areas versus concentrations in µg/mL of the Standard solutions. Calculate the percentage of N-lauroyl-L-arginine in the portion of the sample taken:

Result = C_U / C_SMP × 100

C_U = concentration of N-lauroyl-L-arginine in the Sample solution determined from the standard curve (µg/mL)
C_SMP = concentration of sample in the Sample solution (µg/mL)

Acceptance criteria: NMT 3%

SPECIFIC TESTS
• Ash (Total), Appendix IIIC
Analysis: Proceed as directed, but igniting at 700° instead of 550°.

Acceptance criteria: NMT 2%

• pH, pH Determination, Appendix IIB
  Sample: 10 mg/mL
  Acceptance criteria: Between 3.0 and 5.0

• Water, Water Determination, Method I, Appendix IIB
  Acceptance criteria: NMT 5%

1. Ethyl-N-lauroyl-L-arginate·HCl


3. Symmetry C18 (Waters Corporation, Milford, MA, USA), or equivalent.

4. SIELC Primesep 100 (SIELC Technologies, Prospect Heights, IL), part #100-21.150.0510.

5. L-Arginine·HCl.

6. µBondapack C18 (Waters Corporation, Milford, MA, USA), or equivalent.

7. L-Arginine ethyl ester dihydrochloride.

Symmetry C18 (Waters Corporation, Milford, MA, USA), or equivalent.

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

**Ferrous Ammonium Phosphate.** Because there is no existing *FCC* monograph for this food ingredient, a new monograph is proposed based on the Ferrous Ammonium Phosphate monograph prepared at the 71st Session (2009) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and on the basis of comments received.

1. Acceptance criteria proposed for the Assay section are based on both the GRAS notification and the European Commission Directive for this ingredient. Comments are encouraged, especially those related to methods of analysis to determine quantitatively ammonia and phosphate content to support the limits of these components as stated in the GRAS notification and the European Commission Directive.

2. Impurities proposed are consistent with those specified in JECFA. Comments and data pertaining to methods of analysis for additional impurities proposed in the GRAS notification and the European Commission Directive (aluminum, sulfate, and nickel) are solicited to support the limits stated in these documents for these impurities.

3. The limit tests for Arsenic and Mercury are based on the existing method in *FCC*, because the method referenced by JECFA includes insufficient detail. Interested parties are encouraged to submit comments and data pertinent to these methods.

(FIEC: C. Mejia) C88392

Add the following:

**Ferrous Ammonium Phosphate**

Ammonium Iron (II) Phosphate

Phosphoric acid, ammonium iron (II) salt

FeNH$_4$PO$_4$

*Formula wt 168.85*

*CAS: [10101-60-7]*

**DESCRIPTION**

Ferrous Ammonium Phosphate occurs as a greyish-green fine powder. It consists primarily of the anhydrous salt with small amounts of the hydrate. The final product is obtained by combining iron powder, phosphoric acid, and ammonia solution in demineralized water. The purification and spray drying processes remove any excess of starting materials (e.g. unreacted iron powder), insoluble salts, and volatiles, such as ammonia. The product is then milled into fine powder with the required particle size. It is insoluble in water, but is soluble in dilute mineral acids.

**Function:** Nutrient

**Packaging and Storage:** Store in tightly closed containers. The recommended storage temperature is between 5° and 10°.

**IDENTIFICATION**

- *Ammonium*, Appendix IIIA
Acceptance criteria: Passes test

- Iron (Ferrous Salts), Appendix IIIA
  Acceptance criteria: Passes test

- Phosphate, Appendix IIIA
  Acceptance criteria: Passes test

ASSAY

- Procedure
  Analysis: Weigh 300 g of sample into a 250-mL conical flask, add 25 mL of dilute sulfuric acid (16% v/v), and dissolve with heating. Cool, and add 75 mL of water. Add 0.1 mL of ferroin TS (0.1% w/v in water). Titrate immediately with 0.1 N ceric sulfate VS until the color changes from orange to light bluish-green. Each mL of 0.1 N ceric sulfate is equivalent to 5.585 mg of iron (II).
  Acceptance criteria: Between 22% and 30% expressed as iron (II)

IMPURITIES

Inorganic Impurities

- Arsenic, Elemental Impurities by ICP, Appendix IIIC
  Acceptance criteria: NMT 3 mg/kg

- Cadmium, Elemental Impurities by ICP, Method I, Appendix IIIC
  Acceptance criteria: NMT 1 mg/kg

- Fluoride, Fluoride Limit Test, Method I or Method II, Appendix IIIB
  Acceptance criteria: NMT 0.005%

- Lead, Elemental Impurities by ICP, Method I, Appendix IIIC
  Acceptance criteria: NMT 2 mg/kg

- Mercury, Elemental Impurities by ICP, Appendix IIIC
  Acceptance criteria: NMT 1 mg/kg

SPECIFIC TESTS

- Iron (III)
  Procedure: Transfer 1 g of sample into a 250-mL Erlenmeyer flask, add 20 mL of water and 10 mL of hydrochloric acid TS, diluted, heat to dissolve, and cool to room temperature. Add 3 g of potassium iodide, stopper, swirl to mix, and allow to stand in the dark for 15 min. Remove the stopper, add approximately 100 mL of water, and titrate with 0.1 N sodium thiosulfate, adding starch TS near the endpoint. Each mL of 0.1 N sodium thiosulfate is equivalent to 5.585 mg of iron (III).
  Acceptance criteria: NMT 7%

- Water Determination, Method I (Karl Fischer Titrimetric Method), Appendix IIB
  Acceptance criteria: NMT 3%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>1-301-816-8345</td>
<td></td>
</tr>
</tbody>
</table>
BRIEFING

Isopropyl Alcohol, *FCC 7* page 548 and page 1471 of the *First Supplement*. On the basis of comments received, a revision to remove the refractive index from the *Description* section is proposed. This test and an appropriate requirement already exist under *Identification*. The addition of the FEMA number for this ingredient to the chemical information section is also proposed.

(FIEC: K. Laurvick) C97103

Isopropyl Alcohol

*Change to read:*

2-Propanol

Isopropanol

\[
\text{CH}_3\backslash\text{CH}_2\backslash\text{OH}
\]

\[\text{C}_3\text{H}_8\text{O}\]

*Function:* Extraction solvent

*Packaging and Storage:* Store in tight containers, remote from fire.

**DESCRIPTION**

*Change to read:*

Isopropyl Alcohol occurs as a clear, colorless, flammable liquid. It is miscible with water, with ethyl alcohol, with ether, and with many other organic solvents. Its refractive index at 20° is about 1.377.\[3S\] (FCC7)

*Function:* Extraction solvent

*Packaging and Storage:* Store in tight containers, remote from fire.

**IDENTIFICATION**

*Change to read:*

- **Refractive Index**, Appendix IIB

  [Note—Use an Abbé or other refractometer of equal or greater accuracy.]

  - **Acceptance criteria:** Between 1.377 and 1.380 at 20°.\[1S\] (FCC7)
ASSAY

Change to read:

- **PROCEDURE**

  - **System suitability solution:** USP 2-Propanol System Suitability RS
  
  **Chromatographic system,** Appendix II A
  
  **Mode:** Gas chromatography
  
  **Detector:** Flame ionization
  
  **Column:** 60-m × 0.25-mm fused silica column with 1.4-µm film thickness of 6% cyanopropylphenyl/94% dimethylpolysiloxane stationary phase with a 4-mm straight liner
  
  **Temperature**
  
  **Injector:** 150°C
  
  **Detector:** 200°C
  
  **Column:** Hold at 35°C for 5 min; ramp to 45°C at 1°C/min; ramp to 100°C at 10°C/min; hold at 100°C for 1 min
  
  **Carrier gas:** Helium
  
  **Linear velocity:** 35 cm/s
  
  **Injection size:** 1 µL
  
  **Split ratio:** 50:1
  
  **System suitability**
  
  **Sample:** System suitability solution
  
  [Note—Approximate relative retention times for ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol are 0.7, 0.9, 1.0, 1.4, 1.5, and 2.0, respectively.]
  
  **Suitability requirement 1:** The relative standard deviation for the main isopropyl alcohol peak is NMT 2.0% for replicate injections.
  
  **Suitability requirement 2:** The resolution for the acetone and isopropyl alcohol peaks is NLT 2.0.
  
  **Suitability requirement 3:** The signal-to-noise ratio is NLT 10 for any of the following peaks: ethyl ether, acetone, diisopropyl ether, 1-propanol, and 2-butanol.
  
  **Analysis:** Inject the sample into the chromatograph and record the resulting chromatogram. Determine the percentage of C₃H₈O present in the sample through peak area normalization:

  \[
  \text{Result} = 100 \times \frac{R_i}{R_T}
  \]

  \( R_i \) = peak area for isopropyl alcohol

  \( R_T \) = sum of all of the peak areas

  **Acceptance criteria:** NLT 99.7% C₃H₈O₁S (FCC7)

IMPU RITIES

Inorganic Impurities

- **LEAD, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB**

  **Acceptance criteria:** NMT 1 mg/kg

SPECIFIC TESTS

- **ACIDITY (AS ACETIC ACID)**

  **Sample:** 50 mL (about 39 g)
Analysis: Add 2 drops of phenolphthalein TS to 100 mL of water, then add 0.01 N sodium hydroxide to the first pink color that persists for at least 30 s. Add the Sample to this solution and mix. Continue addition of 0.01 N sodium hydroxide until the pink color is restored.

Acceptance criteria: NMT 0.7 mL of sodium hydroxide is required to restore the pink color (NMT 10 mg/kg).

- DISTILLATION RANGE, Appendix IIB
  Acceptance criteria: Within a range of 1°, including 82.3°

- NONVOLATILE RESIDUE
  Sample: 125 mL (about 100 g)
  Analysis: Evaporate the Sample to dryness in a tared dish on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.
  Acceptance criteria: NMT 10 mg/kg

- SOLUBILITY IN WATER
  Sample: 10 mL
  Analysis: Mix the Sample with 40 mL of water.
  Acceptance criteria: After 1 h, the solution is as clear as an equal volume of water.

- SPECIFIC GRAVITY: Determine by any reliable method (see General Provisions).
  Acceptance criteria: NMT 0.7840 at 25°/25° (equivalent to 0.7870 at 20°/20°)

- SUBSTANCES REDUCING PERMANGANATE
  Sample: 50 mL
  Analysis: Transfer the Sample into a 50-mL glass-stoppered cylinder, add 0.25 mL of 0.1 N potassium permanganate, mix, and allow to stand for 10 min.
  Acceptance criteria: The pink color is not entirely discharged.

- WATER, Water Determination, Appendix IIB
  Acceptance criteria: NMT 0.2%

1 Restek Rtx®-1301, or equivalent. Available at www.restek.com

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 548
FCC Seventh Edition Supplement 1 Page 1471
**Krill Oil.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received.

1. The method used for Arsenic is intended to quantify inorganic arsenic in the Krill Oil. This method is adapted from an existing method in Appendix IIIIB. Validation data for the intended limit in this monograph is requested and should be submitted in the form of comments.
2. The methods used in the tests for Lead and Mercury are used in similar FCC oil monographs.
3. The methods and specifications in the tests for Astaxanthin Esterification and Phospholipids are intended to show that samples exhibit natural properties of authentic krill oil, which should contain naturally occurring krill astaxanthin, primarily in the diester form, and phospholipids. Quantitative methods would be preferred, specifically for the test for Phospholipids, and are specifically requested with supporting data.

(FIEC: K. Laurvick) C88981

---

**Add the following:**

- **Krill Oil**

  *Euphasia superba* Oil

  High Phospholipid Krill Oil

**DESCRIPTION**

Krill Oil occurs as an opaque reddish oil. It is obtained through cold extraction solely from crushed Antarctic krill (*Euphasia superba*) using acetone as the extraction solvent. The whole lipid portion of the extract is then separated and concentrated through evaporation of the acetone, followed by filtration. The major components of Krill Oil are triglycerides and phospholipids, including numerous constituent fatty acids. It is rich in two omega-3 long-chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA, C\textsubscript{20}H\textsubscript{30}O\textsubscript{2}) (C20:5 n-3) and docosahexaenoic acid (DHA, C\textsubscript{22}H\textsubscript{32}O\textsubscript{2}) (C22:6 n-3), in their phospholipid forms. Krill Oil also contains naturally-occurring free and esterified astaxanthin, primarily in the diester form.

**Function:** Source of EPA and DHA, primarily in the phospholipid forms

**Packaging and Storage:** Preserve in tight, light-resistant containers under an inert gas. Store in a cool place.

**IDENTIFICATION**

- **Fatty Acid Composition**, *Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids*, Appendix VII

  **Acceptance criteria:** The retention times of the peaks of the docosahexaenoic acid methyl ester and eicosapentaenoic acid methyl ester from the *Sample Preparation* correspond to those from the *Standard Solution*. Calculate the percentage of each fatty acid listed in the table below present in the sample taken. The amount of each fatty acid determined meets the requirements indicated below.
### Fatty Acid Shorthand Notation

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Shorthand Notation</th>
<th>Lower Limit (%, w/w)</th>
<th>Upper Limit (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monounsaturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-Palmitoleic acid</td>
<td>16:1 n-7 cis</td>
<td>3.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>18:1 n-7</td>
<td>4.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1 n-9</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Eicosenic acid</td>
<td>20:1 n-9</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Erucic acid</td>
<td>22:1 n-9</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18:2 n-6</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>18:3 n-3</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Morotic acid</td>
<td>18:4 n-3</td>
<td>2.2</td>
<td>3.9</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>20:5 n-3</td>
<td>12.0</td>
<td>16</td>
</tr>
<tr>
<td>Docosapentaenoic acid</td>
<td>22:5 n-3</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Docosohexaenoic acid</td>
<td>22:6 n-3</td>
<td>7.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

#### UV-Visible Absorption Spectrum

**Sample solution:** Prepare as directed for the Sample solution in the test for Content of Astaxanthin.

**Analysis:** Using a suitable spectrophotometer, determine the absorption spectrum between 300 and 700 nm.

**Acceptance criteria:** The absorption spectrum of the Sample solution exhibits a single maximum at about 486 nm.

### ASSAY

**DHA and EPA.** Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids, Appendix VII

**Acceptance criteria**

[Note—Separately calculate EPA and DHA as the percent of the fatty acid in the sample taken.]

EPA: NLT 12.0% (w/w)

DHA: NLT 7.0% (w/w)

### IMPURITIES

**Inorganic Impurities**

**Arsenic, Arsenic Limit Test, Appendix IIIB**

**Distillation-reducing solution:** 72 mg/mL of ACS-grade, low-arsenic, ferrous chloride tetrahydrate (FeCl₂·4H₂O) in 6.6 N hydrochloric acid. [Note—Prepare fresh on the day of use.]

**Apparatus:** Refer to the figure Special Apparatus for the Determination of Inorganic Arsenic, Figure 14.

**Control:** 0.2 µg of As (200 µL of Standard Arsenic Solution). [Note—Use this amount rather than the 3.0 mL specified in the Procedure.]

**Sample solution:** Transfer a 2.00-g sample to a distillation flask (A). Add 50 mL of Distillation-reducing solution, connect the flask to the receiver chamber (B), complete the assembly of the apparatus, and begin circulating tap water through the condenser (C). Half-fill the lower two bulbs of the splash head (D) with water.

Maneuver the stopcock to cause the contents of the receiver chamber to drain into the distillation flask, heat the flask until the temperature above the solution reaches 106° to 108°, and continue refluxing at this temperature for 45 min. Close the stopcock, continue heating at 108° to 110°, and collect 30 to 33
mL of distillate in the receiver chamber. Remove the heating source and allow the temperature to drop to about 80°.

Drain the distillate from the receiver chamber into a 250-mL beaker that is contained in an ice-water bath. Close the stopcock, and add a second 50-mL portion of the Distillation-reducing solution through the thermometer opening to the distillation flask. Replace the thermometer, increase the temperature to 108° to 110°, and collect a second 30- to 33-mL portion of distillate in the receiver chamber.

Drain the second distillate into the beaker containing the first portion, and continue cooling in the ice-water bath until the combined distillate cools to room temperature. Remove the splash head, and wash its contents into the beaker. Also, wash down the insides of the condenser and receiver chamber with water, collecting the washings in the beaker. Filter the beaker contents through a Whatman No. 40, or equivalent, filter paper, collecting the filtrate in a 300-mL Erlenmeyer flask having a 24/40 standard-taper joint, to be used later as an arsine generator flask. Wash the filter three times with water so that the final volume of the filtrate measures 200 mL.

Analysis: Add 2 mL of potassium iodide TS and 0.5 mL of Stannous Chloride Solution to the Sample solution contained in the Erlenmeyer flask, and proceed as directed in the Procedure beginning with “Allow the mixture to stand for 30 min at room temperature...”

Acceptance criteria: NMT 0.1 mg/kg, as inorganic arsenic

• Lead

Apparatus

Sample digestion: Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

Sample analysis: See Apparatus in Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB

Calibration standard stock solution: 100 µg/L. Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

Calibration standard solutions: 2.0, 5.0, 10.0, 25.0, and 50.0 µg/L in 2% nitric acid, from the Calibration standard stock solution

10% Ammonium dihydrogen phosphate stock solution: Mix 10 g of ultrapure ammonium dihydrogen phosphate with 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate stock solution: Mix 1 g of ultrapure magnesium nitrate with 40 mL of water and 1 mL of nitric acid in a Teflon beaker, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

[Note—Because of the difficulty in preparing matrix modifier stock solutions with the required purity, purchasing modifier stock solutions and using them to prepare working solutions is recommended.]

Modifier working solution: Transfer 4 mL of 10% Ammonium dihydrogen phosphate stock solution and 2 mL of 1% Magnesium nitrate stock solution to a 10-mL volumetric flask, and dilute with 2% nitric acid to volume. A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Sample solution: ['CAUTION— Wear proper eye protection and protective clothing and gloves during sample preparation. Closely follow the manufacturer’s safety instructions for use of the microwave digestion apparatus. ] Transfer 1.0 g of sample into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest
overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature (a cool water bath may be used to speed the cooling process). Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digestes into 25-mL volumetric flasks, and dilute with deionized water to volume.

**Analysis:** The graphite furnace program is as follows:

1. Dry at 120°C using a 1-s ramp, a 55-s hold, and a 300-mL/min argon flow.
2. Char the sample at 850°C using a 1-s ramp, a 30-s hold, and a 300-mL/min air flow.
3. Cool down and purge the air from the furnace for 10 s using a 20°C set temperature and a 300-mL/min argon flow.
4. Atomize at 2100°C using a 0-s ramp and a 5-s hold with the argon flow stopped.
5. Clean out at 2600°C with a 1-s ramp and a 5-s hold.

Use the autosampler to inject 20-µL aliquots of blanks, Calibration standard solutions, Sample solutions, and 5 µL of Modifier working solution. Inject each solution in duplicate, and average the results. Use peak-area measurement for all quantitation. After ensuring that the furnace is clean by running a 5% nitric acid blank, check instrument sensitivity by running an aliquot of the 25-µg Calibration standard solution. Compare the results obtained with the expected results for the equipment used, and take the necessary steps to correct any problems.

Calculate the characteristic mass, and record and track the integrated absorbance and characteristic mass for reference and quality assurance.

Inject each Calibration standard solution in duplicate. Use the algorithms provided in the instrument software to establish calibration curves. Recheck the calibration periodically, and recalibrate if recheck differs from the original calibration by more than 10%.

Inject the Sample solution in duplicate, and record the integrated absorbance. If the instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into the working range, and note the dilution factor (DF). All sample analyses should be blank corrected using a sample solution blank.

If a computer-based instrument is used, the data output is reported as µg/L. Calculate the concentration, in µg/g (equivalent to mg/kg), of lead in the original sample:

\[
\text{Result} = \left( \frac{C \times DF \times V}{W} \right)
\]

- \(C\) = concentration of lead in the sample aliquot injected (µg/L)
- \(DF\) = dilution factor of the Sample solution
- \(V\) = final volume of the Sample solution (L)
- \(W\) = weight of the sample taken to prepare the Sample solution (g)

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

**Acceptance criteria:** NMT 0.1 mg/kg
**MERCURY**

**Apparatus**

**Sample digestion:** Use a microwave oven equipped with advanced composite vessels with 100-mL Teflon liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes.

**Sample analysis:** Use a suitable atomic absorption spectrophotometer equipped with an atomic vapor assembly. An electrodeless discharge lamp serves as the source, with an inert gas such as argon or nitrogen as the purge gas. Set up the instrument according to manufacturer specifications. Instrument parameters are as follows:
- **Wavelength:** 253.6 nm
- **Slit:** 0.7
- **Reagent setting:** 5
- **Gas flow:** 5 to 6 L/min
- **Reaction time:** 0.5 min

**Glassware:** Acid wash all glass, Teflon, and plastic vessels by soaking them in a nitric acid bath containing a 4:1 solution of water:nitric acid. [**CAUTION—** Wear a full face shield and protective clothing and gloves at all times when working with acid baths.] After acid soaking, rinse acid-washed items in deionized water, dry, and store them in clean, covered cabinets.

**Calibration standard stock solution:** 200 ng/g of mercury. Prepare from a suitable standard, which may be purchased [accuracy certified against National Institute of Standards and Technology (NIST) spectrometric standard solutions].

**Calibration standard solutions:** 20, 60, 100, 200, and 400 ng of mercury in 1 N hydrochloric acid from the Calibration standard stock solution

**Reducing reagent:** 5% Stannous chloride in 25% hydrochloric acid (trace-metal grade). [Note—Prepare daily.]

**Sample solution:** Prepare as directed for the Sample solution in the test for Lead. [**CAUTION—** Wear proper eye protection and protective clothing and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus.]

**Analysis:** Optimize the instrument settings for the spectrophotometer as described in the instrument manual. The instrument parameters for cold vapor generation are as follows:
- **Wavelength:** 253.6 nm
- **Slit:** 0.70 nm
- **Reagent setting:** 5
- **Gas flow:** 5 to 6 L/min
- **Reaction time:** 0.5 min

Use a peak height integration method with a 40-s integration time and a 20-s read delay in an unheated absorption cell. Zero the instrument as follows: Place a Fleaker containing 50 mL of 1 N hydrochloric acid in the sample well of the hydride generator. Press “start” on the vapor generator and “read” on the atomic absorption spectrophotometer. The instrument will automatically flush the sample container with nitrogen, dispense the designated amount of reagent, stir the sample for a designated reaction time, and purge the head volume again with nitrogen, sweeping any vapor into the quartz cell for determination of absorption. The atomic absorption spectrophotometer will automatically zero on this sample when “autozero” is selected from the calibration menu.

Generate a standard curve of concentration versus absorption by analyzing the five Calibration standard solutions prepared as described for daily standards under Calibration standard solutions. Analyze each solution in duplicate, generate the calibration curve, and store, using procedures.
specific for the instrumentation. Transfer an appropriate aliquot of Sample solution (usually 2 mL) in a Fleaker containing 50 mL of 1 N hydrochloric acid. Analyze solutions in duplicate using the procedure specified in the instrument manual. Using the calibration algorithm provided in the instrument software, calculate and report the mercury concentration in ng of mercury in the aliquot analyzed. Calculate the level of mercury as µg/g (equivalent to mg/kg), in the original sample:

\[
\text{Result} = \frac{(A \times DF)}{(W \times F)}
\]

A = amount of mercury in the aliquot analyzed (ng)  
DF = dilution factor (final volume of Sample solution/volume taken for analysis)  
W = weight of the sample taken to prepare the Sample solution (g)  
F = factor converting between ng and µg (1000)

[Note—To monitor recovery and ensure analytical accuracy for proper quality assurance, analyze blanks, spiked blanks, and a spiked oil with each digestion set.]

Acceptance criteria: NMT 0.1 mg/kg

Organic Impurities
- Residual Solvent, Appendix VIII
  Acceptance criteria: NMT 10 ppm of acetone

SPECIFIC TESTS
- Anisidine Value, Appendix VII
  Acceptance criteria: Less than 2.1
- Astaxanthin Esterification, Thin-Layer Chromatography, Appendix IIA
  Standard solution A: 10 mg/mL of USP Astaxanthin Esters from Haematococcus pluvialis RS in acetone
  Standard solution B: 10 mg/mL of USP Astaxanthin (Synthetic) RS in acetone
  Sample solution: 10 mg/mL in acetone
  Adsorbent: 0.25-mm layer of chromatographic silica gel.  [Note—Dry silica gel at 110° for 1 h before use.]
  Developing solvent system: Hexane and acetone (70:30)
  Application volume: 5 µL
  Analysis: Develop the chromatogram in the Developing solvent system until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry in a current of air.
  Acceptance criteria: The principal spot obtained from Standard solution B, located in the bottom half of the plate, is free body astaxanthin. The Sample solution may exhibit a light, minor spot, in the same location. The principal spots obtained from Standard solution A are from monoesters (primary spot, located slightly above the middle of the plate) and diesters (secondary spot, located in the top third of the plate). The principal spot obtained from the Sample solution should correspond in color and R_f value to the diester spot obtained from Standard solution A. The secondary spot obtained from the Sample solution should correspond in color and approximately the same R_f value to the monoster spot obtained from Standard solution A.  [Note—Slight differences in R_f values within monoster spots and within diester spots may exist because these are classes of compounds and not pure compounds.]
- Content of Astaxanthin
  Sample solution: 0.005 g/mL in chloroform.  [Note—if the Sample solution is not clear, transfer it to a 15-mL tube and centrifuge at 1435 g.]
Analysis: Using a suitable UV/vis spectrophotometer, determine the absorbance of the Sample solution versus chloroform at 486 nm in a 1-cm cell. Calculate the percent of astaxanthin in the sample taken:

\[ \text{Result} = \frac{A_U}{(C_U \times a)} \times 100 \]

- \( A_U \) = absorbance of the Sample solution
- \( C_U \) = concentration of Krill Oil in the Sample solution (g/mL)
- \( a \) = absorptivity of astaxanthin in chloroform (169,220 mL·g\(^{-1}\)·cm\(^{-1}\))

Acceptance criteria: 0.10%–0.15%

- IODINE VALUE, Appendix VII
  Acceptance criteria: 130.0–160.0
- PEROXIDE VALUE, Appendix VII
  Acceptance criteria: Less than 0.2 mEq/kg
- PHOSPHOLIPIDS, Thin-Layer Chromatography, Appendix II\(^A\)

Sample: 2.5 g

Total phospholipids extraction: Transfer 25 mL of methanol to a 40-mL centrifuge tube, and add the Sample. Vortex the mixture, then centrifuge for 5 min at 5000 rpm. Carefully transfer the top phase from the centrifuge tube to a 250-mL round-bottomed flask. Avoid the bottom phase, which is rich in triglycerides. Dry evaporate the mixture in the flask at 45°C under vacuum, gradually decreasing pressure from 275 mbar to 30 mbar. Add 8 mL of acetone-saline (9:1) to the flask, and agitate it to achieve good dispersion of the oil. Transfer the resulting mixture into a clean 40-mL centrifuge tube, add 25 mL of acetone, shake well, and centrifuge for 5 min at 5000 rpm. Remove as much of the top phase as possible, and discard it. Add another 25-mL portion of acetone to the tube, shake well, and centrifuge for 5 min at 5000 rpm. Once again, remove as much of the top phase as possible, and discard it. Use the remaining phospholipids to prepare the Sample solution below.

Sample solution: 10 mg/mL of the material obtained from the Total phospholipid extraction in \(n\)-hexane

PA standard solution: 10 mg/mL of 3-sn-phosphaticid acid sodium salt from egg yolk lecithin\(^5\) in \(n\)-hexane

PI standard solution: 10 mg/mL of \(l\)-\(\alpha\)-phosphatidylinositol from glycine max\(^6\) in \(n\)-hexane

PC standard solution: 10 mg/mL of \(l\)-\(\alpha\)-phosphatidylcholine from soybean\(^7\) in \(n\)-hexane

LPC standard solution: 10 mg/mL of \(l\)-\(\alpha\)-lysophosphatidylcholine from egg yolk\(^8\)

[Note—The Standard solutions prepared above allow identification of different classes of phospholipids, identified by the abbreviated title of each solution.]

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent system A: Chloroform, methanol, and 7 N ammonium hydroxide (130:60:8)

Developing solvent system B: Chloroform, methanol, glacial acetic acid, and water (170:25:25:6)

Application volume: 100 µL

Analysis: Apply the Sample solution to the lower right-hand corner of a dry 20-cm \(\times\) 20-cm TLC plate such that the application spot is 2 cm from both edges forming the corner. Using five identical TLC plates, repeat for each of the Standard solutions so that there is only one material applied per TLC plate. Develop the plates in tanks containing Developing solvent system A for 90 min, or until the solvent front has moved nearly the entire length of the plate. Dry the plates for 30 min at room temperature. Next, turn each plate 90° in a clockwise direction (the original application spot of each sample, if visible, will now be at the lower left-hand corner of the plates). Develop each plate again in a tank containing Developing solvent system B for an additional 90 min. Remove all plates from solvent and allow to dry. Once dry, transfer the plates to a tank containing iodine crystals until the spots on the plates are clearly visible. Remove the plates from the tank.
tank, and identify the spots on the plate spotted with the Sample solution by comparing the location and color of each developed spot with those visible on the plates containing each of the Standard solutions.

**Acceptance criteria:** The plate obtained from the Sample solution contains a spot of similar color and location to the principal spots obtained from each of the Standard solutions, indicating the presence of all four classes of phospholipids.

- **Saponification Value**, Appendix VII
  - **Sample:** 4 g
  - **Acceptance criteria:** 170–190
- **Total Oxidation Value**
  - **Analysis:** Calculate:

  \[
  \text{Result} = (2 \times \text{PV}) + \text{AV}
  \]

  \[
  \text{PV} = \text{peroxide value, determined above}
  \]

  \[
  \text{AV} = \text{anisidine value, determined above}
  \]

  **Acceptance criteria:** Less than 2.5

**OTHER REQUIREMENTS**

- **Labeling:** Label to indicate the content of both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in mg/g (%). Indicate the presence of any other oil(s) used to standardize the EPA and DHA content.

---

1. CEM Model MDS-2100, or equivalent.
2. An ammonium dihydrogen phosphate (4%) and magnesium nitrate (0.2%) solution may be purchased from High Purity Standards, or equivalent.
3. This method was developed using a Perkin-Elmer Model 5100 and IL 440 Thermo Jarrell Ash atomic vapor assembly.
4. Based on AOCS Recommended Practice Ja 7-86, “Phospholipids in Lecithin Concentrates by Thin-Layer Chromatography” from the American Oil Chemists Society (2003 Rev.).
5. Catalog No. P9511 from Sigma-Aldrich Co. St. Louis, MO, or equivalent.
6. Catalog No. R6636 from Sigma-Aldrich Co. St. Louis, MO, or equivalent.
7. Catalog No. P7443 from Sigma-Aldrich Co. St. Louis, MO, or equivalent.
8. Catalog No. L4129 from Sigma-Aldrich Co. St. Louis, MO, or equivalent.

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick&lt;br&gt;Scientific Liaison&lt;br&gt;1-301-816-8356</td>
</tr>
</tbody>
</table>
BRIEFING

(-)-Menthyl Acetate, page 1479 of the First Supplement to FCC 7. On the basis on comments and data received, it is proposed to revise the Acceptance criteria range in the test for Angular Rotation. The current range is narrower than that represented by food-grade materials of commerce. The proposed range is consistent with that appearing in the Third Supplement to FCC 4. 

(FIEC: J. Moore) C97313

Change to read:

l- Menthan-3-yl Acetate

l-Menthyl Acetate

C₁₂H₂₀O₂

Formula wt 198.31
FEMA: 2668

DESCRIPTION

Change to read:

(-)-Menthyl Acetate lS (FCC7) occurs as a colorless liquid.

Odor: Mild, minty
Solubility: Soluble in alcohol, propylene glycol, most fixed oils; slightly soluble in water
Boiling Point: ~229° to 230°
Function: Flavoring agent
ASSAY

• Procedure: Proceed as directed under M-1b, Appendix XI.
  Acceptance criteria: NLT 98.0% of C_{12}H_{22}O_{2}

SPECIFIC TESTS

• Acid Value, M-15, Appendix XI
  Acceptance criteria: NMT 2.0

• Refractive Index, Appendix II: At 20°
  Acceptance criteria: Between 1.443 and 1.447

• Specific Gravity: Determine at 25° by any reliable method (see General Provisions).
  Acceptance criteria: Between 0.921 and 0.926

OTHER REQUIREMENTS

Change to read:

• Angular Rotation, Optical (Specific) Rotation, Appendix IIB: Use a 100-mm tube.
  Acceptance criteria: Between -70° and -69° 3S (FCC7)

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D.</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8288</td>
<td></td>
</tr>
</tbody>
</table>
**Meso-Zeaxanthin**, page 1494 of the *First Supplement* to *FCC 7*. On the basis of comments and data received, a revision to add an *Identification* test and specification for *Stereoisomeric Composition* is proposed.

(FIEC: K. Laurvick)  C97089

**Meso-Zeaxanthin**

\[\beta,\beta\text{-Carotene-3,3'}-\text{diol, (3R,3'S)-} \]

\((3R,3'S\text{-meso})\text{-Zeaxanthin}\)

C\(_{40}\)H\(_{56}\)O\(_2\)

**DESCRIPTION**

Meso-Zeaxanthin occurs as a free-flowing, orange to pale-yellow powder. It is the purified fraction obtained from isomerization of lutein from *Tagetes erecta* L., which contains both the (3R,3'S-meso)-zeaxanthin and the (3R,3'R)-zeaxanthin isomers with approximate concentrations of 94% and 6% (of total zeaxanthin), respectively.

**Function:** Source of meso-zeaxanthin; color

**Packaging and Storage:** Store in tight, light-resistant containers in a cool place.

**IDENTIFICATION**

- **A. Ultraviolet Absorption**
  
  **Acceptance criteria:** The *Sample solution* from the test for *Total Carotenoids* shows an absorption maximum at about 453 nm.

- **B. Procedure**
  
  **Acceptance criteria:** The retention time of the major peak in the chromatogram of the *Sample solution* corresponds to that in the chromatogram of the *Standard solution* as obtained in the *Assay for Zeaxanthin*.

**Add the following:**

- **C. Stereoisomeric Composition**

  **Mobile phase:** Hexane, alcohol, and isopropanol (80:5:5)

  **Sample solution:** Transfer 10 mg of sample to a 100-mL volumetric flask, add 50 mL of alcohol, and place the flask in an ultrasonic bath at 60° for 2–5 min to dissolve the sample. Remove the flask from the bath, cool to room temperature, and dilute with hexane to volume. Filter the solution through a 0.45-µm filter membrane.

  **Standard solution:** Prepare a solution containing 0.1 mg/mL of USP Meso-Zeaxanthin RS as follows: dissolve an amount of Reference Standard in an amount of ethanol equal to 50% of the final volume of the solution. Heat in an ultrasonic bath at 60° for 2–5 min to dissolve the Reference Standard. Remove the flask from the bath, cool to room temperature, and dilute with hexane to the desired volume (the volume of
hexane used in the solution should equal the volume of ethanol used to dissolve the standard. Filter the solution through a 0.45-µm filter membrane.

**Chromatographic system**, Appendix II A

**Mode:** High-performance liquid chromatography

**Detector:** 453 nm

**Column:** 4.6-mm × 25-cm column containing amylose tris-3,5-dimethylphenylcarbamate-coated, porous, spherical silica particles, 5-µm in diameter

**Column temperature:** 35°C

**Flow rate:** 0.5 mL/min

**Injection size:** 20 µL

**System suitability**

**Sample:** Standard solution

[Note—The approximate relative retention times for (3S,3'S)-zeaxanthin; (3R,3'S, meso)-zeaxanthin; (3R,3'R)-zeaxanthin; and (3R,3'R,6'R)-lutein are 0.94, 1.00, 1.06, and 1.11, respectively. The chromatogram from the Standard solution should be similar to the Reference Chromatogram provided with the USP Meso-Zeaxanthin RS being used.]

**Suitability requirement:** The resolution between each pair peak due to (3S,3'S)-zeaxanthin, (3R,3'S, meso)-zeaxanthin, (3R,3'R)-zeaxanthin, and (3R,3'R,6'R)-lutein is NLT 1.5.

**Analysis:** Separately inject the Sample solution and the Standard solution into the chromatograph, record the chromatograms, and compare them to the Reference Chromatogram provided with the USP Meso-Zeaxanthin RS being used in order to identify the relevant analyte peaks. Measure the peak areas and calculate the percentage of (3S,3'S)-zeaxanthin, (3R,3'S, meso)-zeaxanthin, and (3R,3'R)-zeaxanthin:

\[ \text{Result} = \left( \frac{r_U}{r_T} \right) \times 100 \]

\( r_U \) = peak area of the analyte of interest
\( r_T \) = total peak area of the chromatogram

**Acceptance criteria**

(3S,3’S)-Zeaxanthin: NMT 1.0%
(3R,3’S, meso)-Zeaxanthin: NLT 85.0%
(3R,3’R)-Zeaxanthin: NMT 15.0%

- 3S (FCC 7)

**ASSAY**

- **ZEA XANTHIN**

[Note—Use low-actinic glassware.]

**Mobile phase:** Hexane and ethyl acetate (75:25); filtered and degassed. Make adjustments if necessary.

**Standard solution:** 150 µg/mL of USP Meso-Zeaxanthin RS prepared as follows: dissolve 15.0 mg of USP Meso-Zeaxanthin RS in 10 mL of chloroform, swirling briefly, and dilute with Mobile phase to 100 mL.

**Sample solution:** Transfer 15.0 mg of sample to a 100-mL volumetric flask, add 10 mL of chloroform, and place the flask in an ultrasonic bath at 30°C for 2–5 min to obtain a clear solution. Dilute with Mobile phase to volume.

**Chromatographic system**, Appendix II A

**Mode:** High-performance liquid chromatography

**Detector:** 453 nm

**Column:** 4.6-mm × 25-cm column that contains 5- to 10-µm porous silica packing
Flow rate: 1.5 mL/min
Injection size: 10 µL

System suitability
Sample: Standard solution

[Note—The approximate relative retention times for zeaxanthin and lutein are about 1.0 and 0.95, respectively.]

Suitability requirement 1: The resolution between zeaxanthin and lutein is NLT 1.0.
Suitability requirement 2: The tailing factor is NMT 2.
Suitability requirement 3: The relative standard deviation for replicate injections is NMT 2.0%.

Analysis: Inject the Sample solution into the chromatograph, record the chromatogram, and measure the peak area responses. [Note—The peak area of zeaxanthin is NLT 90.0% of the total detected area of peaks in the chromatogram.]

Calculate the percentage of zeaxanthin in the sample taken:

\[
\text{Result} = T \times \left( \frac{r_U}{r_T} \right)
\]

\[T = \text{percentage of Total Carotenoids determined below}\]
\[r_U = \text{peak response of zeaxanthin}\]
\[r_T = \text{sum of the responses of all of the peaks}\]

Acceptance criteria: NLT 74.0%

Total Carotenoids

[Note—Use low-actinic glassware.]

Sample stock solution: Transfer 25.0 mg of sample to a 100-mL volumetric flask, add 20 mL of chloroform, and place the flask in an ultrasonic bath at 30°C for 2–5 min to obtain a clear solution. Dilute with cyclohexane to volume to obtain a solution containing 250 µg/mL.

Sample solution: 2.5 µg/mL in cyclohexane; from the Sample stock solution
Blank: Cyclohexane

Analysis: Determine the absorbance of the Sample solution against that of the Blank at the wavelength of maximum absorbance at about 453 nm, with a suitable spectrophotometer.
Calculate the percentage of total carotenoids as zeaxanthin (C_{40}H_{56}O_{2}):

\[
\text{Result} = \frac{A}{(C \times F)}
\]

\[A = \text{absorbance of the Sample solution}\]
\[C = \text{concentration of the Sample solution (g/mL)}\]
\[F = \text{absorptivity of zeaxanthin in cyclohexane (2540 mL·g}^{-1}·\text{cm}^{-1})\]

Acceptance criteria: NLT 80.0%

IMPURITIES

Inorganic Impurities

• Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB

Sample: 10 g

Acceptance criteria: NMT 1 mg/kg

Organic Impurities

• Lutein and Other Related Compounds

[Note—Use low-actinic glassware.]
**Mobile phase:** Hexane and ethyl acetate (75:25); filtered and degassed. Make adjustments if necessary.

**Standard solution:** 150 µg/mL of USP Meso-Zeaxanthin RS prepared as follows: dissolve 15.0 mg of USP Meso-Zeaxanthin RS in 10 mL of chloroform, swirling briefly, and dilute with Mobile phase to 100 mL.

**Sample solution:** Transfer 15.0 mg of sample to a 100-mL volumetric flask, add 10 mL of chloroform, and place the flask in an ultrasonic bath at 30° for 2–5 min to obtain a clear solution. Dilute with Mobile phase to volume.

**Chromatographic system,** Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** 453 nm
- **Column:** 4.6-mm × 25-cm column that contains 5- to 10-µm porous silica packing
- **Flow rate:** 1.5 mL/min
- **Injection size:** 10 µL

**System suitability**

- **Sample:** Standard solution
  
  [Note—The approximate relative retention times for zeaxanthin and lutein are about 1.0 and 0.95, respectively.]

  **Suitability requirement 1:** The resolution between zeaxanthin and lutein is NLT 1.0.
  **Suitability requirement 2:** The tailing factor is NMT 2.
  **Suitability requirement 3:** The relative standard deviation for replicate injections is NMT 2.0%.

**Analysis:** Inject the Sample solution into the chromatograph, record the chromatogram, and measure the peak area responses.  

[Note—The peak area of lutein is NMT 9.0% of the total detected area of peaks in the chromatogram of the Sample solution.]

Calculate the percentage of lutein in the portion of the sample taken:

\[
\text{Result} = T \times \left(\frac{r_U}{r_T}\right)
\]

- \(T\) = percentage of Total Carotenoids determined above
- \(r_U\) = peak response of lutein
- \(r_T\) = sum of the responses of all of the peaks

Calculate the percentage of other related compounds in the sample taken:

\[
\text{Result} = 100 \times \left(\frac{r_O}{r_T}\right)
\]

- \(r_O\) = individual peak response of any other peak in the chromatogram, excluding zeaxanthin and lutein
- \(r_T\) = sum of the responses of all of the peaks

**Acceptance criteria**

- **Lutein:** NMT 8.5%
- **Other related compounds:** NMT 1.0% of any other single related compound

**SPECIFIC TESTS**

- **Residue on Ignition (Sulfated Ash), Appendix IIC**
  
  **Analysis:** Proceed as directed, but igniting at 600 ± 50°.
  **Acceptance criteria:** NMT 1.0%

- **Water, Water Determination, Appendix IIB**
  
  **Acceptance criteria:** NMT 1.0%
1 Chiralpak AD-H from Chiral Technologies, or equivalent.

2 Agilent Zorbax Rx-SIL, or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>

_FCC Seventh Edition Page 1494_

_FCC Seventh Edition Supplement 1 Page 1494_
BRIEFING

Methyl Salicylate, FCC 7 page 675. On the basis of efforts to modernize Identification test methods in FCC, it is proposed to change the existing infrared method in the Identification section to one that utilizes a USP Reference Standard instead of comparison to a printed spectrum. It is also proposed to move the existing printed spectrum for Methyl Salicylate to the Infrared Spectra section of FCC’s General Information section.

(FIEC: J. Moore) C94783

Methyl Salicylate

\[ \text{C}_8\text{H}_8\text{O}_3 \]

Formula wt 152.15
FEMA: 2745

DESCRIPTION

Methyl Salicylate occurs as a colorless to yellow liquid.

Odor: Wintergreen

Solubility: Soluble in alcohol, glacial acetic acid; slightly soluble in water

Boiling Point: \( \sim 222^\circ \text{C} \) (decomp)

Solubility in Alcohol, Appendix VI: One mL dissolves in 7 mL of 70% alcohol, and may be slightly cloudy.

Function: Flavoring agent

IDENTIFICATION

Change to read:

- Infrared Spectra, Spectrophotometric Identification Tests, Appendix III C
  - Reference standard: USP Methyl Salicylate RS
  - Sample and standard preparation: \( F_{\text{3S}} \) (FCC7)
  - Acceptance criteria: The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below. The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard. \( F_{\text{3S}} \) (FCC7)
ASSAY
• Procedure: Proceed as directed under M-1b, Appendix XI.
  Acceptance criteria: NLT 98.0% of C₈H₈O₃

SPECIFIC TESTS
• Acid Value, M-15, Appendix XI
  [Note—Use phenol red TS as the indicator.]
  Acceptance criteria: NMT 1.0

• Refractive Index, Appendix II: At 20°
  Acceptance criteria: Between 1.535 and 1.538

• Specific Gravity: Determine at 25° by any reliable method (see General Provisions).
  Acceptance criteria: Between 1.180 and 1.185

Auxiliary Information—Please check for your question in the FAQs before contacting USP.
BRIEFING

Methylparaben, FCC 7 page 686. Based on FCC's efforts to modernize the Identification and Assay procedures, the following revisions are proposed. These revisions also harmonize this monograph with the monograph for Methylparaben published in Pharmacopeial Forum 36(5) [Nov.–Dec. 2010].

1. Add a test for Infrared Absorption to the Identification section which utilizes comparison to a USP Reference Standard. The existing Identification test is not sufficient for complete identification of this ingredient.
2. Replace the existing titrimetric Assay method with a liquid chromatographic method. The LC method provides improved specificity over the existing titrimetric method.

Comments on both revisions are encouraged.

(FIEC: K. Laurvick) C97088

Methylparaben

Methyl p-Hydroxybenzoate

\[
\text{C}_{8}\text{H}_{8}\text{O}_{3} \text{ Formula wt 152.15}
\]

INS: 218 CAS: [99-76-3]

DESCRIPTION

Methylparaben occurs as small, colorless crystals or as a white, crystalline powder. One g dissolves in about 400 mL of water at 25°, in about 50 mL of water at 80°, in about 2.5 mL of alcohol, in about 7 mL of ether, and in about 4 mL of propylene glycol. It is slightly soluble in glycerin and in fixed oils.

Function: Preservative; antimicrobial agent

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION

Add the following:

- **Infrared Absorption**, Spectrophotometric Identification Tests, Appendix III C

  Reference standard: USP Methylparaben RS

  Sample and standard preparation: M

  Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard. 3S (FCC7)
Sample preparation: Dissolve 500 mg of sample in 10 mL of 1 N sodium hydroxide, boil for 30 min, allow the solution to evaporate to a volume of about 5 mL, and cool. Acidify the solution with 2 N sulfuric acid, collect the resulting p-hydroxybenzoic acid crystals on a filter, wash several times with small portions of water, and dry in a desiccator over silica gel.

Acceptance criteria: The Sample preparation so obtained melts between 212° and 217°.

ASSAY

Change to read:

Sample: 2 g

Analysis: Transfer the Sample into a flask, add 40.0 mL of 1 N sodium hydroxide and rinse the sides of the flask with water. Cover the flask with a watch glass, boil gently for 1 h, cool and titrate the excess sodium hydroxide with 1 N sulfuric acid to pH 6.5. Perform a blank determination (see General Provisions) with the same quantities of the same reagents in the same manner, and make any necessary correction.

Each mL of 1 N sodium hydroxide is equivalent to 152.2 mg of C₈H₈O₃.

Acceptance criteria: NLT 99.0% and NMT 100.5% of C₈H₈O₃, calculated on the dried basis.

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)

Sample solution: Dissolve 50.0 mg in 2.5 mL of methanol, and dilute with Mobile phase to 50 mL. Dilute 10.0 mL of this solution with Mobile phase to 100 mL.

Standard solution: Dissolve 50.0 mg of USP Methylparaben RS in 2.5 mL of methanol, and dilute with Mobile phase to 50 mL. Dilute 10.0 mL of this solution with Mobile phase to 100 mL.

Chromatographic system, Appendix IIA

Mode: High-performance liquid chromatography

Detector: UV 272 nm

Column: 4.6-mm × 15-cm column packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles 5-µm in diameter

Flow rate: 1.3 mL/min

[Note—The run time is about 5 times the retention time of methylparaben.]

Injection size: 10 µL

System suitability

Sample: Standard solution

Suitability requirement: The relative standard deviation for 6 injections is NMT 0.85%.

Analysis: Separately inject the Sample solution and the Standard solution into the chromatograph. Record the chromatograms and calculate the percentage of methylparaben in the sample taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100 \]

\( r_U \) = peak area of methylparaben from the chromatogram of the Sample solution

\( r_S \) = peak area of methylparaben from the chromatogram of the Standard solution

\( C_S \) = concentration of USP Methylparaben RS in the Standard solution, corrected for purity (mg/mL)

\( C_U \) = concentration of the Sample solution (mg/mL)

Acceptance criteria: 99.0%–100.5%, calculated on the dried basis.
IMPURITIES
Inorganic Impurities
• Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB
  Sample: 10 g
  Acceptance criteria: NMT 2 mg/kg

SPECIFIC TESTS
• Acidity
  Sample: 750 mg
  Analysis: Mix the Sample with 15 mL of water, heat at 80° for 1 min, cool, and filter. The filtrate is acid or neutral to litmus. Add 0.2 mL of 0.1 N sodium hydroxide and 2 drops of methyl red TS to 10 mL of the filtrate.
  Acceptance criteria: The solution is yellow, without even a light cast of pink.
• Loss on Drying, Appendix IIC: Over silica gel for 5 h
  Acceptance criteria: NMT 0.5%
• Melting Range or Temperature, Appendix IIB
  Acceptance criteria: Between 125° and 128°
• Residue on Ignition (Sulfated Ash), Appendix IIC
  Sample: 4 g
  Acceptance criteria: NMT 0.05%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 686
BRIEFING

**Monk Fruit Extract.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received.

1. The Assay was developed using a validated literature method along with a method and data submitted by the sponsor. System suitability information was not included. Comments and data are requested specifically regarding appropriate system suitability parameters.

2. This monograph does not provide information regarding the separation, detection, or quantitation of other mogroside components of monk fruit besides mogroside V. Comments and data are requested specifically regarding whether or not this monograph should contain requirements relating to the content of other mogrosides or naturally occurring components of this ingredient. Commenters are encouraged to submit methods and data relating to mogrosides limits that may be appropriate for future addition to this monograph.

3. Ethanol is identified as an extraction solvent used in the manufacture of this ingredient, but no test or limit for ethanol is being included in this monograph. It seems unlikely that residual ethanol could remain in the material after heating under vacuum and spray drying of the extract. Comments regarding the lack of an ethanol limit are requested.

(FIEC: K. Laurvick) C95452

---

**Add the following:**

- Monk Fruit Extract
- Luo Han Fruit Concentrate
- Luo Han Guo Concentrate
- Luo Han Guo Extract
- Monk Fruit Concentrate
- *Siraitia grosvenorii* Extract

- \( C_{60}H_{102}O_{29} \) (Mogroside V)

- Formula wt, Mogroside V 1286
- CAS: Mogroside V [88901-36-4]
DESCRIPTION
Monk Fruit Extract occurs as an off-white to light yellow powder. It is an extract of the fruit luo han guo (Siraitia grosvenorii Swingle, also known as monk fruit) that has been concentrated to optimize the concentration of mogroside V. It is obtained through water extraction of the mechanically crushed or shredded pulp of the fruit. Precipitate is removed by decanting, and the supernatant is cooled and passed through a food-grade copolymer resin which binds the target compounds. The resin is flushed with cold ethanol to release the extracted compounds, and the effluent is heated under vacuum to remove the ethanol, then spray dried. Monk Fruit Extract is composed primarily of cucurbitane glycosides, known as mogrosides, with mogroside V being the principal sweetening component. Other components are mogroside II, mogroside III, mogroside IV, mogroside VI, flavonoids, melanoidins, and protein fragments. Monk Fruit Extract is freely soluble in water.

Function: Non-nutritive sweetener

Packaging and Storage: Store in tight, light-resistant containers. Avoid exposure to excessive heat.

IDENTIFICATION

- Thin-Layer Chromatography, Appendix IIA
  
  **Standard solution A:** 7.5 mg/mL of USP Mogroside V RS in methanol. Sonicate to aid dissolution.
  
  **Standard solution B:** Prepare a solution by dissolving 50 mg of USP Monk Fruit Extract RS in 20 mL of water. Extract the aqueous solution twice, using 10 mL of n-butanol each time, and combine the n-butanol extracts. Evaporate the combined n-butanol extract at room temperature, and dissolve the residue in 2 mL of methanol.
  
  **Sample solution:** Prepare a solution by dissolving 50 mg of the sample in 20 mL of water. Extract the aqueous solution twice, using 10 mL of n-butanol each time, and combine the n-butanol extracts. Evaporate the combined n-butanol extract at room temperature, and dissolve the residue in 2 mL of methanol.
  
  **Adsorbent:** 0.25-mm layer of chromatographic silica gel, prepared with a gypsum (calcium sulfate hemihydrate) binder
  
  **Developing solvent system:** n-Butanol, acetic acid, and water (4:1:1)
  
  **Spray reagent:** 10% sulfuric acid solution
  
  **Application volume:** 2 µL
  
  **Analysis:** After developing the plate in the Developing solvent system, remove the plate from the developing chamber, and allow the solvent to evaporate. Spray the plate with the Spray reagent, and heat at 105°C to allow color development.
  
  **Acceptance criteria:** The principal spot obtained from the Sample solution corresponds in color and Rf value to that obtained from Standard solution A. The chromatogram obtained from the Sample solution corresponds to that obtained from Standard solution B in the placement and color of the spots.

ASSAY

- Mogroside V Content
  
  **Mobile phase:** Acetonitrile and water (22:78)
  
  **Standard solution:** 0.10 mg/mL of USP Mogroside V RS in Mobile phase
  
  **Sample solution:** Dissolve 30 mg of the sample in Mobile phase in a 50-mL volumetric flask. Sonicate the solution for 40 min, cool to room temperature, and dilute with Mobile phase to volume. Filter the solution through a 0.45-µm membrane.
  
  **Chromatographic system,** Appendix IIA
  
  **Mode:** High-performance liquid chromatography
Detector: UV 203 nm

Column: 4.6-mm × 250-mm column that contains 5-µm porous silica microparticles chemically bonded to octadecylsilane

Column temperature: 25°C
Flow rate: 1.0 mL/min
Injection size: 20 µL

Analysis: Separately inject the Standard solution and the Sample solution into the chromatograph, record the chromatograms, and measure the areas for the major peaks in the respective chromatograms. [Note —The approximate retention time for mogroside V is 15.7 min.] Using the peak area obtained from the chromatogram of the Standard solution, determine the percent of mogroside V in the Sample solution:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\[r_U = \text{peak area response for mogroside V obtained from the chromatogram of the Sample solution}\]
\[r_S = \text{peak area response for mogroside V obtained from the chromatogram of the Standard solution}\]
\[C_S = \text{concentration of mogroside V in the Standard solution (mg/mL)}\]
\[C_U = \text{concentration of the Sample solution (mg/mL)}\]

Acceptance criteria: NLT 30.0%

IMPURITIES

Inorganic Impurities
- **Arsenic**, *Elemental Impurities by ICP*, Appendix III C
  Acceptance criteria: NMT 0.5 mg/kg
- **Cadmium**, *Elemental Impurities by ICP*, Appendix III C
  Acceptance criteria: NMT 1.0 mg/kg
- **Lead**, *Elemental Impurities by ICP*, Appendix III C
  Acceptance criteria: NMT 1.0 mg/kg

SPECIFIC TESTS
- **Ash (Total)**, Appendix II C
  Sample: 2 g
  Analysis: Proceed as directed, holding the furnace at 600°C for 2 h.
  Acceptance criteria: NMT 5.0%
- **Loss on Drying**, Appendix II C
  Acceptance criteria: NMT 6.0%

3S (FCC7)

1 ZORBAX SB-C18 (Agilent Technologies); Symmetry Shield RP 18 (Waters Corporation); or equivalent. Use a compatible guard column (C18, 5-µm, 4.6 mm × 7.5 mm).

Auxiliary Information— Please check for your question in the FAQs before contacting USP.
**BRIEFING**

**Potassium Nitrate, FCC 7 page 854.** On the basis of comments and data received, a revision to the Assay is proposed to replace the existing Kjeldahl nitrogen method with a titrimetric procedure appearing in FCC 3. Users reported difficulties and inherent sources of error in the existing Assay method.

(FIEC: K. Laurick) C97343

---

**Potassium Nitrate**

KNO$_3$

Formula wt 101.10

INS: 252

CAS: [7757-79-1]

---

**DESCRIPTION**

Potassium Nitrate occurs as colorless, transparent prisms, as white granules, or as a white, crystalline powder. It is slightly hygroscopic in moist air. One g dissolves in 3 mL of water at 25$^\circ$, in 0.5 mL of boiling water, and in about 620 mL of alcohol. Its solutions are neutral to litmus.

**Function:** Antimicrobial agent; preservative

**Packaging and Storage:** Store in tight containers.

---

**IDENTIFICATION**

- **Nitrate**, Appendix IIIA
  - **Sample solution:** 100 mg/mL
  - **Acceptance criteria:** Passes tests

- **Potassium, Appendix IIIA**
  - **Sample solution:** 100 mg/mL
  - **Acceptance criteria:** Passes tests

---

**ASSAY**

**Change to read:**

- **Procedure**
  - **Sample:** 0.4 g, previously dried at 105$^\circ$ for 4 h
  - **Analysis:** Dissolve the Sample in 300 mL of water contained in a 500 mL round-bottom flask. Add 3 g of a powder of Deverda’s alloy and 15 mL of a 40% sodium hydroxide solution, and connect with a spray-preventing device and condenser to the flask. Allow to stand for 2 h. Transfer 50 mL of 0.1 N sulfuric acid into a receptacle; use this to collect 250 mL of the distillate. Titrate the excess sulfuric acid with 0.1 N sodium hydroxide, using 3 drops of methyl red–methylene blue TS as the indicator. Perform a blank determination (see General Provisions) and make any necessary correction. Each mL of 0.1 N sulfuric acid is equivalent to 10.11 mg of KNO$_3$. Weigh the Sample into a 250-mL iodine flask. Add 10 mL of hydrochloric acid to dissolve the Sample, then evaporate to dryness on a steam bath. Dissolve the residue in 10 mL of hydrochloric acid, and again evaporate to dryness. After evaporating to dryness the second time, heat the flask on a hot plate at a medium to high setting for 1 h. Allow the flask to cool to room temperature, then dissolve the residue in 25 mL of water. Add, separately, to the flask 50.0 mL of 0.1 N silver nitrate, 3 mL of nitric acid, and 10 mL of nitrobenzene, then stopper the flask and shake it vigorously.

[CAUTION—Nitrobenzene is highly flammable and is a poison which can cause cyanosis. Handle with
caution and avoid inhaling or contact with skin. Sonicate the contents of the flask for 5 min. Remove the flask from the sonicator and add 3 mL of ferric ammonium sulfate TS as the indicator. Titrate the excess silver nitrate in the solution with 0.1 N ammonium thiocyanate to the first appearance of a reddish-brown color. Calculate the percentage of potassium nitrate in the sample taken:

\[
\text{Result} = [(V \times N) - (V_T \times N_T)] \times (F/W)
\]

- \(V\) = volume of 0.1 N silver nitrate added to the flask (50 mL)
- \(N\) = exact normality of the 0.1 N silver nitrate used (mol/L)
- \(V_T\) = volume of 0.1 N ammonium thiocyanate used to titrate the solution (mL)
- \(N_T\) = exact normality of the 0.1 N ammonium thiocyanate used (mol/L)
- \(F\) = factor representing the amount of potassium nitrate, in mg, equivalent to 1 mL of 0.1 N silver nitrate (10.11)
- \(W\) = quantity of the Sample (g)

Acceptance criteria: NLT 99.0% and NMT 100.5% KNO₃, on the dried basis

IMPURITIES
Inorganic Impurities
- CHLORATE
  Sample: 100 mg
  Analysis: Sprinkle the dried Sample on 1 mL of sulfuric acid.
  Acceptance criteria: The mixture does not turn yellow.
- LEAD, Lead Limit Test, Appendix IIIB
  Sample solution: 100 mg/mL made to 10 mL
  Control: 4 µg Pb (4 mL of the Diluted Standard Lead Solution)
  Acceptance criteria: NMT 4 mg/kg

SPECIFIC TESTS
- LOSS ON DRYING, Appendix IIC: 105°C for 4 h
  Acceptance criteria: NMT 1%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 854
1,3-Propanediol. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed. A method to determine the biobased content of this ingredient is also proposed as an optional method for interested users (See Briefing under Appendix XIV: Markers for Authenticity Testing). (FIEC: J. Moore) C86302

Add the following:

- 1,3-Propanediol
- 1,3-Dihydroxypropane
- Propane, 1-3-diol
- Trimethylene Glycol

\[ \text{C}_3\text{H}_8\text{O}_2 \]

Formula wt 76.09
CAS: [504-63-2]

DESCRIPTION
1,3-Propanediol is a clear, colorless, hygroscopic liquid with a mild, sweet odor. It is produced from corn-derived glucose in a multi-step fermentation process using a non-pathogenic strain of Escherichia coli K-12 as a biocatalyst. Principle steps include fermentation, separation, and distillation. It is soluble in water, alcohols, and acetone, and miscible with many polar solvents. 1,3-Propanediol produced from a biobased carbon source (modern carbon source such as plant-derived glucose) can be distinguished from 1,3-propanediol produced from fossil carbon sources (such as fossil fuel) using carbon isotope analysis.

[Note—An informational (not a monograph requirement) carbon isotope analysis method is available to determine the biobased content of 1,3-propanediol, see Biobased Content of 1,3-Propanediol, Markers for Authenticity Testing, Appendix XIV.]

Function: Solvent; wetting agent; humectant

Packaging and storage: Store in tight containers.

IDENTIFICATION
- **A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix III C**
  - Reference standard: USP 1,3-Propanediol RS
  - Sample and Standard preparation: F
  - Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

- **B. PROEDURE**
  - Acceptance criteria: The retention time of the major peak in the chromatogram of the Sample solution corresponds to the 1,3-propanediol peak in the chromatogram of the System suitability solution.
ASSAY

• Procedure

System suitability solution: Mix quantities of USP Propylene Glycol RS and USP 1,3-Propanediol RS to obtain about a 5% propylene glycol/95% propanediol solution.

Sample solution: Neat

Chromatographic system, Appendix II A

Mode: Gas chromatography

Detector: Flame ionization

Column: 0.25-mm × 30-m; bonded with a 0.25-µm layer of polyethylene glycol compound

Carrier gas: Helium

Temperature

Inlet: 250 °C

Detector: 250 °C

Column: See the temperature program table below.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Rate (°/min)</th>
<th>Hold Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50→200</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>200→250</td>
<td>40</td>
<td>17</td>
</tr>
</tbody>
</table>

Split ratio: 18:1

Flow rate: About 1.0 mL/min

Injection size: 0.2 µL

System suitability

Sample: System suitability solution

Suitability requirement: The resolution between 1,3-propanediol and propylene glycol is NLT 1.5

Analysis: Inject the Sample solution into the chromatograph, record the chromatogram, and measure all peak areas. Calculate the percent 1,3-propanediol in the portion of the sample taken:

\[ \text{Result} = \left( \frac{A}{B} \right) \times 100 \]

A = response of 1,3-propanediol

B = sum of the responses of all the peaks

Acceptance criteria: NLT 99.9%

IMPURITIES

Inorganic Impurities

• Cobalt, Elemental Impurities by ICP, Appendix II C

Acceptance criteria: NMT 1.0 mg/kg

• Lead, Elemental Impurities by ICP, Appendix II C

Acceptance criteria: NMT 1.0 mg/kg

• Nickel, Elemental Impurities by ICP, Appendix II C

Acceptance criteria: NMT 1.0 mg/kg

SPECIFIC TESTS

• Specific Gravity: Determine by any reliable method (see General Provisions).

Acceptance criteria: 1.040–1.065

• Water, Water Determination, Method I, Appendix II B

Acceptance criteria: NMT 0.1%
OTHER REQUIREMENTS

- **Labeling:** 1,3-Propanediol may be labeled as “biobased”, indicating that it was produced using a plant derived carbon source. The biobased content of 1,3-propanediol can be determined using carbon isotope analysis, see *Biobased Content of 1,3-Propanediol, Markers for Authenticity Testing*, Appendix XIV.

1 ZB Wax (Phenomenex, Torrance, CA), or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D.</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8288</td>
<td></td>
</tr>
</tbody>
</table>
**BRIEFING**

**Propylparaben** , *FCC 7* page 877. On the basis of efforts to modernize *Identification* and *Assay* procedures, the following revisions are proposed. These revisions also harmonize this monograph with the *Propylparaben* monograph in *Pharmacopeial Forum* 36(5) [Nov. – Dec. 2010].

1. Add an *Infrared Absorption* test to the *Identification* section which utilizes comparison to a USP Reference Standard. The existing *Identification* test is not sufficient for complete identification of this ingredient.

2. Replace the existing titrimetric *Assay* method with a liquid chromatographic method. The LC method provides improved specificity over the existing titrimetric method.

Comments on both revisions are encouraged.

(FIEC: K. Laurvick) C97087

---

**Propylparaben**

Propyl $p$-Hydroxybenzoate

![Propylparaben structure](image)

$\text{C}_{10}\text{H}_{12}\text{O}_3$  

Formula wt 180.20

INS: 216

CAS: [94-13-3]

**DESCRIPTION**

Propylparaben occurs as small, colorless crystals or as a white powder. One g dissolves in about 2500 mL of water at 25°, in about 400 mL of boiling water, in about 1.5 mL of alcohol, and in about 3 mL of ether.

**Function:** Preservative; antimicrobial agent

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

*Add the following:*

- **Infrared Absorption**, Spectrophotometric Identification Tests, Appendix III C  
  **Reference standard:** USP Propylparaben RS  
  **Sample and standard preparation:** $M$  
  **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard. $3S$ (*FCC7*)

- **Melting Range or Temperature**, Appendix IIB  
  **Sample preparation:** Dissolve 500 mg of sample in 10 mL of 1 N sodium hydroxide, boil for 30 min, allow the solution to evaporate to a volume of about 5 mL, and cool. Acidify the solution with 2 N sulfuric acid,
collect the resulting, liberated \( p \)-hydroxybenzoic acid crystals on a filter, wash several times with small portions of water, and dry in a desiccator over silica gel.

**Acceptance criteria:** The \( p \)-hydroxybenzoic acid thus obtained melts between 212° and 217°.

## ASSAY

**Change to read:**

### Procedure

- **Sample:** 2 g

**Analysis:** Transfer the Sample into a flask, add 40.0 mL of 1 N sodium hydroxide, and rinse the sides of the flask with water. Cover with a watch glass, boil gently for 1 h, and cool. Titrate the excess sodium hydroxide with 1 N sulfuric acid to pH 6.5. Perform a blank determination (see General Provisions) and make any necessary correction. Each mL of 1 N sodium hydroxide is equivalent to 180.2 mg of \( C_{10}H_{12}O_3 \).

**Acceptance criteria:** NLT 99.0% and NMT 100.5% of \( C_{10}H_{12}O_3 \), calculated on the dried basis

- **Mobile phase:** Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)

- **Standard solution:** Dissolve 50.0 mg of USP Propylparaben RS in 2.5 mL of methanol, and dilute with Mobile phase to 50 mL. Dilute 10.0 mL of this solution with Mobile phase to 100 mL.

- **Sample solution:** Dissolve 50.0 mg in 2.5 mL of methanol, and dilute with Mobile phase to 50 mL. Dilute 10.0 mL of this solution with Mobile phase to 100 mL.

### Chromatographic system, Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** UV 272 nm
- **Column:** 4.6-mm \( \times \) 15-cm column packed with octadecyl silane chemically bonded to porous silica or ceramic microparticles 5-µm in diameter
- **Flow rate:** 1.3 mL/min
  
  [Note—The run time is about 2.5 times the retention time of propylparaben.]

- **Injection size:** 10 µL

### System suitability

- **Sample:** Standard solution
- **Suitability requirement:** The relative standard deviation for 6 injections is NMT 0.85%.

**Analysis:** Separately inject the Sample solution and the Standard solution into the chromatograph. Record the chromatograms and calculate the percentage of propylparaben in the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \( r_U \) = peak area of propylparaben from the chromatogram of the Sample solution
- \( r_S \) = peak area of propylparaben from the chromatogram of the Standard solution
- \( C_S \) = concentration of USP Propylparaben RS in the Standard solution, corrected for purity (mg/mL)
- \( C_U \) = concentration of the Sample solution (mg/mL)

**Acceptance criteria:** 99.0%–100.5%, calculated on the dried basis

## IMPURITIES

### Inorganic Impurities
• **LEAD, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**  
  **Sample**: 10 g  
  **Acceptance criteria**: NMT 2 mg/kg

**SPECIFIC TESTS**

• **ACIDITY**  
  **Sample**: 750 mg  
  **Analysis**: Mix the **Sample** with 15 mL of water, heat at 80° for 1 min, cool, and filter. The filtrate is acid or neutral to litmus. Add 0.2 mL of 0.1 N sodium hydroxide and 2 drops of methyl red TS to 10 mL of the filtrate.  
  **Acceptance criteria**: The resulting solution is yellow, without even a light cast of pink.

• **LOSS ON DRYING, Appendix IIC: Over silica gel for 5 h**  
  **Acceptance criteria**: NMT 0.5%

• **MELTING RANGE OR TEMPERATURE, Appendix IIB**  
  **Acceptance criteria**: Between 95° and 98°

• **RESIDUE ON IGNITION (SULFATED ASH), Appendix IIC**  
  **Sample**: 4 g  
  **Acceptance criteria**: NMT 0.05%

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td>1-301-816-8356</td>
</tr>
</tbody>
</table>
BRIEFING

Sodium Chloride, FCC 7 page 929. On the basis of comments received, the following revisions are proposed.

1. It is proposed to include calcium and potassium ferrocyanide as anticaking agents added alone or in combination with sodium ferrocyanide. This is consistent with the Food Additive provisions for ferrocyanides of the Codex Alimentarius General Standard for Food Additives, Stan 192 1995, adopted in 2006 (FAO/WHO). The level of anticaking agents in dendritic salt is also consistent with the Codex Alimentarius. Comments and data supporting the limit are requested.

2. The test for Sodium Ferrocyanide was also modified to accommodate the proposed levels of anticaking agents. Comments and data are requested related to the Note that suggests the substitution of decahydrate ferrocyanide by anhydrous ferrocyanide.

Comments and data are also encouraged if they are related to the addition of green ferric ammonium citrate as a crystal-modifying and anticaking agent.

(FIEC: C. Mejia) C95752

Sodium Chloride

Salt

NaCl Formula wt 58.44
CAS: [7647-14-5]

DESCRIPTION

Change to read:

Sodium Chloride occurs as a transparent to opaque, white crystalline solid of variable particle size. Salt is a generic term applied to commercially produced Sodium Chloride. It is available in various crystalline forms, referred to as evaporated salt, rock salt (may be white to off-white), solar salt, or simply salt. It may contain up to 2% (total) of suitable food-grade anticaking, free-flowing, or conditioning agents, either singly or in combination. It may contain not more than 13 mg/kg of sodium ferrocyanide, or not more than 25 mg/kg of green ferric ammonium citrate as crystal-modifying and anticaking agents.\[dendritic salt, evaporated salt, rock salt (may be white to off-white), solar salt, or simply salt. It may contain up to 2% (total) of suitable food-grade anticaking, free-flowing, or conditioning agents, either singly or in combination. It may contain not more than 14 mg/kg of calcium, potassium, or sodium ferrocyanide alone or in combination, expressed as anhydrous sodium ferrocyanide, or not more than 25 mg/kg of green ferric ammonium citrate as a crystal-modifying and anticaking agent. Food-grade dendritic salt can contain NMT 29 mg/kg of calcium, potassium, or sodium ferrocyanide, expressed as anhydrous sodium ferrocyanide. If labeled as iodized, it contains not less than 0.006% and not more than 0.010% of potassium iodide. Sodium Chloride remains dry in air at a relative humidity below 75%, but becomes deliquescent at higher humidity. One g is soluble in 2.8 mL of water at 25°, in 2.7 mL of boiling water, and in about 10 mL of glycerin. Sodium Chloride containing water-insoluble anticaking, free-flowing, and conditioning agents may produce cloudy solutions or may dissolve incompletely. A 1:20 aqueous solution usually has a pH between 5.5 and 8.5 (the pH may be higher if alkaline conditioning agents have been added).

Function: Nutrient; preservative; flavoring agent and intensifier; curing agent; dough conditioner
Packaging and Storage: Store in well-closed containers.

IDENTIFICATION
- **SODIUM**, Appendix IIIA
  Acceptance criteria: Passes tests
- **CHLORIDE**, Appendix IIIA
  Acceptance criteria: Passes test
  [Note—In the following procedures under Assay, Impurities, and Specific Tests, it may be necessary to filter the sample solutions to avoid interference from insoluble or suspended anticaking, free-flowing, or conditioning agents.]

ASSAY

Change to read:
- **Procedure**
  Sample: 250 mg, previously dried at 625°C for 2 h
  Analysis: Dissolve the Sample in 50 mL of water in a glass-stoppered flask. Add, while agitating, 3 mL of nitric acid, 5 mL of nitrobenzene, 50.0 mL of 0.1 N silver nitrate, and 2 mL of ferric ammonium sulfate TS. Shake well, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of NaCl.
  Acceptance criteria
  Evaporated salt with up to 2% of suitable free-flowing or conditioning agents and anticaking agents such as calcium, potassium, or sodium ferrocyanide: 97.5%–100.5% of NaCl, on the dried basis
  Evaporated salt with only anticaking agents such as calcium, potassium, or sodium ferrocyanide: 99.0%–100.5%, on the dried basis
  Rock or solar salt: 97.5%–100.5% of NaCl, on the dried basis, the remainder consisting chiefly of minor amounts of naturally occurring components such as alkaline and/or alkaline earth sulfates and chlorides

IMPURITIES

Change to read:
Inorganic Impurities
- **ARSENIC**, Arsenic Limit Test, Appendix III B
  Sample solution: 3 g in 25 mL of water
  Acceptance criteria: NMT 1 mg/kg
- **CALCIUM AND MAGNESIUM**
  Standard EDTA solution: 4.0 mg/mL of disodium EDTA (C\textsubscript{10}H\textsubscript{14}N\textsubscript{2}Na\textsubscript{2}O\textsubscript{6}·2H\textsubscript{2}O)
  Magnesium sulfate solution: 2.6 mg/mL of magnesium sulfate (MgSO\textsubscript{4}·7H\textsubscript{2}O)
  Buffer solution A: Transfer 67.5 mg of ammonium chloride into a 1000-mL volumetric flask, and dissolve in 570 mL of ammonium hydroxide. Use 2 mL of this solution as directed under Titer determination.
  Buffer solution B: Pipet 50.0 mL of Magnesium sulfate solution into the Buffer solution A flask, add exactly the volume, T, in mL, of Standard EDTA solution, determined as directed under Titer determination, then dilute with water to volume, and mix.
  Titer determination (T): Pipet 50 mL of Magnesium sulfate solution into a 400-mL beaker, add 200 mL of water, 2 mL of Buffer solution A, 1.0 mL of 1:20 potassium cyanide solution, and 5 drops of eriochrome
black TS or another suitable indicator. While stirring with a magnetic stirrer, titrate with the Standard EDTA solution to a true blue endpoint. Record the volume, T, in mL, of Standard EDTA solution equivalent to 50.0 mL of Magnesium sulfate solution.

**Standardization of EDTA solution:** Transfer 1 g of primary standard calcium carbonate (CaCO₃) into a 1000-mL volumetric flask, dissolve in 800 mL of water containing 5 mL of hydrochloric acid, dilute with water to volume, and mix. Pipet 25.0 mL of this solution into a 400-mL beaker, and add 200 mL of water, 2 mL of Buffer solution B, 1.0 mL of 1:20 potassium cyanide solution, and 20 drops of eriochrome black TS or another suitable indicator. While stirring with a magnetic stirrer, titrate with the Standard EDTA solution to a true blue endpoint.

Calculate the factor, F, giving the number of mg of calcium (Ca) equivalent to 1.0 mL of Standard EDTA solution:

Result = 10.011 × w/v

w = exact weight of the primary standard calcium carbonate taken (g)

v = volume of Standard EDTA solution required in the titration (mL)

**Sample solution**

**Rock and solar salts:** Transfer 50.0 g of sample into a 500-mL volumetric flask, dissolve in 400 mL of water containing 2 mL of hydrochloric acid, dilute to volume, and mix. Filter a 50-mL aliquot, pipet 10.0 mL of the filtrate into a 400-mL beaker, and add 190 mL of water.

**Evaporated salt:** Transfer 10.0 g of sample into a 400-mL beaker, and dissolve in 100 mL of water. If free-flowing agents are present, filter and rinse quantitatively. Dilute the solution or filtrate with water to 200 mL.

**Analysis:** Add 5.0 mL of Buffer solution B, 1 mL of a 1:20 potassium cyanide solution, and 5 drops of eriochrome black TS or another suitable indicator to the Sample solution. Begin stirring with a magnetic stirrer, and titrate with Standard EDTA solution to a true blue endpoint, recording the volume, in mL, required as V. Calculate the mg/kg of total calcium and magnesium (both expressed as Ca) in the sample:

Result = V × F × 1000/W

V = volume of Standard EDTA solution required for the titration (mL)

F = standardization factor determined above

W = weight of sample in the final solution titrated (g)

**Acceptance criteria**

**Salt other than evaporated salt with only anticaking agents such as calcium, potassium, or sodium ferrocyanide:** NMT 0.9%

**Evaporated salt with only anticaking agents such as calcium, potassium, or sodium ferrocyanide:** NMT 0.35%

**HEAVY METALS (as Pb)**

[Note—This test is designed to limit the content of common metallic impurities colored by sulfide ion (Ag, As, Bi, Cd, Cu, Hg, Pb, Sb, Sn) by comparing the color with a standard containing lead (Pb) ion under the specified test conditions. It demonstrates that the test substance is not grossly contaminated by such heavy metals, and within the precision of the test, that it does not exceed the Heavy Metals limit given as determined by concomitant visual comparison with a control solution. In the specified pH range, the optimum concentration of lead (Pb) ion for matching purposes by this method is 20 µg in 50 mL of solution. The most common limitation of the Heavy Metals test is that the color the sulfide ion produces in the...}
Sample solution depends on the metals present and may not match the color in the dilution of the Standard lead solution used for matching purposes. Lead sulfide is brown, as are Ag, Bi, Cu, Hg, and Sn sulfides. While it is possible that ions not mentioned here may also yield nonmatching colors, among the nine common metallic impurities listed above, the sulfides with different colors are those of As and Cd, which are yellow, and that of Sb, which is orange. If a yellow or orange color is observed, the following action is indicated: because this monograph includes an arsenic requirement, any As found should not exceed 1 mg/kg. If these criteria are met, Cd may be a contributor to the yellow color, so the Cd content should be determined. If an orange color is observed, the Sb content should be determined. These additional tests are in accord with the section on Trace Impurities in the General Provisions of this book, as follows: "if other possible impurities may be present, additional tests may be required, and should be applied, as necessary, by the manufacturer, vendor, or user to demonstrate that the substance is suitable for its intended application."

Determine the amount of heavy metals by Method I or Method II as the following criteria specify: Use Method I for samples that yield clear, colorless solutions before adding sulfide ion. Use Method II for samples that do not yield clear, colorless solutions under the test conditions specified for Method I. Use Method III, a wet digestion method, only in those cases where neither Method I nor Method II can be used.

**Lead nitrate stock solution:** Dissolve 159.8 mg of Reagent-Grade ACS Lead Nitrate [Pb(NO₃)₂] in 100 mL of water containing 1 mL of nitric acid, dilute to 1000.0 mL, and mix. [Note—Prepare and store this solution in glass containers that are free from lead salts.]

**Standard lead solution:** Dilute 10.0 mL of Lead nitrate stock solution with water to 100.0 mL. [Note —Prepare on the day of use.] Each mL is equivalent to 10 µg of lead (Pb) ion.

Note—In the following tests, failure to accurately adjust the pH of the solution within the specified limits may result in a significant loss of test sensitivity.

- **Method I**
  - **Sample solution:** 10 g in 35 mL of water
  - **Solution A:** Pipet 2.0 mL of Standard lead solution (20 µg of Pb) into a 50-mL color-comparison tube, and add water to make 25 mL. Adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper) by adding 1 N acetic acid or 6 N ammonia, dilute with water to 40 mL, and mix.
  - **Solution B:** Transfer 25 mL of the Sample solution into a 50-mL color-comparison tube that matches the one used for Solution A, adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper) by adding 1 N acetic acid or 6 N ammonia, dilute with water to 40 mL, and mix.
  - **Solution C:** Transfer 25 mL of the Sample solution into a third color-comparison tube that matches those used for Solutions A and B, and add 2.0 mL of Standard lead solution. Adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper) by adding 1 N acetic acid or 6 N ammonia, dilute with water to 40 mL, and mix.
  - **Analysis:** Add 10 mL of freshly prepared hydrogen sulfide TS to each tube, mix, allow to stand for 5 min, and view downward over a white surface.
  - **Acceptance criteria:** The color of Solution B is not darker than that of Solution A, and the intensity of the color of Solution C is equal to or greater than that of Solution A. (NMT 2 mg/kg). [Note—If the color of Solution C is lighter than that of Solution A, the sample is interfering with the test procedure and Method II must be used.]

- **Method II**
  - **Solution A:** Prepare as directed in Method I.
  - **Solution B:** Place a quantity of sample into a suitable crucible, add sufficient sulfuric acid to wet the sample, and carefully ignite at a low temperature until thoroughly charred, covering the crucible loosely.
with a suitable lid during the ignition. After the sample is thoroughly carbonized, add 2 mL of nitric acid and 5 drops of sulfuric acid, cautiously heat until white fumes no longer evolve, then ignite, preferably in a muffle furnace, at 500° to 600° until all of the carbon is burned off. Cool, add 4 mL of 1:2 hydrochloric acid, cover, and digest on a steam bath for 10 to 15 min. Uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 min. Add 6 N ammonia dropwise until the solution is just alkaline to litmus paper, dilute with water to 25 mL, and adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper) by adding 1 N acetic acid. Filter if necessary, rinse the crucible and the filter with 10 mL of water, transfer the solution and rinsings into a 50-mL color-comparison tube, dilute with water to 40 mL, and mix.

**Analysis:** Add 10 mL of freshly prepared hydrogen sulfide TS to each tube, mix, allow to stand for 5 min, and view downward over a white surface.

**Acceptance criteria:** The color of Solution B is not darker than that of Solution A. (NMT 2 mg/kg)

**Method III**

**Sample:** 4 mg

**Solution A:** Transfer an 8:10 (v/v) mixture of sulfuric acid and nitric acid into a 100-mL Kjeldahl flask, clamp the flask at an angle of 45°, and then add, in small increments, an additional volume of nitric acid equal to that added in the preparation of Solution B. Heat the solution to dense, white fumes, cool, and cautiously add 10 mL of water. Add a volume of 30% hydrogen peroxide equal to that added in the preparation of Solution B, then boil gently to dense, white fumes, and cool. Cautiously add 5 mL of water, mix, and boil gently to dense, white fumes. Continue boiling until the volume is reduced to about 2 or 3 mL, then cool, and dilute cautiously with a few mL of water. Pipet 2.0 mL of Standard lead solution into this solution, and mix. Transfer the solution into a 50-mL color-comparison tube, rinse the flask with water, add the rinsings to the tube until the volume is 25 mL, and mix. Adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper), initially with ammonium hydroxide and then with 6 N ammonia as the desired range is neared, dilute with water to 40 mL, and mix.

**Solution B:** Transfer the Sample into a 100-mL Kjeldahl flask (or into a 300-mL flask if the reaction foams excessively), clamp the flask at an angle of 45°, and add a sufficient amount of an 8:10 (v/v) mixture of sulfuric acid:nitric acid to moisten the sample thoroughly. Warm gently until the reaction begins, allow the reaction to subside, and then add additional portions of the acid mixture, heating after each addition, until all of the 18 mL of acid mixture has been added. Increase the heat, and boil gently until the reaction mixture darkens. Remove the flask from the heat, add 2 mL of nitric acid, and heat to boiling again. Continue the intermittent heating and addition of 2-mL portions of nitric acid until no further darkening occurs, then heat strongly to dense, white fumes, and cool. Cautiously add 5 mL of water, mix, boil gently to dense, white fumes, and continue heating until the volume is reduced to about 2 or 3 mL. Cool, cautiously add 5 mL of water, and examine. If the solution is yellow, cautiously add 1 mL of 30% hydrogen peroxide, and again evaporate to dense, white fumes and to a volume of about 2 or 3 mL. Cool, dilute cautiously with a few mL of water, and mix. Transfer into a 50-mL color-comparison tube, rinse the flask with water, add the rinsings to the tube until the volume is 25 mL, and mix. Adjust the pH to between 3.0 and 4.0 (using short-range pH indicator paper), initially with ammonium hydroxide and then with 6 N ammonia as the desired range is neared, dilute with water to 40 mL, and mix.

**Analysis:** Add 10 mL of freshly prepared hydrogen sulfide TS to each tube, mix, allow to stand for 5 min, and view downward over a white surface.

**Acceptance criteria:** The color of Solution B is not darker than that of Solution A. (NMT 2 mg/kg)

**SPECIFIC TESTS**

- **Loss on Drying, Appendix II C: 110° for 2 h**
Acceptance criteria: NMT 0.5%

**Iodine**

[Note—This specification applies only to iodized salt.]

**Sample:** 20 g

**Analysis:** Dissolve the Sample in about 300 mL of water in a 600-mL beaker. Add a few drops of methyl orange TS, neutralize the solution with 85% phosphoric acid, and then add 1 mL excess of the acid. Add 25 mL of bromine TS and a few glass beads, boil until the solution is clear, then boil for an additional 5 min. Add about 50 mg of salicylic acid crystals, 1 mL of phosphoric acid, and 10 mL of a 1:20 potassium iodide solution, and titrate to a pale yellow color with 0.01 N sodium thiosulfate. Add 1 mL of starch TS, and continue the titration to the disappearance of the blue color. Each mL of 0.01 N sodium thiosulfate is equivalent to 0.277 mg of potassium iodide (KI).

Acceptance criteria: 0.006%–0.010% of potassium iodide

**Iron**

[Note—This specification applies only to products to which green ferric ammonium citrate has been added.]

**Sample solution:** Dissolve 625.0 mg of sample in 10 mL of 2.7 N hydrochloric acid, and dilute with water to 50 mL. Add 40 mg of ammonium persulfate crystals and 10 mL of ammonium thiocyanate TS.

**Control solution:** Dissolve 10 mL of Iron Standard Solution (10 µg of Fe) (see Standard Solutions for the Preparation of Controls and Standards, Solutions and Indicators) in 2 mL of 2.7 N hydrochloric acid, and dilute with water to 50 mL.

**Analysis:** To the Sample solution add 40 mg of ammonium persulfate crystals and 10 mL of ammonium thiocyanate TS. Repeat the preceding using the Control solution in place of the Sample solution.

Acceptance criteria: Any red or pink color produced by the Sample solution does not exceed that produced by the Control solution. (NMT 0.0016% of iron (Fe))

**Change to read:**

**Sodium ferrocyanides**

[Note—This specification applies only to products to which calcium, potassium, or sodium ferrocyanide have been added.]

**Sample:** 9.62 g

**Control solution:** 425 µg/mL of sodium ferrocyanide [Na₄Fe(CN)₆] prepared as follows: Dissolve 99.5 mg of decahydrate ferrocyanide [Na₄Fe(CN)₆·10 H₂O] in 500 mL of water. 135 µg/mL of sodium ferrocyanide [Na₄Fe(CN)₆] prepared as follows: dissolve 107.5 mg of decahydrate ferrocyanide [Na₄Fe(CN)₆·10 H₂O] in 500 mL of water.

[Note—if dendritic salt is analyzed, dissolve 230 mg of decahydrate ferrocyanide in 500 mL of water to obtain a solution with 290 µL/mL of sodium ferrocyanide [Na₄Fe(CN)₆].]

[Note—Although decahydrate ferrocyanide is stable, it may lose hydration water over time. To avoid this, a prior drying step (105 °C, overnight) could be incorporated to obtain anhydrous sodium ferrocyanide. Once dried, use 62.5 mg in the Control solution instead of the decahydrate ferrocyanide.]

**Analysis:** Dissolve the Sample in 80 mL of water in a 150-mL glass-stoppered cylinder or flask. Transfer 1.0 mL of Control solution into a separate 150-mL container similar to that used for the Sample. Add 2 mL of ferrous sulfate TS and 1 mL of 2 N sulfuric acid to each container, dilute with water to 100 mL, and mix.

Transfer 50-mL portions of the resulting solutions into matched color-comparison tubes.

Acceptance criteria: The solution resulting from treatment of the Sample shows no more blue color than...
the solution resulting from treatment of the *Standard solution*. (NMT 0.0013% of anhydrous sodium ferrocyanide \([\text{Na}_4\text{Fe(CN)}_6]\); NMT 0.0014% of ferrocyanides, expressed as anhydrous sodium ferrocyanide \([\text{Na}_4\text{Fe(CN)}_6]\); for dendritic salt NMT 0.0029% of ferrocyanides, expressed as anhydrous sodium ferrocyanide \([\text{Na}_4\text{Fe(CN)}_6]\)\(_3\text{S (FCC7)}\)

**OTHER REQUIREMENTS**

- **LABELING**: Indicate whether the article is iodized.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 929*
BRIEFING

**Sodium Cyclamate.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Sodium Cyclamate monograph prepared at the 63rd Session (2004) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Sodium Cyclamate monograph prepared by the *British Pharmacopoeia* (Vol. I, II, 2010) and *European Pharmacopoeia* (6th Ed.), and on the basis of comments received. Interested parties are encouraged to submit comments.

(FIEC: C. Mejia) C93555

---

*Add the following:*

- **Sodium Cyclamate**
- Sodium Cyclohexanesulfamate
- Sodium Cyclohexylsulfamate

![Chemical Structure of Sodium Cyclamate]

\[
\text{C}_6\text{H}_{12}\text{NNaO}_3\text{S}
\]

Formula wt 201.22

INS: 952(iv)

CAS: [139-05-9]

**DESCRIPTION**

Sodium Cyclamate occurs as colorless to white crystals or crystalline powder. It is soluble in water and practically insoluble in ethanol.

**Function:** Sweetener

**Packaging and Storage:** Store in tight containers in a cool, dry place.

**IDENTIFICATION**

- **Sodium, Appendix IIIA**
  - **Acceptance criteria:** Passes test

- **Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC**
  - **Reference standard:** USP Sodium Cyclamate RS
  - **Sample and standard preparation:** K
  - **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the *Reference standard*.

**ASSAY**

- **Procedure**
  - **Sample:** 0.4 g
  - **Analysis:** Dissolve the *Sample* in a mixture of 50 mL of water and 5 mL of hydrochloric acid TS, diluted.
Titrate the solution with 0.1 M sodium nitrite. Add the last mL of titrant dropwise until a blue color is produced immediately when a glass rod dipped into the titrated solution is streaked on a piece of starch iodide test paper. Alternatively, the endpoint may be determined potentiometrically. When the titration is complete, the endpoint is reproducible after the mixture has been allowed to stand for 1 min. Each mL of 0.1 M sodium nitrite is equivalent to 20.12 mg of C_6H_12NNaO_3S.

**Acceptance criteria:** 99.0%–101.0%, calculated on the dried basis

**IMPURITIES**

**Inorganic Impurities**
- **Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**
  - **Sample:** 5 g
  - **Acceptance criteria:** NMT 1.0 mg/kg

**Organic Impurities**
- **Cyclohexanamine, Aniline, and N-Cyclohexylcyclohexanamine**
  - **Internal standard solution:** Dissolve 0.02 µL/mL of tetradecane in methylene chloride.
  - **Solution A:** Dissolve 10 mg of cyclohexanamine, 1 mg of N-cyclohexylcyclohexanamine, and 1 mg of aniline in water, then dilute with the same solvent to 1000 mL. Dilute 10 mL of this solution with water to 100 mL.
  - **Solution B:** 42% w/v sodium hydroxide solution
  - **Standard solution:** To 20 mL of Solution A, add 0.5 mL of Solution B, and extract with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of water and an acetic acid solution (12% w/v). Separate the lower layer, add 0.5 mL of Solution B and 0.5 mL of the Internal standard solution, and shake. Use the lower layer immediately after separation.
  - **Sample solution:** Dissolve 2 g of sample in 20 mL of water, add 0.5 mL of Solution B, and shake with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of an acetic acid solution (12% w/v) and water. Separate the lower layer, add 0.5 mL of Solution B and 0.5 mL of the Internal standard solution, and shake. Use the lower layer immediately after separation.

**Chromatographic system, Appendix IIA**
- **Mode:** Gas chromatography
- **Detector:** Flame ionization
- **Column:** 25-m × 0.32-mm (i.d.) fused-silica column with poly(dimethyl)(diphenyl)siloxane containing 95% of methyl groups and 5% of phenyl groups (DB-5, SE52) as stationary phase (film thickness 0.51 µm)
- **Carrier gas:** Helium
- **Flow rate:** 1.8 mL/min
- **Temperature**
  - **Injection port:** 250°
  - **Detector:** 270°
  - **Column:** See the temperature program in the table below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>85</td>
</tr>
<tr>
<td>1–9</td>
<td>85–150</td>
</tr>
<tr>
<td>9–13</td>
<td>150</td>
</tr>
</tbody>
</table>

**Injection volume:** 1.5 µL. Use a split vent at a flow rate of 20 mL/min.

**Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph, record the chromatograms, and measure the responses. [Note—The approximate
retention times (relative to cyclohexanamine, which has a retention time of about 2.3 min) for aniline, tetradecane, and N-cyclohexylcyclohexanamine are about 1.4, 4.3, and 4.5, respectively.]

Acceptance criteria
Cyclohexanamine: NMT 10.0 mg/kg
Aniline: NMT 1.0 mg/kg
N-Cyclohexylcyclohexanamine: NMT 1.0 mg/kg

SPECIFIC TESTS

- **Loss on Drying, Appendix IIC**: 105° for 4 h
  
  **Acceptance criteria**: NMT 1.0%

3S (FCC7)

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
Spice Oleoresins, FCC 7 page 985. In order to add modern methods of analysis that reflect current practices in the industry, and on the basis of comments received, it is proposed to add a new test to determine total capsaicinoids content, with acceptance criteria for Oleoresin Capsicum and Oleoresin Paprika. The Specific Tests section references the proposed new test for Total Capsaicinoids Content, Appendix VIII (see the Briefing for Oleoresins, Appendix VIII, appearing elsewhere in this Forum). Additionally, it is proposed to move the test for Scoville Heat Units in the Specific Tests section of this monograph to the General Information chapter to avoid confusion and redundancy. Comments by interested parties are encouraged.

(FIEC: C. Mejia) C92015

Spice Oleoresins

DESCRIPTION

Spice Oleoresins used in foods are derived from spices and contain the total sapid, odorous, and related characterizing principles normally associated with the respective spices. The oleoresins are produced by one of the following processes: (1) by extraction of the spice with any suitable solvent or solvents, in combination or sequence, followed by removal of the solvent or solvents in conformance with applicable residual solvent regulations (see the Identification section and test for Residual Solvent); or (2) by removal of the volatile portion of the spice by distillation, followed by extraction of the nonvolatile portion, which after solvent removal is combined with the total volatile portion.

Spice Oleoresins are frequently used in commerce with added suitable food-grade diluents, preservatives, antioxidants, and other substances consistent with good manufacturing practices, as provided for under Added Substances (see General Provisions). When added substances are used, they must be declared on the label in accordance with current U.S. regulations or with the regulations of other countries that recognize the Food Chemicals Codex.

Oleoresin Angelica Seed: Obtained by the solvent extraction of the dried seed of Angelica archangelica L. (Fam. Umbelliferae) as a dark brown or green liquid.

Oleoresin Anise: Obtained by the solvent extraction of the dried ripe fruit of anise, Pimpinella anisum L., or star anise, Illicium verum Hooker (Fam. Umbelliferae) as a dark brown or green liquid.

Oleoresin Basil: Obtained by the solvent extraction of the dried plant of Ocimum basilicum L. (Fam. Labiatae) as a dark brown or green semisolid.

Oleoresin Black Pepper: Obtained by the solvent extraction of the dried fruit of Piper nigrum L. (Fam. Piperaceae) as a dark green, olive green, or olive drab extract usually consisting of an upper oily layer and a lower crystalline layer. It may appear as a homogeneous emulsion if examined shortly after the oleoresin has been homogenized, but the product separates on standing. It may be decolorized by partial removal of chlorophyll.

Oleoresin Capsicum: Obtained by the solvent extraction of dried pods of Capsicum frutescens L. or Capsicum annum L. (Fam. Solanaceae) as a clear red to dark red, somewhat viscous liquid of characteristic odor, flavor, and bite. It may be decolorized through good manufacturing practices. It is partly soluble in alcohol (with oily separation and/or sediment) and is soluble in most fixed oils. The bite is usually standardized according to the label declaration.

Oleoresin Caraway: Obtained by the solvent extraction of the dried seeds of Carum carvi L. (Fam. Umbelliferae) as a green-yellow to brown liquid.

Oleoresin Cardamom: Obtained by the solvent extraction of the dried seeds of Elettaria cardamomum Maton (Fam. Zingiberaceae) as a dark brown or green liquid.

Oleoresin Celery: Obtained by the solvent extraction of the dried ripe seed of Apium graveolens L. (Fam. Umbelliferae) as a dark green or brown semisolid.
Umbelliferae) as a dark green, somewhat viscous, nonhomogeneous liquid with the characteristic odor and flavor of celery. It may be decolorized by the partial removal of chlorophyll. It is partly soluble in alcohol (with oily separation), and is soluble in most fixed oils.

**Oleoresin Coriander:** Obtained by the solvent extraction of the dried seeds of *Coriandrum sativum* L. (Fam. Umbelliferae) as a brown-yellow to green liquid.

**Oleoresin Cubeb:** Obtained by the solvent extraction of the dried fruit of *Piper cubeba* L. (Fam. Piperaceae) as a green or green-brown liquid.

**Oleoresin Cumin:** Obtained by the solvent extraction of the dried seeds of *Cuminum cyminum* L. (Fam. Umbelliferae) as a brown to yellow-green liquid.

**Oleoresin Dillseed:** Obtained by the solvent extraction of the dried seeds of *Anethum graveolens* L. (Fam. Umbelliferae) as a brown or green liquid.

**Oleoresin Fennel:** Obtained by the solvent extraction of the dried fruit of *Foeniculum vulgare* P. Miller (Fam. Umbelliferae) as a brown-green liquid.

**Oleoresin Ginger:** Obtained by the solvent extraction of the dried rhizomes of *Zingiber officinale* Roscoe (Fam. Zingiberaceae) as a dark brown, viscous to highly viscous liquid with the characteristic odor and flavor of ginger. It is soluble in alcohol (with sediment).

**Oleoresin Hop:** Obtained by the solvent extraction of the dried membranous cones of the female hop plants of *Humulus lupulus* L. or *Humulus americanus* Nutt. (Fam. Moraceae), using a food-grade solvent such as liquid carbon dioxide. It occurs as a light golden to black liquid to semisolid with a characteristic odor. It is soluble in methanol and is slightly soluble in acidified water. It may be reduced with sodium borohydride or with hydrogen and palladium catalyst. It conforms to U.S. Food and Drug Administration regulations pertaining to the specifications for extraction solvents for modified hop extract.

**Oleoresin Laurel Leaf:** Obtained by the solvent extraction of the dried leaves of *Laurus nobilis* L. (Fam. Lauraceae) as a dark brown or green semisolid.

**Oleoresin Marjoram Sweet:** Obtained by the solvent extraction of the dried herb of the marjoram shrub *Majorana hortensis* Moench (Fam. Labiatae) as a dark green to brown viscous liquid or semisolid.

**Oleoresin Origanum:** Obtained by the solvent extraction of the dried flowering herb *Origanum spp.* (Fam. Labiatae) as a dark brown-green semisolid.

**Oleoresin Paprika:** Obtained by the solvent extraction of the pods of *Capsicum annuum* L. (Fam. Solanaceae) as a deep red to deep purple-red, somewhat viscous liquid of characteristic odor and flavor. It frequently occurs as a two-phase mixture. The color is usually standardized according to the label declaration. It is partly soluble in alcohol (with oily separation), and is soluble in most fixed oils.

**Oleoresin Parsley Leaf:** Obtained by the solvent extraction of the dried herb of *Petroselinum crispum* (P. Miller) Nyman ex A.W. Hill (Fam. Umbelliferae) as a brown to green liquid.

**Oleoresin Parsley Seed:** Obtained by the solvent extraction of the dried seeds of *Petroselinum crispum* (P. Miller) Nyman ex A.W. Hill (Fam. Umbelliferae) as a deep green, semi-viscous liquid.

**Oleoresin Pimenta Berries:** Obtained by the solvent extraction of the dried fruit of *Pimenta officinalis* Lindl (Fam. Myrtaceae) as a brown-green to dark green liquid.

**Oleoresin Rosemary:** Obtained by the solvent extraction of the dried leaves of *Rosmarinus officinalis* L. (Fam. Labiatae). It is a thick, green paste that can be diluted with food-grade water- or oil-dispersible solvents. It may have a reduced chlorophyll content. The volatile oil content varies depending on its intended effect from a highly camphoraceous note to a subtle herbal note.

**Oleoresin Thyme:** Obtained by the solvent extraction of the dried flowering plant *Thymus vulgaris* L. or *Thymus zygis* L. and its var. *gracilis* Boissier (Fam. Labiatae) as a dark brown to green, viscous semisolid.

**Oleoresin Turmeric:** Obtained by the solvent extraction of the dried rhizomes of *Curcuma longa* L. (Fam. Zingiberaceae) as a yellow-orange to red-brown, viscous liquid with a characteristic odor and flavor. The content of curcumin normally varies, and the product is generally standardized according to the label declaration.
Function:  Flavored agent; color (oleoresins paprika and turmeric only)

Packaging and Storage: Store in a cool place protected from light in full, tight containers that are made from steel or aluminum and that are suitably lined.

Identification

Volatile Oil Content, Appendix VIII
Acceptance criteria: The volatile oil distilled from an oleoresin is similar in its physical and chemical properties, including its infrared spectrum, to that distilled from the spice of the same origin.

Impurities

Inorganic Impurities

Lead, Lead Limit Test, Appendix IIIB
Sample solution: Prepare as directed for organic compounds.
Control: 5 µg of Pb (5 mL of Diluted Standard Lead Solution)
Acceptance criteria: NMT 5 mg/kg

Organic Impurities

Residual Solvent, Appendix VIII
Acceptance criteria
Chlorinated hydrocarbons (total): NMT 0.003%
Acetone: NMT 0.003%
Isopropanol: NMT 0.003%
Methanol: NMT 0.005%
Hexane: NMT 0.0025%

Specific Tests

Color Value, Appendix VIII
Acceptance criteria
Oleoresin Paprika: Between 500 and 4500 units, as specified on the label (according to the method of analysis)

Curcumin, Curcumin Content, Appendix VIII
Acceptance criteria
Oleoresin Turmeric (or Color Value equivalent): Between 1% and 45%, as specified on the label

Piperine, Piperine Content, Appendix VIII
Acceptance criteria
Oleoresin Black Pepper: NLT 36%

Delete the following:

Scoville Heat Units, Appendix VIII
Acceptance criteria
Oleoresin Capsicum: Between 100,000 and 2,000,000, as specified on the label
Oleoresin Paprika (pungency): NMT 3000
3S (FCC7)

Add the following:

Total Capsaicinoids Content, Appendix VIII
Acceptance criteria
Oleoresin Capsicum: Between 6.7 and 133 mg/g, as specified on the label
Oleoresin Paprika (pungency): NMT 0.2 mg/g

3S (FCC7)

- Volatile Oil Content, Appendix VIII

Acceptance criteria

Oleoresin Angelica Seed: Between 2 mL and 7 mL/100 g
Oleoresin Anise: Between 9 mL and 22 mL/100 g
Oleoresin Basil: Between 4 mL and 17 mL/100 g
Oleoresin Black Pepper: Between 15 mL and 35 mL/100 g
Oleoresin Caraway: Between 10 mL and 20 mL/100 g
Oleoresin Cardamom: Between 50 mL and 80 mL/100 g
Oleoresin Celery: Between 7 mL and 20 mL/100 g
Oleoresin Coriander: Between 2 mL and 12 mL/100 g
Oleoresin Cumin: Between 10 mL and 30 mL/100 g
Oleoresin Dillseed: Between 10 mL and 20 mL/100 g
Oleoresin Fennel: Between 3 mL and 20 mL/100 g
Oleoresin Ginger: Between 18 mL and 35 mL/100 g
Oleoresin Hop: NMT 30 mL/100 g
Oleoresin Laurel Leaf: Between 5 mL and 25 mL/100 g
Oleoresin Marjoram Sweet: Between 8 mL and 20 mL/100 g
Oleoresin Origanum: Between 20 mL and 45 mL/100 g
Oleoresin Parsley Leaf: Between 2 mL and 10 mL/100 g
Oleoresin Parsley Seed: Between 2 mL and 7 mL/100 g
Oleoresin Pimenta Berries: Between 20 mL and 50 mL/100 g
Oleoresin Rosemary: NMT 15 mL/100 g
Oleoresin Thyme: Between 5 mL and 12 mL/100 g

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FL2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 985
**BRIEFING**

Sucromalt. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received.

1. The method referenced in the Identification test is a new General Test and Assay being proposed elsewhere in this FCC Forum for addition to Appendix IIC. This method is based on Nuclear Magnetic Resonance \( 761 \) as published in USP 32–NF 27.

2. The data submitted in support of the Identification specifications was generated using a Bruker DRX 500 MHz nuclear magnetic resonance spectrometer \( (\text{H field} = 500 \text{ MHz}) \). Additional parameters: 1 s relaxation delay; 0.30 Hz line broadening; 10330 Hz spectral width.

(FIEC: K. Laurick) C95175

*Add the following:*

- Sucromalt

Low Glycemic Carbohydrate

**DESCRIPTION**

Sucromalt occurs as a clear, colorless to light yellow, slightly cloudy liquid. It is the product of an enzyme-catalyzed reaction of sucrose and maltose, combined at a specific ratio. The reaction is pH- and temperature-controlled and utilizes a food-grade glucosyltransferase enzyme. The resulting syrup may be further treated to deactivate the enzyme, purified, and evaporated under vacuum to obtain an optimal dry solids content. Sucromalt is composed of 35%–45% fructose, 7%–15% leucrose, less than 5% other mono- and disaccharides, and 40%–60% higher glucooligosaccharides of 12+ DP.

**Function:** Source of slowly digestible nutritive carbohydrate

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

- \( ^1 \text{H NMR Spectroscopy, Nuclear Magnetic Resonance Spectroscopy, Appendix IIC} \)

**Solvent:** Use deuterium oxide (D\(_2\)O, 99.999% atom D) that contains 0.01% (v/v) dimethylsulfoxide (DMSO) as an internal intensity standard and 0.01% (w/v) sodium 3,3,4,4,5,5-hexadeutero-2,2-dimethyl-2-silapentane-5-sulfonate (DSS-d\(_6\)) as an internal chemical shift standard.

**Standard solution:** Prepare a solution in Solvent containing 5 mg/mL of USP Sucromalt RS. Vortex the mixture for 10 min at room temperature, then centrifuge at 7200 g for 10 min. Transfer 600-µL aliquots of the supernatant to 5-mm NMR tubes, and sonicate for 10 min to eliminate air bubbles.

**Sample solution:** Prepare a solution in Solvent containing 5 mg/mL of Sucromalt. Vortex the mixture for 10 min at room temperature, then centrifuge at 7200 g for 10 min. Transfer 600-µL aliquots of the supernatant to 5-mm NMR tubes, and sonicate for 10 min to eliminate air bubbles.

**Analysis:** Collect \(^1\text{H NMR spectra of the Standard solution and the Sample solution at } 25^\circ\). Compare the data collected between 4.80 ppm and 5.50 ppm within the anomeric region of the spectrum. For the spectrum obtained from the Sample solution, identify the signals due to \( \alpha-(1,6) \) and \( \alpha-(1,3) \) glycosidic linkages by comparison to the Reference Spectrum provided with the USP Sucromalt RS. Calculate the ratio of the intensities of the \(^1\text{H signals due to the } \alpha-(1,6) \) and \( \alpha-(1,3) \) glycosidic linkages in the spectrum
obtained for the Sample solution.

Acceptance criteria: The spectrum obtained for the Sample solution exhibits a chemical shift pattern with signal locations and intensities that match those obtained from the Standard solution within the defined chemical shift range of 4.80–5.50 ppm. The $\alpha$-(1,6)/$\alpha$-(1,3) signal ratio for the Sample solution is within the range 1.4–1.9.

ASSAY

• Procedure

Mobile phase: Degassed, purified water passed through a 0.22-µm filter before use. The conductivity should be NMT 1 megaohm. [Note—Maintain the water at 85$^\circ$ during operation of the chromatograph.]

Standard solution: Prepare a solution containing a total of about 10% solids, using sugars of known purity (e.g., USP Fructose RS, USP Dextrose RS, USP Maltose Monohydrate RS, or NIST Standard Reference Material; leucrose;¹ or equivalent) that approximates, on the dry basis, the composition of the sample to be analyzed. Dissolve each standard sugar in 20 mL of purified water contained in a 50-mL beaker. Heat on a steam bath until all sugars are dissolved, then cool, and transfer to a 100-mL volumetric flask. Dilute with water to volume, and mix. [Note—Freeze the solution if it is to be reused.]

Sample solution: Dilute to approximately 7%–12% solids and filter through a syringe filter system.

Chromatographic system, Appendix IIA

[Note—Use a suitable high-performance liquid chromatography system.²]

Mode: High-performance liquid chromatography

Detector: Differential refractometer

Column: 22- to 31-cm stainless steel column, or equivalent with a stationary phase of prepacked macroreticular polystyrene sulfonated divinylbenzene cation-exchange resin (2%–8% cross-linked, 8- to 25-µm particle size), preferably in the calcium or silver form. Examples of acceptable resins are Bio-Rad Aminex HPX-87C, or equivalent, for separating DP$_1$–DP$_4$ saccharides, and Aminex HPX-42C and HPX-42A, or equivalent, for separating DP$_1$–DP$_7$ saccharides. [Note—Condition the column before use by pumping solvent at 0.1 mL/min through the column while bringing it to operating temperature. Increase the flow rate to 0.5 mL/min and allow to equilibrate for 45 min prior to use.]

Column temperature: 85$^\circ$

Detector temperature: 45$^\circ$

Flow rate: 0.5–1.0 mL/min

Injection volume: 10 µL

Standardization: If a corn syrup or maltodextrin is used to supply a DP$_{4+}$ fraction, take care to include all saccharides in the standard composition calculation.

Calculate the dry-basis concentration, in percent, of each individual component in the Standard solution:

\[
\text{Result} = \left( \frac{W_C}{\sum W_i} \right) \times 100
\]

$W_C$ = weight of the sugar of interest

$\sum W_i$ = sum of the weights of all sugar components

Standardize by injecting 10–20 µL (about 1.0–2.0 mg of solids) of the Standard solution into the chromatograph. Integrate the peaks and normalize. Sum the individual DP$_{4+}$ responses from the normalized printout to obtain the total DP$_{4+}$ normalized response. Calculate the response factors:

\[
R_i = \frac{\text{known concentration, dry basis %}}{\text{measured concentration, normalized %}}
\]
R_i = \text{response factor for component } i

Compute the response factor for each component relative to glucose (R'_i):

\[ R'_i = \frac{R_i}{R_G} \]

R'_i = \text{response factor relative to glucose for component } i
R_i = \text{response factor for component } i
R_G = \text{response factor for glucose}

The R'_i for DP_4+ should be programmed as a default value (if automated equipment is used) and used to compute the concentration of higher saccharides.

**Analysis:** Inject a volume of the Sample solution into the chromatograph and record the resulting chromatogram. Calculate the concentration of each component:

\[ C_i = \frac{(A_i \times R_i \times 100)}{\left(\sum A_i R_i\right)} \]

C_i = \text{concentration of component } i
A_i = \text{peak area recorded for component } i
R_i = \text{response factor for component } i
\Sigma A_i R_i = \text{sum of the product of the areas (A) and response factors (R) for all components detected}

**Acceptance criteria**

**Fructose:** 35%–45%, calculated on the dry basis
**Leucrose:** 7%–15%, calculated on the dry basis
**Higher saccharides and polymer:** NLT 40%, calculated on the dry basis
**DP_2:** NMT 5%, calculated on the dry basis

**IMPURITIES**

**Inorganic Impurities**

- **Lead,** Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIIB
  
  **Sample:** 5 g
  
  **Acceptance criteria:** NMT 0.1 mg/kg
  
- **Sulfur Dioxide,** Sulfur Dioxide Determination, Appendix X
  
  **Sample:** 50 g

  [Note—Alternatively, the following method may be used.]

  **Solution A:** Dissolve 40 g of potassium iodide in 200 mL of water in a 1-L volumetric flask. Allow the solution to come to room temperature, add 12.7 g of crystalline iodine, and stir until the iodine is completely dissolved. Add 3 drops of concentrated hydrochloric acid, and dilute with water to volume. Mix the solution thoroughly, and store in an actinic glass bottle.

  **Solution B:** Dilute 2 mL of Solution A with water to 100 mL (0.002 N iodine).

  **Indicator solution:** Prepare a slurry of 10 g of soluble starch (Lintner) in 50 mL of cold water. Quantitatively transfer the slurry to 1 L of boiling water and stir, with boiling, until dissolved. Allow the solution to cool to room temperature before use.

  **Sample:** 50 g

  **Analysis:** Transfer the Sample to a 250-mL beaker, add 75 mL of water, and mix well. Transfer the solution to a 250-mL Erlenmeyer flask, and cool to 25°. Add 10 mL of cold 1.3 N potassium hydroxide to the flask,
and stir, then immediately add 10 mL of cold 1.5 N sulfuric acid, and stir. Add a few drops of the Indicator solution, and titrate immediately with Solution B until the blue color remains for 30 s. Perform a blank titration (see General Provisions).

Calculate the sulfur dioxide content, in ppm, of the sample:

\[
\text{Result} = (V_U - V_B) \times N_I \times F_1 \times (1/W_U) \times F_2
\]

- \( V_U \) = volume of titrant required for the sample titration (mL)
- \( V_B \) = volume of titrant required for the blank titration (mL)
- \( N_I \) = normality of the iodine solution, Solution B
- \( F_1 \) = milliequivalent weight of sulfur dioxide (64.071/2 \times 1000), 0.032
- \( W_U \) = weight of the Sample (g)
- \( F_2 \) = factor converting g to mg and g to kg, 1,000,000

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

- **Moisture**
  - **Filter aid:** Use a celite diatomite filter aid such as Hyflo Super-Cel, or equivalent. [Note—Do not substitute an “acid-washed” diatomaceous earth filter aid.] Wash a large quantity of the filter aid by percolation on a Buchner funnel with water acidified with hydrochloric acid (1 mL of concentrated hydrochloric acid per L of water). Continue washing until the effluent is acid to litmus, then wash the filter aid with water until the effluent is pH 4 or above. Air dry the washed filter aid for storage. Before use, dry a quantity of the prepared filter aid overnight in an oven maintained at 105°C, then store in a closed container.
  - **Sample:** Amount of material equivalent to 4–7 g of dry substance
  - **Apparatus:** Use a vacuum oven with uniform heat distribution and that is capable of maintaining the vacuum for several hours when the pump is shut off. The air inlet of the oven should be attached to a drying tower filled with a calcium sulfate desiccant with added moisture indicator. The tower should be connected in series to a gas scrubber containing concentrated sulfuric acid.
  - **Stirrers:** Use 100-mm \( \times \) 13-mm borosilicate glass test tubes equipped with extensions. The extensions should be made from 8-mm \( \times \) 180-mm stainless steel rods. Near one end of each rod, place two rubber rings (use appropriately-sized rubber o-rings or rings cut from rubber tubing) spaced such that when the rod is inserted into the test tube, it fits snugly at the top and bottom of the tube.
  - **Analysis:** Transfer about 30 g of Filter aid to a 3-in diameter aluminum drying dish. Use dishes that are about 3.5-in high that are fitted with aluminum lids. Prepare two dishes: one for the sample analysis and one for a blank. Place one of the test tube Stirrers (without the stainless steel rod) in each sample dish. Dry the dishes uncovered in the vacuum oven described under Apparatus at 100°C and NMT 25 torr while bleeding a small current of air through the oven and its drying tower. Continue drying for 5 h, then shut off the vacuum and allow the oven to slowly fill with air drawn through the drying tower. Open the oven, quickly cover the dishes with their lids, then place the dishes in a desiccator and allow them to cool to room temperature before weighing. Once the dishes are completely cooled, remove them from the desiccator, release the closure, and immediately weigh. Record all weights to the nearest mg.
  - Weigh the Sample into a 45-mL weighing bottle equipped with a a cap style ground glass stopper. Add 10 mL of warm water, and stir with a glass rod. Pour the diluted material onto the prepared Filter aid in one of the aluminum weighing dishes, using three 5-mL portions of warm water to assist the quantitative transfer. Insert the steel extension rod described under Stirrers into the sample dish, and stir until the
mixture is homogenous and evenly dispersed. Remove the rod from the test tube, leaving the stirring tube in the dish. Place dishes for the blank and the sample, uncovered, in the vacuum oven. [Note—Set the oven at 70° for samples with a DE of over 58 (and for all samples containing fructose) and at 100° for samples with a DE of 58 and below.] Allow the materials to dry for 5 h in the vacuum oven, then remove the dishes from the oven. Re-insert the stainless steel rods into the test tube Stirrers, and stir the material in the dish until a fine powder free of lumps is obtained. Return the dishes to the oven and continue heating for an additional 15–16 h. Once the heating period is over, shut off the vacuum line and allow the oven to slowly fill with air drawn through the drying tower. Open the oven, quickly cover the dishes with their lids, then place the dishes in a desiccator and allow them to cool to room temperature before weighing. Once the dishes are completely cooled, remove them from the desiccator, release the closure, and immediately weigh. Record all weights to the nearest mg, and calculate the percent moisture in the Sample.

**Acceptance criteria:** NMT 30.0%

- **pH, pH Determination, Appendix IIB**
  - **Sample solution:** 10% (w/w)
  - **Analysis:** Proceed as directed using pH 4.00 and 7.00 standard buffer solutions to calibrate the pH meter.
  - **Acceptance criteria:** 3.5–6.0

---

1 Aldrich Chemical Company.

2 Such as the one described in Standard Analytical Methods of the Corn Refiners Association, available from the Corn Refiners Association, Washington, DC.

3 Available from Johns-Manville Products Corp., Lompoc, CA.

4 Drierite®, or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(Fl2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td>1-301-816-8356</td>
</tr>
</tbody>
</table>
BRIEFING

L-Theanine. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the L-theanine monograph prepared by Japan's Specifications and Standards for Food Additives (JSFA) 7th Edition (2000), and on comments and data received. Interested parties are encouraged to submit comments.

(FIEC: C. Mejia) C94329

Add the following:

- L-Theanine

5-N-Ethyl-L-glutamine

L-Glutamic acid-γ-monoethylamide

Gamma-glutamylethylamide

C₇H₁₄N₂O₃

Formula wt 174.20
CAS: [3081-61-6]

DESCRIPTION

L-Theanine occurs as a white, odorless, crystalline powder, with a slight sweet taste. L-Theanine is produced by enzymatic synthesis from L-glutamine and ethylamine using glutaminase derived from either Pseudomonas nitroreducens or Bacillus amyloliquefaciens. The microorganisms are cultured, immobilized in a gel and placed into reaction columns where the substrate and environmental conditions are controlled. The resultant mixture is cooled, purified, concentrated, and dried into the final product. It is soluble in water and forms a transparent and colorless solution. It is insoluble in ethanol.

Function: Nutrient
Packaging and Storage: Store in well-closed, light-resistant containers at room temperature.

IDENTIFICATION

- A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix IIIC
  Reference standard: USP L-Theanine RS
  Sample and standard preparation: K
  Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

- B. PROCEDURE
  Acceptance criteria: The retention time of the major peak (excluding the solvent peak) in the chromatogram of the Sample solution is the same as that observed in the Standard solution.
ASSAY

**PROCEDURE**

**Mobile phase:** 2% (v/v) methanol solution containing 0.1% trifluoroacetic acid

**Internal standard solution:** 10 mg/mL of nicotinamide solution

**Standard solutions:** 0.5, 1, and 2 mg/mL of USP L-Theanine RS in water. Add the Internal standard solution to the Standard solutions for a final Internal standard concentration of 1 mg/mL.

**Sample solution:** 1 mg/mL in water. Add the Internal standard solution to the Sample solution for a final Internal standard concentration of 1 mg/mL.

**Chromatographic system**, Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** UV 210 nm
- **Column:** 4.0 mm I.D. × 150 mm C18 analytical column and 4.0 mm I.D. × 10 mm C18 precolumn
- **Column temperature:** 30°C
- **Flow rate:** 0.5 mL/min
- **Injection volume:** 11 µL

**System suitability**

- **Sample:** 0.5 mg/mL of the Standard solution
- **Suitability requirements**
  - **Suitability requirement 1:** The signal-to-noise ratio is NLT 10.
  - **Suitability requirement 2:** The relative standard deviation is NLT 1.96% for the peak area and retention time from 0.5 mg/mL of the Standard solution.
  - **Suitability requirement 3:** The retention time for the L-theanine peak from 0.5 mg/mL of the Standard solution is NMT 8.5 min.

**Analysis:** Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph and measure the responses for the major peaks on the resulting chromatograms. Prepare a standard curve by plotting the ratio of the concentration of the Standard solutions and the Internal standard versus the ratio of the peak area of the Standard solutions and the Internal standard. From the regression of the standard curve, calculate the concentration of L-theanine in the Sample. Calculate the percentage of L-Theanine in the Sample taken by the formula:

\[ \text{Result} = \frac{C_O}{C_{SMP}} \times 100\% \]

- \( C_O \) = concentration of L-Theanine in the sample determined from the standard curve (mg/mL)
- \( C_{SMP} \) = concentration of the sample in the Sample solution (mg/mL)

**Acceptance criteria:** 98%–102% on the dried basis

**IMPURITIES**

**Inorganic Impurities**

- **ARSENIC**, Arsenic Limit Test, Appendix IIIB
  - **Sample:** 0.5 g
  - **Acceptance criteria:** NMT 4 mg/Kg

- **CHLORIDE**, Chloride and Sulfate Limit Tests, Appendix IIIB
  - **Sample:** 1.0 g
  - **Control:** 210 µg of chloride (21 mL of the Standard Chloride Solution)
  - **Acceptance criteria:** Any turbidity produced by the Sample does not exceed that shown in the Control (NMT 210 ppm)

- **LEAD**, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB
Acceptance criteria: NMT 1 mg/kg

SPECIFIC TESTS

- **Loss on Drying**, Appendix IIIC: 105°, 3h
  
  *Sample:* 1 g
  
  *Acceptance criteria:* NMT 1%

- **Residue on Ignition (Sulfated Ash),** Appendix IIIC
  
  *Sample:* 1 g
  
  *Acceptance criteria:* NMT 0.2%

- **pH**, Appendix IIB
  
  *Analysis:* Analyze 1% solution with a pH meter.
  
  *Acceptance criteria:* Between 5 and 6

- **Optical (Specific) Rotation,** Appendix IIB
  
  *Sample:* 50 mg/mL sample previously dried
  
  *Acceptance criteria:* \( [\alpha]_D^{20} \) between +7.7° and +8.5°, on the dry basis

- **Melting Range or Temperature,** Appendix IIB
  
  *Acceptance criteria:* Between 214° and 215°

1 Develosil ODS-HG-50; Nomura Chemical Co. Ltd., or equivalent.

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

Trehalose, FCC 7 page 1051 and FCC Forum [June 2010]. On the basis of comments and data received, it is proposed to harmonize the standards in the tests for Color in Solution and Turbidity of a 30% Solution with those appearing in the Trehalose monograph published in USP 32–NF 27.
(FIEC: J. Moore) C91957

Trehalose

\(\alpha\)-d-Glucopyranosyl-\(\alpha\)-d-glucopyranoside, dihydrate

\[\text{C}_{12}\text{H}_{22}\text{O}_{11}\cdot2\text{H}_{2}\text{O}\]

Formula wt 378.33
CAS: dihydrate [6138-23-4]

DESCRIPTION

Change to read:

Trehalose occurs as a nonhygroscopic, white, crystalline powder. It is obtained through enzymatic conversion of food-grade starch into a stable, nonreducing disaccharide with two glucose molecules linked in an \(\alpha\), \(\alpha\)-1,1 configuration. The powder is freely soluble or readily dispersible in water. \(^{\text{2S (FCC7)}}\)

Trehalose is typically used in its dihydrate form.

Function: Humectant; nutritive sweetener, stabilizer; thickener; texturizer

Packaging and Storage: Store in tight containers in a dry place.

IDENTIFICATION

Add the following:

\(\text{\textbullet\  Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC}\)

Reference standard: USP Trehalose RS
Sample and Standard preparations: M
Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard. \(^{\text{2S (FCC7)}}\)

Delete the following:

\(\text{\textbullet\  Microscopic Examination}\)

Analysis: Observe a sample with a light microscope at 50x.
Acceptance criteria: The sample is composed of colorless, rectangular crystals with a prismatic structure. \(^{\text{2S (FCC7)}}\)
ASSAY

Change to read:

- **PROCEDURE**

  **Sample:** 3 g

  **Sample stock solution:** 30 mg/mL, passed through a 0.45-µm membrane filter

  **Sample solution:** Combine 3.7 mL of Sample stock solution with 10 mL of acetonitrile.

  **Standard stock solution:** 40 mg/mL Trehalose \(^1\) reference standard (on an anhydrous basis). [Note — Determine the water content of the Trehalose reference standard as directed under Water Determination, Appendix II B. Using the water content, calculate the weight of Trehalose reference standard needed to prepare the solution.]

  **Standard solution:** Combine 3.7 mL of Standard stock solution with 10 mL of acetonitrile.

  **Mobile phase:** Acetonitrile and water [73:27] (v/v)

  **Chromatographic system, Appendix IIA**

    - **Mode:** High-performance liquid chromatography
    - **Detector:** Differential-refractometer
    - **Column:** 300-mm x 10-mm (id); (Shodex Ionpack KS-801, or equivalent)
    - **Column temperature:** 35 °C
    - **Injection volume:** 20 µL

  **Analysis:** Pass the Standard solution and Sample solution through a 0.45-µm membrane filter, and separately inject the solutions into the chromatograph. Calculate the percent Trehalose in the sample using the equation:

    \[
    \%\text{Trehalose} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100\%
    \]

    \(r_U\) = peak response from the Sample solution

    \(r_S\) = peak response from the Standard solution

    \(C_S\) = concentration of Standard solution (mg/mL)

    \(C_U\) = concentration of Sample solution (mg/mL)

- **Mobile phase:** Water

  **Standard solution:** 10 mg/mL of USP Trehalose RS, calculated on the basis of the USP RS label claim

  **Sample solution:** 10 mg/mL, calculated on the anhydrous basis

  **Chromatographic system, Appendix IIA**

    - **Mode:** High-performance liquid chromatography
    - **Detector:** Refractive index
    - **Column:** 8-mm x 30-cm\(^1\)

  **Temperature**

    - **Detector:** 40 °C

    - **Column:** 80 °C

  **Flow rate:** Adjust so that the retention time of trehalose is about 15 min.

  **Injection size:** 20 µL

  **System suitability**

    - **Sample:** Standard solution

    - **Suitability requirement:** The relative standard deviation of the trehalose area responses from replicate injections is NMT 2.0%.

  **Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the
chromatograph, and measure the responses for the major peaks on the resulting chromatograms. Calculate the percentage of trehalose in the portion of the sample taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100 \]

- \( r_U \) = peak response from the Sample solution
- \( r_S \) = peak response from the Standard solution
- \( C_S \) = concentration of the Standard solution, calculated based on the USP Trehalose RS label claim (mg/mL)
- \( C_U \) = concentration of the Sample solution (mg/mL)

Acceptance criteria: NLT 98.0%, calculated on the dried anhydrous 2S (FCC7) basis

IMPURITIES

Inorganic Impurities

- Lead, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB

Sample: 5 g

Acceptance criteria: NMT 0.1 mg/kg

SPECIFIC TESTS

Change to read:

- Color in Solution

Sample solution: Dissolve 33 g of the sample in 67 g of recently boiled water.

Analysis: Determine the absorbance of the Sample solution at 420 and 720 nm using a 1-cm 10-cm 3S (FCC7) cuvette. Calculate the Color in Solution by the formula:

\[ \text{Result} = A_{420} - A_{720} \]

- \( A_{420} \) = absorbance at 420 nm
- \( A_{720} \) = absorbance at 720 nm

Acceptance criteria: NMT 0.100

Delete the following:

- Loss on Drying, Appendix IIC: 60° for 5 h

Acceptance criteria: NMT 1.5% 2S (FCC7)

Add the following:

- Water, Water Determination, Appendix IIB

Acceptance criteria: NMT 11.0% 2S (FCC7)

- pH, pH Determination, Appendix IIB

Sample solution: Dissolve 33 g of the sample in 67 g of recently boiled water.

Acceptance criteria: Between 4.5 and 6.5

- Residue on Ignition (Sulfated Ash), Appendix IIC

Sample: 5 g

Acceptance criteria: NMT 0.05%
Change to read:

- **Turbidity of a 30% Solution**

**Sample solution:**
Dissolve 33 g of the sample in 67 g of recently boiled water.

**Analysis:** Using a suitable spectrophotometer, determine the absorbance of the Sample solution at 720 nm using a 10-cm cuvette. \( \text{3S (FCC7)} \)

**Acceptance criteria:** NMT 0.050

---


1 Shodex SUGAR KS-801, or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 1051*
BRIEFING

2-Tridecenal, FCC 7 page 1053. On the basis of comments and data received, the food-grade materials of commerce for this ingredient have a different Refractive Index range than the current requirement. It is proposed to change the Acceptance criteria in the test for Refractive Index to a range that represents the current materials of commerce. Comments by interested parties are encouraged.
(FIEC: C. Mejia) C97213

2-Tridecenal

![Chemical Structure](image)

C_{13}H_{24}O

Formula wt 196.33
FEMA: 3082

DESCRIPTION

2-Tridecenal occurs as a white or slightly yellow liquid. It may contain a suitable antioxidant.

Odor: Oily, citrus

Solubility: Soluble in alcohol, most fixed oils; insoluble or practically insoluble in water

Solubility in Alcohol, Appendix VI: One mL dissolves in 1 mL of 95% ethanol.

Function: Flavoring agent

IDENTIFICATION

- Infrared Spectra, Spectrophotometric Identification Tests, Appendix IIIC
  - Acceptance criteria: The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

ASSAY

- Procedure: Proceed as directed under M-1a, Appendix XI.
  - Acceptance criteria: NLT 92.0% of C_{13}H_{24}O

SPECIFIC TESTS

Change to read:

- Refractive Index, Appendix II: At 20°
  - Acceptance criteria: Between 1.455 and 1.460, 1.457 and 1.462, 3S (FCC7)

- Specific Gravity: Determine at 25° by any reliable method (see General Provisions).
Acceptance criteria: Between 0.842 and 0.862

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D.</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8571</td>
<td></td>
</tr>
</tbody>
</table>
BRIEFING

Yeast Extract, FCC 7 page 1097 and FCC Forum [June 2010]. It is proposed to remove the Glutamic Acid test and specification from this monograph. Comments and data submitted indicate that some yeast extracts permitted for use in foods contain higher levels glutamic acid than allowed in the current monograph. Comments also indicate that the glutamic acid content should not be limited in this monograph because it is a desirable constituent.

(FIEC: J. Moore) C94701

Yeas t Extract

Autolyzed Yeast Extract

DESCRIPTION

Yeast Extract occurs as a liquid, paste, powder, or granular substance. It comprises the water-soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates, and salts. Yeast Extract is produced through the hydrolysis of peptide bonds by the naturally occurring enzymes present in edible yeasts or by the addition of food-grade enzymes. Food-grade salts may be added during processing.

Function: Flavoring agent; flavor enhancer

Packaging and Storage: Store in well-closed containers.

[Note—Perform all calculations on the dried basis. In a suitable tared container, evaporate liquid and paste samples to dryness on a steam bath, then, as for the powdered and granular forms, dry to constant weight at 105°C (see General Provisions).]

ASSAY

• PROTEIN, Nitrogen Determination, Appendix IIIC
  Analysis: Calculate the percent protein:

  \[ \text{Result} = N \times F \]

  \[ N = \text{percent nitrogen} \]
  \[ F = \text{nitrogen-to-protein conversion factor, 6.25} \]

  Acceptance criteria: NLT 42.0% protein

IMPURITIES

Inorganic Impurities

• LEAD, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB
  Sample: 10 g
  Acceptance criteria: NMT 2 mg/kg

• MERCURY, Mercury Limit Test, Appendix IIIB
  Acceptance criteria: NMT 3 mg/kg

SPECIFIC TESTS

• α-AMINO NITROGEN/TOTAL NITROGEN (AN/TN) PERCENT RATIO, α-AMINO NITROGEN (AN) DETERMINATION, Appendix IIIC and Total Nitrogen (TN), Nitrogen Determination, Appendix IIIC
  Analysis: Calculate the AN/TN percent ratio, where AN is the percent of α-Amino Nitrogen and TN is the
percent of Total Nitrogen.

**Acceptance criteria:** Between 15.0% and 55.0%

- **Ammonia Nitrogen,** Ammonia Nitrogen (NH₃-N) Determination, Appendix IIIC

  **Acceptance criteria:** NMT 2.0%, calculated on the sodium chloride-free basis

**Delete the following:**

- **Glutamic Acid,** Appendix IIIG

  **Acceptance criteria:** NMT 12.0% as C₅H₉NO₄ and NMT 28.0% of the total amino acids•protein

- **Insoluble Matter**

  **Sample:** 5 g

  **Analysis:** Transfer the Sample into a 250-mL Erlenmeyer flask, add 75 mL of water, cover the flask with a watch glass, and boil gently for 2 min. Filter the solution through a tared filtering crucible, dry the crucible at 105° for 1 h, cool, and weigh.

  **Acceptance criteria:** NMT 2%

- **Microbial Limits**

  [Note—Current methods for the following tests may be found in the Food and Drug Administration's Bacteriological Analytical Manual online at www.cfsan.fda.gov.]

  **Acceptance criteria**

  - **Aerobic plate count:** NMT 50,000 cfu/g
  - **Coliforms:** NMT 10 cfu/g
  - **Salmonella:** Negative in 25 g
  - **Yeasts and molds:** NMT 50 cfu/g

- **Potassium**

  **Standard stock solution:** 200 µg/mL of potassium in deionized water prepared as follows: Transfer 38.20 mg of reagent-grade potassium chloride into a 100-mL volumetric flask, add deionized water to dissolve the salt, dilute with deionized water to volume, and mix.

  **Standard solution:** 1.0 µg/mL of potassium in deionized water from the **Standard stock solution**

  **Sample stock solution:** Transfer 2.33 g of the sample into a silica or porcelain dish. Ash it in a muffle furnace at 550° for 2–4 h. Allow the ash to cool and add 5 mL of 20% hydrochloric acid. If necessary, warm the mixture to completely dissolve the residue. Filter the solution through acid-washed filter paper into a 1000-mL volumetric flask. Wash the filter paper with hot water, dilute the solution to volume, and mix.

  **Sample solution:** 1:300 dilution of the **Sample stock solution**

  **Analysis:** Using a suitable spectrophotometer, measure the absorbances of the **Sample solution** and the **Standard solution** at 766.5 nm.

  **Acceptance criteria:** The absorbance of the **Sample solution** does not exceed that of the **Standard solution.** (NMT 13.0%)

- **Sodium Chloride**

  **Standard stock solution:** 500 µg/mL of sodium chloride in deionized water

  **Standard solution:** 5.0 µg/mL of sodium chloride in deionized water from the **Standard stock solution**

  **Sample stock solution:** Transfer 1.0 ± 0.05 g of the sample into a silica or porcelain dish. Ash in a muffle furnace at 550° for 2–4 h. Allow the ash to cool and add 5 mL of 20% hydrochloric acid. If necessary, warm the mixture to completely dissolve the residue. Filter the solution through acid-washed filter paper into a 100-mL volumetric flask. Wash the filter paper with hot water, dilute the solution to volume, and mix.

  **Sample solution:** 1:100 dilution of the **Sample stock solution**
**Analysis:** Using a suitable spectrophotometer, measure the absorbances of the Sample solution and the Standard solution at 589.0 nm.

**Acceptance criteria:** The absorbance of the Sample solution does not exceed that of the Standard solution. (NMT 50.0%)

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
</table>
| Monograph      | Jeffrey Moore, Ph.D.  
Scientific Liaison  
1-301-816-8288 | (FI2010) Monographs - Food Ingredients |

*FCC Seventh Edition* Page 1097
APPENDIX II: PHYSICAL TESTS AND DETERMINATIONS

A. CHROMATOGRAPHY

[Note—Chromatographic separations may also be characterized according to the type of instrumentations or apparatus used. The types of chromatography that may be used in the Food Chemicals Codex (FCC) are column, thin-layer, gas, and high-pressure or high-performance liquid chromatography.

The Committee on Food Chemicals Codex recognizes that the field of chromatography continues to advance. Accordingly, the use of equivalent or improved systems is acceptable with appropriate validation.]

For the purposes of the FCC, chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase, is a gas or liquid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures by selectively retarding the passage of some compounds through the stationary phase while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the $R_F$, or retardation factor, for each of the eluted substances. The $R_F$ is a measure of that fraction of its total elution time that any compound spends in the mobile phase. Because this fraction is directly related to the fraction of the total amount of the solute that is in the mobile phase, the $R_F$ can be expressed as

$$ R_F = \frac{V_mC_m}{V_mC_m + V_SC_s} $$

in which $V_m$ and $V_s$ are the volumes of the mobile and stationary phase, respectively, and $C_m$ and $C_s$ are the concentrations of the solute in either phase at any time. This can be simplified to
\[ R_F = \frac{V_m}{V_m + K V_s} \]

in which \( K = \frac{C_s}{C_m} \) and is an equilibrium constant that indicates this differential affinity of the solute for the phases. Alternatively, a new constant, \( k \), the capacity factor, may be introduced, giving another form of the expression:

\[ R_F = \frac{1}{1 + k} \]

in which \( k = \frac{K V_s}{V_m} \). The capacity factor, \( k \), which is normally constant for small samples, is a parameter that expresses the ability of a particular chromatographic system to interact with a solute. The larger the \( k \) value, the more the sample is retarded.

Both the retardation factor and the capacity factor may be used for qualitative identification of a solute or for developing strategies for improving separation. In terms of parameters easily obtainable from the chromatogram, the \( R_F \) is defined as the ratio of the distance traveled by the solute band to the distance traveled by the mobile solvent in a particular time. The capacity factor, \( k \), can be evaluated by the expression

\[ k = \frac{(t_r - t_o)}{t_o} \]

in which \( t_r \), the retention time, is the elapsed time from the start of the chromatogram to the elution maximum of the solute, and \( t_o \) is the retention time of a solute that is not retained by the chromatographic system.

Retardation of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by adsorption, either physical adsorption, in which the binding forces are weak and easily reversible, or chemisorption, in which strong bonding to the surface can occur. Another important mechanism of retardation is partition, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert particle or chemically bonded to it. If the liquid phase is a polar substance (e.g., polyethylene glycol) and the mobile phase is nonpolar, the process is termed normal-phase chromatography. When the stationary phase is nonpolar (e.g., octadecylsilane) and the mobile phase is polar, the process is reversed-phase chromatography. For the separation of mixtures of ionic species, insoluble polymers called ion exchangers are used as the stationary phase. Ions of the solutes contained in the mobile phase are adsorbed onto the surface of the ion exchanger while at the same time displacing an electrically equivalent amount of less strongly bound ions to maintain the electroneutrality of both phases. The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called size exclusion chromatography. The stationary phases used are highly cross-linked polymers that have imbibed a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solvated solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules permeate into the pores of the gel and are eluted later. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used in the FCC are column, thin-layer, gas, and high-performance liquid chromatography.

**COLUMN CHROMATOGRAPHY**

**Apparatus**  The equipment needed for column chromatography is not elaborate, consisting only of a cylindrical
glass or Teflon tube that has a restricted outflow orifice. The dimensions of the tube are not critical and may vary from 10 to 40 mm in inside diameter and from 100 to 600 mm in length. For a given separation, greater efficiency may be obtained with a long narrow column, but the resultant flow rate will be lower. A fitted-glass disk may be seated in the end of the tube to act as a support for the packing material. The column is fitted at the end with a stopcock or other flow-restriction device to control the rate of delivery of the eluant.

**Procedure**  The stationary phase is introduced into the column either as a dry powder or as a slurry in the mobile phase. Because a homogeneous bed free of void spaces is necessary to achieve maximum separation efficiency, the packing material is introduced in small portions and allowed to settle before further additions are made. Settling may be accomplished by allowing the mobile phase to flow through the bed, by tapping or vibrating the column if a dry powder is used, or by compressing each added portion using a tamping rod. The rod can be a solid glass, plastic, or metal cylinder whose diameter is slightly smaller than that of the column, or it can be a thinner rod onto the end of which has been attached a disk of suitable diameter. Ion-exchange resins and exclusion polymers are never packed as dry powders because after introduction of the mobile phase, they will swell and create sufficient pressure to shatter the column. When the packing has been completed, the sample is introduced onto the top of the column. If the sample is soluble, it is dissolved in a minimum amount of the mobile phase, pipetted onto the column, and allowed to percolate into the top of the bed. If it is not soluble or if the volume of solution is too large, it may be mixed with a small amount of the column packing. This material is then transferred to the chromatographic tube to form the top of the bed.

The chromatogram is then developed by adding the mobile phase to the column in small portions and allowing it to percolate through the packed bed either by gravity or under the influence of pressure or vacuum. Development of the chromatogram takes place by selective retardation of the components of the mixture as a result of their interaction with the stationary phase. In column chromatography, the stationary phase may act by adsorption, partition, ion exchange, exclusion of the solutes, or a combination of these effects.

When the development is complete, the components of the sample mixture may be detected and isolated by either of two procedures. The entire column may be extruded carefully from the tube, and if the compounds are colored or fluorescent under ultraviolet light, the appropriate segments may be cut from the column using a razor blade. If the components are colorless, they may be visualized by painting or spraying a thin longitudinal section of the surface of the chromatogram with color-developing reagents. The chemical may then be separated from the stationary phase by extraction with a strong solvent such as methanol and subsequently quantitated by suitable methods.

In the second procedure, the mobile phase may be allowed to flow through the column until the components of the mixture successively appear in the effluent. This eluate may be collected in fractions and the mobile phase evaporated if desired. The chemicals present in each fraction may then be determined by suitable analytical techniques.

**PAPER CHROMATOGRAPHY**

In this type of chromatography, the stationary phase ordinarily consists of a sheet of paper of suitable texture and thickness. The paper used is made from highly purified cellulose, which has a great affinity for water and other polar solvents since it has many hydroxyl functional groups. The tightly bound water acts as the stationary phase, and therefore the mechanism that predominates is liquid–liquid or partition chromatography. Adsorption of solutes to the cellulose surface may also occur, but this is of lesser importance. Papers especially impregnated to permit ion-exchange or reverse-phase chromatography are also available.

**Apparatus**  The essential equipment for paper chromatography consists of the following:
**Vapor-Tight Chamber**  The chamber is constructed preferably of glass, stainless steel, or porcelain. It is provided with inlets for the addition of solvent or for releasing internal pressure, and it is designed to permit observation of the progress of the chromatographic run without being opened. Tall glass cylinders are convenient if they are made vapor-tight with suitable covers and a sealing compound.

**Supporting Rack**  The rack serves as a support for the solvent troughs and antisiphoning rods. It is constructed of a corrosion-resistant material about 5 cm shorter than the inside height of the chamber.

**Solvent Troughs**  The troughs, made of glass, are designed to be longer than the width of the chromatographic sheets and to contain a volume of solvent greater than that required for one chromatographic run.

**Antisiphoning Rods**  Constructed of heavy glass, the rods are placed on the rack and arranged to run outside of, parallel to, and slightly above the edge of the glass trough.

**Chromatographic Sheets**  Special chromatographic filter paper is cut to length approximately equal to the height of the chamber. The sheet is at least 2.5 cm wide but not wider than the length of the trough. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few cm below the rods. Care is necessary to avoid contaminating the paper by excessive handling or by contact with dirty surfaces.

**Procedure for Descending Chromatography**  Separation of substances by descending chromatography is accomplished by allowing the mobile phase to flow downward on the chromatographic sheet.

The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes of the resulting solution, normally containing 1 to 20 µg of the compound, are placed in 6- to 10-mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6 to 10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphoning rod and an additional heavy glass rod that holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with a mixture containing both phases of the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with solvent vapor. Any excess pressure is released as necessary. For large chambers, equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed, and the mobile phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated components of the mixture.

**Procedure for Ascending Chromatography**  In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet.

The test materials are applied to the chromatographic sheet as directed under Procedure for Descending Chromatography.
Chromatography. Enough of both phases of the solvent mixture to cover the bottom of the chamber is added. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheet is suspended so that the end near which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under Procedure for Descending Chromatography. Then the solvent is added through the inlet to the trough in excess of the quantity of solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.

Small cylinders may be used without troughs so that only the mobile phase is placed on the bottom. The chromatographic sheet is suspended during equilibration with the lower end just above the solvent, and chromatography is started by lowering the sheet so that it touches the solvent.

Detection of Chromatographic Bands After the chromatogram has been fully developed, the bands corresponding to the various solutes may be detected by means similar to those described in Column Chromatography. If the compounds are colored or fluorescent under ultraviolet light, they may be visualized directly. Colorless compounds may be detected by spraying the paper with color-developing reagents. The bands corresponding to the individual components can be cut from the paper, and the chemical substances eluted from the cellulose by the use of a strong solvent such as methanol.

Identification of Solutes Since the chromatographic mobilities of the solutes may change from run to run due to varying experimental conditions, presumptive identification of a substance should be based on comparison with a reference standard. The \( R_F \) values of the unknown substance and the standard on the same chromatogram must be identical. Alternatively, the ratio between the distances traveled by a given compound and a reference substance, the \( R_r \) value, must be 1.0. Identification may also be made by mixing a small amount of the reference substance with the unknown and chromatographing. The resulting chromatogram should contain only one spot. Definitive identification of solutes may be achieved by eluting them from the paper and subjecting them to IR, NMR, or mass spectrometry.

THIN-LAYER CHROMATOGRAPHY

In thin-layer chromatography (TLC), the stationary phase is a uniform layer of a finely divided powder that has been coated on the surface of a glass or plastic sheet and that is held in place by a binder. The capacity of the system is dependent on the thickness of the layer, which may range from 0.1 to 2.0 mm. The thinner layers are used primarily for analytical separations, while the thicker layers, because of their greater sample-handling ability, are useful for preparative work.

Substances that are used as coatings in TLC include silica gel, alumina, cellulose, and reversed-phase packings. Separations occur because of adsorption of the solutes from the mobile phase onto the surface of the thin layer. However, adsorption of water from the air or solvent components from the mobile phase can give rise to partition or liquid–liquid chromatography. Specially coated plates are available that permit ion-exchange or reversed-phase separations.

Apparatus Acceptable apparatus and materials for thin-layer chromatography consist of the following:

Glass Plates Flat glass plates of uniform thickness throughout their areas. The most common sizes are 20, 10, and 5 cm × 20 cm. (Aluminum plates also are commonly used.)

Aligning Tray An aligning tray or other suitable flat surface is used to align and hold plates during application
Adsorbent  The adsorbent may consist of finely divided adsorbent materials for chromatography. It can be applied directly to the glass plate, or it can be bonded to the plate by means of plaster of Paris or with starch paste. Pretreated chromatographic plates are available commercially.

Spreader  A suitable spreading device that, when moved over the glass plate, applies a uniform layer of adsorbent of the desired thickness over the entire surface of the plate.

Storage Rack  A rack of convenient size to hold the prepared plates during drying and transportation.

Developing Chamber  A glass chamber that can accommodate one or more plates and can be properly closed and sealed. It is fitted with a plate-support rack that can support the plates when the lid of the chamber is in place.

[Note—Preformed TLC plates available commercially may also be used.]

Procedure  Clean the plates scrupulously, as by immersion in a chromic acid cleansing mixture, rinse them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and dry.

Arrange the plate or plates on the aligning tray, and secure them so that they will not slip during the application of the adsorbent. Mix an appropriate quantity of adsorbent and liquid, usually water, which when shaken for 30 s gives a smooth slurry that will spread evenly with the aid of a spreader. Transfer the slurry to the spreader, and apply the coating at once before the binder begins to harden. Move the spreader smoothly over the plates from one end of the tray to the other. Remove the spreader, and wipe away excess slurry.

Allow the plates to set for 10 min, and then place them in the storage rack and dry at 105° for 30 min or as directed in the individual monograph. Store the finished plates in a desiccator.

Equilibrate the atmosphere in the Developing Chamber by placing in it a volume of the mobile phase in excess of that required for complete development of the chromatogram, cover the chamber with its lid, and allow it to stand for at least 30 min.

Apply the Sample Solution and the Standard Solution at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moves in the application of the adsorbent layer), and allow to dry. A template will aid in determining the spot points and the 10- to 15-cm distance through which the solvent front should move.

Arrange the plate on the supporting rack (sample spots on the bottom), and introduce the rack into the developing chamber. The solvent in the chamber must be deep enough to reach the lower edge of the adsorbent, but must not touch the spot points. Seal the cover in place, and maintain the system until the solvent ascends to a point 10 to 15 cm above the initial spots; this usually requires 15 min to 1 h. Remove the plates, and dry them in air. Measure and record the distance of each spot from the point of origin. If so directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

Detection and Identification  Detection and identification of solute bands is done by methods essentially the same as those described in Column Chromatography. However, in TLC an additional method called fluorescence quenching is also used. In this procedure, an inorganic phosphor is mixed with the adsorbent before it is coated on the plate. When the developed chromatogram is irradiated with ultraviolet light, the surface of the plate fluoresces with a characteristic color, except in those places where ultraviolet-absorbing solutes are situated. These quench the fluorescence and are detectable as dark spots.
Detection with an ultraviolet light source suitable for observations with short (254-nm) and long (360-nm) ultraviolet wavelengths may be called for in some cases.

**Quantitative Analysis** Two methods are available if quantitation of the solute is necessary. In the first, the bands are detected and their positions marked. Those areas of adsorbent containing the compounds of interest are scraped from the surface of the plate into a centrifuge tube. The chemicals are extracted from the adsorbent with the aid of a suitable strong solvent, the suspension is centrifuged, and the supernatant layer is subjected to appropriate methods of quantitative analysis.

The second method involves the use of a scanning densitometer. This is a spectrophotometric device that directs a beam of monochromatic radiation across the surface of the plate. After interaction with the solutes in the adsorbent layer, the radiation is detected as transmitted or reflected light and a recording of light intensity versus distance traveled is produced. The concentration of a particular species is proportional to the area under its peak and can be determined accurately by comparison with standards.

**GAS CHROMATOGRAPHY**

The distinguishing features of gas chromatography are a gaseous mobile phase and a solid or immobilized liquid stationary phase. Liquid stationary phases are available in packed or capillary columns. In the packed columns, the liquid phase is deposited on a finely divided, inert solid support, such as diatomaceous earth or porous polymer, which is packed into a column that typically has a 2- to 4-mm id and is 1 to 3 m long. In capillary columns, which contain no particles, the liquid phase is deposited on the inner surface of the fused silica column and may be chemically bonded to it. In gas–solid chromatography, the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase.

When a volatile compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retarded to a greater or lesser extent by sorption and desorption in the stationary phase. The elution of the compound is characterized by the partition ratio, k, a dimensionless quantity also called the capacity factor. It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the retention time of a nonretarded compound. The value of the capacity factor depends on the chemical nature of the compound; the nature, amount, and surface area of the liquid phase; and the column temperature. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors.

**Apparatus** A gas chromatograph consists of a carrier gas source, an injection port, column, detector, and recording device. The injection port, column, and detector are carefully temperature controlled. The typical carrier gas is helium or nitrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder and passes through suitable pressure-reducing valves to the injection port and column. Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending on the configuration of the apparatus, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas before entering the column.

Once in the column, compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend on their vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution and retention times of components of the test mixture, is also
temperature dependent. The use of temperature-programmable column ovens takes advantage of this
dependence to achieve efficient separation of compounds differing widely in vapor pressure.

As resolved compounds emerge from the column, they pass through a detector, which responds to the
amount of each compound present. The type of detector to be used depends on the nature of the compounds
to be analyzed, and is specified in the individual monograph. Detectors are heated above the maximum column
operating temperature to prevent condensation of the eluting compounds.

Detector output is recorded as a function of time, producing a chromatogram, which consists of a series of
peaks on a time axis. Each peak represents a compound in the vaporized test mixture, although some peaks
may overlap. The elution time is characteristic of the individual compounds (qualitative analysis), and the peak
area is a function of the amount present (quantitative analysis).

Injectors Sample injection devices range from simple syringes to fully programmable automatic injectors. The
amount of sample that can be injected into a capillary column without overloading is small compared with the
amount that can be injected into a packed column, and may be less than the smallest amount that can be
manipulated satisfactorily by syringe. Capillary columns are therefore used with injectors able to split samples
into two fractions, a small one that enters the column and a large one that goes to waste (split injector). Such
injectors may also be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are
carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed
into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid
samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile
components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or
headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the
headspace in the sample container into the gas chromatograph.

Columns Capillary columns, which are usually made of fused silica, have a 0.2- to 0.53-mm id and are 5 to
30 m long. The liquid or stationary phase is 0.1 to 1.0 µm thick, although nonpolar stationary phases may be
up to 5 µm thick.

Packed columns, made of glass or metal, are 1 to 3 m long, with a 2- to 4-mm id. Those used for analysis
typically have liquid phase loadings of about 5% (w/w) on a solid support.

Supports for analysis of polar compounds on low-capacity, low-polarity liquid phase columns must be inert to
avoid peak tailing. The reactivity of support materials can be reduced by silanizing before coating with liquid
phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are
available in various mesh sizes, with 80- to 100-mesh and 100- to 120-mesh being more commonly used with
2- to 4-mm columns. Because of the absence of a solid support, capillary compounds are much more inert
than packed columns.

Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly
proportional to column length, while resolution is proportional to the square root of the column length. For
packed columns, the carrier gas flow rate is usually expressed in milliliters per minute at atmospheric
pressure and room temperature. It is measured at the detector outlet with a soap film flow meter while the
column is at operating temperature. Unless otherwise specified in the individual monograph, flow rates for
packed columns are 60 to 75 mL/min for 4-mm id columns and ~30 mL/min for 2-mm id columns.

For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. Typical linear velocities are 20 to 60 cm/s for helium. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called “bleeding.”

**Detectors** Flame-ionization detectors are used for most analyses, with lesser use made of thermal conductivity, electron-capture, nitrogen–phosphorus, and mass spectrometric detectors. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations. Flame-ionization detectors have a wide linear range (~10^6) and are sensitive to organic compounds. Unless otherwise specified in individual monographs, flame-ionization detectors with either helium or nitrogen carrier gas are to be used for packed columns, and helium is used for capillary columns.

The thermal conductivity detector detects changes in the thermal conductivity of the gas stream as solutes are eluted. Although its linear dynamic range is smaller than that of the flame-ionization detector, it is quite rugged and occasionally used with packed columns, especially for compounds that do not respond to flame-ionization detectors.

The alkali flame-ionization detector, sometimes called an NP or nitrogen—phosphorus detector, contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal, that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons.

The electron-capture detector contains a radioactive source (usually ^{63}Ni) of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.

**Data Collection Devices** Modern data stations receive the detector output, calculate peak areas, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation.

Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible reprocessing.

**Procedure** Capillary columns must be tested to ensure that they comply with the manufacturers’ specifications before they are used. These tests consist of the following injections: a dilute methane sample to determine the linear flow velocity; a mixture of alkanes (e.g., C_{14}, C_{15}, and C_{16}) to determine resolution; and a polarity test mixture to check for active sites on the column. The latter mixture may include a methyl ester, an unsaturated compound, a phenol, an aromatic amine, a diol, a free carboxylic acid, and a polycyclic aromatic compound, depending on the samples to be analyzed.

Packed columns must be conditioned before use until the baseline and other characteristics are stable. This may be done by operation at a temperature above that called for by the method or by repeated injections of the compound or mixture to be chromatographed. A suitable test for support inertness should be done. Very polar molecules (like free fatty acids) may require a derivatization step.
Before any column is used for assay purposes, a calibration curve should be constructed to verify that the instrumental response is linear over the required range and that the curve passes through the origin. If the compound to be analyzed is adsorbed within the system, the calibration curve will intersect the abscissa at a nonzero value. This may result in error, particularly for compounds at low concentrations determined by a procedure based on a single reference point. At high concentrations, the liquid phase may be overloaded, leading to loss of peak height and symmetry.

Assays require quantitative comparison of one chromatogram with another. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. The effects of variability can be minimized by addition of an internal standard, a noninterfering compound present at the same concentration as in the sample and standard solutions. The ratio of peak response of the analyte to that of the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being determined, there is also compensation for minor variations in column and detector characteristics. In some cases, the internal standard may be carried through the sample preparation procedure before gas chromatography to control other quantitative aspects of the assay. Automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards.

Many monographs require that system suitability requirements be met before samples are analyzed, see *System Suitability* below.

**HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**

High-performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, exclusion, or ion-exchange processes, depending on the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of nonvolatile organic compounds. Compounds to be analyzed are dissolved in a liquid, and most separations take place at room temperature.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, $k$, which depends on the chemical nature of the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

**Apparatus**  A liquid chromatograph consists of one, two, or more reservoirs containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder. Short, 3-, 5-, 10-, and 25-cm, small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

**Pumping Systems**  HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 5000 psi with delivery rates up to about 10 mL/min are typical. Pumps used for quantitative analysis should be
constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

Injectors  After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a calibrated, fixed-volume loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

Some valve systems incorporate a calibrated sample loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, test solution is transferred to a cavity by syringe and then switched into the mobile phase.

Columns  For most analyses, separation is achieved by partition of compounds in the test solution between the mobile and stationary phases. Systems consisting of polar stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reversed-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of a molecular weight that is less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reversed-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3, 5, or 10 µm in diameter, but sizes may range up to 50 µm for preparative columns. Small particles thinly coated with organic phase allow fast mass transfer and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups.

Columns used for analytical separations usually have internal diameters of 2 to 4.6 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60°C because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at an ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weights that are less than 2000. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines; while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column (total exclusion). Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to remove high molecular weight matrices or to characterize the molecular weight distribution of a polymer.
Detectors  Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of ultraviolet radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

Fixed, variable, and photodiode array (PDA) detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done before chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just before its entering the detector.

Potentiometric, voltammetric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulseless pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

Data Collection Devices  Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation.

Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity, from those providing a printout of peak areas to those providing a printout of peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

Procedure  The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. Composition has a much greater effect than
temperature on the capacity factor, $k$.

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength as well as changes in the composition of the mobile phase affect capacity factors. The technique of continuously increasing mobile phase strength during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

For accurate quantitative work, high-purity, “HPLC-grade” solvents and reagents must be used. The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of the analyte and internal standard are compared.

Because of normal variations in equipment, supplies, and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable. The main features of System Suitability tests are described below.

For information on the interpretation of results, see the section Interpretation of Chromatograms.

Interpretation of Chromatograms  Fig. 1 represents a typical chromatographic separation of two substances, 1 and 2, in which $t_R(1)$ and $t_R(2)$ are the respective retention times; $h$, $h/2$, and $W_{h/2}$ are the height, the half-height, and the width at half-height, respectively, for peak 1; and $W_1$ and $W_2$ are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

![FIGURE 1 Chromatographic Separation of Two Substances.](image)

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next. Comparisons are normally made in terms of relative retention, which is calculated by the equation

$$\alpha = \frac{(t_{R(2)} - t_{R(0)})/t_{R(1)} - t_0}{(t_{R(2)} - t_{R(0)})/t_{R(1)} - t_0}$$
in which \( t_{R(2)} \) and \( t_{R(1)} \) are the retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column, and \( t_O \) is the retention time of a nonretained substance, such as methane in this case, of gas chromatography.

In this and the following expressions, the corresponding retention volumes or linear separations on the chromatogram, both of which are directly proportional to retention time, may be substituted in the equations. Where the value of \( t_O \) is small, \( R_r \) may be estimated from the retention times measured from the point of injection \( (t_{R(2)}/t_{R(1)}) \).

The number of theoretical plates, \( N \), is a measure of column efficiency. For Gaussian peaks, it is calculated by the equations

\[
N = 16(t_R/W)^2 \quad \text{or} \quad N = 5.54(t_R/W_{1/2})^2
\]

in which \( t_R \) is the retention time of the substance and \( W \) is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. \( W_{1/2} \) is the peak width at half-height, obtained directly by electronic integrators. The value of \( N \) depends on the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column, and for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution, \( R \), is determined by the equation

\[
R = 2(t_{R(2)} - t_{R(1)})/(W_2 + W_1)
\]

in which \( t_{R(2)} \) and \( t_{R(1)} \) are the retention times of the two components, and \( W_2 \) and \( W_1 \) are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided (see Fig. 2). The relative standard deviation is expressed by the equation

\[
S_R(\%) = \left(\frac{100}{\bar{X}}\right)\left[\frac{\sum_{i=1}^{N}(X_i - \bar{X})^2}{(N-1)}\right]^{1/2}
\]

in which \( S_R \) is the relative standard deviation in percent, \( \bar{X} \) is the mean of the set of \( N \) measurements, and \( X_i \) is an individual measurement. When an internal standard is used, the measurement \( X_i \) usually refers to the measurement of relative area, \( A_s \),

\[
X_i = A_s = a_i/a_r
\]

in which \( a_r \) is the area of the peak corresponding to the standard substance and \( a_i \) is the area of the peak.
corresponding to the internal standard. When peak heights are used, the measurement $X_i$ refers to the measurement of relative heights, $H_s$,

$$X_i = H_s = h_r/h_i$$

in which $h_r$ is the height of the peak corresponding to the standard substance and $h_i$ is the height of the peak corresponding to the internal standard.

**System Suitability** Such tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The resolution, $R$, is a function of column efficiency, $N$, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the analyte. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

The tailing factor, $T$, a measure of peak symmetry, is unity for perfectly symmetrical peaks, and its value increases as tailing becomes more pronounced. In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. The calculation is expressed by the equation

$$tailing \ factor = T = W_{0.05}/2f$$

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions (see Procedures under Tests and Assays in General Provisions). Adjustments of operating conditions to meet system suitability requirements may be necessary.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test before use and during testing whenever there is a significant change in equipment or in a critical reagent or when a
B. PHYSICOCHEMICAL PROPERTIES

DISTILLATION RANGE

Scope  This method is to be used for determining the distillation range of pure or nearly pure compounds or mixtures having a relatively narrow distillation range of about 40° or less. The result so determined is an indication of purity, not necessarily of identity. Products having a distillation range of greater than 40° may be determined by this method if a wide-range thermometer, such as ASTM E1, 1C, 2C, or 3C, is specified in the individual monograph.

Definitions

Distillation Range  The difference between the temperature observed at the start of a distillation and that observed at which a specified volume has distilled, or at which the dry point is reached.

Initial Boiling Point  The temperature indicated by the distillation thermometer at the instant the first drop of condensate leaves the end of the condenser tube.

Dry Point  The temperature indicated at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, disregarding any liquid on the side of the flask.

Apparatus

Distillation Flask  A 200-mL round-bottom distilling flask of heat-resistant glass is preferred when sufficient sample (in excess of 100 mL) is available for the test. If a sample of less than 100 mL must be used, a smaller flask having a capacity of at least double the volume of the liquid taken may be employed. The 200-mL flask has a total length of 17 to 19 cm, and the inside diameter of the neck is 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side arm 10 to 12.7 cm long and 5 mm in internal diameter, which forms an angle of 70° to 75° with the lower portion of the neck.

Condenser  Use a straight glass condenser of heat-resistant tubing, 56 to 60 cm long and equipped with a water jacket so that about 40 cm of the tubing is in contact with the cooling medium. The lower end of the condenser may be bent to provide a delivery tube or it may be connected to a bent adapter that serves as the delivery tube.

[Note—All-glass apparatus with standard-taper ground joints may be used alternatively if the assembly employed provides results equal to those obtained with the flask and condenser described above.]

Receiver  The receiver is a 100-mL cylinder that is graduated in 1-mL subdivisions and calibrated “to contain.” It is used for measuring the sample as well as for receiving the distillate.

Thermometer  An accurately standardized partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended to avoid the necessity for an emergent stem correction. Suitable thermometers are available as the ASTM E1 Series 37C through 41C, and 102C through 107C, or as the MCA types R-1 through R-4 (see Thermometers, Appendix I).
Source of Heat  A Bunsen burner is the preferred source of heat. An electric heater may be used, however, if it is shown to give results comparable to those obtained with the gas burner.

Shield  The entire burner and flask assembly should be protected from external air currents. Any efficient shield may be employed for this purpose.

Flask Support  A heat-resistant board, 5 to 7 mm in thickness and having a 10-cm circular hole, is placed on a suitable ring or platform support and fitted loosely inside the shield to ensure that hot gases from the source of heat do not come in contact with the sides or neck of the flask. A second 5- to 7-mm thick heat-resistant board, 14- to 16-cm square and provided with a 30- to 40-mm circular hole, is placed on top of the first board. This board is used to hold the 200-mL distillation flask, which should be fitted firmly on the board so that direct heat is applied to the flask only through the opening in the board.

Procedure  
[Note—For materials boiling below 50°, cool the liquid to below 10° before sampling, receive the distillate in a water bath cooled to below 10°, and use water cooled to below 10° in the condenser.]

Measure 100 ± 0.5 mL of the liquid in the 100-mL graduate, and transfer the sample, together with an efficient antibumping device, into the distilling flask. Do not use a funnel in the transfer or allow any of the sample to enter the side arm of the flask. Place the flask on the heat-resistant boards, which are supported on a ring or platform, and position the shield for the flask and burner. Connect the flask and condenser, place the graduate under the outlet of the condenser tube, and insert the thermometer. The thermometer should be located in the center of the neck so that the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side arm. Regulate the heating so that the first drop of liquid is collected within 5 to 10 min. Read the thermometer at the instant the first drop of distillate falls from the end of the condenser tube, and record as the initial boiling point. Continue the distillation at the rate of 4 or 5 mL of distillate per minute, noting the temperature as soon as the last drop of liquid evaporates from the bottom of the flask (dry point) or when the specified percentage has distilled over. Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm) by allowing 0.1° for each 2.7 mm of variation, adding the correction if the pressure is lower, or subtracting if higher, than 760 mm.

When a total-immersion thermometer is used, correct for the temperature of the emergent stem by the formula

\[
\text{Result} = 0.00015 \times N(T - t)
\]

in which N represents the number of degrees of emergent stem from the bottom of the stopper, T represents the observed temperatures of the distillation, and t represents the temperature registered by an auxiliary thermometer, the bulb of which is placed midway of the emergent stem, adding the correction to the observed readings of the main thermometer.

MELTING RANGE OR TEMPERATURE  
For purposes of the FCC, the melting range or temperature of a solid is defined as those points of temperature within which or the point at which the solid coalesces and is completely melted when determined as directed below. Any apparatus or method capable of equal accuracy may be used. The accuracy should be checked frequently by the use of one or more of the six USP Melting Point Reference Standards, preferably the one that melts nearest the melting temperature of the compound to be tested.
Five procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for Class I.

The procedure known as the mixed melting point determination, whereby the melting range of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture usually constitutes reliable evidence of chemical identity.

**Apparatus** The melting range apparatus consists of a glass container for a bath of colorless fluid, a suitable stirring device, an accurate thermometer (see Appendix I), and a controlled source of heat. The bath fluid is selected consistent with the temperature required, but light paraffin is used generally, and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied electrically or by an open flame. The capillary tube is about 10 cm long, with an internal diameter of 0.8 to 1.2 mm, and with walls 0.2 to 0.3 mm thick.

The thermometer is preferably one that conforms to the specifications provided under Thermometers, Appendix I, selected for the desired accuracy and range of temperature.

**Procedure for Class I** Reduce the sample to a very fine powder, and unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or when the substance contains no water of hydration, dry it over a suitable desiccant for 16 to 24 h.

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube 2.5 to 3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until a temperature approximately 30°C below the expected melting point is reached, attach the capillary tube to the thermometer, and adjust its height so that the material in the capillary is level with the thermometer bulb. Return the thermometer to the bath, continue the heating, with constant stirring, at a rate of rise of approximately 3°C/min until a temperature 3°C below the expected melting point is attained, then carefully regulate the rate to about 1°C to 2°C/min until melting is complete.

The temperature at which the column of the sample is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of melting. The two temperatures fall within the limits of the melting range.

**Procedure for Class Ia** Prepare the sample and charge the capillary glass tube as directed for Class I. Heat the bath until a temperature 10°C ± 1°C below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3°C ± 0.5°C/min until melting is complete. Record the melting range as for Class I.

**Procedure for Class Ib** Place the sample in a closed container, and cool to 10°C or lower for at least 2 h. Without previous powdering, charge the cooled material into the capillary tube as directed for Class I, immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm Hg for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube. As soon as is practicable, proceed with the determination of the melting range as follows: Heat the bath until a temperature of 10°C ± 1°C below the expected melting range is reached, then introduce the charged tube, and heat at a rate
of rise of $3^\circ \pm 0.5^\circ$/min until melting is complete. Record the melting range as directed in Class I.

If the particle size of the material is too large for the capillary, precool the sample as directed above, then with as little pressure as possible, gently crush the particles to fit the capillary, and immediately charge the tube.

**Procedure for Class II** Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube that is left open at both ends to a depth of about 10 mm. Cool the charged tube at $10^\circ$ or lower, for 24 h, or in contact with ice for at least 2 h. Then attach the tube to the thermometer by means of a rubber band, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for Class I, except within $5^\circ$ of the expected melting temperature, regulate the rate of rise of temperature to $0.5^\circ$ to $1.0^\circ$/min. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

**Procedure for Class III** Melt a quantity of the substance slowly, while stirring, until it reaches a temperature of $90^\circ$ to $92^\circ$. Remove the source of heat, and allow the molten substance to cool to a temperature of $8^\circ$ to $10^\circ$ above the expected melting point. Chill the bulb of an ASTM 14C thermometer (see Appendix I) to $5^\circ$, wipe it dry, and while it is still cold, dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 min into a water bath having a temperature not higher than $16^\circ$.

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about $16^\circ$, and raise the temperature of the bath at the rate of $2^\circ$/min to $30^\circ$, then change to a rate of $1^\circ$/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the sample. If the variation of three determinations is less than $1^\circ$, take the average of the three as the melting point. If the variation of three determinations is greater than $1^\circ$, make two additional determinations and take the average of the five.

**OPTICAL (SPECIFIC) ROTATION**

Many chemicals in a pure state or in solution are optically active in the sense that they cause incident polarized light to emerge in a plane forming a measurable angle with the plane of the incident light. When this effect is large enough for precise measurement, it may serve as the basis for an assay or an identity test. In this connection, the optical rotation is expressed in degrees, as either *angular rotation* (observed) or *specific rotation* (calculated with reference to the specific concentration of 1 g of solute in 1 mL of solution, measured under stated conditions).

Specific rotation of a liquid substance usually is expressed by the equation $[\alpha]_t^\lambda = a/ld$, and for solutions of solid substances, expressed by the equation $[\alpha]_t^\lambda = 100a/lpd = 100a/lc$, in which $a$ is the corrected observed rotation, in degrees, at temperature $t$; $x$ is the wavelength of the light used; $l$ is the length of the polarimeter cell, in dm; $d$ is the specific gravity of the liquid or solution at the temperature of observation; $p$ is the concentration of the solution expressed as the number of grams of substance in 100 g of solution; and $c$ is the concentration of the solution expressed as the number of grams of substance in 100 mL of solution. The concentrations $p$ and $c$
should be calculated on the dried or anhydrous basis, unless otherwise specified. Spectral lines most frequently employed are the D line of sodium (doublet at 589.0 and 589.6 nm) and the yellow-green line of mercury at 546.1 nm. The specific gravity and the rotatory power vary appreciably with the temperature.

The accuracy and precision of optical rotatory measurements will be increased if they are carried out with due regard for the following general considerations.

Supplement the source of illumination with a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1-nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably colored liquids may be employed as filters (see also A. Weissberger and B. W. Rossiter, Techniques of Chemistry, Vol. I: Physical Methods of Chemistry, Part 3, Wiley-Interscience, New York, 1972).

Pay special attention to temperature control of the solution and of the polarimeter. Make accurate and reproducible observations to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), calculated in terms of either specific rotation or angular rotation, whichever is appropriate, do not exceed one-fourth of the range given in the individual monograph for the rotation of the article being tested. Generally, a polarimeter accurate to 0.05° of angular rotation, and capable of being read with the same precision, suffices for FCC purposes; in some cases, a polarimeter accurate to 0.01°, or less, of angular rotation, and read with comparable precision, may be required.

Fill polarimeter tubes in such a way as to avoid creating or leaving air bubbles, which interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, tubes of uniform bore, such as semimicro- or micro-tubes, require care for proper filling. At the time of filling, the tubes and the liquid or solution should be at a temperature not higher than that specified for the determination to guard against the formation of a bubble upon cooling and contraction of the contents.

In closing tubes having removable end plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end plate and the body of the tube. Excessive pressure on the end plate may set up strains that result in interference with the measurements. In determining the specific rotation of a substance of low rotatory power, loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero point. Differences arising from end plate strain thus generally will be revealed and appropriate adjustments to eliminate the cause may be made.

Procedure In the case of a solid, dissolve the substance in a suitable solvent, reserving a separate portion of the latter for a blank determination. Make at least five readings of the rotation of the solution, or of the substance itself if liquid, at 25° or the temperature specified in the individual monograph. Replace the solution with the reserved portion of the solvent (or, in the case of a liquid, use the empty tube), make the same number of readings, and use the average as the zero point value. Subtract the zero point value from the average observed rotation if the two figures are of the same sign, or add if opposite in sign, to obtain the corrected observed rotation.

Calculation Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

1. for liquid substances,
\[
\left[ \alpha \right]_x^t = \frac{a}{ld}
\]

2. for solutions of solids,
\[
\left[ \alpha \right]_x^t = 100a/lpd = 100a/lc
\]

in which \( a \) is the corrected observed rotation, in degrees, at temperature \( t \); \( x \) is the wavelength of the light used; \( l \) is the length, in dm, of the polarimeter cell; \( d \) is the specific gravity of the liquid or solution at the temperature of observation; \( p \) is the concentration of the solution expressed as the number of grams of substance in 100 g of solution; and \( c \) is the concentration of the solution expressed as the number of grams of substance in 100 mL of solution. The concentrations \( p \) and \( c \) should be calculated on the dried or anhydrous basis, unless otherwise specified.

**pH DETERMINATION**

**Principle**  The definition of pH is the negative log of the hydrogen ion concentration in moles per liter of aqueous solutions. Measure pH potentiometrically by using a pH meter or colorimetrically by using pH indicator paper.

**Scope**  This method is suitable to determine the pH of aqueous solutions. While pH meters, calibrated with aqueous solutions, are sometimes used to make measurements in semiaqueous solutions or in nonaqueous polar solutions, the value obtained is the apparent pH value only and should not be compared with the pH of aqueous solutions. For nonpolar solutions, pH has no meaning, and pH electrodes may be damaged by direct contact with these solutions. References to the pH of nonpolar solutions or liquids usually indicate the pH of a water extract of the nonpolar liquid or the apparent pH of a mixture of the nonpolar liquid in a polar liquid such as alcohol or alcohol–water mixtures.

**Procedure [Potentiometric Method (pH Meter)]**

**Calibration**  Select two standard buffers to bracket, if possible, the anticipated pH of the unknown substances. These commercially available standards and the sample should be at the same temperature, within \( 2^\circ \). Set the temperature compensator of the pH meter to the temperature of the samples and standards. Follow the manufacturer's instructions for setting temperature compensation and for adjusting the output during calibration. Rinse the electrodes with distilled or deionized water, and blot them dry with clean, absorbent laboratory tissue. Place the electrode(s) in the first standard buffer solution, and adjust the standardization control so that the pH reading matches the stated pH of the standard buffer. Repeat this procedure with fresh portions of the first buffer solution until two successive readings are within \( \pm 0.02 \) pH units with no further adjustment. Rinse the electrodes, blot them dry, and place them in a portion of the second standard buffer solution. Following the manufacturer's instructions, adjust the slope control (not the standardization control) until the output displays the pH of the second standard buffer.

Repeat the sequence of standardization with both buffers until pH readings are within \( \pm 0.02 \) pH units for both buffers without adjustments to either the slope or standardization controls. The pH of the unknown may then
be measured, using either a pH electrode in combination with a reference electrode or a single combination electrode. Select electrodes made of chemically resistant glass when measuring samples of either low or high pH.

**pH Indicator Paper**  Test papers impregnated with acid–base indicators, although less accurate than pH meters, offer a convenient way to determine the pH of an aqueous solution. They may be purchased in rolls or strips covering all or part of the pH range; papers covering a narrow part of the pH range can be sensitive to differences of 0.2 pH units. Some test papers comprise a plastic strip with small squares of test paper attached. The different squares are sensitive to different pH ranges. When using this type of test paper, wet all of the squares with the test sample to ensure a correct pH reading.

Test paper can contaminate the sample being tested; therefore, do not dip it into the sample. Either use a clean glass rod to remove a drop of the test solution and place it on the test paper, or transfer a small amount of the sample to a small container, dip the test paper into this portion, and compare the developed color with the color comparison chart provided with the test paper to determine the pH of the sample.

### READILY CARBONIZABLE SUBSTANCES

**Reagents**

**Sulfuric Acid, 95%**  Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to between 94.5% and 95.5% of \( \text{H}_2\text{SO}_4 \). Because the acid concentration may change upon standing or upon intermittent use, check the concentration frequently and either adjust solutions assaying more than 95.5% or less than 94.5% by adding either diluted or fuming sulfuric acid, as required, or discard them.

**Cobaltous Chloride CS**  Dissolve about 65 g of cobaltous chloride (CoCl\(_2\)·6H\(_2\)O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 5 mL of this solution into a 250-mL iodine flask, add 5 mL hydrogen peroxide TS (3%) and 15 mL of a 1:5 solution of sodium hydroxide, boil for 10 min, cool, and add 2 g of potassium iodide and 20 mL of 1:4 sulfuric acid. When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate. The titration is sensitive to air oxidation and should be blanketed with carbon dioxide. Each milliliter of 0.1 N sodium thiosulfate is equivalent to 23.79 mg of CoCl\(_2\)·6H\(_2\)O. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each milliliter contains 59.5 mg of CoCl\(_2\)·6H\(_2\)O.

**Cupric Sulfate CS**  Dissolve about 65 g of cupric sulfate (CuSO\(_4\)·5H\(_2\)O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 40 mL of water, 4 mL of acetic acid, and 3 g of potassium iodide; and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Each milliliter of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of CuSO\(_4\)·5H\(_2\)O. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each milliliter contains 62.4 mg of CuSO\(_4\)·5H\(_2\)O.

**Ferric Chloride CS**  Dissolve about 55 g of ferric chloride (FeCl\(_3\)·6H\(_2\)O) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 15 mL of water, 5 mL of hydrochloric acid, and 3 g of potassium iodide; and allow the mixture to stand for 15 min. Dilute with 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate,
adding starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents and in the same manner, and make any necessary correction. Each milliliter of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of FeCl$_3$·6H$_2$O. Adjust the final volume of the solution by adding the mixture of hydrochloric acid and water so that each milliliter contains 45.0 mg of FeCl$_3$·6H$_2$O.

Platinum–Cobalt CS Transfer 1.246 g of potassium chloroplatinate (K$_2$PtCl$_6$) and 1.00 g of crystallized cobaltous chloride (CoCl$_2$·6H$_2$O) into a 1000-mL volumetric flask, dissolve in about 200 mL of water and 100 mL of hydrochloric acid, dilute with water to volume, and mix. This solution has a color of 500 APHA units. [Note—Use this solution only when specified in an individual monograph.]

Procedure Unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of 95% Sulfuric Acid.

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 min, unless otherwise directed, and compare the color of the solution with that of the specified matching fluid in a comparison container that also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed to effect solution of the substance in the 95% Sulfuric Acid, mix the sample and the acid in a test tube, heat as directed, cool, and transfer the solution to the comparison container for matching.

Matching Fluids For purposes of comparison, a series of 20 matching fluids, each designated by a letter of the alphabet, is provided, the composition of each being as indicated in the accompanying table. To prepare the matching fluid specified, pipet the prescribed volumes of the colorimetric test solutions (CS) and water into one of the matching containers, and mix the solutions in the container.
### Matching Fluids

<table>
<thead>
<tr>
<th>Matching Fluid</th>
<th>Parts of Cobaltous Chloride CS</th>
<th>Parts of Ferric Chloride CS</th>
<th>Parts of Cupric Sulfate CS</th>
<th>Parts of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>B</td>
<td>0.3</td>
<td>0.9</td>
<td>0.3</td>
<td>8.5</td>
</tr>
<tr>
<td>C</td>
<td>0.1</td>
<td>0.6</td>
<td>0.1</td>
<td>4.2</td>
</tr>
<tr>
<td>D</td>
<td>0.3</td>
<td>0.6</td>
<td>0.4</td>
<td>3.7</td>
</tr>
<tr>
<td>E</td>
<td>0.4</td>
<td>1.2</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>F</td>
<td>0.3</td>
<td>1.2</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>G</td>
<td>0.5</td>
<td>1.2</td>
<td>0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>H</td>
<td>0.2</td>
<td>1.5</td>
<td>0.0</td>
<td>3.3</td>
</tr>
<tr>
<td>I</td>
<td>0.4</td>
<td>2.2</td>
<td>0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>J</td>
<td>0.4</td>
<td>3.5</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>K</td>
<td>0.5</td>
<td>4.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L</td>
<td>0.8</td>
<td>3.8</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>M</td>
<td>0.1</td>
<td>2.0</td>
<td>0.1</td>
<td>2.8</td>
</tr>
<tr>
<td>N</td>
<td>0.0</td>
<td>4.9</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>O</td>
<td>0.1</td>
<td>4.8</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>P</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Q</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>4.4</td>
</tr>
<tr>
<td>R</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>4.1</td>
</tr>
<tr>
<td>S</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
<td>4.7</td>
</tr>
<tr>
<td>T</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>3.6</td>
</tr>
</tbody>
</table>


---

**REFRACTIVE INDEX**

The refractive index of a transparent substance is the ratio of the velocity of light in air to its velocity in that material under like conditions. It is equal to the ratio of the sine of the angle of incidence made by a ray in air to the sine of the angle of refraction made by the ray in the material being tested. The refractive index values specified in this Codex are for the D line of sodium (589 nm) unless otherwise specified. The determination should be made at the temperature specified in the individual monograph, or at 25° if no temperature is specified. This physical constant is used as a means for identification of, and detection of impurities in, volatile oils and other liquid substances. The Abbé refractometer, or other refractometers of equal or greater accuracy, may be employed at the discretion of the operator.
**SOLIDIFICATION POINT**

**Scope** This method is designed to determine the solidification point of food-grade chemicals having appreciable heats of fusion. It is applicable to chemicals having solidification points between $-20^\circ$ and $+150^\circ$. Necessary modifications will be noted in individual monographs.

**Definition** Solidification Point is an empirical constant defined as the temperature at which the liquid phase of a substance is in approximate equilibrium with a relatively small portion of the solid phase. It is measured by noting the maximum temperature reached during a controlled cooling cycle after the appearance of a solid phase.

The solidification point is distinguished from the freezing point in that the latter term applies to the temperature of equilibrium between the solid and liquid state of pure compounds.

Some chemical compounds have more than one temperature at which there may be an equilibrium between the solid and liquid state depending on the crystal form of the solid that is present.

**Apparatus** The apparatus illustrated in Figs. 3 and 4 consists of the components described in the following paragraphs.

![FIGURE 3 Apparatus for Determination of Solidification Point.](image)
Thermometer  A thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for 76-mm immersion should be employed. A satisfactory series of thermometers, covering a range from −20° to +150°, is available as ASTM-E1 89C through 96C (see Thermometers, Appendix I). A thermometer should be chosen such that the solidification point is not obscured by the cork stopper of the sample container.

Sample Container  Use a standard glass 25- × 150-mm test tube with a lip, fitted with a two-hole cork stopper to hold the thermometer in place and to allow adequate stirring with a stirrer.

Air Jacket  For the air jacket, use a standard glass 38- × 200-mm test tube with a lip and fitted with a cork or rubber stopper bored with a hole into which the sample container can easily be inserted up to the lip.

Cooling Bath  Use a 2000-mL beaker or a similar, suitable container as a cooling bath. Fill it with an appropriate cooling medium such as glycerin, mineral oil, water, water and ice, or alcohol–dry ice.

Stirrer  The stirrer (Fig. 4) consists of a 1-mm in diameter (B & S gauge 18), corrosion-resistant wire bent into a series of three loops about 25 mm apart. It should be made so that it will move freely in the space between the thermometer and the inner wall of the sample container. The shaft of the stirrer should be of a convenient length designed to pass loosely through a hole in the cork holding the thermometer. Stirring may be hand operated or mechanically activated at 20 to 30 strokes/min.

Assembly  Assemble the apparatus in such a way that the cooling bath can be heated or cooled to control the desired temperature ranges. Clamp the air jacket so that it is held rigidly just below the lip, and immerse it in the cooling bath to a depth of 160 mm.

Sample Preparation  The solidification point of chemicals is usually determined as they are received. Some may be hygroscopic, however, and will require special drying. If this is necessary, it will be noted in the individual monographs.
Products that are normally solid at room temperature must be carefully melted at a temperature about 10° above the expected solidification point. Care should be observed to avoid heating in such a way as to decompose or distill any portion of a sample.

**Procedure**  Adjust the temperature of the cooling bath to about 5° below the expected solidification point. Fit the thermometer and stirrer with a cork stopper so that the thermometer is centered and the bulb is about 20 mm from the bottom of the sample container. Transfer a sufficient amount of the sample, previously melted if necessary, into the sample container to fill it to a depth of about 90 mm when in the molten state. Place the thermometer and stirrer in the sample container, and adjust the thermometer so that the immersion line will be at the surface of the liquid and so that the end of the bulb is 20 ± 4 mm from the bottom of the sample container. When the temperature of the sample is about 5° above the expected solidification point, place the assembled sample tube in the air jacket.

Allow the sample to cool while stirring, at the rate of 20 to 30 strokes/min, in such a manner that the stirrer does not touch the thermometer. Stir the sample continuously during the remainder of the test.

The temperature at first will gradually fall, then will become constant as crystallization starts and continues under equilibrium conditions, and finally will start to drop again. Some chemicals may supercool slightly below (0.5°) the solidification point; as crystallization begins, the temperature will rise and remain constant as equilibrium conditions are established. Other products may cool more than 0.5° and cause deviation from the normal pattern of temperature change. If the temperature rise exceeds 0.5° after the initial crystallization begins, repeat the test, and seed the melted compound with small crystals of the sample at 0.5° intervals as the temperature approaches the expected solidification point. Crystals for seeding may be obtained by freezing a small sample in a test tube directly in the cooling bath. It is preferable that seed of the stable phase be used from a previous determination.

Observe and record the temperature readings at regular intervals until the temperature rises from a minimum, due to supercooling, to a maximum and then finally drops. The maximum temperature reading is the solidification point. Readings 10 s apart should be taken to establish that the temperature is at the maximum level and should continue until the drop in temperature is established.

**VISCOSITY**

Viscosity is a fluid’s measured internal resistance to flow. Thick, slow-moving fluids have higher viscosities than thin, free-flowing fluids. The basic unit of measure for viscosity is the poise or Pascal second, Pa·s, in SI units. The relationship between poise and Pa·s is 1 poise = 0.1 Pa·s. Since commonly encountered viscosities are often fractions of 1 poise, viscosities are commonly expressed as centipoises (one centipoise = 0.01 poise). Poise or centipoise is the unit of measure for absolute viscosity. Kinematic viscosity also is commonly used and is determined by dividing the absolute viscosity of the test liquid by the density of the test liquid at the same temperature as the viscosity measurement and is expressed as stokes or centistokes (poise/density = stokes). The specified temperature is important: viscosity varies greatly with temperature, generally decreasing with increasing temperature.

Absolute viscosity can be determined directly if accurate dimensions of the measuring instruments are known. It is common practice to calibrate an instrument with a fluid of known viscosity and to determine the unknown viscosity of another fluid by comparison with that of the known viscosity.
Many substances, such as gums, have a variable viscosity, and most of them are less resistant to flow at higher flow (more correctly, shear) rates. In such cases, select a given set of conditions for measurement, and consider the measurement obtained to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the operator must closely adhere to the instrument dimensions and conditions for measurement.

**Measuring Viscosity** Several common methods are available for measuring viscosity. Two very common ones are the use of capillary tubes such as Ubbelohde, Ostwald, or Cannon-Fenske viscometer tubes and the use of a rotating spindle such as the Brookfield viscometer.

Determine the viscosity in capillary tubes by measuring the amount of time it takes for a given volume of liquid to flow through a calibrated capillary tube. Calibrate the capillary tube by using liquids of known viscosity. The calibration may be supplied with the viscometer tube when purchased along with specific instructions for its use. Many types of capillary viscometer tubes are available, and exact procedures will vary with the type of tube chosen. Examples of procedures are in the following sections: *Viscosity of Dimethylpolysiloxane* and *Viscosity of Methylcellulose*. In general, calibrate capillary viscometers by filling the viscometers per the manufacturer’s instructions and allowing the filled tube to equilibrate to the given temperature in a constant-temperature bath. Draw the liquid to the top graduation line, and measure the time, in seconds, it takes for the liquid to flow from the upper mark to the lower mark in the capillary tube. Calculate the viscometer constant, \( k \), by the equation

\[
k = \frac{v}{dt}
\]

in which \( v \) is the known viscosity, in centipoises, of the standard liquid; \( d \) is the density, at the specified temperature, of the liquid; and \( t \) is the time, in seconds, for the liquid to pass from the upper mark to the lower mark. It is not necessary to recalibrate the tube unless changes or repairs are made to it. To measure viscosity, introduce the unknown liquid into the viscometer tube in the same way as the calibration standard was introduced, and measure the time, in seconds, it takes for the liquid to flow from the upper mark to the lower mark. Calculate viscosity by the equation

\[
v = kdt
\]

in which \( v \) is the viscosity to be determined, \( k \) is the viscometer constant, and \( d \) is the density of the liquid being measured.

Using rotational viscometers provides a particularly rapid and convenient method for determining viscosity. They employ a rotating spindle or cup immersed in the liquid, and they measure the resistance of the liquid to the rotation of the spindle or cup. A wide range of viscosities can be measured with one instrument by using spindles or cups of different sizes and by rotating them at different speeds. The manufacturer supplies the calibration of viscosity versus the spindle size and speed, which can be checked by using fluids of known viscosity. Take a measurement by allowing the sample to come to the desired temperature in a constant-temperature bath and immersing the spindle or cup to the depth specified by the manufacturer. Allow the spindle or cup to rotate until a constant reading is obtained. Multiply the reading by a factor supplied by the manufacturer for a given spindle or cup and given rotational speed to obtain the viscosity. The exact procedures will vary with the particular instrument. An example is given in the section on *Viscosity of Cellulose Gum*.

Another method to determine viscosity uses the falling-ball viscometer. Determine viscosity by noting the time it takes for a ball to fall through the distance between two marks on a tube filled with the unknown liquid (the tube is generally in a constant-temperature bath). Use balls of different weights to measure a wide range of viscosities. Calculate the viscosity by using manufacturer-supplied constants for the ball used. These
instruments can be quite precise for Newtonian liquids, that is, liquids that do not have viscosities that vary with flow (more correctly, shear) rate.

Three specific methods are described below:

**Viscosity of Dimethylpolysiloxane**

**Apparatus** The Ubbelohde suspended level viscometer, shown in Fig. 5 is preferred to determine the viscosity of dimethylpolysiloxane. Alternatively, a Cannon-Ubbelohde viscometer may be used.

Select a viscometer having a minimum flow time of at least 200 s. Use a No. 3 size Ubbelohde, or a No. 400 size Cannon-Ubbelohde, viscometer for the range of 300 to 600 centistokes. The viscometer should be fitted with holders that satisfy the dimensional positions of the separate tubes as shown in the diagram and that hold the viscometer vertically. Filling lines in bulb A indicate the minimum and maximum volumes of liquid to be used for convenient operation. The volume of bulb B is approximately 5 mL.

**Calibration of the Viscometer** Determine the viscosity constant, C, for each viscometer by using an oil of known viscosity. Charge the viscometer by tilting the instrument about 30 degrees from the vertical, with bulb A below the capillary, and then introduce enough of the sample into tube I to bring the level up to the lower filling line. The level should not be above the upper filling line when the viscometer is returned to the vertical position and the sample has drained from tube I. Charge the viscometer in such a manner that the U-tube at the bottom fills completely without trapping air.

After the viscometer has been in a constant-temperature bath (25° ± 0.2°) long enough for the sample to reach temperature equilibrium, place a finger over tube 3, and apply suction to tube 2 until the liquid reaches the center of bulb C. Remove suction from tube 2, then remove the finger from tube 3, and place it over tube 2 until the sample drops away from the lower end of the capillary. Remove the finger from tube 2, and measure the time, to the nearest 0.1 s, required for the meniscus to pass from the first timing mark (T₁) to the second (T₂).

Calculate the viscometer constant, C, by the equation

\[ C = \frac{c_s t_1}{t_1} \]

in which \( c_s \) is the viscosity, in centistokes, and \( t_1 \) is the efflux time, in seconds, for the standard liquid.
Determination of the Viscosity of Dimethylpolysiloxane

Charge the viscometer with the sample in the same manner as described for the calibration procedure; determine the efflux time, $t_2$; and calculate the viscosity of the dimethylpolysiloxane by the formula

$$V = C \times t_2$$

Viscosity of Methylcellulose

Apparatus

Viscometers used to determine the viscosity of methylcellulose and some related compounds are illustrated in Fig. 6 and consist of three parts: a large filling tube, A; an orifice tube, B; and an air vent to the reservoir, C.

Calibration of the Viscometer

Determine the viscometer constant, $K$, for each viscometer by using an oil of known viscosity. Place an excess of the liquid that is to be tested (adjusted to $20^\circ \pm 0.1^\circ$) in the filling tube, A, and transfer it to the orifice tube, B, by gentle suction, taking care to keep the liquid free from air bubbles by closing the air vent tube, C. Adjust the column of liquid in tube B so it is even with the top graduation line. Open both tubes B and C to permit the liquid to flow into the reservoir against atmospheric pressure. [Note—Failure to open air vent tube C before determining the viscosity will yield false values.] Record the time, in seconds, for the liquid to flow from the upper mark to the lower mark in tube B.

Calculate the viscometer constant, $K$, from the equation

$$K = \frac{V}{d \times t}$$

in which $V$ is the viscosity, in centipoises, of the liquid; $K$ is the viscometer constant; $d$ is the specific gravity of the liquid tested at $20^\circ /20^\circ$; and $t$ is the time, in seconds, for the liquid to pass from the upper to the lower mark.
For the calibration, all values in the equation are known or can be determined except \( K \), which must be solved. If a tube is repaired, it must be recalibrated to avoid obtaining significant changes in the value of \( K \).

**Determination of the Viscosity of Methylcellulose** Prepare a 2% solution of methylcellulose or other cellulose derivative, by weight, as directed in the monograph. Place the solution in the proper viscometer and determine the time, \( t \), required for the solution to flow from the upper mark to the lower mark in orifice tube B. Separately determine the specific gravity, \( d \), at 20\(^\circ\)/20\(^\circ\). Viscosity, \( V = Kdt \).

**Viscosity of Cellulose Gum**

**Apparatus** Use a Brookfield Model LV series viscometer, analog or digital, or equivalent type viscometer for the determination of viscosity of aqueous solutions of cellulose gum within the range of 25 to 10,000 centipoises at 25\(^\circ\). Rotational viscometers of this type have spindles for use in determining the viscosity of different viscosity types of cellulose gum. The spindles and speeds for determining viscosity within different ranges are tabulated below.

<table>
<thead>
<tr>
<th>Viscosity Range (centipoises)</th>
<th>Spindle No.</th>
<th>Speed (rpm)</th>
<th>Scale</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>10–100</td>
<td>1</td>
<td>60</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>100–200</td>
<td>1</td>
<td>30</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>200–1000</td>
<td>2</td>
<td>30</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>1000–4000</td>
<td>3</td>
<td>30</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>4000–10,000</td>
<td>4</td>
<td>30</td>
<td>100</td>
<td>200</td>
</tr>
</tbody>
</table>

**Mechanical Stirrer** Use an agitator essentially as shown in Fig. 7 that can be attached to a variable-speed motor capable of operating at 900 ± 100 rpm under varying load conditions.

![FIGURE 7 Agitator for Viscosity of Cellulose Gum.](image)

[Note—The agitator may be fabricated from stainless steel (Hercules, Inc., Wilmington, Delaware, or...
equivalent.) or glass as shown in Fig. 7. Where this procedure is specified for viscosity measurements by reference in other monographs, equivalent three-blade agitators may be used.

**Sample Container**  Use a glass jar about 152 mm deep having an od of approximately 64 mm and a capacity of about 340 g.

**Water Bath**  Use a water bath capable of maintaining a constant temperature. Set the temperature to 25°, and maintain it within ±0.2°.

**Thermometer**  Use an ASTM Saybolt Viscosity Thermometer having a range from 19° to 27° and conforming to the requirements for Thermometer 17C as described in ASTM Specification E1.

**Sample Preparation**  Accurately weigh an amount of sample equivalent to 4.8 g of cellulose gum on the dried basis, and record the actual quantity required, in grams, as S. Transfer an accurately measured volume of water equivalent to 240 − S g into the sample container. Position the stirrer in the sample container, allowing minimal clearance between the stirrer and the bottom of the container. Begin stirring, and slowly add the sample. Adjust the stirring speed to approximately 900 ± 100 rpm. Mix for exactly 2 h. Do not allow the stirring speed to exceed 1200 rpm. Remove the stirrer, cap the sample container, and transfer the sample container into a constant-temperature water bath, maintained at 25° ± 0.2°, for 1 h. Check the sample temperature with a thermometer at the end of 1 h to ensure that the test temperature has been reached.

**Procedure**  Remove the sample container from the water bath, shake vigorously for 10 s, and measure the viscosity with the Brookfield viscometer, using the proper spindle and speed indicated in the accompanying table. Be sure to use the viscometer guard, and allow the spindle to rotate for 3 min before taking the reading. Calculate the viscosity, in centipoises, by multiplying the reading observed by the appropriate factor from the table.

**WATER DETERMINATION**

**Method I (Karl Fischer Titrimetric Method)**  Determine the water by Method Ia, unless otherwise specified in the individual monograph.

**Method Ia (Direct Titration)**

**Principle**  The titrimetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. Pyridine-free reagents are more commonly used now. The test specimen may be titrated with the Karl Fischer Reagent directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of the determination depends on such factors as the relative concentrations of the Karl Fischer Reagent ingredients, the nature of the inert solvent used to dissolve the test specimen, the apparent pH of the final mixture, and the technique used in the particular determination. Therefore, an empirically standardized technique is used to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens.
Substances that may interfere with the test results are ferric ion, chlorine, and similar oxidizing agents, as well as significant amounts of strong acids or bases, phosgene, or anything that will reduce iodide to iodine, poison the reagent, and show the sample to be bone dry when water may be present (false negative). Hydroxyquinoline may be added to the vessel to eliminate interference from ferric ion. Chlorine interference can be eliminated with SO\(_2\) or unsaturated hydrocarbon. Excess pyridine or other amines may be added to the vessel to eliminate the interference of strong acids. Excess acetic acid or other carboxylic acid can be added to reduce the interference of strong bases. Aldehydes and ketones may react with the solution, showing the sample to be wet while the detector never reaches an endpoint (false positive).

**Apparatus** Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and for determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 mm\(^2\) in area and about 2.5 cm apart) immersed in the solution to be titrated. At the endpoint of the titration, a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending on the solution being titrated. The time is shortest for substances that dissolve in the reagent. The longer times are required for solid materials that do not readily go into solution in the Karl Fischer Reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. A commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or a current of dry air.

**Reagent** The Karl Fischer Reagent may be prepared as follows: Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One milliliter of this solution, when freshly prepared, is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily in continual use. Protect the solution from light while in use. Store any bulk stock of the solution in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration.

A commercially available, stabilized solution of a Karl Fischer-type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol also may be used. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted Karl Fischer Reagent called for in some monographs should be diluted as directed by the manufacturer. Either methanol, or another suitable solvent such as ethylene glycol monomethyl ether, may be used as the diluent.

**Test Preparation** Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10 to 250 mg of water.

Where the monograph specifies that the specimen under test is hygroscopic, accurately weigh a sample of the specimen into a suitable container. Use a dry syringe to inject an appropriate volume of methanol, or other
suitable solvent, accurately measured, into the container and shake to dissolve the specimen. Dry the syringe, and use it to remove the solution from the container and transfer it to a titration vessel prepared as directed under Procedure. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed under Standardization of Water Solution for Residual Titration, and subtract this value from the water content, in milligrams, obtained in the titration of the specimen under test.

Standardization of the Reagent  Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient Karl Fischer Reagent to give the characteristic color or 100 ± 50 microamperes of direct current at about 200 mV of applied potential. Pure methanol can make the detector overly sensitive, particularly at low ppm levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar nonconducting solvent will retard this sensitivity and can improve the analysis.

For determination of trace amounts of water (less than 1%), quickly add 25 µL (25 mg) of pure water, using a 25- or 50-µL syringe, and titrate to the endpoint. The water equivalence factor F, in milligrams of water per milliliter of reagent, is given by the formula

\[
\text{Result} = 25/V
\]

in which V is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration.

For the precise determination of significant amounts of water (more than 1%), quickly add between 25 and 250 mg (25 to 250 µL) of pure water, accurately weighed by difference from a weighing pipet or from a precalibrated syringe or micropipet, the amount of water used being governed by the reagent strength and the buret size, as referred to under Volumetric Apparatus. Titrate to the endpoint. Calculate the water equivalence factor, F, in milligrams of water per milliliter of reagent by the formula

\[
\text{Result} = W/V
\]

in which W is the weight, in milligrams, of the water, and V is the volume, in milliliters, of the Karl Fischer Reagent required.

Procedure  Unless otherwise specified, transfer 35 to 40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the Test Preparation, mix, and again titrate with the Karl Fischer Reagent to the electrometric or visual endpoint. Calculate the water content of the specimen, in milligrams, by the formula

\[
\text{Result} = SF
\]

in which S is the volume, in milliliters, of the Karl Fischer Reagent consumed in the second titration, and F is the water equivalence factor of the Karl Fischer Reagent.

Method Ib (Residual Titration)

Principle  See the information in the section entitled Principle under Method Ia. In the residual titration, add excess Karl Fischer Reagent to the test specimen, allow sufficient time for the reaction to reach completion, and titrate the unconsumed Karl Fischer Reagent with a standard solution of water in a solvent such as methanol. The residual titration procedure is generally applicable and avoids the difficulties that may be
encountered in the direct titration of substances from which the bound water is released slowly.

**Apparatus, Reagent, and Test Preparation**  Use those in Method Ia.

**Standardization of Water Solution for Residual Titration**  Prepare a Water Solution by diluting 2 mL of pure water to 1000 mL with methanol or another suitable solvent. Standardize this solution by titrating 25.0 mL with the Karl Fischer Reagent, previously standardized as directed under Standardization of the Reagent. Calculate the water content, in milligrams per milliliter, of the Water Solution with the formula

\[ \text{Result} = \frac{VF}{25} \]

in which \( V \) is the volume of the Karl Fischer Reagent consumed, and \( F \) is the water equivalence factor of the Karl Fischer Reagent. Determine the water content of the Water Solution weekly, and standardize the Karl Fischer Reagent against it periodically as needed. Store the Water Solution in a tightly capped container.

**Procedure**  Where the individual monograph specifies the water content is to be determined by Method Ib, transfer 35 to 40 mL of methanol or other suitable solvent into the titration vessel, and titrate with the Karl Fischer Reagent to the electrometric or visual endpoint. Quickly add the Test Preparation, mix, and add an accurately measured excess of the Karl Fischer Reagent. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed Karl Fischer Reagent with standardized Water Solution to the electrometric or visual endpoint. Calculate the water content of the specimen, in milligrams, with the formula

\[ \text{Result} = F(X - XR) \]

in which \( F \) is the water equivalence factor of the Karl Fischer Reagent; \( X \) is the volume, in milliliters, of the Karl Fischer Reagent added after introduction of the specimen; \( X \) is the volume, in milliliters, of standardized Water Solution required to neutralize the unconsumed Karl Fischer Reagent; and \( R \) is the ratio \( V/25 \) (milliliters of Karl Fischer Reagent/milliliters of Water Solution), determined from the Standardization of Water Solution for Residual Titration.

**Method Ic (Coulometric Titration)**

**Principle**  Use the Karl Fischer reaction in the coulometric determination of water. In this determination, iodine is not added in the form of a volumetric solution, but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with the water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which can be detected potentiometrically, thus indicating the endpoint. Pre-electrolysis, which can take several hours, eliminates moisture from the system. Therefore, changing the Karl Fischer Reagent after each determination is not practical. Individual determinations may be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen be compatible with the other components and that no side reactions take place. Samples may be transferred into the vessel as solids or as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. For the water determination of solids, another common technique is to dissolve the solid in a suitable solvent and then inject a portion of this solution into the cell. In the case of insoluble solids, water may be extracted using suitable solvents, and then the extracts injected into the coulometric cell. Alternatively, an evaporation technique may be used in which the sample is heated in a tube and the water is evaporated and carried into the cell by means of a stream of dry, inert gas. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of
the system may be monitored by measuring the amount of baseline drift. The titration of water in solid test specimens is usually carried out with the use of anhydrous methanol as the solvent. Other suitable solvents may be used for special or unusual test specimens. This method is particularly suited to chemically inert substances such as hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method. The method uses extremely small amounts of current. It is predominantly used for substances with a very low water content (0.1% to 0.0001%).

**Apparatus** Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary as the current consumed can be measured absolutely. Proper operation of the instrument can be confirmed by injecting 1 µL of water into the vessel. The instrument should read 1000 µg of water on reaching the endpoint.

**Reagent** See Reagent under Method Ia.

**Test Preparation** Using a dry syringe, inject an appropriate volume of test specimen estimated to contain 0.5 to 5 mg of water, accurately measured, into the anolyte solution. The sample may also be introduced as a solid, accurately weighed, into the anolyte solution. Perform coulometric titration, and determine the water content of the specimen under test.

Alternatively, when the specimen is a suitable solid, dissolve an appropriate quantity, accurately weighed, in anhydrous methanol or another suitable solvent, and inject a suitable portion into the anolyte solution.

When the specimen is an insoluble solid, extract the water by using a suitable anhydrous solvent from which an appropriate quantity, accurately weighed, may be injected into the anolyte solution. Alternatively use an evaporation technique.

**Procedure** Quickly inject the Test Preparation, or transfer the solid sample, into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the Test Preparation directly from the instrument's display, and calculate the percent that is present in the substance.

**Method II (Toluene Distillation Method)**

**Principle** This method determines water by distillation of a sample with an immiscible solvent, usually toluene.

**Apparatus** Use a glass distillation apparatus (see Fig. 8) provided with 24/40 ground-glass connections. The components consist of a 500-mL short-neck, round-bottom flask connected by means of a trap to a 400-mm water-cooled condenser. The lower tip of the condenser should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established (see Procedure).
The trap should be constructed of well-annealed glass, the receiving end of which is graduated to contain 5 mL and subdivided into 0.1-mL divisions, with each 1-mL line numbered from 5 mL beginning at the top. Calibrate the receiver by adding 1 mL of water, accurately measured, to 100 mL of toluene contained in the distillation flask. Conduct the distillation, and calculate the volume of water obtained as directed in the Procedure. Add another milliliter of water to the cooled apparatus, and repeat the distillation. Continue in this manner until five 1-mL portions of water have been added. The error at any indicated capacity should not exceed 0.05 mL. The source of heat is either an oil bath or an electric heater provided with a suitable means of temperature control. The distillation may be better controlled by insulating the tube leading from the flask to the receiver. It is also advantageous to protect the flask from drafts. Clean the entire apparatus with potassium dichromate-sulfuric acid cleaning solution, rinse thoroughly, and dry completely before using.

Procedure  Place in the previously cleaned and dried flask a quantity of the substance, weighed accurately to the nearest 0.01 g, that is expected to yield from 1.5 to 4 mL of water. If the substance is of a pastelike consistency, weigh it in a boat of metal foil that will pass through the neck of the flask. If the substance is likely to cause bumping, take suitable precautions to prevent it. Transfer about 200 mL of ACS reagent-grade toluene into the flask, and swirl to mix it with the sample. Assemble the apparatus, fill the receiver with toluene by pouring it through the condenser until it begins to overflow into the flask, and insert a loose cotton plug in the top of the condenser. Heat the flask so that the distillation rate will be about 200 drops/min, and continue distilling until the volume of water in the trap remains constant for 5 min. Discontinue the heating, use a copper or nichrome wire spiral to dislodge any drops of water that may be adhering to the inside of the condenser tube or receiver, and wash down with about 5 mL of toluene. Disconnect the receiver, immerse it in water at 25°C for at least 15 min or until the toluene layer is clear, and then read the volume of water. Conduct a blank determination using the same volume of toluene as used when distilling the sample mixture, and make any necessary correction (see General Provisions).
C. OTHERS

ASH (Acid-Insoluble)
Boil the ash obtained as directed under Ash (Total), below, with 25 mL of 2.7 N hydrochloric acid for 5 min, collect the insoluble matter on a tared, porous-bottom porcelain filter crucible or ashless filter, wash it with hot water, ignite to constant weight at $675^\circ \pm 25^\circ$, and weigh. Calculate the percent acid-insoluble ash from the weight of the sample taken.

[Note—Avoid exposing the crucible to sudden temperature changes.]

ASH (Total)
Unless otherwise directed, accurately weigh about 3 g of the sample in a tared crucible, ignite it at a low temperature (about $550^\circ$), not to exceed a very dull redness, until it is free from carbon, cool it in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, collect the insoluble residue on an ashless filter paper, and ignite the residue and filter paper until the ash is white or nearly so. Finally, add the filtrate, evaporate it to dryness, and heat the whole to a dull redness. If a carbon-free ash is still not obtained, cool the crucible, add 15 mL of ethanol, break up the ash with a glass rod, then burn off the ethanol, again heat the whole to a dull redness, cool it in a desiccator, and weigh.

HYDROCHLORIC ACID TABLE

<table>
<thead>
<tr>
<th>°Bé</th>
<th>Sp. Gr.</th>
<th>Percent HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>1.0069</td>
<td>1.40</td>
</tr>
<tr>
<td>2.00</td>
<td>1.0140</td>
<td>2.82</td>
</tr>
<tr>
<td>3.00</td>
<td>1.0211</td>
<td>4.25</td>
</tr>
<tr>
<td>4.00</td>
<td>1.0284</td>
<td>5.69</td>
</tr>
<tr>
<td>5.00</td>
<td>1.0357</td>
<td>7.15</td>
</tr>
<tr>
<td>5.25</td>
<td>1.0375</td>
<td>7.52</td>
</tr>
<tr>
<td>5.50</td>
<td>1.0394</td>
<td>7.89</td>
</tr>
<tr>
<td>5.75</td>
<td>1.0413</td>
<td>8.26</td>
</tr>
<tr>
<td>6.25</td>
<td>1.0450</td>
<td>9.02</td>
</tr>
<tr>
<td>6.50</td>
<td>1.0469</td>
<td>9.40</td>
</tr>
<tr>
<td>6.75</td>
<td>1.0488</td>
<td>9.78</td>
</tr>
<tr>
<td>7.00</td>
<td>1.0507</td>
<td>10.17</td>
</tr>
<tr>
<td>7.25</td>
<td>1.0526</td>
<td>10.55</td>
</tr>
<tr>
<td>7.50</td>
<td>1.0545</td>
<td>10.94</td>
</tr>
<tr>
<td>7.75</td>
<td>1.0564</td>
<td>11.32</td>
</tr>
<tr>
<td>°Bé</td>
<td>Sp. Gr.</td>
<td>Percent HCl</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>8.00</td>
<td>1.0584</td>
<td>11.71</td>
</tr>
<tr>
<td>8.25</td>
<td>1.0603</td>
<td>12.09</td>
</tr>
<tr>
<td>8.50</td>
<td>1.0623</td>
<td>12.48</td>
</tr>
<tr>
<td>8.75</td>
<td>1.0642</td>
<td>12.87</td>
</tr>
<tr>
<td>9.00</td>
<td>1.0662</td>
<td>13.26</td>
</tr>
<tr>
<td>9.25</td>
<td>1.0681</td>
<td>13.65</td>
</tr>
<tr>
<td>9.50</td>
<td>1.0701</td>
<td>14.04</td>
</tr>
<tr>
<td>9.75</td>
<td>1.0721</td>
<td>14.43</td>
</tr>
<tr>
<td>10.00</td>
<td>1.0741</td>
<td>14.83</td>
</tr>
<tr>
<td>10.25</td>
<td>1.0761</td>
<td>15.22</td>
</tr>
<tr>
<td>10.50</td>
<td>1.0781</td>
<td>15.62</td>
</tr>
<tr>
<td>10.75</td>
<td>1.0801</td>
<td>16.01</td>
</tr>
<tr>
<td>11.00</td>
<td>1.0821</td>
<td>16.41</td>
</tr>
<tr>
<td>11.25</td>
<td>1.0841</td>
<td>16.81</td>
</tr>
<tr>
<td>11.50</td>
<td>1.0861</td>
<td>17.21</td>
</tr>
<tr>
<td>11.75</td>
<td>1.0881</td>
<td>17.61</td>
</tr>
<tr>
<td>12.00</td>
<td>1.0902</td>
<td>18.01</td>
</tr>
<tr>
<td>12.25</td>
<td>1.0922</td>
<td>18.41</td>
</tr>
<tr>
<td>12.50</td>
<td>1.0943</td>
<td>18.82</td>
</tr>
<tr>
<td>12.75</td>
<td>1.0964</td>
<td>19.22</td>
</tr>
<tr>
<td>13.00</td>
<td>1.0985</td>
<td>19.63</td>
</tr>
<tr>
<td>13.25</td>
<td>1.1006</td>
<td>20.04</td>
</tr>
<tr>
<td>13.50</td>
<td>1.1027</td>
<td>20.44</td>
</tr>
<tr>
<td>13.75</td>
<td>1.1048</td>
<td>20.86</td>
</tr>
<tr>
<td>19.2</td>
<td>1.1526</td>
<td>30.00</td>
</tr>
<tr>
<td>19.3</td>
<td>1.1535</td>
<td>30.18</td>
</tr>
<tr>
<td>19.4</td>
<td>1.1544</td>
<td>30.35</td>
</tr>
<tr>
<td>19.5</td>
<td>1.1554</td>
<td>30.53</td>
</tr>
<tr>
<td>19.6</td>
<td>1.1563</td>
<td>30.71</td>
</tr>
<tr>
<td>19.7</td>
<td>1.1572</td>
<td>30.90</td>
</tr>
<tr>
<td>19.8</td>
<td>1.1581</td>
<td>31.08</td>
</tr>
<tr>
<td>19.9</td>
<td>1.1590</td>
<td>31.27</td>
</tr>
<tr>
<td>20.0</td>
<td>1.1600</td>
<td>31.45</td>
</tr>
<tr>
<td>20.1</td>
<td>1.1609</td>
<td>31.64</td>
</tr>
<tr>
<td>20.2</td>
<td>1.1619</td>
<td>31.82</td>
</tr>
<tr>
<td>20.3</td>
<td>1.1628</td>
<td>32.01</td>
</tr>
<tr>
<td>20.4</td>
<td>1.1637</td>
<td>32.19</td>
</tr>
<tr>
<td>20.5</td>
<td>1.1647</td>
<td>32.38</td>
</tr>
<tr>
<td>°Bé</td>
<td>Sp. Gr.</td>
<td>Percent HCl</td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>20.6</td>
<td>1.1656</td>
<td>32.56</td>
</tr>
<tr>
<td>20.7</td>
<td>1.1666</td>
<td>32.75</td>
</tr>
<tr>
<td>20.8</td>
<td>1.1675</td>
<td>32.93</td>
</tr>
<tr>
<td>20.9</td>
<td>1.1684</td>
<td>33.12</td>
</tr>
<tr>
<td>21.0</td>
<td>1.1694</td>
<td>33.31</td>
</tr>
<tr>
<td>21.1</td>
<td>1.1703</td>
<td>33.50</td>
</tr>
<tr>
<td>21.2</td>
<td>1.1713</td>
<td>33.69</td>
</tr>
<tr>
<td>21.3</td>
<td>1.1722</td>
<td>33.88</td>
</tr>
<tr>
<td>21.4</td>
<td>1.1732</td>
<td>34.07</td>
</tr>
<tr>
<td>21.5</td>
<td>1.1741</td>
<td>34.26</td>
</tr>
<tr>
<td>21.6</td>
<td>1.1751</td>
<td>34.45</td>
</tr>
<tr>
<td>21.7</td>
<td>1.1760</td>
<td>34.64</td>
</tr>
<tr>
<td>21.8</td>
<td>1.1770</td>
<td>34.83</td>
</tr>
<tr>
<td>21.9</td>
<td>1.1779</td>
<td>35.02</td>
</tr>
<tr>
<td>22.0</td>
<td>1.1789</td>
<td>35.21</td>
</tr>
<tr>
<td>22.1</td>
<td>1.1798</td>
<td>35.40</td>
</tr>
<tr>
<td>22.2</td>
<td>1.1808</td>
<td>35.59</td>
</tr>
<tr>
<td>22.3</td>
<td>1.1817</td>
<td>35.78</td>
</tr>
<tr>
<td>22.4</td>
<td>1.1827</td>
<td>35.97</td>
</tr>
<tr>
<td>22.5</td>
<td>1.1836</td>
<td>36.16</td>
</tr>
<tr>
<td>22.6</td>
<td>1.1846</td>
<td>36.35</td>
</tr>
<tr>
<td>22.7</td>
<td>1.1856</td>
<td>36.54</td>
</tr>
<tr>
<td>22.8</td>
<td>1.1866</td>
<td>36.73</td>
</tr>
<tr>
<td>22.9</td>
<td>1.1875</td>
<td>36.93</td>
</tr>
<tr>
<td>23.0</td>
<td>1.1885</td>
<td>37.14</td>
</tr>
<tr>
<td>23.1</td>
<td>1.1895</td>
<td>37.36</td>
</tr>
<tr>
<td>23.2</td>
<td>1.1904</td>
<td>37.58</td>
</tr>
<tr>
<td>23.3</td>
<td>1.1914</td>
<td>37.80</td>
</tr>
<tr>
<td>23.4</td>
<td>1.1924</td>
<td>38.03</td>
</tr>
<tr>
<td>23.5</td>
<td>1.1934</td>
<td>38.26</td>
</tr>
<tr>
<td>23.6</td>
<td>1.1944</td>
<td>38.49</td>
</tr>
<tr>
<td>23.7</td>
<td>1.1953</td>
<td>38.72</td>
</tr>
<tr>
<td>23.8</td>
<td>1.1963</td>
<td>38.95</td>
</tr>
<tr>
<td>23.9</td>
<td>1.1973</td>
<td>39.18</td>
</tr>
<tr>
<td>°Bé</td>
<td>Sp. Gr.</td>
<td>Percent HCl</td>
</tr>
<tr>
<td>-----</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>24.0</td>
<td>1.1983</td>
<td>39.41</td>
</tr>
<tr>
<td>24.1</td>
<td>1.1993</td>
<td>39.64</td>
</tr>
<tr>
<td>24.2</td>
<td>1.2003</td>
<td>39.86</td>
</tr>
<tr>
<td>24.3</td>
<td>1.2013</td>
<td>40.09</td>
</tr>
<tr>
<td>24.4</td>
<td>1.2023</td>
<td>40.32</td>
</tr>
<tr>
<td>24.5</td>
<td>1.2033</td>
<td>40.55</td>
</tr>
<tr>
<td>24.6</td>
<td>1.2043</td>
<td>40.78</td>
</tr>
<tr>
<td>24.7</td>
<td>1.2053</td>
<td>41.01</td>
</tr>
<tr>
<td>24.8</td>
<td>1.2063</td>
<td>41.24</td>
</tr>
<tr>
<td>24.9</td>
<td>1.2073</td>
<td>41.48</td>
</tr>
<tr>
<td>25.0</td>
<td>1.2083</td>
<td>41.72</td>
</tr>
<tr>
<td>25.1</td>
<td>1.2093</td>
<td>41.99</td>
</tr>
<tr>
<td>25.2</td>
<td>1.2103</td>
<td>42.30</td>
</tr>
<tr>
<td>25.3</td>
<td>1.2114</td>
<td>42.64</td>
</tr>
<tr>
<td>25.4</td>
<td>1.2124</td>
<td>43.01</td>
</tr>
<tr>
<td>25.5</td>
<td>1.2134</td>
<td>43.40</td>
</tr>
</tbody>
</table>

Specific gravity determinations were made at 60°F, compared with water at 60°F.

From the specific gravities, the corresponding degrees Baumé were calculated by the following formula:

\[
\text{degrees Baumé} = 145 - \frac{145}{\text{sp. gr.}}
\]

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale.

**Allowance for Temperature**

10° to 15°Bé: 1/40 °Bé or 0.0002 sp. gr. for 1°F
15° to 22°Bé: 1/30 °Bé or 0.0003 sp. gr. for 1°F
22° to 25°Bé: 1/28 °Bé or 0.00035 sp. gr. for 1°F

**LOSS ON DRYING**

This procedure is used to determine the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include material other than adsorbed moisture, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. For substances appearing to contain water as the only volatile constituent, the Direct (Karl Fischer) Titration Method, provided under Water, Appendix IIB, is usually appropriate.

**Procedure**  Unless otherwise directed in the monograph, conduct the determination on 1 to 2 g of the substance, previously mixed and accurately weighed. If the sample is in the form of large crystals, reduce the
particle size to about 2 mm, quickly crushing the sample to avoid absorption or loss of moisture. Tare a glassstoppered, shallow weighing bottle that has been dried for 30 min under the same conditions to be used in the determination. Transfer the sample to the bottle, replace the cover, and weigh the bottle and its contents. By gentle sideways shaking, distribute the sample as evenly as possible to a depth of about 5 mm for most substances and not over 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber, and dry at the temperature and for the length of time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature, preferably in a desiccator, before weighing.

Where drying in vacuum is specified in the monograph, use a pressure as low as that obtainable by an aspirating water pump (not higher than 20 mm Hg).

If the test substance melts at a temperature lower than that specified for the determination, preheat the bottle and its contents for 1 to 2 h at a temperature 5° to 10° below the melting range, then continue drying at the specified temperature for the determination. When drying the sample in a desiccator, ensure that the desiccant is kept fully effective by replacing it frequently.

Add the following:

**NUCLEAR MAGNETIC RESONANCE**

Nuclear magnetic resonance (NMR) spectroscopy is an analytical procedure based on the magnetic properties of certain atomic nuclei. It is similar to other types of spectroscopy in that absorption or emission of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs in that the discrete energy levels between which the transitions take place are created artificially by placing the nuclei in a magnetic field.

Atomic nuclei are charged and behave as if they were spinning on the nuclear axis, thus creating a magnetic dipole of moment \( \mu \) along this axis. The angular momentum of the spinning nucleus is characterized by a spin quantum number \( I \). If the mass number is odd, \( I \) is \( \frac{1}{2} \) or an integer plus \( \frac{1}{2} \); otherwise, it has a value of 0 or a whole number.

Nuclei having a spin quantum number \( I \neq 0 \), when placed in an external uniform static magnetic field of strength, \( H_0 \), align with respect to the field in \((2I + 1)\) possible orientations. Thus, for nuclei with \( I = \frac{1}{2} \), which include most isotopes of analytical significance, as shown in the table below, there are two possible orientations, corresponding to two different energy states. A nuclear resonance is the transition between these states, by absorption or emission of the corresponding amount of energy. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the external field axis. The precessional angular velocity, \( \omega_0 \), is related to the external magnetic field strength through the equation:

\[
\omega_0 = \gamma H_0
\]

in which \( \gamma \) is the magnetogyric ratio and is a constant for all nuclei of a given isotope. If energy from an oscillating radio-frequency field is introduced, the absorption of radiation takes place according to the relationship:

\[
\Delta E = h\nu = \mu H_0 I
\]
where h is Planck’s constant, and

\[ \nu = \omega y/2\pi = \gamma H y/2\pi \]

Thus, when the frequency \( \nu_0 \) of the external energy field \( E = h\nu \) is the same as the precessional angular velocity, resonance is achieved.

The energy difference between the two levels corresponds to electromagnetic radiation in the radio-frequency range. It is a function of \( \gamma \), which is a property of the nucleus, and \( H_0 \), the external field strength. As shown in the table below, the resonance frequency of a nucleus increases with the increase of the magnetic field strength.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the excited and the ground states (0.02 calories at 15 to 20 kilogauss field strength), which results in a population difference between the two levels of only a few parts per million. Another important aspect of the NMR phenomenon, with negative effects on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, especially in pulsed repetitive experiments. Simultaneous acquisition of the entire spectrum instead of frequency-swept spectra can give sensitivity enhancement.

### Properties of Some Nuclei Amenable to NMR Study

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>I</th>
<th>Natural Abundance, %</th>
<th>Sensitivity</th>
<th>Resonance Frequency (MHZ) at</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H</td>
<td>½</td>
<td>99.980</td>
<td>1.000</td>
<td>1.4093 T 60.000 100.000 200.000</td>
</tr>
<tr>
<td>13C</td>
<td>½</td>
<td>1.108</td>
<td>0.0159</td>
<td>2.3488 T 15.087 25.144 50.288</td>
</tr>
<tr>
<td>19F</td>
<td>½</td>
<td>100.000</td>
<td>0.830</td>
<td>4.6975 T 56.446 94.077 188.154</td>
</tr>
<tr>
<td>31P</td>
<td>½</td>
<td>100.000</td>
<td>0.0663</td>
<td>60.000 24.289 40.481 80.961</td>
</tr>
<tr>
<td>11B</td>
<td>(3/2)</td>
<td>80.420</td>
<td>0.170</td>
<td>1.4093 T 19.250 32.084 64.167</td>
</tr>
</tbody>
</table>

* T = tesla, 1 T = 10,000 Gauss.

**Apparatus** The distinctive components of an NMR spectrometer are a magnet and a source of radio frequency. The instruments are described by the approximate resonance frequency of the analytical nucleus, e.g., \(^1\)H NMR. More recently, instruments are being referred to by their field strengths. Some spectrometers are dedicated to the analysis of one type of nucleus; others are designed to obtain spectra of different nuclei.

There are two types of commercial NMR spectrometers: the classical continuous wave (CW) instruments and the more modern pulse Fourier-transform (FT) instruments. The CW spectrometers use a technique similar to that of classical optical spectrometers: a slow scan of the radio frequency (at fixed magnetic field) or the magnetic field (at fixed radio frequency) over a domain corresponding to the resonance of the nuclei being studied. The signal generated by the absorption of energy is detected, amplified, and recorded.

Various instrument configurations are possible. The arrangement of a typical double-coil spectrometer, as one might see in the lower resolution 60-MHz and 100-MHz CW instruments, is illustrated in Fig. NMR-1.
The limitations of the CW spectrometers are low sensitivity and long analysis time. In pulsed NMR spectrometers, a single pulse of radio frequency energy is used to simultaneously activate all nuclei. The excited nuclei returning to the lower energy level generate a free induction decay (FID) signal that contains in a time domain all the information obtained in a frequency domain with a CW spectrometer. The time domain and the frequency domain responses form a pair of FTs; the mathematical operation is performed by a computer after analog-to-digital conversion. After a delay allowing for relaxation of the excited nuclei, the pulse experiment (transient) may be repeated and the response coherently added in the computer memory, with random noise being averaged out. (A similar signal-to-noise increase can be obtained by combining CW spectrometers with computers that average transients.)

The block diagram of a typical high-resolution pulsed spectrometer is shown in Fig. NMR-2.

It is a typical configuration of the high-resolution spectrometer that uses a superconducting (cryogenic) solenoid as the source of the magnetic field. Introduction of the pulsed NMR spectrometer has made the acquisition of spectra of many nuclei, other than protons, routine. It has also allowed proton spectra to be obtained in much less time, and with smaller amounts of specimen, as compared to CW techniques.

NMR spectrometers have strict stability and homogeneity requirements. Stability is often achieved by a field-frequency locking system that “locks” the magnetic field to the resonance frequency of a reference signal. The lock signal can be homonuclear or heteronuclear. In the latter case, the reference resonance is usually a deuterium signal from a deuterated solvent. On older spectrometers, using deuterium as a locking nucleus permits noise decoupling of protons to be carried out while studying nuclei like $^{13}$C. While internal homonuclear locks are still used in CW proton spectrometers (where tetramethylsilane at about 0.5% provides a convenient lock), they are hardly ever used in pulsed FT spectrometers.

No type of magnet is capable of producing a homogeneous field over the space occupied by the specimen. Two techniques are usually employed to compensate for this lack of homogeneity: specimen spinning and the use of additional (shim) coils. Because of design, particularly probe design, the spinning in the case of the electromagnet or permanent magnet is perpendicular to the basic field. In the superconducting magnet, the axis of rotation can only be parallel to the basic magnetic field. The spin rate should be sufficient to produce averaging of the field, but not fast enough to produce an extended vortex in the specimen tube. A vortex extended near the region exposed to the radio-frequency coils decreases resolution. The shim coils are adjusted by the operator until instrumental contributions to the observed line width are minimized.
An electronic integrator is a feature of most NMR spectrometers. On a CW instrument ($^1$H and $^{19}$F) the integrator, connected to the spectrometer output stage, determines the relative areas of the resonance peaks and presents these areas as a series of stepped horizontal lines when a sweep is made in the integration mode. On FT-NMR spectrometers, an integration algorithm is included in the spectrometer software, and the resonance peak areas may be presented graphically as stepped lines or tabulated as numeric values. The use of computer-generated tabulated/numeric integration data should not be accepted without a specific demonstration of precision and accuracy on the spectrometer in question.

The Spectrum  The signals (peaks) in an NMR spectrum are characterized by four attributes: resonance frequency, multiplicity, line width, and relative intensity. The analytical usefulness of the NMR technique resides in the fact that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field experienced by a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. (The latter is generally opposed to the external field and the phenomenon is called “shielding.”) In contrast with other spectroscopic methods, it is not possible to measure accurately the absolute values of transition frequencies. However, it is possible to measure accurately the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called chemical shift.

The chemical shift, being the difference between two resonance frequencies, is directly proportional to the magnetic field strength (or to the frequency of the oscillator). However, the ratio between the chemical shift, in frequency units, and the instrument frequency is constant. This allows definition of a dimensionless chemical shift parameter ($\delta$) that is independent of the instrument frequency:

$$\delta = (\nu_s - \nu_r)/\nu_o + \delta_r$$

in which $\nu_s$ is the test substance line frequency, $\nu_r$ is the reference line frequency, $\nu_o$ is the instrument frequency, in mHz, and $\delta_r$ is the chemical shift of the reference.

By employing the above equation, it is possible to use (with appropriate caution) the chemical shift of any known species (such as the residual $^1$H-containing species in deuterated solvent) as a chemical shift reference. The above equation, now in common use, is applicable to nearly all methods except in the relatively rare cases where extremely precise chemical shift values must be determined, and is readily adaptable to nuclei where non-zero reference standards are the only practical method of chemical shift determinations.

For CW instruments, tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one line, which is at a higher field than most signals, and is volatile, thus allowing for ready specimen recovery. Sodium 3-(trimethylsilyl)propionate (TSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) are used as NMR references for aqueous solutions. The resonance frequency of the TSP or DSS methyl groups closely approximate that of the TMS signal; however, DSS has the disadvantage of showing a number of methylene multiplets that may interfere with signals from the test substance. Where the use of an internal NMR reference material is not desirable, an external reference may be used.

Conventional NMR spectra are shown with the magnetic field strength increasing from left to right. Nuclei that resonate at high magnetic field strengths (to the right) are said to be more shielded (greater electron density) than those that resonate at lower magnetic field strengths: these are said to be de-shielded (lower electron
Fig. NMR-3 shows the proton NMR spectrum of 2,3-dimethyl-2-butene methyl ether. This compound contains protons in a methylene group (marked in the graphic formula) and in four methyl groups (a, a, b, and c). Methyl groups b and c are situated in distinctly different molecular environments than the two a methyl groups. Three different methyl proton resonances are observed as spectral peaks in addition to the peak corresponding to methylene proton resonance. The two a methyl groups, being in very similar environments, have the same chemical shift. Interaction between magnetically active nuclei situated within a few bond lengths of each other leads to coupling, which results in a mutual splitting of the respective signals into sets of peaks or multiplets.

The coupling between two nuclei may be described in terms of the spin-spin coupling constant, J, which is the separation (in hertz) between the individual peaks of the multiplet. Where two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting multifolds are equal. Furthermore, J is independent of magnetic field strength.

In a first-order, comparatively noncomplex spin system, the number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by \(2n+1\), where \(n\) is the number of nuclei on adjacent groups that are active in splitting. For protons this becomes \((n+1)\) peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion \((a+b)^n\). These coefficients may conveniently be found by use of Pascal’s triangle, which produces the following relative areas for the specified multifolds: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. This orderly arrangement, generally referred to as first-order behavior, may be expected when the ratio of \(D\) to \(J\) is greater than about 10; \(D\) is the chemical shift difference between two nuclei or two groups of equivalent nuclei. Two examples of idealized spectra arising from first-order coupling are shown in Fig. NMR-4.
**FIGURE NMR-4** Diagrammatic representation of simple first-order coupling of adjacent protons.

Fig. NMR-5 shows a spectrum displaying triplet signals resulting from the mutual splitting of two adjacent methylene groups.

**FIGURE NMR-5** NMR spectrum of 3-keto-tetrahydrofuran (10% in CCl₄) showing three nonequivalent protons, with a normal integral trace (peak area ratio from low H₀ to high H₀ of 1:1:1). Note two sets of methylene groups coupled to each other at 4.2 and 2.4 ppm. (Tetramethylsilane, the NMR Reference, appears at 0 ppm.)

Coupling may occur between ¹H and other nuclei, such as ¹⁹F, ¹³C, and ³¹P. In some cases, e.g., in the CW mode, the coupling constants may be large enough so that part of the multiplet is off scale at either the upfield or downfield end. This type of coupling may occur over the normal “three-bond distance,” as for ¹H-¹H coupling.

Magnetically active nuclei with I ≥ 1, such as ¹⁴N, possess an electrical quadrupole moment, which produces line-broadening of the signal due to neighboring nuclei.

Another characteristic of the signal, its relative intensity, has wide analytical applications. In carefully designed experiments (see General Method, below), the area or intensity of a signal is directly proportional to the number of protons giving rise to the signal. As a result, it is possible to determine the relative ratio of the different kinds of protons or other nuclei in a specimen or to perform NMR assays with the aid of an internal
The NMR spectra may contain extraneous signals due to the inhomogeneity of the magnetic field throughout the specimen. These artifacts, called spinning side bands, appear as minor lines symmetrically located around each signal. The presence of large spinning side bands indicates that the non-spinning shims require adjustment. The separation is equal to the frequency of the specimen tube spin rate or some integral multiple of that frequency. Thus, spinning side bands are readily identifiable.

**General Method**  Inadequate specimen preparation or incorrect instrumental adjustments and parameters may lead to poor resolution, decreased sensitivity, spectral artifacts, and erroneous data. It is preferable that the operator be familiar with the basic theory of NMR, the properties of the specimen, and the operating principles of the instruments. Strict adherence to the instruction manuals provided by the manufacturer and frequent checks of the performance of the instrument are essential.

The method and procedures discussed here refer specifically to $^1$H (proton) and $^{19}$F NMR. They are applicable, with modification, to other nuclei. The discussion presumes that the NMR spectra are obtained from liquid test substances or solutions in suitable solvents.

**Selection of Solvent**  In addition to having good solubility properties, suitable solvents do not exhibit resonance peaks that obscure resonance peaks of the specimen being analyzed. The most commonly used solvents for proton and carbon NMR are listed in the table below. Deuterated solvents also provide the signal for the heteronuclear system lock. If solvent peaks might interfere with any signals from the specimen, then the isotopic purity of the solvent should be as high as possible. Deuterium ($I = 1$) does not exhibit resonance under $^1$H conditions but may cause J-coupling to be observed. The residual protons generate solvent peaks whose chemical shifts are shown in the table below.

**Solvents Commonly Used for Proton NMR**
<table>
<thead>
<tr>
<th>Solvent</th>
<th>Residual Proton Signal, $\delta^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCl$_4^b$</td>
<td>—</td>
</tr>
<tr>
<td>CS$_2^b$</td>
<td>—</td>
</tr>
<tr>
<td>SO$_2$ (liquid)</td>
<td>—</td>
</tr>
<tr>
<td>(CF$_3$)$_2$CO</td>
<td>—</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>7.27</td>
</tr>
<tr>
<td>CD$_3$OD</td>
<td>3.35, 4.8$^c$</td>
</tr>
<tr>
<td>(CD$_3$)$_2$CO</td>
<td>2.05</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>4.7$^c$</td>
</tr>
<tr>
<td>DMSO-$d_6^d$</td>
<td>2.50</td>
</tr>
<tr>
<td>C$_6$D$_6$</td>
<td>7.20</td>
</tr>
<tr>
<td>p-Dioxane-$d_8$</td>
<td>3.55</td>
</tr>
<tr>
<td>CD$_3$CO$_2$D</td>
<td>2.05, 8.5$^c$</td>
</tr>
<tr>
<td>DMF-$d_7^e$</td>
<td>2.77, 2.93, 8.05</td>
</tr>
</tbody>
</table>

$^a$ $\delta$ in ppm relative to tetramethylsilane arbitrarily taken as 0 $\delta$ or 0 ppm.

$^b$ Spectrophotometric grade.

$^c$ Highly variable; depends on solute and temperature.

$^d$ Dimethyl sulfoxide-$d_6$.

$^e$ N,N-Dimethylformamide-$d_7$ per Aldrich, Alfa, Fluka, and Sigma catalogs.

Some solvents (e.g., D$_2$O or CD$_3$OD) enter into fast exchange reactions with protons and may eliminate resonance signals from –COOH, –OH, and –NH$_2$ structural groups. The protons in alcohols and amines do not take part in rapid exchange unless catalyzed by small concentrations of acid or base, except in the presence of D$_2$O and some other solvents (e.g., CD$_3$OD).

For $^{19}$F NMR, most solvents used in proton NMR may be employed, the most common ones being CHCl$_3$, CCl$_4$, H$_2$O, CS$_2$, aqueous acids and bases, and dimethylacetamide. In general, any nonfluorinated solvent may be used, provided that it is of spectral quality. Obviously, there is no interference from the protonated functional groups of the solvent. However, unless they are decoupled, protonated functional groups on the $^{19}$F-containing specimen will provide J-coupling.

**Specimen Preparation** Directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment and on the type of instrument. Detection of minor contaminants may require higher concentrations. The solutions are prepared in separate vials and transferred to the NMR specimen tube. The volume required depends on the size of the specimen tube and on the geometry of the instrument. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe and spun.
The NMR specimen tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5- or 10-mm outside diameter and a length of between 15 and 20 cm. Microtubes are available for the analysis of small amounts of specimen.

**Procedure**  The specimen tube is placed in a probe located in the magnetic field. The probe contains electronic circuitry including the radio-frequency coil(s), and is provided with attachments for the air supply that spins the specimen tubes.

Instrument adjustments are made before each experiment. The spinning rate of the specimen tube is adjusted so that spinning side bands do not interfere with the peaks of interest and the vortex does not extend beyond the coils in the probe. To optimize the instrument performance, the magnetic shim gradients on FT-NMR spectrometers are adjusted. In adjusting resolution on CW spectrometers, a good indicator is the definite “ringing” of the TMS peak. The phenomenon of ringing is the oscillation of the recorder trace after the magnetic field has passed through a resonance frequency. Ringing, evident on a number of the peaks in Figs. NMR-5 and NMR-6, arises during rapid scans and decays exponentially to the baseline value.

![FIGURE NMR-6 Continuous wave proton spectrum of ethyl ether.](image)

*Fig. NMR-7* clearly indicates the absence, in an FT experiment, of the ringing phenomenon. Ringing will not appear because the spectrum obtained is the result of analysis of the FID by Fourier transformation and not a magnetic field or frequency sweep through the individual resonance positions.

![FIGURE NMR-7 Proton NMR spectrum of ethyl ether in deuterated chloroform.](image)
With proton CW instruments the spectrum is scanned from 0 ppm to about 10 ppm with a scan time of about 1 to 5 min. The amplification is adjusted so that all peaks remain on scale. If the response is low at reasonable amplitude, the radio-frequency power is increased to obtain the highest possible peak response without peak broadening. After the initial scan, the presence of peaks downfield of 10 ppm is quickly checked by offsetting the instrument response by about 5 ppm. With CW instrumentation, it is common for the TMS peak to shift slightly during an extended scan. The extent of the shift is usually obtained by comparing the relative positions of another peak in the initial scan with the same peak in the offset scan.

The operation of an FT-NMR spectrometer is a much more elaborate experiment. The computer serves to control the spectrometer, to program the experiment, and to store and process the data. Programming the experiment involves setting values for a large number of variables including the spectral width to be examined, the duration ("width") of the excitation pulse, the time interval over which data will be acquired, the number of transients to be accumulated, and the delay between one acquisition and the next. The analysis time for one transient is in the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment. At the end of the experiment, the FID signal is stored in digitized form in the computer memory and is displayed on the video screen. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum. The instrument provides a plot of the spectrum. The integration routine, accessed through keyboard commands, results in a stepped-line plot. Considerably more accurate integrals are obtained if the signals or regions of interest are separately integrated.

FT-NMR spectrometers may yield qualitative and quantitative data from the same experiment, but this is seldom done in practice. In quantitative FT experiments, special precautions must be taken for the signal areas to be proportional to the number of protons. The delays between pulses must be long enough to allow complete relaxation of all excited nuclei. This results in a considerable increase in analysis time and in some loss of resolution. Qualitative analysis is usually performed in nonquantitative conditions, with the design of the experiment directed to fast analysis with maximum resolution or sensitivity.

**Qualitative and Quantitative Analysis** NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All five characteristics of the signal (chemical shift, multiplicity, line width, coupling constants, and relative intensity) contribute analytical information.

**Qualitative Applications** Comparison of a spectrum from the literature or from an authentic specimen with that of a test specimen may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the numeric value of the chemical shifts and coupling constants, and by the number of protons under each signal. (The software of modern instruments includes programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, the specimen concentration, and the chemical shift reference, must also be provided.

For unknown specimens, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important stereochemical information. Mutual signal splitting of functional groups indicates close proximity. The magnitude of the coupling constant, $J$, between residual protons on substituted aromatic, olefinic, or cycloalkyl structures is used to identify the relative position of the substituents.
Several special techniques (double resonance, chemical exchange, use of shift reagents, two-dimensional analysis, etc.) are available to simplify some of the more complex spectra, to identify certain functional groups, and to determine coupling correlations.

Double resonance, or spin decoupling, is a technique that removes the coupling between nuclei and thus simplifies the spectrum and identifies the components in a coupling relationship. For example, in a simple two-proton system, generally designated an AX system (see Fig. NMR-4), each proton appears as a doublet. If a strong radio-frequency field is introduced at the frequency of X, while the normal radio-frequency field is maintained at the frequency that causes A to resonate, the coupling between A and X is removed (homonuclear decoupling). A is no longer split, but instead appears as a singlet. Routine $^{13}$C spectra are obtained under proton decoupling conditions that remove all heteronuclear $^{13}$C-$^1$H couplings. As a result of this decoupling, the carbon signals appear as singlets, unless other nuclei that are not decoupled are present (e.g., $^{19}$F, $^{31}$P).

Functional groups containing exchangeable protons bound to hetero-atoms such as –OH, –NH$_2$, or –COOH groups may be identified by taking advantage of the rapid exchange of these protons with D$_2$O. To determine the presence and position of these groups, scan the test substance in CDCl$_3$ or DMSO-$d_6$, then add a few drops of D$_2$O to the specimen tube, shake, and scan again. The resonance peaks from these groups collapse in the second scan and are replaced by the HDO singlet between 4.7 and 5.0 ppm.

This chemical exchange is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra. If a proton can experience different environments by virtue of such a process (tautomerism, rotation about a bond, exchange equilibria, ring inversion, etc.), the appearance of the spectrum will be a function of the rate of the process. Slow processes (on an NMR time scale) result in more than one signal, fast processes average these signals to one line, and intermediate processes produce broad signals.

The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear two-dimensional analysis, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

Quantitative Applications If appropriate instrument settings for quantitative analysis have been made, the areas (or intensities) of two signals are proportional to the total number of protons generating the signals.

\[
\frac{A_1}{A_2} = \frac{N_1}{N_2} \quad (1)
\]

If the two signals originate from two functional groups of the same molecule, the equation can be simplified to

\[
\frac{A_1}{A_2} = \frac{n_1}{n_2} \quad (2)
\]

in which $n_1$ and $n_2$ are the number of protons in the respective functional groups.

If the two signals originate from different molecular species,

\[
\frac{A_1}{A_2} = \frac{n_1m_1}{n_2m_2} = \frac{(n_1W_1M_1)/(n_2W_2M_2)} \quad (3)
\]

where $m_1$ and $m_2$ are the numbers of moles; $W_1$ and $W_2$ are the masses; and $M_1$ and $M_2$ are the molecular weights of compounds 1 and 2, respectively.

Examination of Equations 2 and 3 shows that NMR quantitative analysis can be performed in an absolute or
relative manner. In the absolute method, an internal standard is added to the specimen and a resonance peak area arising from the test substance is compared with a resonance peak area from the internal standard. If both test substance and internal standard are accurately weighed, the absolute purity of the substance may be calculated. A good internal standard has the following properties: it presents a reference resonance peak, preferably a singlet, at a field position removed from all specimen peaks; it is soluble in the analytical solvent; its proton equivalent weight, i.e., the molecular weight divided by the number of protons giving rise to the reference peak, is low; and it does not interact with the compound being tested. Typical examples of useful standards are 1,2,4,5-tetrachlorobenzene, 1,4-dinitrobenzene, benzyl benzoate, and maleic acid. The choice of a standard will be dictated by the spectrum of the specimen.

The relative method may be used to determine the molar fraction of an impurity in a test substance (or of the components in a mixture) as calculated by Equation 3.

Quantitative analysis, as well as detection of trace impurities, is markedly improved with modern instrumentation. Stronger magnetic fields and the ability to accumulate and/or average signals over long periods of time greatly enhance the sensitivity of the method.

**Absolute Method of Quantitation**  Where the individual monograph directs that the Absolute Method of Quantitation be employed, proceed as follows.

*Solvent, Internal Standard, and NMR Reference*  Use as directed in the individual monograph.

*Test Preparation*  Transfer an accurately weighed quantity of the test substance, containing about 4.5 proton mEq, to a glass-stoppered, graduated centrifuge tube. Add about 4.5 proton mEq of *Internal Standard*, accurately weighed, and 3.0 mL of Solvent, insert the stopper, and shake. When dissolution is complete, add about 30 µL (30 mg if a solid) of *NMR Reference*, provided that it does not interfere with subsequent measurements, and shake.

*Procedure*  Transfer an appropriate amount (0.4 to 0.8 mL) of *Test Preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area of the *Internal Standard* peak as $A_S$ and that of the *Test Preparation* peak as $A_U$.

Calculate the quantity, in mg, of the analyte in the *Test Preparation* by the formula:

$$W_S(A_U/A_S)(E_U/E_S)$$

in which $W_S$ is the weight, in mg, of *Internal Standard* taken; and $E_U$ and $E_S$ are the proton equivalent weights (i.e., the molecular weights divided by the number of protons giving rise to the reference peak) of the analyte and the *Internal Standard*, respectively.

**Relative Method of Quantitation**  Where the individual monograph directs that the Relative Method of Quantitation be employed, proceed as follows.

*Solvent, NMR Reference, and Test Preparation*  Use as directed under Absolute Method of Quantitation.

*Procedure*  Transfer an appropriate amount (0.4 to 0.8 mL) of *Test Preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area or intensity under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area or intensity resulting from the resonances of the groups designated in the individual monograph as $A_1$ and $A_2$.

Calculate the quantity, in mole percent, of the analyte in the *Test Preparation* by the formula:
\[ (100 \times (A_1/n_1)/(A_1/n_1 + A_2/n_2)) \]

in which \( n_1 \) and \( n_2 \) are, respectively, the numbers of protons in the designated groups.

Cover 3S (FCC7)

**OIL CONTENT OF SYNTHETIC PARAFFIN**

**Apparatus**

*Filter Stick*  Use either a 10-mm diameter sintered-glass filter stick of 10- to 15-\(\mu\)m maximum pore diameter, or a filter stick made of stainless steel and having a 0.5-in. disk of 10- to 15-\(\mu\)m maximum pore diameter. Determine conformance with the pore diameter specified as follows: Clean sintered-glass filter sticks by soaking in hydrochloric acid, or stainless steel sticks by soaking in nitric acid, wash with water, rinse with acetone, and dry in air followed by drying in an oven at 105\(^\circ\)C for 30 min.

Thoroughly wet the clean filter stick by soaking in water, and then connect it with an apparatus (see Fig. 9) consisting of a mercury-filled manometer, readable to 0.5 mm; a clean and filtered air supply; a drying bulb filled with silica gel; and a needle-valve type air pressure regulator. Apply pressure slowly from the air source, and immerse the filter just below the surface of water contained in a beaker.

![Diagram of apparatus](image)

**FIGURE 9 Assembly for Checking Pore Diameter of Filter Sticks.**

[Note—If a head of liquid is noted above the surface of the filter after it is inserted into the water, the back pressure thus produced should be subtracted from the observed pressure when the pore diameter is calculated as directed below.]

Increase the air pressure to 10 mm below the acceptable pressure limit, and then increase the pressure at a slow, uniform rate of about 3 mm Hg per minute until the first bubble passes through the filter. This can be conveniently observed by placing the beaker over a mirror. Read the manometer when the first bubble passes off the underside of the filter. Calculate the pore diameter, in micrometers, by the formula

\[ \text{Result} = 2180/p \]

in which \( p \) is the observed pressure, in millimeters, corrected for any back pressure as mentioned above.

*Filtration Assembly*  Connect the *Filter Stick* with an air pressure inlet tube and delivery nozzle and ground-glass joint to fit a 25-\(\times\)170-mm test tube as shown in Fig. 10. If a stainless steel *Filter Stick* is used, make the connection to the test tube by means of a cork.
Cooling Bath  Use a suitable insulated box having 1-in. holes in the center to accommodate any desired number of test tubes. The bath may be filled with a suitable medium such as kerosene and may be cooled by circulating a refrigerant through coils, or by using solid carbon dioxide, to produce a temperature of 30 ± 2°F.

Air Pressure Regulator  Use a suitable pressure-reduction valve, or other suitable regulator, that will supply air to the Filtration Assembly at the volume and pressure required to give an even flow of filtrate (see Procedure). Connect the regulator with rubber tubing to the end of the Filter Stick in the Filtration Assembly.

Thermometer  Use an ASTM Oil in Wax Thermometer having the range of −35° to +70°F and conforming to the requirements for an ASTM 71F thermometer (see Thermometers, Appendix I).

Weighing Bottles  Use glass-stoppered conical bottles having a capacity of 15 mL. The bottles are used as evaporating flasks in the Procedure.

Evaporation Assembly  The assembly consists of an evaporating cabinet capable of maintaining a temperature of 95° ± 2°F around the evaporation flasks, and air jets (4 ± 0.2 mm id) for delivering a stream of clean, dry air vertically downward into the flasks. In the Procedure below, support each jet so that the tip is 15 ± 5 mm above the surface of the liquid at the start of the evaporation. Supply the air (purified by passage through a tube of 1-cm bore packed loosely to a height of 20 cm with absorbent cotton) at the rate of 2 to 3 L/min per jet. The cleanliness of the air should be checked periodically to ensure that not more than 0.1 mg of residue is obtained when 4 mL of methyl ethyl ketone is evaporated as directed in the Procedure.

Wire Stirrer  Use a 250-mm length of stiff iron or ni-chrome wire of about No. 20 B & S gauge. Form a 10-mm diameter loop at each end, and bend the loop at the bottom end so that the plane of the loop is perpendicular to the length of the wire.

Sample Selection  If the sample weighs about 1 kg or less, obtain a representative portion by melting the entire sample and stirring thoroughly. For samples heavier than about 1 kg, exercise special care to ensure that a truly representative portion is obtained, noting that the oil may not be distributed uniformly throughout the sample and that mechanical operations may have expressed some of the oil.

Procedure  Melt a representative portion of the sample in a beaker, using a water bath or oven maintained at 160° to 210°F. As soon as the sample is completely melted, thoroughly mix it by stirring. Preheat a dropper
pipet, provided with a rubber bulb and calibrated to deliver 1 ± 0.05 g of molten sample, and withdraw a 1-g portion of the sample as soon as possible after it has melted. Hold the pipet in a vertical position, and carefully transfer its contents into a clean, dry test tube previously weighed to the nearest milligram. Evenly coat the bottom of the tube by swirling, allow the tube to cool, and weigh to the nearest milligram. Calculate the sample weight, in grams, and record it as B (see Calculation). Pipet 15 mL of methyl ethyl ketone (ASTM Specification D 740, or equivalent) into the tube, and immerse the tube up to the top of the liquid in a hot water or steam bath. Stir with an up-and-down motion with the wire stirrer, and continue heating and stirring until a homogeneous solution is obtained, exercising care to avoid loss of solvent by prolonged boiling. [Note—If it appears that a clear solution will not be obtained, stir until any undissolved material is well dispersed so as to produce a slightly cloudy solution.]

After the sample solution is prepared, plunge the test tube into an 800-mL beaker of ice water, and continue to stir until the contents are cold. Remove the stirrer, then remove the test tube from the bath, dry the outside of the tube with a cloth, and weigh to the nearest 100 mg. Calculate the weight, in grams, of solvent in the test tube, and record it as C (see Calculation). Place the tube in the cooling bath, maintained at −30° ± 2°F, and stir continuously with the thermometer until the temperature reaches −25° ± 0.5°F, maintaining the slurry at a uniform consistency and taking precautions to prevent the sample from setting up on the walls of the tube or forming crystals.

Place the filter stick in a test tube and cool at −30° ± 2°F in the cooling bath for a minimum of 10 min. Immerse the cooled filter stick in the sample, then connect the filtration assembly, seating the ground-glass joint of the filter so as to make an airtight seal. Place an unstoppered weighing bottle, previously weighed together with the glass stopper to the nearest 0.1 mg, under the delivery nozzle of the filtration assembly. [Note—Suitable precautions and proper analytical technique should be applied to ensure the accuracy of the weight of the bottle. Before determining its weight, the bottle and its stopper should have been cleaned and dried, then rinsed with methyl ethyl ketone, wiped dry on the outside, dried in the evaporation assembly for about 5 min, and cooled. Then allow it to stand for about 10 min near the balance before weighing.]

Apply air pressure to the filtration assembly, immediately collect about 4 mL of filtrate in the weighing bottle, and release the air pressure to permit the liquid to drain back slowly from the delivery nozzle. Stopper the bottle, and weigh it to the nearest 10 mg without waiting for it to come to room temperature. Remove the stopper, transfer the bottle to the evaporation assembly maintained at 95° ± 2°F, and place it under an air jet centered inside the neck, with the tip 15 ± 5 mm above the surface of the liquid. After the solvent has evaporated (usually less than 30 min), stopper the bottle, and allow it to stand near the balance for about 10 min before it is weighed to the nearest 0.1 mg. Repeat the evaporation procedure for 5-min periods until the loss between successive weighings is not more than 0.2 mg. Determine the weight of the oil residue, in grams, by subtracting the weight of the empty stoppered bottle from the weight of the stoppered bottle plus the oil residue after the evaporation procedure, and record the results as A (see Calculation). Determine the weight of solvent evaporated, in grams, by subtracting the weight of the bottle plus oil residue from the weight of the bottle plus filtrate, and record the result as D (see Calculation).

Calculation Calculate the percent, by weight, of oil in the sample by the formula

\[
\text{Result} = \left(\frac{100 AC}{BD}\right) - 0.15
\]

in which 0.15 is a factor to correct for solubility of the sample in the solvent at −25°F.
PLASMA SPECTROCHEMISTRY

Plasma-based instrumental techniques that are useful for food ingredient analyses fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. An inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma–atomic emission spectroscopy (ICP–AES), also known as inductively coupled plasma–optical emission spectroscopy (ICP–OES); or the excited or ground state ions can be determined by a technique known as inductively coupled plasma–mass spectrometry (ICP–MS). ICP–AES and ICP–MS may be used for either single- or multi-element analysis, and they provide good general-purpose procedures for either sequential or simultaneous analyses over an extended linear range with good sensitivity.

An emerging technique in plasma spectrochemistry is laser-induced breakdown spectroscopy (LIBS). In LIBS, a solid, liquid, or gaseous sample is heated directly by a pulsed laser, or indirectly by a plasma generated by the laser. As a result, the sample is volatilized at the laser beam contact point, and the volatilized constituents are reduced to atoms, molecular fragments, and larger clusters in the plasma that forms at or just above the surface of the sample. Emission from the atoms and ions in the sample is collected, typically using fiber optics or a remote viewing system, and is measured using an array detector such as a charge-coupled device (CCD). LIBS can be used for qualitative analysis or against a working standard curve for quantitative analysis. Although LIBS is not currently in wide use, it might be suited for at-line or on-line measurements in a production setting, as well as in the laboratory. Because of its potential, it should be considered a viable technique for plasma spectrochemistry in the laboratory. However, because LIBS is still an emerging technique, details will not be further discussed here.3

Sample Preparation  Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP–AES or ICP–MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP–AES and ICP–MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid may also be used, but great care must be taken to ensure the safety of the analyst, as well as to protect the quartz sample introduction equipment when using this acid; specifically, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP–AES and ICP–MS analyses in cases where accuracy and precision are not adequate. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can
be employed. These include hot-plate digestion and microwave-assisted digestions, including open- and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open- or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP–MS is employed. Deionized water must be at least 18 megaohm. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical labware used in ICP–AES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can also lead to inaccurate results.

The use of labware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

Sample Introduction There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred, and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same, irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli or Venturi effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1% to 2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP. As with nebulizers, there is more than one type of spray chamber available for use with ICP–AES or ICP–MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent, and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered.

Gas and liquid chromatography systems can be interfaced with ICP–AES and ICP–MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass
Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA–ICP–AES and LA–ICP–MS are better suited for qualitative analyses of compounds, because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated through appropriate method validation that the available standards are adequate.  

**Standard Preparation**  
Single- or multi-element standard solutions, which have concentrations traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials and their concentrations, determined independently, as appropriate. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. As a general rule, working standard solutions should be retained for no more than 24 h, unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP–AES or ICP–MS. Internal standards can also be introduced through a T connector into the sample uptake tubing. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP–AES and ICP–MS selenium and arsenic quantification measurements, which can be remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

**ICP**  
The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in an ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas.
The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting. An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000 to 10,000 K, so most covalent bonds and analyte-to-analyte interactions have been eliminated.

ICP–AES  An inductively coupled plasma can use either an optical or a mass spectral detection system. In the former case, ICP–AES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP–AES systems are available, each with different capabilities, as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next to perform analyses, and often provide a larger number of analytical lines from which to choose. Array detectors, including charge-coupled devices and charge-injection devices, with detectors on a chip, make it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Methods validated on an instrument with a radial configuration will probably not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, analyte in question, analytical wavelength(s) used, cost of instrumentation, required sensitivity, and type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP–AES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known standards over the concentration range of interest. It is, however, also possible to perform a single-point calibration under certain circumstances, such as with limit tests, if the methodology has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in an ICP are
rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because “wings” of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP–AES. The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at some point away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP–AES spectrometers.

The selection of the analytical spectral line is critical to the success of an ICP–AES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used, and the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument, and select alternative wavelengths based on manufacturer recommendations or published wavelength tables. Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

In general, it is desirable to operate the ICP under robust conditions, which can be gauged on the basis of the MgII/MgI line pair at (280.270 nm/285.213 nm). If that ratio of intensities is above 6.0 in an aqueous solution, the ICP is said to be robust, and is less susceptible to matrix interferences. A ratio of about 10.0 is generally what is sought. Note that the term robust conditions is unrelated to robustness as applied to analytical method validation. Operation of an instrument with an MgII/MgI ratio greater than 6.0 is not mandated, but is being suggested as a means of optimizing instrument parameters in many circumstances.

The analysis of the Group I elements can be an exception to this strategy. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization, which can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. When using organic solvents, it may also be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

**Calibration** The wavelength accuracy for ICP–AES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–AES instrument should be followed. These might include, but are not limited to, use of a multi-element wavelength calibration with a reference solution, internal mercury (Hg) wavelength calibration, and peak search. The analyst should perform system checks in accordance with the manufacturer's recommendations.
Standardization  The instrument must be standardized for quantification at time of use. However, because ICP–AES is a technique generally considered to be linear over a range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products if the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of a single-point standardization is also acceptable for qualitative ICP–AES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantification.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. However, it is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of use of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantification, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working curve. Here, too, however, the nature of the sample matrix, the analyte(s), the desired sensitivity, and the type of instrumentation available may dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis, and should employ additional working standards.

To demonstrate the stability of the system's initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. The reassayed standard should agree with its expected value to within ±10%, or as specified in an individual monograph, for single-element analyses when analytical wavelengths are between 200 and 500 nm, or concentrations are >1 µg per mL. The reassayed standard should agree with its theoretical value to within ±20%, or as specified in an individual monograph, for multi-element analyses, when analytical wavelengths are <200 nm or >500 nm, or at concentrations of <1 µg per mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

Procedure  Follow the procedure as directed in the individual monograph for the instrumental parameters. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions may be used and modified as needed. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. This calculation is often performed directly by the instrument.

ICP–MS  When an inductively coupled plasma uses a mass spectral detection system, the technique is referred to as inductively coupled plasma–mass spectrometry (ICP–MS). In this technique, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS,
the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–AES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Gaining in interest is time-of-flight ICP–MS. Although still not in widespread use, this approach may see greater use in the future. Additionally, high-resolution sector field instruments are available.

Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample. Ions are generated from the analyte atoms by the plasma. The analyte ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held near a milliTorr. Lastly, the skimmed ions pass a third-stage orifice to enter a zone held near a microTorr, where they encounter ion optics and are passed into the mass spectrometer. The mass spectrometer separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units (amu). Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used.

ICP–MS generally offers considerably lower (better) detection limits than ICP–AES, largely because of the extremely low background that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–AES.

The selection of the analytical mass to use is critical to the success of an ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts might choose to start with masses recommended by the manufacturer of their particular instrument, and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes.10

Optimization of an ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer...
cone may also be required in order to reduce cone degradation with some organic solvents.

**Calibration**  The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument. These may include, but are not limited to, tuning on a reference mass or masses, peak search, and mass calibration. The analyst should perform system checks recommended by the instrument manufacturer.

**Standardization**  The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working standard curve. Here, too, however, the nature of the sample matrix, the analyte, the desired sensitivity, and the type of instrumentation available might dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis and should employ additional working standards.

To demonstrate the stability of the system since initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. Appropriate intervals may be established as occurring after every fifth or tenth sample, or as deemed adequate by the analyst, on the basis of the analysis being performed. The reassayed standard should agree with its expected value to within ±10% for single-element analyses when analytical masses are free of interferences and when concentrations are >1 ng per mL. The reassayed standard should agree with its expected value to within ±20% for multi-element analyses, or when concentrations are <1 ng per mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

**Procedure**  Follow the procedure as directed in the individual monograph for the detection mode and instrument parameters. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers’ equipment configurations, the analyst may wish to begin with the manufacturer’s suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as
a single result. Sample concentrations are calculated versus the working curve generated by plotting the
detector response versus the concentration of the analyte in the standard solutions. With modern instruments,
this calculation is often performed by the instrument.

Glossary

**Auxiliary Gas**  See *Intermediate (or Auxiliary) Gas*.

**Axial Viewing**  A configuration of the plasma for AES in which the plasma is directed toward the
spectrometer optical path, also called “end-on viewing.”

**Central (or Nebulizer) Gas**  One of three argon gas flows in an ICP torch. The central gas is used to help
create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then
directed through the central tube of the torch and into the plasma.

**Collision Cell**  A design feature of some ICP–MS instruments. Collision cells are used to reduce
interferences from argon species or polyatomic ions and to facilitate the analysis of elements that might be
affected by those interferences.

**Cool Plasma**  Plasma conditions used for ICP–MS that result in a plasma that is cooler than that normally
used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas
flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.

**Coolant Gas**  See *Outer (or Coolant or Plasma) Gas*.

**Forward Power**  The number of watts used to ignite and sustain the plasma during an analysis. Forward
power requirements may vary, depending on sample matrix and analyte.

**Intermediate (or Auxiliary) Gas**  Gas used to “lift” the plasma off the surface of the torch, thereby preventing
melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.

**Internal Standard**  An element added to or present in the same concentration in blanks, standards, and
samples to act as an intensity reference for the analysis. An internal standard should be used for ICP–AES
work and must always be used for quantitative ICP–MS analyses.

**Lateral Viewing**  See *Radial Viewing*.

m  The ion mass of interest.

**Multiply-Charged Ions**  Atoms that, when subjected to the high-ionization temperature of the ICP, can form
doubly or triply charged ions (X^{++}, X^{+++}, etc.). When detected by MS, the apparent mass of these ions will be
half or one-third that of the atomic mass.

**Nebulizer**  Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently
sent into the ICP.

**Outer (or Coolant or Plasma) Gas**  The main gas supply for the plasma.

**Plasma Gas**  See *Outer (or Coolant or Plasma) Gas*.

**Radial Viewing**  A configuration of the plasma for AES in which the plasma is viewed orthogonal to the
spectrometer optic path. Also called “side-on viewing.” See also *Lateral Viewing*.

**Reaction Cell**  Similar to *Collision Cell*, but operating on a different principle. Designed to reduce or eliminate
spectral interferences.
Sampling Cone A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma.

Sequential A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.

Simultaneous A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.

Skimmer Cone A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP–MS.

Standard Additions A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.

Torch A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

RESIDUE ON IGNITION (Sulfated Ash)

Method I (for Solids)
Transfer the quantity of the sample directed in the individual monograph onto a tared 50- to 100-mL platinum dish or other suitable container, and add sufficient 2 N sulfuric acid to moisten the entire sample. Heat gently, using a hot plate, an Argand burner, or an infrared heat lamp, until the sample is dry and thoroughly charred, then continue heating until all of the sample has been volatilized or nearly all of the carbon has been oxidized, and cool. Moisten the residue with 0.1 mL of sulfuric acid, and heat in the same manner until the remainder of the sample and any excess sulfuric acid have been volatilized. To promote volatilization of sulfuric acid, add a few pieces of ammonium carbonate just before completing ignition. Finally, ignite to constant weight in a muffle furnace at 800° ± 25° for 15 min, or longer if necessary to complete ignition, cool in a desiccator, and weigh.

Method II (for Liquids)
Unless otherwise directed, transfer the required weight of the sample onto a tared 75- to 100-mL platinum dish. Heat gently, using an Argand or Meker burner, until the sample ignites, then allow the sample to burn until it self-extinguishes. Cool, then wet the residue with 2 mL of concentrated sulfuric acid, and heat the sample over a low flame until dry. Ignite to constant weight in a muffle furnace at 800° ± 25° for 30 min, or longer if necessary for complete ignition, cool in a desiccator, and weigh.

SIEVE ANALYSIS OF GRANULAR METAL POWDERS (Based on ASTM Designation: B 214)\textsuperscript{11}

Apparatus

Sieves Use a set of standard sieves, ranging from 80-mesh to 325-mesh, conforming to the specifications in

**Sieve Shaker** Use a mechanically operated sieve shaker that imparts to the set of sieves a horizontal rotary motion of between 270 and 300 rotations/min and a tapping action of between 140 and 160 taps/min. The sieve shaker is fitted with a plug to receive the impact of the tapping device. The entire apparatus is rigidly mounted —bolted to a solid foundation, preferably of concrete. Preferably a time switch is provided to ensure the accuracy of test duration.

**Procedure** Assemble the sieves in consecutive order by opening size, with the coarsest sieve (80-mesh) at the top, and place a solid-collecting pan below the bottom sieve (325-mesh). Place 100.0 g of the test sample, W, on the top sieve, and close the sieve with a solid cover. Securely fasten the assembly to the sieve shaker, and operate the shaker for 15 min. Remove the most coarse sieve from the nest, gently tap its contents to one side, and pour the contents onto a tared, glazed paper. Using a soft brush, transfer onto the next finer sieve any material adhering to the bottom of the sieve and frame. Place the sieve just removed upside down on the paper containing the retained portion, and tap the sieve. Accurately weigh the paper and its contents, and record the net weight of the fraction, F, obtained. Repeat this process for each sieve in the nest and for the portion of the sample that has been collected in the bottom pan. Record the total of the fractions retained on the sieves as T and that portion collected in the pan as t. The combined total, S, of T + t is the amount of the sample, W, recovered in the test. Calculate the percent recovery by the formula

\[
\text{Result} = \frac{S}{W} \times 100
\]

If the percent recovery is less than 99.0%, check the condition of the sieves and for possible errors in weighing, and repeat the test. If the percent recovery is not less than 99.0%, calculate the percent retained on each sieve by the formula

\[
\text{Result} = \frac{F}{W} \times 100
\]

Calculate the percent through the smallest mesh sieve from the portion collected in the pan by the formula

\[
\text{Result} = \left(\frac{(100 - t)}{W}\right) \times 100
\]
# SULFURIC ACID TABLE

<table>
<thead>
<tr>
<th>°Bé</th>
<th>Sp. Gr.</th>
<th>Percent H₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0000</td>
<td>0.00</td>
</tr>
<tr>
<td>1</td>
<td>1.0069</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>1.0140</td>
<td>2.08</td>
</tr>
<tr>
<td>3</td>
<td>1.0211</td>
<td>3.13</td>
</tr>
<tr>
<td>4</td>
<td>1.0284</td>
<td>4.21</td>
</tr>
<tr>
<td>5</td>
<td>1.0357</td>
<td>5.28</td>
</tr>
<tr>
<td>6</td>
<td>1.0432</td>
<td>6.37</td>
</tr>
<tr>
<td>7</td>
<td>1.0507</td>
<td>7.45</td>
</tr>
<tr>
<td>8</td>
<td>1.0584</td>
<td>8.55</td>
</tr>
<tr>
<td>9</td>
<td>1.0662</td>
<td>9.66</td>
</tr>
<tr>
<td>10</td>
<td>1.0741</td>
<td>10.77</td>
</tr>
<tr>
<td>11</td>
<td>1.0821</td>
<td>11.89</td>
</tr>
<tr>
<td>12</td>
<td>1.0902</td>
<td>13.01</td>
</tr>
<tr>
<td>13</td>
<td>1.0985</td>
<td>14.13</td>
</tr>
<tr>
<td>14</td>
<td>1.1069</td>
<td>15.25</td>
</tr>
<tr>
<td>15</td>
<td>1.1154</td>
<td>16.38</td>
</tr>
<tr>
<td>16</td>
<td>1.1240</td>
<td>17.53</td>
</tr>
<tr>
<td>17</td>
<td>1.1328</td>
<td>18.71</td>
</tr>
<tr>
<td>18</td>
<td>1.1417</td>
<td>19.89</td>
</tr>
<tr>
<td>19</td>
<td>1.1508</td>
<td>21.07</td>
</tr>
<tr>
<td>20</td>
<td>1.1600</td>
<td>22.25</td>
</tr>
<tr>
<td>21</td>
<td>1.1694</td>
<td>23.43</td>
</tr>
<tr>
<td>22</td>
<td>1.1789</td>
<td>24.61</td>
</tr>
<tr>
<td>23</td>
<td>1.1885</td>
<td>25.81</td>
</tr>
<tr>
<td>24</td>
<td>1.1983</td>
<td>27.03</td>
</tr>
<tr>
<td>25</td>
<td>1.2083</td>
<td>28.28</td>
</tr>
<tr>
<td>26</td>
<td>1.2185</td>
<td>29.53</td>
</tr>
<tr>
<td>27</td>
<td>1.2288</td>
<td>30.79</td>
</tr>
<tr>
<td>28</td>
<td>1.2393</td>
<td>32.05</td>
</tr>
<tr>
<td>29</td>
<td>1.2500</td>
<td>33.33</td>
</tr>
<tr>
<td>°Bé</td>
<td>Sp. Gr.</td>
<td>Percent H₂SO₄</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
<td>---------------</td>
</tr>
<tr>
<td>30</td>
<td>1.2609</td>
<td>34.63</td>
</tr>
<tr>
<td>31</td>
<td>1.2719</td>
<td>35.93</td>
</tr>
<tr>
<td>32</td>
<td>1.2832</td>
<td>37.26</td>
</tr>
<tr>
<td>33</td>
<td>1.2946</td>
<td>38.58</td>
</tr>
<tr>
<td>34</td>
<td>1.3063</td>
<td>39.92</td>
</tr>
<tr>
<td>35</td>
<td>1.3182</td>
<td>41.27</td>
</tr>
<tr>
<td>36</td>
<td>1.3303</td>
<td>42.63</td>
</tr>
<tr>
<td>37</td>
<td>1.3426</td>
<td>43.99</td>
</tr>
<tr>
<td>38</td>
<td>1.3551</td>
<td>45.35</td>
</tr>
<tr>
<td>39</td>
<td>1.3679</td>
<td>46.72</td>
</tr>
<tr>
<td>40</td>
<td>1.3810</td>
<td>48.10</td>
</tr>
<tr>
<td>41</td>
<td>1.3942</td>
<td>49.47</td>
</tr>
<tr>
<td>42</td>
<td>1.4078</td>
<td>50.87</td>
</tr>
<tr>
<td>43</td>
<td>1.4216</td>
<td>52.26</td>
</tr>
<tr>
<td>44</td>
<td>1.4356</td>
<td>53.66</td>
</tr>
<tr>
<td>45</td>
<td>1.4500</td>
<td>55.07</td>
</tr>
<tr>
<td>46</td>
<td>1.4646</td>
<td>56.48</td>
</tr>
<tr>
<td>47</td>
<td>1.4796</td>
<td>57.90</td>
</tr>
<tr>
<td>48</td>
<td>1.4948</td>
<td>59.32</td>
</tr>
<tr>
<td>49</td>
<td>1.5104</td>
<td>60.75</td>
</tr>
<tr>
<td>50</td>
<td>1.5263</td>
<td>62.18</td>
</tr>
<tr>
<td>51</td>
<td>1.5426</td>
<td>63.66</td>
</tr>
<tr>
<td>52</td>
<td>1.5591</td>
<td>65.13</td>
</tr>
<tr>
<td>53</td>
<td>1.5761</td>
<td>66.63</td>
</tr>
<tr>
<td>54</td>
<td>1.5934</td>
<td>68.13</td>
</tr>
<tr>
<td>55</td>
<td>1.6111</td>
<td>69.65</td>
</tr>
<tr>
<td>56</td>
<td>1.6292</td>
<td>71.17</td>
</tr>
<tr>
<td>57</td>
<td>1.6477</td>
<td>72.75</td>
</tr>
<tr>
<td>58</td>
<td>1.6667</td>
<td>74.36</td>
</tr>
<tr>
<td>59</td>
<td>1.6860</td>
<td>75.99</td>
</tr>
</tbody>
</table>
Specific gravity determinations were made at 60° F, compared with water at 60° F. The values given above for aqueous sulfuric acid solutions were adopted as standard in 1904 by the Manufacturing Chemists’ Association of the United States.

From the specific gravities, the corresponding degrees Baumé were calculated by the following equation:

\[ \text{°Baumé} = 145 - (145/\text{sp. gr.}) \]

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale. Acids stronger than 66° Bé should have their percentage compositions determined by chemical analysis.

Add the following:

\section*{WATER-INSOLUBLE MATTER}

\textbf{Sample Preparation} Add 5 g of sample (if a different amount of sample is specified in the individual monograph, use that amount) to 100 mL of water and stir until the sample is dissolved.

\textbf{Procedure} Dry a membrane filter (cellulose nitrate, 0.45-µm porosity) at 110° C for 1 hour, allow to cool in a desiccator, and weigh to the nearest 0.1 mg. Pass the Sample Preparation through the dried membrane filter and wash with three successive 10-mL portions of water. Dry the membrane filter at 110° C for 1 hour. Cool in a desiccator and weigh the membrane filter to the nearest 0.1 mg. Calculate the insoluble matter as percentage.

\footnote{1 Oils of known viscosities may be obtained from the Cannon Instrument Co., P.O. Box 812, State College, PA 16801. For determining the viscosity of dimethylpolysiloxane, choose an oil with a viscosity as close as possible to that of the type of sample to be tested.}
Oils of known viscosities may be obtained from the Cannon Instrument Co., P.O. Box 812, State College, PA 16801. For determining the viscosity of methylcellulose, choose an oil that has a viscosity as close as possible to that of the type of sample to be tested.


Harrison GR. Massachusetts Institute of Technology Wavelength Tables [also referred to as MIT Wavelength Tables]. Cambridge, MA: MIT Press; 1969.


Adapted from ASTM B214 Standard Test Method for Sieve Analysis of Metal Powders. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9585, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.

Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(F2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

FCC Seventh Edition Page 1111
BRIEFING

Appendix III: Chemical Tests and Determinations, First Supplement to FCC 7 page 1497; and FCC Forum [June 2010]. On the basis of comments received, two revisions are proposed to the tests for Nickel under B. Limit Tests.

1. A revision is proposed to the Test Preparation under Method I to replace the use of strong acetic acid TS (as the diluent) with dilute acetic acid TS. Comments received indicated difficulties were encountered in producing the required separation of the methyl isobutyl ketone layer. This revision harmonizes the preparation of the test sample with the current nickel limit test used for polyols (including Sorbitol) in the USP 33–NF 28 Reissue.

2. A revision is proposed to the Procedure in Method II to change the wavelength used from 352.0 nm to 232.0 nm. Comments received indicated that 352.0 nm is not a default wavelength for some instruments, which presents difficulties in performing the analysis. This revision is supported by the use of the 232.0-nm wavelength in the current nickel test used for polyols in the USP 33–NF 28 Reissue.

(FIEC: K. Laurvick) C97206

APPENDIX III: CHEMICAL TESTS AND DETERMINATIONS

A. IDENTIFICATION TESTS
The identification tests described in section A of this Appendix are frequently referred to in the Food Chemicals Codex for the presumptive identification of FCC-grade chemicals taken from labeled containers. These tests are not intended to be applied to mixtures unless so specified.

Acetate
Acetic acid or acetates, when warmed with sulfuric acid and alcohol, form ethyl acetate, recognizable by its characteristic odor. With neutral solutions of acetates, ferric chloride TS produces a deep red color that is destroyed by the addition of a mineral acid.

Aluminum
Solutions of aluminum salts yield with 6 N ammonia a white, gelatinous precipitate that is insoluble in an excess of the 6 N ammonia. The same precipitate is produced by 1 N sodium hydroxide, but it dissolves in an excess of this reagent.

Ammonium
Ammonium salts are decomposed by 1 N sodium hydroxide with the evolution of ammonia, recognizable by its alkaline effect on moistened red litmus paper. The decomposition is accelerated by warming.
**Benzoate**
Neutral solutions of benzoates yield a salmon colored precipitate with ferric chloride TS. From moderately concentrated solutions of benzoate, 2 N sulfuric acid precipitates free benzoic acid, which is readily soluble in ether.

**Bicarbonate**
*See Carbonate.*

**Bisulfite**
*See Sulfite.*

**Bromide**
Free bromine is liberated from solutions of bromides upon the dropwise addition of chlorine TS. When shaken with chloroform, the bromine dissolves, coloring the chloroform red to red-brown. A yellow-white precipitate, which is insoluble in nitric acid and slightly soluble in 6 N ammonia, is produced when solutions of bromides are treated with silver nitrate TS.

**Calcium**
Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner: using 2 drops of methyl red TS as the indicator, neutralize a 1:20 solution of a calcium salt with 6 N ammonia, then add 2.7 N hydrochloric acid, dropwise, until the solution is acid. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

Calcium salts moistened with hydrochloric acid impart a transient yellow-red color to a nonluminous flame.

**Carbonate**
Carbonates and bicarbonates effervesce with acids, yielding a colorless gas that produces a white precipitate immediately when passed into calcium hydroxide TS. Cold solutions of soluble carbonates are colored red by phenolphthalein TS, whereas solutions of bicarbonates remain unchanged or are slightly changed.

**Chloride**
Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate that is insoluble in nitric acid but soluble in a slight excess of 6 N ammonia.

**Citrate**
To 15 mL of pyridine add a few milligrams of a citrate salt, dissolved or suspended in 1 mL of water, and shake. Add 5 mL of acetic anhydride to this mixture, and shake. A light red color appears.

**Cobalt**
Solutions of cobalt salts (1:20) in 2.7 N hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared 1:10 solution of 1-nitroso-2-naphthol in 9 N acetic acid.
Solutions of cobalt salts yield a yellow precipitate when saturated with potassium chloride and treated with potassium nitrite and acetic acid.

Copper
When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited on a bright untarnished surface of metallic iron. An excess of 6 N ammonia, added to a solution of a cupric salt, produces first a blue precipitate and then a deep blue colored solution. Solutions of cupric salts yield with potassium ferrocyanide TS a red-brown precipitate, insoluble in diluted acids.

Hypophosphite
Hypophosphites evolve spontaneously flammable phosphine when strongly heated. Solutions of hypophosphites yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present. Hypophosphite solutions, acidified with sulfuric acid and warmed with copper sulfate TS, yield a red precipitate.

Iodide
Solutions of iodides, upon the dropwise addition of chlorine TS, liberate iodine, which colors the solution yellow to red. Chloroform is colored violet when shaken with this solution. The iodine thus liberated gives a blue color with starch TS. In solutions of iodides, silver nitrate TS produces a yellow, curdy precipitate that is insoluble in nitric acid and in 6 N ammonia.

Iron
Solutions of ferrous and ferric compounds yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold 2.7 N hydrochloric acid with the evolution of hydrogen sulfide.

Ferric Salts: Potassium ferrocyanide TS (10%) produces a dark blue precipitate in acid solutions of ferric salts. With an excess of 1 N sodium hydroxide, a red-brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS (1.0 N) a deep red color that is not destroyed by diluted mineral acids.

Ferrous Salts: Potassium ferricyanide TS (10%) produces a dark blue precipitate in solutions of ferrous salts. This precipitate, which is insoluble in dilute hydrochloric acid, is decomposed by 1 N sodium hydroxide. Solutions of ferrous salts yield with 1 N sodium hydroxide a green-white precipitate, the color rapidly changing to green and then to brown when shaken.

Lactate
When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS (0.1 N) is added, and the mixture is heated, acetaldehyde is evolved. This can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS. A blue color is produced.

Magnesium
Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate with ammonium carbonate TS, but a white crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed on the subsequent addition of sodium phosphate TS (6%).
Manganese
Solutions of manganous salts yield with ammonium sulfide TS a salmon colored precipitate that dissolves in acetic acid.

Nitrate
When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture cooled, and a solution of ferrous sulfate superimposed, a brown color is produced at the junction of the two liquids. Brown-red fumes are evolved when a nitrate is heated with sulfuric acid and metallic copper. Nitrates do not decolorize acidified potassium permanganate TS (0.1 N) (distinction from nitrites).

Nitrite
Nitrites yield brown-red fumes when treated with diluted mineral acids or acetic acid. A few drops of potassium iodide TS (15%) and a few drops of 2 N sulfuric acid added to a solution of nitrite liberate iodine, which colors starch TS blue.

Peroxide
Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color on the addition of potassium dichromate TS. On shaking the mixture with an equal volume of diethyl ether and allowing the liquids to separate, the blue color is transferred to the ether layer.

Phosphate
Neutral solutions of orthophosphates yield with silver nitrate TS (0.1 N) a yellow precipitate, which is soluble in 1.7 N nitric acid or in 6 N ammonium hydroxide. With ammonium molybdate TS, a yellow precipitate, which is soluble in 6 N ammonium hydroxide, is formed.

Potassium
Potassium compounds impart a violet color to a nonluminous flame if not masked by the presence of small quantities of sodium. In neutral, concentrated or moderately concentrated solutions of potassium salts, sodium bitartrate TS (10%) slowly produces a white, crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides or carbonates. The precipitation may be accelerated by stirring or rubbing the inside of the test tube with a glass rod or by the addition of a small amount of glacial acetic acid or alcohol.

Sodium
Dissolve 0.1 g of the sodium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimonate TS, and heat to boiling. Allow to cool in ice water, and if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed. Sodium compounds impart an intense yellow color to a nonluminous flame.

Change to read:
**Sulfate**

Solutions of sulfates yield with barium chloride TS a white precipitate that is insoluble in hydrochloric and nitric acids. Sulfates yield with lead acetate TS a white precipitate that is soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

**Sulfite**

When treated with 2.7 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic odor. This gas blackens filter paper moistened with mercurous nitrate TS.

**Tartrate**

When a few milligrams of a tartrate are added to a mixture of 15 mL of pyridine and 5 mL of acetic anhydride, an emerald green color is produced.

**Thiosulfate**

With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, liberating sulfur dioxide, recognizable by its odor. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that quickly disappears.

**Zinc**

Zinc salts, in the presence of sodium acetate, yield a white precipitate with hydrogen sulfide. This precipitate, which is insoluble in acetic acid, is dissolved by 2.7 N hydrochloric acid. A similar precipitate is produced by ammonium sulfide TS in neutral or alkaline solutions. Solutions of zinc salts yield with potassium ferrocyanide TS (10%) a white precipitate that is insoluble in 2.7 N hydrochloric acid.

**B. LIMIT TESTS**

**ALUMINUM LIMIT TEST**

[Note—The Standard Solutions and Sample Solution may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

**Nitric Acid Diluent**  Dilute 40 mL of nitric acid with water to 1000 mL.

**Standard Aluminum Solutions**  Treat a quantity of aluminum wire with 6 N hydrochloric acid at 80° for a few min. Dissolve 100 mg of the treated wire in a mixture consisting of 10 mL of hydrochloric acid and 2 mL of nitric acid, by heating at 80° for about 30 min. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, and dilute with water to volume (1 mg/mL aluminum). Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, and dilute with water to volume (100 µg/mL aluminum). Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, and dilute with water to volume (1 µg/mL aluminum).  [Note—if more diluted Standard Aluminum Solutions are required,
transfer 1.0-, 2.0-, and 4.0-mL portions of the 1 µg/mL Standard Aluminum Solution to separate 100-mL volumetric flasks, dilute with Nitric Acid Diluent to volume, and mix. These solutions contain 0.01, 0.02, and 0.04 µg/mL of aluminum, respectively.]

Sample Solution  Transfer the amount of sample specified in the monograph to a plastic 100-mL volumetric flask. Add 50 mL of water, and sonicate for 30 min. Add 4 mL of nitric acid, and dilute with water to volume.

Procedure  Determine the absorbances of the Standard Aluminum Solutions and the Sample Solution at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the Nitric Acid Diluent as the blank. Plot the absorbances of the Standard Solutions versus the concentration of aluminum, in µg/mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the concentration, in µg/mL, of aluminum in the Sample Solution.

Calculate the amount of aluminum in the sample taken, in µg/g, using the following formula:

\[
\text{Result} = \frac{C_A}{C_S}
\]

where \( C_A \) is the concentration of aluminum in the Sample Solution, in µg/mL, obtained from the standard curve; and \( C_S \) is the concentration of the Sample Solution, in g/mL.

ARSENIC LIMIT TEST

Silver Diethyldithiocarbamate Colorimetric Method  
[Note—All reagents used in this test should be very low in arsenic content.]

Apparatus  Use the general apparatus shown in Fig. 11 unless otherwise specified in an individual monograph. It consists of a 125-mL arsine generator flask (a) fitted with a scrubber unit (c) and an absorber tube (e), with a 24/40 standard-taper joint (b) and a ball-and-socket joint (d), secured with a No. 12 clamp, connecting the units. The tubing between d and e and between d and c is a capillary having an id of 2 mm and an od of 8 mm. Alternatively, an apparatus embodying the principle of the general assembly described and illustrated may be used.
FIGURE 11 General Apparatus for Arsenic Limit Test. (Courtesy of the Fisher Scientific Co., Pittsburgh, PA.)

[Note—The special assemblies shown in Figs. 12, 13, and 14 are to be used only when specified in certain monographs.]

FIGURE 12 Modified Bethge Apparatus for the Distillation of Arsenic Tribromide.
FIGURE 13 Special Apparatus for the Distillation of Arsenic Trichloride. (Flask A contains 150 mL of hydrochloric acid; flasks D and F contain 20 mL of water. Flask D is placed in an ice water bath, E.)

FIGURE 14 Special Apparatus for the Determination of Inorganic Arsenic. (A, 250-mL distillation flask; B, 250 mL.)
receiver chamber, approximately 50-mL capacity; C, reflux condenser; D, splash head.)

**Standard Arsenic Solution** Accurately weigh 132.0 mg of arsenic trioxide that has been previously dried at 105° for 1 h, and dissolve it in 5 mL of a 1:5 sodium hydroxide solution. Neutralize the solution with 2 N sulfuric acid, add 10 mL in excess, and dilute with recently boiled water to 1000.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with recently boiled water to volume, and mix. Use this final solution, which contains 1 µg of arsenic in each milliliter, within 3 days.

**Silver Diethyldithiocarbamate Solution** Dissolve 1 g of ACS reagent-grade silver diethyldithiocarbamate in 200 mL of recently distilled pyridine. Store this solution in a light-resistant container and use within 1 month.

**Stannous Chloride Solution** Dissolve 40 g of stannous chloride dihydrate (SnCl₂·2H₂O) in 100 mL of hydrochloric acid. Store the solution in glass containers and use within 3 months.

**Lead Acetate-Impregnated Cotton** Soak cotton in a saturated solution of lead acetate trihydrate, squeeze out the excess solution, and dry in a vacuum at room temperature.

**Sample Solution** Use directly as the Sample Solution in the Procedure the solution obtained by treating the sample as directed in an individual monograph. Prepare sample solutions of organic compounds in the generator flask (a), unless otherwise directed, according to the following general procedure:

[CAUTION— Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times. ]

[Note—If halogen-containing compounds are present, use a lower temperature while heating the sample with sulfuric acid; do not boil the mixture; and add the peroxide, with caution, before charring begins to prevent loss of trivalent arsenic.] Transfer 1.0 g of sample into the generator flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, preferably using a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the acid has initially decomposed the sample, cautiously add, dropwise, hydrogen peroxide (30%), allowing the reaction to subside and reheating the sample between drops. Add the first few drops very slowly with sufficient mixing to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during digestion.

[Note—Maintain oxidizing conditions at all times during the digestion by adding small quantities of the peroxide whenever the mixture turns brown or darkens.] Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° to 300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, heat again to strong fuming, and cool. Cautiously add 10 mL of water, mix, wash the sides of the flask with a few milliliters of water, and dilute to 35 mL.

**Procedure** If the Sample Solution was not prepared in the generator flask, transfer to the flask a volume of the solution, prepared as directed, equivalent to 1.0 g of the substance being tested, and add water to make 35 mL. Add 20 mL of 1:5 sulfuric acid, 2 mL of potassium iodide TS, 0.5 mL of Stannous Chloride Solution, and 1 mL of isopropyl alcohol, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber unit (c) with two plugs of Lead Acetate-Impregnated Cotton, leaving a small air space between the two plugs, lubricate joints b and d with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube (e). Transfer 3.0 mL of Silver Diethyldithiocarbamate Solution to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint (b) into the
flask. Allow the evolution of hydrogen and color development to proceed at room temperature (25 ± 3°C) for 45 min, swirling the flask gently at 10-min intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the Silver Diethyldithiocarbamate Solution to a 1-cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 and 540 nm, with a suitable spectrophotometer or colorimeter, using Silver Diethyldithiocarbamate Solution as the blank. The absorbance due to any red color from the solution of the sample does not exceed that produced by 3.0 mL of Standard Arsenic Solution (3 µg As) when treated in the same manner and under the same conditions as the sample.

The room temperature during the generation of arsine from the standard should be held to within ±2°C of that observed during the determination of the sample.

Interferences  Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. Antimony, which forms stibine, is the only metal likely to produce a positive interference in the color development with the silver diethyldithiocarbamate. Stibine forms a red color with silver diethyldithiocarbamate that has a maximum absorbance at 510 nm, but at 535 to 540 nm, the absorbance of the antimony complex is so diminished that the results of the determination would not be altered significantly.

CADMIUM LIMIT TEST

Spectrophotometer  Use any suitable atomic absorption spectrophotometer equipped with a Boling-type burner, an air–acetylene flame, and a hollow-cathode cadmium lamp. The instrument should be capable of operating within the sensitivity necessary for the determination.

Standard Solution  Transfer 100 mg of cadmium chloride crystals (CdCl₂·2½H₂O), accurately weighed, into a 1000-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Pipet 25 mL of this solution into a 100-mL volumetric flask, add 1 mL of hydrochloric acid, dilute with water to volume, and mix. Each milliliter contains 12.5 µg of cadmium.

Sample Solution  Transfer 10 g of sample, accurately weighed, into a 50-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Test Solutions  Transfer 5.0 mL of Sample Solution into each of five separate 25-mL volumetric flasks. Dilute the contents of Flask 1 with water to volume, and mix. Add 1.00, 2.00, 3.00, and 4.00 mL of Standard Solution, to Flasks 2, 3, 4, and 5, respectively, then dilute each flask with water to volume, and mix. The Test Solutions contain, respectively, 0, 0.5, 1.0, 1.5, and 2.0 µg/mL of cadmium.

Procedure  Determine the absorbance of each Test Solution at 228.8 nm, setting the instrument to previously established optimum conditions, using water as a blank. Plot the absorbance of the Test Solutions versus their contents of cadmium, in micrograms per milliliter. Draw the straight line best fitting the five points, and extrapolate the line until it intercepts the concentration axis. From the intercept, determine the amount, in micrograms, of cadmium in each milliliter of the Test Solution containing 0 mL of the Standard Preparation. Calculate the quantity, in milligrams per kilogram, of cadmium in the sample by multiplying this value by 25.

CHLORIDE AND SULFATE LIMIT TESTS

Where limits for chloride and sulfate are specified in the individual monograph, compare the Sample Solution and control in appropriate glass cylinders of the same dimensions and matched as closely as practicable with
respect to their optical characteristics.

If the solution is not perfectly clear after acidification, pass it through filter paper that has been washed free of chloride and sulfate. Add identical quantities of the precipitant (silver nitrate TS or barium chloride TS) in rapid succession to both the Sample Solution and the control solution.

Experience has shown that visual turbidimetric comparisons are best made between solutions containing from 10 to 20 µg of chloride (Cl) ion or from 200 to 400 µg of sulfate (SO₄²⁻) ion in 50 mL. Weights of samples are specified on this basis in the individual monographs in which these limits are included.

**Chloride Limit Test**

**Standard Chloride Solution**  Dissolve 165 mg of sodium chloride in water and dilute to 100.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix. Each milliliter of the final solution contains 10 µg of chloride (Cl) ion.

**Procedure**  Unless otherwise directed, dissolve the specified amount of the test substance in 30 to 40 mL of water; neutralize to litmus external indicator with nitric acid, if necessary; and add 1 mL in excess. Add 1 mL of silver nitrate TS to the clear solution or filtrate, dilute with water to 50 mL, mix, and allow to stand for 5 min protected from direct sunlight. Compare the turbidity, if any, with that produced similarly in a control solution containing the required volume of Standard Chloride Solution and the quantities of the reagents used for the sample.

**Sulfate Limit Test**

**Standard Sulfate Solution**  Dissolve 148 mg of anhydrous sodium sulfate in water, and dilute to 100.0 mL. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. Each milliliter of the final solution contains 10 µg of sulfate (SO₄²⁻).

**Procedure**  Unless otherwise directed, dissolve the specified amount of the test substance in 30 to 40 mL of water; neutralize to litmus external indicator with hydrochloric acid, if necessary; then add 1 mL of 2.7 N hydrochloric acid. Add 3 mL of barium chloride TS to the clear solution or filtrate, dilute with water to 50 mL, and mix. After 10 min compare the turbidity, if any, with that produced in a solution containing the required volume of Standard Sulfate Solution and the quantities of the reagents used for the sample.

**1,4-DIOXANE LIMIT TEST**

**Vacuum Distillation Apparatus**  Assemble a closed-system vacuum distillation apparatus employing glass vacuum stopcocks (A, B, and C), as shown in Fig. 15.

![FIGURE 15 Closed-System Vacuum Distillation Apparatus for 1,4-Dioxane.](image)
The concentrator tube ($D$) is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate and marked so that the analyst can accurately dilute to 2.0 mL (available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ, Catalog No. K42560-0000).

**Standard Preparation** Prepare a solution of 1,4-dioxane in water containing 100 µg/mL. Keep the solution refrigerated, and prepare fresh weekly.

**Sample Preparation** Transfer 20 g of the sample, accurately weighed, into a 50-mL round-bottom flask ($E$) having a 24/40 ground-glass neck. Semisolid or waxy samples should be liquefied by heating on a steam bath before making the transfer. Add 2.0 mL of water to the flask for crystalline samples, and 1.0 mL for liquid, semisolid, or waxy samples. Place a small Teflon-covered stirring bar in the flask, stopper, and stir to mix. Immerse the flask in an ice bath, and chill for about 1 min.

Wrap heating tape around the tube connecting the Chromaflex tube ($D$) and the round-bottom flask ($E$), and apply about 10 V to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the Chromaflex tube to the 10/30 joint and the round-bottom flask to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks $A$ and $B$, open stopcock $C$, and begin evacuating the system with a vacuum pump. Prepare a slush bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for about 10 min, and when the vacuum system is operating at 0.05 mm pressure or lower, open stopcock $A$ for 20 s, and then close it. Remove the slush bath, and allow the flask to warm in air for about 1 min. Immerse the flask in a water bath at 20° to 25°, and after about 5 min warm the water in the bath to 35° to 40° (sufficient to liquefy most samples) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 min. Replace the water bath with the slush bath, freeze the contents of the flask for about 10 min, then open stopcock $A$ for 20 s, and close it. Remove the slush bath, and repeat the heating steps as before, this time reaching a final temperature of 45° to 50° or a temperature necessary to melt the sample completely. If there is any condensation in the tube connecting the round-bottom flask to the Chromaflex tube, slowly increase the voltage to the heating tape and heat until condensation disappears.

Stir with the magnetic stirrer throughout the following steps: very slowly immerse the Chromaflex tube in the Dewar flask containing liquid nitrogen. [CAUTION—When there is liquid distillate in the Chromaflex tube, the tube must be immersed in the nitrogen very slowly, or the tube will break.]

Water will begin to distill into the tube. As ice forms in the tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the Chromaflex tube, remove the Dewar flask and let the ice melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until at least 0.9 mL of water has been collected. Freeze the tube once again for about 2 min, and release the vacuum first by opening stopcock $B$, followed by stopcock $A$. Remove the Chromaflex tube from the apparatus, close it with a greased stopper, and let the ice melt without heating. Mix the contents of the tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary. Use this Sample Preparation as directed under Chromatography.

**Chromatography** (See Chromatography, Appendix IIA.) Use a gas chromatograph equipped with a flame-ionization detector. Under typical conditions, the instrument contains a 4-mm (id) × 6-ft glass column, or equivalent, packed with 80-100- or 100-120-mesh Chromosorb 104, or equivalent. The column is maintained isothermally at about 140°, the injection port at 200°, and the detector at 250°. Nitrogen is the carrier gas, flowing at a rate of about 35 mL/min. Install an oxygen scrubber between the carrier gas line and the column. The column should be conditioned for about 72 h at 250° with 30 to 40 mL/min carrier flow.
[Note—Chromosorb 104 is oxygen sensitive. Both new and used columns should be flushed with carrier gas for 30 to 60 min before heating each time they are installed in the gas chromatograph.]

Inject a volume of the Standard Preparation, accurately measured, to give about 20% of maximum recorder response. Where possible, keep the injection volume in the range of 2 to 4 µL, and use the solvent-flush technique to minimize errors associated with injection volumes. In the same manner, inject an identical volume of the Sample Preparation. The height of the peak produced by the Sample Preparation does not exceed that produced by the Standard Preparation.¹

**FLUORIDE LIMIT TEST**

**Method I (Thorium Nitrate Colorimetric Method)**

Use this method unless otherwise directed in the individual monograph.

[CAUTION—When applying this test to organic compounds, rigidly control at all times the temperature at which the distillation is conducted to the recommended range of 135° to 140° to avoid the possibility of explosion.]

[Note—To minimize the distillation blank resulting from fluoride leached from the glassware, treat the distillation apparatus as follows: treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15 to 20 mL of 1:2 sulfuric acid until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see AOAC method 944.08.]

Unless otherwise directed, place a 5.0-g sample and 30 mL of water in a 125-mL Pyrex distillation flask having a side arm and trap. The flask is connected with a condenser and carries a thermometer and a capillary tube, both of which must extend into the liquid. Slowly add, with continuous stirring, 10 mL of 70% perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads. Connect a small dropping funnel or a steam generator to the capillary tube. Support the flask on a flame-resistant mat or shielding board, with a hole that exposes about one-third of the flask to the low, “clean” flame of a Bunsen burner.

[Note—The shielding is essential to prevent the walls of the flask from overheating above the level of its liquid contents.]

Distill until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary, maintaining the temperature between 135° and 140° at all times. Continue the distillation until 100 mL of distillate has been collected. After the 100-mL portion (Distillate A) is collected, collect an additional 50-mL portion of distillate (Distillate B) to ensure that all of the fluorine has been volatilized.

Place 50 mL of Distillate A in a 50-mL Nessler tube. In another, similar Nessler tube, place 50 mL of water distilled through the apparatus as a control. Add to each tube 0.1 mL of a filtered 1:1000 solution of sodium alizarinsulfonate and 1 mL of a freshly prepared 1:4000 solution of hydroxylamine hydrochloride, and mix well. Add, dropwise and with stirring, either 1 N or 0.05 N sodium hydroxide, depending on the expected volume of volatile acid distilling over, to the tube containing the distillate until its color just matches that of the control, which is faintly pink. Then add to each tube 1.0 mL of 0.1 N hydrochloric acid, and mix well. From a buret, graduated in 0.05 mL, add slowly to the tube containing the distillate enough of a 1:4000 solution of thorium nitrate so that, after mixing, the color of the liquid just changes to a faint pink. Note the volume of the solution
added, then add exactly the same volume to the control, and mix. Now add to the control solution sodium fluoride TS (10 µg F per milliliter) from a buret to make the colors of the two tubes match after dilution to the same volume. Mix well, and allow all air bubbles to escape before making the final color comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in color should take place. Note the volume of sodium fluoride TS added.

Dilute Distillate B to 100 mL, and mix well. Place 50 mL of this solution in a 50-mL Nessler tube, and follow the procedure used for Distillate A. The total volume of sodium fluoride TS required for the solutions from both Distillate A and Distillate B should not exceed 2.5 mL.

**Method II (Ion-Selective Electrode Method A)**

**Buffer Solution** Dissolve 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in sufficient 1 N sodium hydroxide to make 200 mL. Transfer 20 mL of this solution (equivalent to 4 g of disodium CDTA) into a 1000-mL beaker containing 500 mL of water, 57 mL of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to between 5.0 and 5.5 by the addition of 5 N sodium hydroxide, then cool to room temperature, dilute with water to 1000 mL, and mix.

**Procedure** Unless otherwise directed in the individual monograph, transfer 8.0 g of sample and 20 mL of water into a 250-mL distilling flask, cautiously add 20 mL of perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads.

> [CAUTION— Handle perchloric acid in an appropriate fume hood.]

Following the directions, and observing the Caution and Notes, as given under Method I, distill the solution until 200 mL of distillate has been collected.

Transfer a 25.0-mL aliquot of the distillate into a 250-mL plastic beaker, and dilute with the Buffer Solution to 100 mL. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) of a suitable ion-selective electrode apparatus in the solution. Adjust the calibration control until the indicator needle points to the center on the logarithmic concentration scale, allowing sufficient time for equilibration (about 20 min), and stirring constantly during the equilibration period and throughout the remainder of the procedure. Pipet 1.0 mL of a solution containing 100 µg of fluoride (F) ion per milliliter (prepared by dissolving 22.2 mg of sodium fluoride, previously dried at 200° for 4 h, in sufficient water to make 100.0 mL) into the beaker, allow the electrode to come to equilibrium, and record the final reading on the logarithmic concentration scale.

> [Note—Follow the instrument manufacturer's instructions regarding precautions and interferences, electrode filling and check, temperature compensation, and calibration.]

**Calculations** Calculate the fluoride content, in milligrams per kilogram, of the sample taken:

\[
\text{Result} = \left[ \frac{IA}{R-I} \right] \times 100 \times \left( \frac{200}{25W} \right)
\]

in which I is the initial scale reading before the addition of the sodium fluoride solution; A is the concentration, in micrograms per milliliter, of fluoride in the sodium fluoride solution added to the sample solution; R is the final scale reading after addition of the sodium fluoride solution; and W is the original weight, in grams, of the sample.

*Change to read:*

**Method III (Ion-Selective Electrode Method B)**
Sodium Fluoride Solution (5 µg F per milliliter) Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-mL plastic beaker, add 200 mL of water, and stir until dissolved. Quantitatively transfer this solution into a 1000-mL volumetric flask with the aid of water, dilute with water to volume, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 mL of the stock solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Calibration Curve Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the Sodium Fluoride Solution into separate 250-mL plastic beakers; add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of 1 M sodium citrate, and 10 mL of 0.2 M disodium EDTA to each beaker; and mix. Transfer each solution into separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution into separate 125-mL plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode apparatus (such as the Orion Model No. 94-09, with solid-state membrane), using a suitable reference electrode (such as the Orion Model No. 90-01, with single junction). Plot the calibration curve on two-cycle semilogarithmic paper (such as K & E No. 465130) or with the use of a suitable graphing calculator or spreadsheet program. Estimate the micrograms of F per 100 mL solution on the logarithmic scale.

Procedure Transfer 1.00 g of sample into a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of 1 M sodium citrate and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary; transfer into a 100-mL volumetric flask; dilute with water to volume; and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and measure the potential of the solution with the apparatus described under Calibration Curve. Determine the fluoride content, in micrograms, of the sample from the Calibration Curve. Estimate the percentage of fluoride in the sample taken:

\[
\text{Result} = (C/W_S) \times 0.000001 \times 100\%
\]

in which C is the content of fluoride, in µg, in the sample, determined from the Calibration Curve; W_S is the sample weight, in g; and 0.000001 is a factor converting micrograms to grams.

Change to read:

Method IV (Ion-Selective Electrode Method C)
[Note—Unless directed otherwise by the individual monograph, use Buffer Solution A for samples with a neutral to higher pH, and use Buffer Solution B for samples with a neutral to lower pH.]

Buffer Solution A Add 2 volumes of 6 N acetic acid to 1 volume of water, and adjust the pH to 5.0 with 50% potassium hydroxide solution.

Buffer Solution B Dissolve 150 g of sodium citrate dihydrate and 10.3 g of disodium EDTA dihydrate in 800 mL of water, adjust the pH to 8.0 with 50% sodium hydroxide solution, and dilute with water to 1000 mL.

Fluoride Standard Solutions

1000 mg/kg Fluoride Standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask and dissolve in and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per milliliter.

50 mg/kg Fluoride Standard: Pipet 50 mL of the 1000 mg/kg Fluoride Standard into a 1000-mL volumetric flask. Dilute with water to volume.
10 mg/kg Fluoride Standard: Pipet 100 mL of the 50 mg/kg Fluoride Standard into a 500-mL volumetric flask. Dilute with water to volume.

Fluoride Limit Solutions (for a 1-g sample)

50 mg/kg Fluoride Limit Solution (1 mg/kg fluoride standard): Pipet 50 mL of the 10 mg/kg Fluoride Standard into a 500-mL volumetric flask, and dilute with water to volume.

10 mg/kg Fluoride Limit Solution (0.2 mg/kg fluoride standard): Pipet 10 mL of the 10 mg/kg Fluoride Standard into a 500-mL volumetric flask, and dilute with water to volume.

Fluoride Limit Solutions (for a 2-g sample)

50 mg/kg Fluoride Limit Solution (2 mg/kg fluoride standard): Pipet 100 mL of the 10 mg/kg Fluoride Standard into a 500-mL volumetric flask, and dilute with water to volume.

10 mg/kg Fluoride Limit Solution (0.4 mg/kg fluoride standard): Pipet 20 mL of the 10 mg/kg Fluoride Standard into a 500-mL volumetric flask, and dilute with water to volume.

[Note—Store all standard and limit solutions in plastic containers.]

Sample Preparation Accurately weigh the amount of sample specified in the monograph, transfer it into a 100-mL volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 mL of the appropriate Buffer Solution, dilute with water to volume, and mix.

Electrode Calibration Pipet 50 mL of the appropriate Buffer Solution into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. At 5-min intervals, add 100 µL and 1000 µL of the 1000 mg/kg Fluoride Standard and read the potential, in millivolts, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 63 to 70 mV at 25°C for Buffer Solution A and in the range of 54 to 60 mV at 25°C for Buffer Solution B. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Alternatively, the electrode calibration should be performed according to the manufacturer's instructions and should comply with the manufacturer's calibration range at 25°C. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Procedure Transfer the entire sample into a plastic beaker. Place the electrode into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential, in millivolts. Remove and rinse the electrode(s) with water. In another beaker, using a pipet, add 50 mL of the appropriate Buffer Solution followed by 50 mL of the Fluoride Limit Solution that best reflects the fluoride limit of the sample. Place the electrode in the beaker, equilibrate for 3 min, and read the potential in millivolts. If the potential of the Fluoride Limit Solution is less than that of the sample, the sample passes the test criteria for maximum acceptable fluoride level limit.

Method V

Lime Suspension Carefully shake about 56 g of low-fluorine calcium oxide (about 2 mg/kg of F) with 250 mL of water, and while stirring, slowly add 250 mL of 60% perchloric acid. Add a few glass beads, and boil until copious fumes of perchloric acid evolve, then cool, add 200 mL of water, and boil again.

[CAUTION—Handle perchloric acid in an appropriate fume hood.]

Repeat the dilution and boiling once more, cool, dilute considerably, and if precipitated silicon dioxide forms, pass through a fritted-glass filter. While stirring, pour the clear solution into 1000 mL of a 1:10 solution of
sodium hydroxide, allow the precipitate to settle, and siphon off the supernatant liquid. Remove the sodium salts from the precipitate by washing five times with water in large centrifuge bottles, shaking the mass thoroughly each time. Finally, shake the precipitate into a suspension and dilute with water to 2000 mL. Store in paraffin-lined bottles, and shake well before use.

[Note—100 mL of this suspension should give no appreciable fluoride blank when evaporated, distilled, and titrated as directed under Method I.]

**Procedure**  Assemble the distilling apparatus as described under Method I, and add 1.67 g of sample, accurately weighed, and 25 mL of 1:2 sulfuric acid to the distilling flask. Distill until the temperature reaches 160°, then maintain at 160° to 165° by adding water from the funnel, collecting 300 mL of distillate. Oxidize the distillate by cautiously adding 2 or 3 mL of fluorine-free 30% hydrogen peroxide (to remove sulfates), allow to stand for a few minutes, and evaporate in a platinum dish with an excess of Lime Suspension. Ignite briefly at 600°, then cool and wet the ash with about 10 mL of water. Cover the dish with a watch glass, and cautiously introduce under the watch glass just sufficient 60% perchloric acid to dissolve the ash. Add the contents of the dish through the dropping funnel of a freshly prepared distilling apparatus (the distilling flask should contain a few glass beads), using a total of 20 mL of the 60% perchloric acid to dissolve the ash and transfer the solution. Add 10 mL of water and a few drops of a 1:2 solution of silver perchlorate through the dropping funnel, and continue as directed under Method I, beginning with “Distill until the temperature reaches 135° …”

**LEAD LIMIT TEST**
[Note—Unless otherwise specified in the monograph, use the Dithizone Method to determine lead levels.]

**Dithizone Method**

**Special Reagents**  Select reagents having as low a lead content as practicable, and store all solutions in containers of borosilicate glass. Rinse all glassware thoroughly with warm, 1:2 nitric acid followed by water.

**Ammonia–Cyanide Solution**  Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

**Ammonium Citrate Solution**  Dissolve 40 g of citric acid in 90 mL of water, add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a red color. Extract it with 20-mL portions of Dithizone Extraction Solution until the dithizone solution retains its green color or remains unchanged.

**Diluted Standard Lead Solution** (1 µg Pb in 1 mL)

- **Lead Nitrate Stock Solution:**  Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate \([\text{Pb(NO}_3\text{)}_2]\) in 100 mL of water containing 1 mL of nitric acid, dilute with water to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts.

- **Standard Lead Solution:**  On the day of use, dilute 10.0 mL of Lead Nitrate Stock Solution with water to 100.0 mL. Each milliliter of Standard Lead Solution contains the equivalent of 10 µg of lead (Pb) ion.

- **Diluted Standard Lead Solution:**  Immediately before use, transfer 10.0 mL of Standard Lead Solution into a 100-mL volumetric flask, dilute with 1:100 nitric acid to volume, and mix.

**Dithizone Extraction Solution**  Dissolve 30 mg of dithizone in 1000 mL of chloroform, add 5 mL of alcohol,
and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid.

**Hydroxylamine Hydrochloride Solution** Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of a 1:25 solution of sodium diethylthiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding 1 or 2 drops more of thymol blue TS if necessary, then dilute with water to 100 mL, and mix.

**Potassium Cyanide Solution** Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from the solution by extraction with successive portions of *Dithizone Extraction Solution* as described under *Ammonium Citrate Solution*, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally, dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

**Standard Dithizone Solution** Dissolve 10 mg of dithizone in 1000 mL of chloroform, keeping the solution in a glass-stoppered, lead-free bottle suitably wrapped to protect it from light and stored in a refrigerator.

**Sample Solution** Use the solution obtained by treating the sample as directed in an individual monograph as the *Sample Solution* in the *Procedure*. Sample solutions of organic compounds are prepared, unless otherwise directed, according to the following general method: [**CAUTION**—Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times.]

Transfer 1.0 g of sample into a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, using preferably a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the sample has initially been decomposed by the acid, add with caution, dropwise, hydrogen peroxide (30%), allowing the reaction to subside and reheating between drops. The first few drops must be added very slowly with sufficient mixing to prevent a rapid reaction, and heating should be discontinued if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during the digestion.

[**Note**—Add small quantities of the peroxide when the solution begins to darken.]

Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° to 300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, again evaporate to strong fuming, and cool. Quantitatively transfer the solution into a separator with the aid of small quantities of water.

**Procedure** Transfer the *Sample Solution*, prepared as directed in the individual monograph, into a separator, and unless otherwise directed, add 6 mL of *Ammonium Citrate Solution* and 2 mL of *Hydroxylamine Hydrochloride Solution*. (Use 10 mL of the citrate solution when determining lead in iron salts.) Add 2 drops of phenol red TS to the separator, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution, if necessary, under a stream of tap water, then add 2 mL of *Potassium Cyanide Solution*. Immediately extract the solution with 5-mL portions of *Dithizone Extraction Solution*, draining each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 s with 20 mL of 1:100 nitric acid, discard the chloroform layer, add 5.0 mL of
Standard Dithizone Solution and 4 mL of Ammonia–Cyanide Solution to the acid solution, and shake for 30 s. The purple hue in the chloroform solution of the sample caused by any lead dithizonate present does not exceed that in a control, containing the volume of Diluted Standard Lead Solution equivalent to the amount of lead specified in the monograph, when treated in the same manner as the sample.

**Flame Atomic Absorption Spectrophotometric Method**
Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm, 1:2 nitric acid followed by water.

**Lead Nitrate Stock Solution** (100 µg/mL) Dissolve 159.8 mg of reagent-grade lead nitrate \([\text{Pb(NO}_3\text{)}_2]\) in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

**Standard Lead Solution** (10 µg/mL) On the day of use, transfer 10 mL of Lead Nitrate Stock Solution into a 100-mL volumetric flask, and dilute with water to volume.

**Diluted Standard Lead Solutions** On the day of use, prepare a set of standard lead solutions that corresponds to the lead limit specified in the monograph:
- **1 mg/kg Lead Limit** (0.5, 1.0, and 1.5 µg/mL standards): On the day of use, transfer 5.0, 10.0, and 15.0 mL of Standard Lead Solution into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.
- **5 mg/kg Lead Limit** (1.0, 5.0, and 10.0 µg/mL standards): On the day of use, transfer 10.0 and 50.0 mL of Standard Lead Solution into two separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume. The final standard, 10.0 µg/mL, is taken directly from the Standard Lead Solution.
- **10 mg/kg Lead Limit** (5.0, 10.0, and 15.0 µg/mL standards): On the day of use, transfer 5.0, 10.0, and 15.0 mL of Lead Nitrate Stock Solution into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.

**25% Sulfuric Acid Solution (by volume)** Cautiously add 100 mL of sulfuric acid to 300 mL of water with constant stirring while cooling in an ice bath.

**Sample Preparation** Transfer the sample weight as specified in the monograph, weighed to the nearest 0.1 mg, into an evaporating dish. Add a sufficient amount of 25% Sulfuric Acid Solution, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Prepare a Sample Blank by ashing 5 mL of 25% sulfuric acid. Cool and cautiously wash down the inside of each evaporation dish with water.

Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer each solution quantitatively to a 10-mL volumetric flask, dilute to volume, and mix.

**Procedure** Concomitantly determine the absorbances of the Sample Blank, the Diluted Standard Lead Solutions, and the Sample Preparation at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrodeless discharge lamp (EDL), an air–acetylene flame, and a 4-in burner head. Use water as the blank.

**Calculations** Determine the corrected absorbance values by subtracting the Sample Blank absorbance from each of the Diluted Standard Lead Solutions and from the Sample Preparation absorbances. Prepare a
standard curve by plotting the corrected Diluted Standard Lead Solutions absorbance values versus their corresponding concentrations expressed as micrograms per milliliter. Determine the lead concentration in the Sample Preparation by reference to the calibration curve. Calculate the quantity of lead, in milligrams per kilogram, in the sample taken:

\[
\text{Result} = 10C/W_S
\]

in which C is the concentration, in micrograms per milliliter, of lead from the standard curve; and \( W_S \) is the weight, in grams, of the sample taken.

**Atomic Absorption Spectrophotometric Graphite Furnace Method**

The following methods are primarily intended for the analysis of applicable substances containing less than 1 mg/kg of lead.

**Method I**

This method is intended for the quantitation of lead in substances that are soluble in water, such as sugars and sugar syrups, at levels as low as 0.03 mg/kg. The method detection limit is approximately 5 ng/kg.

**Apparatus** Use a suitable graphite furnace atomic absorption spectrophotometer set at 283.3 nm and equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. Zeeman effect or Smith-Hieftje background correction is preferred, but deuterium arc background correction should be acceptable. (This method was developed on a Perkin-Elmer Model Z5100, 0.7-nm slit, HGA-600 furnace, AS-60 autosampler with Zeeman background correction.) If the instrument does not have a well-defined calibration function, a separate calculator or computer is required for linear least squares, nonlinear, or quadratic calibrations. Use either a hollow cathode lamp or an electrode-less discharge lamp as the source, and use argon as the purge gas and breathing-quality air (for oxygen ashing to avoid residue build up during the char step) as the alternate gas. Set up the instrument according to the manufacturer's specifications with consideration of current good GFAAS practices—addressing such factors as line voltage, cooling water temperature, graphite part specifications, and furnace temperature. If an optical pyrometer or thermocouple is not available to check the furnace controller temperature calibration, dim the room lights, and observe the furnace emission through the sample introduction port while increasing the furnace temperature. A characteristic cherry red glow should begin to appear at 800°C. If it glows at a lower temperature, then the furnace is hotter, and temperatures must be adjusted downward accordingly.

Use acid-cleaned [in a mixture of 5% sub-boiling, distilled nitric acid and 5% sub-boiling, distilled hydrochloric acid made up in deionized, distilled water (18 megohm), and thoroughly rinsed with deionized, distilled water (18 megohm)] autosampler cups (PE 800-7600 Teflon, or equivalent) to avoid contamination. Use micropipets with disposable tips free of lead contamination for dilution. Ensure accuracy and precision of micropipets and tips by dispensing and weighing 5 to 10 replicate portions of water onto a microbalance. Use acid-cleaned volumetric glassware to prepare standards and dilute samples to a final volume. For digestion, use acid-cleaned, high-density polyethylene tubes, polypropylene tubes, Teflon tubes, or quartz tubes. Store final diluted samples in plastic tubes.

**Standard Solutions** Prepare all lead solutions in 5% sub-boiling distilled nitric acid. Use a single-element 1000- or 10,000-µg/mL lead stock to prepare (weekly) an intermediate 10-µg/mL standard in 5% nitric acid. Prepare (daily) a Lead Standard Solution (1 µg/mL) by diluting the intermediate 10-µg/mL stock solution 1:10. Prepare Working Calibration Standards of 100.0, 50.0, 25.0, and 10.0 ng/mL from this, using appropriate
dilutions. Store standards in acid-cleaned polyethylene test tubes or bottles. If the GFAAS autosampler is used to automatically dilute standards, ensure calibration accuracy by pipetting volumes of 3 µL or greater.

**Modifier Stock Solution**  Weigh 20 g of ultrapure magnesium nitrate hexahydrate and dilute to 100 mL. Just before use, prepare a *Modifier Working Solution* by diluting stock solution 1:10. A volume of 5 µL will provide 0.06 mg of magnesium nitrate.

**Sample Digestion**  [CAUTION— Perform the procedure in a fume hood, and wear safety glasses. ] Obtain a representative subsample to be analyzed. For liquid samples such as sugar syrups, ultrasonicate and/or vortex mix before weighing. For solid samples such as crystalline sucrose, make a sugar solution using equal weights of sample (5-g minimum) and deionized, distilled (18 megohm) water. Mix samples until completely dissolved. Transfer approximately 1.5 g (record to nearest mg) of sample (or 3.0 g of sugar solution), accurately weighed, into a digestion tube. Run a *Sample Preparation Blank* of 1.5 g of deionized, distilled (18 megohm) water through the entire procedure with each batch of samples. Add 0.75 mL of sub-boiling, distilled nitric acid. Heat plastic tubes in a water bath, quartz tubes in a water bath or heating block, warming slowly to between 90° and 95° to avoid spattering. Monitor the temperature by using a “dummy” sample. Heat until all brown vapors have dissipated and any rust-colored tint is gone (20 to 30 min). Cool. Add 0.5 mL of 50% hydrogen peroxide dropwise, heat at 90° to 95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide, dropwise, and heat at 90° to 100° for 5 to 10 min until clear. Cool, and dilute to a final volume of 10 mL.

**Procedure**  The furnace program is as follows: (1) dry at 200°, using a 20-s ramp and a 30-s hold and a 300-mL/min argon flow; (2) char the sample at 750°, using a 40-s ramp and a 40-s hold and a 300-mL/min air flow; (3) cool down, and purge the air from the furnace for 60 s, using a 20° set temperature and a 300-mL/min argon flow; (4) atomize at 1800°, using a 0-s ramp and a 10-s hold with the argon flow stopped; (5) clean out at 2600°, with a 1-s ramp and a 7-s hold; (6) cool down the furnace (if necessary) at 20°, with a 1-s ramp and a 5-s hold with a 300-mL/min argon flow.

Use the autosampler to inject 20 µL of blanks, calibration standards, and sample solutions and 5 µL of *Modifier Working Solution*. Inject each respective solution in triplicate, and average results. Use peak area measurements for all quantitation. After ensuring that the furnace is clean by running a 5% nitric acid blank, check the instrument sensitivity by running the 25-ng/mL calibration standard. If the integrated absorbance is less than 0.14 abs-sec for a standard, 28-mm × 6-mm, end-heated furnace tube, correct the cause of insufficient sensitivity before proceeding. If the integrated absorbance is greater than 0.25 abs-sec, contamination is likely, and the source should be investigated. Calculate the characteristic mass (m₀) (mass of Pb pg necessary to produce an integrated absorbance of 0.0044 abs-sec) as follows:

\[ m₀ = \frac{(0.0044 \text{ abs-sec})(25 \text{ pg/µL})(20 \text{ µL})}{(\text{measured 25 pg/µL abs-sec})} \]

Record and track the integrated absorbance and m₀ for reference and quality assurance.

**Standard Curve:**  Inject each calibration standard in triplicate. Normal instrument linearity extends to 25 ng/mL. If nonlinear calibration capability is not available, limit the working calibration curve to ≤25 ng/mL.

Use the calibration algorithms provided in the instrument software. Recheck calibration periodically (≤15 samples) by running a 25- or 50-ng/mL calibration standard interspersed with samples. If recheck differs from calibration by >10%, recalibrate the instrument. The instrumental detection limit (DL) and quantitation limit (QL), in picograms, may be based on 7 to 10 replicates of the *Sample Preparation Blank* and
calculated as follows:

\[ DL = (3)(s.d. \text{ blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})/(\text{abs-sec 10 ng/mL std}) \]

\[ QL = (10)(s.d. \text{ blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})(\text{abs-sec 10 ng/mL std}) \]

During method development, detection limits were typically 10 to 14 pg, corresponding to 0.5 to 0.7 ng/mL for 20 µL. This corresponds to a method detection limit of 3.3 to 4.7 ng/g of sugar.

**Sample Analyses:** Inject each sample digest in triplicate, and record the integrated absorbance. If instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into working range, and note the dilution factor (DF). Sample solutions having a final concentration of >25 ng/mL should be diluted 1:10 to facilitate analysis in the linear range for systems not equipped with nonlinear calibration. All sample analyses should be blank corrected using the sample preparation blank. This can typically be done automatically by the software after identifying and running a representative sample preparation blank. Use the calibration algorithm provided in the instrument software to calculate a blank-corrected, digest lead concentration (in nanograms per milliliter).

**Calculation of Lead Content:** Calculate the lead level in the original sample as follows:

\[ \text{Pb (ng/g)} = (\text{blank-corrected Pb ng/mL})(\text{DF})[\text{sample vol (10 mL)}]/[\text{sample wt (approx. 1.5 g)}]^2 \]

**Quality Assurance** To ensure analytical accuracy, NIST SRM 1643c acidified water or a similar material should be analyzed before the unknown samples are. The certified content of SRM 1643c is 35.3 ± 0.9 ng/mL. If the concentration determined is not within 10% of the mean reference value (31.8 to 38.8 ng/mL), the reason for inaccuracy should be evaluated, and unknown samples should not be analyzed until acceptable accuracy is achieved. Also prepare an in-house control solution made from uncontaminated table sugar or reagent-grade sucrose (or other appropriate substance with a Pb content <5 ng/g as received) mixed with an equal volume of water. Spike this solution with Pb to produce a concentration of 100 ng/g. Analyze with each batch of samples. Recoveries should be 100 ± 20%, and the precision for complete replicate digestions should be <5% RSD. Periodically, a sample digest should be checked using the method of standard additions to ensure that there are no multiplicative or chemical interferences. Spiking samples and checking recoveries is always a good practice.

**Method II**
This method is primarily intended for the determination of lead at levels of less than 1 mg/kg in substances immiscible with water, such as edible oils.

**Apparatus** Use a suitable atomic absorption spectrophotometer (Perkin-Elmer Model 3100 or equivalent) fitted with a graphite furnace (Perkin-Elmer HGA 600 or equivalent). Use a lead hollow-cathode lamp (Perkin-Elmer or equivalent) with argon as the carrier gas. Follow the manufacturers' directions for setting the appropriate instrument parameters for lead determination.

[Note—For this test, use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with high-purity water, preferably obtained from a mixed-bed strong-acid, strong-base ion-exchange cartridge capable of producing water with an electrical resistivity of 12 to 15 megohms.]

**Hydrogen Peroxide–Nitric Acid Solution** Dissolve equal volumes of 10% hydrogen peroxide and 10% nitric acid.

[Note—Use caution.]
**Lead Nitrate Stock Solution**  Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate (alternatively, use NIST Standard Reference Material, containing 10 mg of lead per kilogram, or equivalent) in 100 mL of Hydrogen Peroxide–Nitric Acid Solution. Dilute with Hydrogen Peroxide–Nitric Acid Solution to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts. Each milliliter of this solution contains the equivalent of 100 µg of lead (Pb) ion.

**Standard Lead Solution**  On the day of use, dilute 10.0 mL of Lead Nitrate Stock Solution with Hydrogen Peroxide–Nitric Acid Solution to 100.0 mL, and mix. Each milliliter of Standard Lead Solution contains the equivalent of 10 µg of lead (Pb) ion.

**Butanol–Nitric Acid Solution**  Slowly add 50 mL of nitric acid to approximately 500 mL of butanol contained in a 1000-mL volumetric flask. Dilute with butanol to volume, and mix.

**Standard Solutions**  Prepare a series of lead standard solutions serially diluted from the Standard Lead Solution in Butanol–Nitric Acid Solution. Pipet into separate 100-mL volumetric flasks 0.2, 0.5, 1, and 2 mL, respectively, of Standard Lead Solution, dilute with Butanol–Nitric Acid Solution to volume, and mix. The Standard Solutions contain, respectively, 0.02, 0.05, 0.1, and 0.2 µg of lead per milliliter. (For lead limits greater than 1 mg/kg, prepare a series of standard solutions in a range encompassing the expected lead concentration in the sample.)

**Sample Solution**  [CAUTION—Perform this procedure in a fume hood, and wear safety glasses.] Transfer 1 g of sample, accurately weighed, into a large test tube. Add 1 mL of nitric acid. Place the test tube in a rack in a boiling water bath. As soon as the rusty tint is gone, add 1 mL of 30% hydrogen peroxide dropwise to avoid a vigorous reaction, and wait for bubbles to form. Stir with an acid-washed plastic spatula if necessary. Remove the test tube from the water bath, and let it cool. Transfer the solution into a 10-mL volumetric flask, and dilute with Butanol–Nitric Acid Solution to volume, and mix. Use this solution for analysis.

**Procedure**  
**Tungsten Solution:** Transfer 0.1 g of tungstic acid (H₂WO₄) and 5 g of sodium hydroxide pellets into a 50-mL plastic bottle. Add 5.0 mL of high-purity water, and mix. Heat the mixture in a hot water bath until complete solution is achieved. Cool, and store at room temperature.  
**Procedure:** Place the graphite tube in the furnace. Inject a 20-µL aliquot of the Tungsten Solution into the graphite tube, using a 300-mL/min argon flow and the following sequence of conditions: dry at 110° for 20 s, char at 700° to 900° for 20 s, and with the argon flow stopped, atomize at 2700° for 10 s; repeat this procedure once more using a second 20-µL aliquot of the Tungsten Solution. Clean the quartz windows.  

**Standard Curve:**  
[Note—The sample injection technique is the most crucial step in controlling the precision of the analysis; the volume of the sample must remain constant. Rinse the µL pipet tip (Eppendorf or equivalent) three times with either the Standard Solutions or Sample Solution before injection. Use a fresh pipet tip for each injection, and start the atomization process immediately after injecting the sample. Between injections, flush the graphite tube of any residual lead by purging at a high temperature as recommended by the manufacturer.]  
With the hollow cathode lamp properly aligned for maximum absorbance and the wavelength set at 283.3 nm, atomize 20-µL aliquots of the four Standard Solutions, using a 300-mL/min argon flow and the following sequence of conditions: dry at 110° for 30 s, with a 20-s ramp period and a 10-s hold time; then char at 700° for 42 s, with a 20-s ramp period and a 22-s hold time; and then, with the argon flow stopped, atomize at 2300° for 7 s.
Plot a standard curve using the concentration, in micrograms per milliliter, of each Standard Solution versus its maximum absorbance value compensated for background correction as directed for the particular instrument, and draw the best straight line.

Atomize 20 µL of the Sample Solution under identical conditions, and measure its corrected maximum absorbance. From the Standard Curve, determine the concentration, C, in micrograms per milliliter, of the Sample Solution. Calculate the quantity, in milligrams per kilogram, of lead in the sample by the formula:

\[ \text{Result} = 10C/W \]

in which W is the weight, in grams, of the sample taken.

APDC Extraction Method
Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm, 1:2 nitric acid followed by water.

2% APDC Solution Dissolve 2.0 g of ammonium pyrrolidinedithiocarbamate (APDC) in 100 mL of water. Filter any slight residue of insoluble APDC from the solution before use.

Lead Nitrate Stock Solution (100 µg/mL) Dissolve 159.8 mg of reagent-grade lead nitrate [Pb(NO\(_3\))\(_2\)] in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

Standard Lead Solutions
2 mg/kg Lead Standard: On the day of use, transfer 2.0 mL of Lead Nitrate Stock Solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 2 µg of lead per milliliter.

3 mg/kg Lead Standard: On the day of use, transfer 3.0 mL of Lead Nitrate Stock Solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 3 µg of lead per milliliter.

4 mg/kg Lead Standard: On the day of use, transfer 4.0 mL of Lead Nitrate Stock Solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 4 µg of lead per milliliter.

10 mg/kg Lead Standard: On the day of use, transfer 10.0 mL of Lead Nitrate Stock Solution into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 10 µg of lead per milliliter.

Sample Preparation Transfer a 10.0-g sample to a clean 150-mL beaker, and 10 mL of water to a second 150-mL beaker to serve as the blank. Add to each 30 mL of water and the minimum amount of hydrochloric acid needed to dissolve the sample, plus an additional 1 mL of hydrochloric acid to ensure the dissolution of any lead present. Heat to boiling, and boil for several minutes. Allow to cool, and dilute with deionized water to about 100 mL. Adjust the pH of the resulting solution to between 1.0 and 1.5 with 25% NaOH. Quantitatively transfer the pH-adjusted solution to a clean 250-mL separatory funnel, and dilute with water to about 200 mL. Add 2 mL of 2% APDC Solution, and mix. Extract with two 20-mL portions of chloroform, collecting the extracts in a clean 50-mL beaker. Evaporate to dryness on a steam bath. Add 3 mL of nitric acid to the residue, and heat to near dryness. Then add 0.5 mL of nitric acid and 10 mL of deionized water to the beaker, and heat until the volume is reduced to about 3 to 5 mL. Transfer the digested extract to a clean 10-mL volumetric flask, and dilute with water to volume.

Procedure Concomitantly determine the absorbances of the appropriate Standard Lead Solution and the
Sample Preparation against the blank at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrode-less discharge lamp (EDL), or equivalent; an air–acetylene flame; and a 4-in burner head. Use water as the blank. The absorbance of the Sample Preparation is not greater than that of the Standard Lead Solution.

MANGANESE LIMIT TEST

Manganese Detection Instrument Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder or other readout device and capable of measuring the radiation absorbed by manganese atoms at the manganese resonance line of 279.5 nm.

Standard Preparations Transfer 1000 mg, accurately weighed, of manganese metal powder into a 1000-mL volumetric flask, dissolve by warming in a mixture of 10 mL of water and 10 mL of 0.5 N hydrochloric acid, cool, dilute with water to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with water to volume, and mix. Finally, pipet 5.0 mL, 10.0 mL, 15.0 mL, and 25.0 mL of this solution into separate 1000-mL volumetric flasks, dilute each flask with water to volume, and mix. The final solutions contain 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, and 2.5 mg/kg of Mn, respectively.

Sample Preparation Transfer 10.000 g of the sample into a 200-mL Kohlrausch volumetric flask, previously rinsed with 0.5 N hydrochloric acid, add 140 mL of 0.5 N hydrochloric acid, and shake vigorously for 15 min, preferably with a mechanical shaker. Dilute with 0.5 N hydrochloric acid to volume, and shake. Centrifuge approximately 100 mL of the sample mixture in a heavy-walled centrifuge tube at 2000 rpm for 5 min, and use the clear supernatant liquid in the following Procedure.

Procedure Aspirate 0.5 N hydrochloric acid through the air–acetylene burner for 5 min, and obtain a baseline reading at 279.5 nm, following the manufacturer's instructions for operating the atomic absorption spectrophotometer being used for the analysis. Aspirate a portion of each Standard Preparation in the same manner, note the readings, then aspirate a portion of the Sample Preparation, and note the reading. Prepare a standard curve by plotting the mg/kg of Mn in each Standard Preparation against the respective readings. From the graph determine the mg/kg of Mn in the Sample Preparation, and multiply this value by 20 to obtain the mg/kg of Mn in the original sample taken for analysis.

MERCURY LIMIT TEST

Method I

Mercury Detection Instrument Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. A simple mercury vapor meter or detector equipped with a variable span recorder also is satisfactory.

[Note—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

Aeration Apparatus The apparatus, shown in Fig. 16, consists of a flowmeter (a), capable of measuring flow rates from 500 to 1000 mL/min, connected via a three-way stopcock (b), with a Teflon plug, to 125-mL gas washing bottles (c and d), followed by a drying tube (e), and finally a suitable quartz liquid absorption cell (f), terminating with a vent (g) to a fume hood.
The absorption cell will vary in optical pathlength depending on the type of mercury detection instrument used.

FIGURE 16 Aeration Apparatus for Mercury Limit Test.

Bottle $c$ is fitted with an extra-coarse fritted bubbler (Corning 31770 125 EC, or equivalent), and the bottle is marked with a 60-mL calibration line. The drying tube $e$ is lightly packed with magnesium perchlorate. Bottle $c$ is used for the test solution, and bottle $d$, which remains empty throughout the procedure, is used to collect water droplets.

Alternatively, an apparatus embodying the principle of the assembly described and illustrated may be used. The aerating medium may be either compressed air or compressed nitrogen.

**Standard Preparation** Transfer 1.71 g of mercuric nitrate [Hg(NO$_3$)$_2$·H$_2$O] into a 1000-mL volumetric flask, dissolve in a mixture of 100 mL of water and 2 mL of nitric acid, dilute with water to volume, and mix. Discard after 1 month. Transfer 10.0 mL of this solution into a second 1000-mL volumetric flask, acidify with 5 mL of a 1:5 sulfuric acid solution, dilute with water to volume, and mix. Discard after 1 week. On the day of use, transfer 10.0 mL of the second solution into a 100-mL volumetric flask, acidify with 5 mL of 1:5 sulfuric acid, dilute with water to volume, and mix. Each milliliter of this solution contains 1 µg of mercury. Transfer 2.0 mL of this solution (2 µg Hg) into a 50-mL beaker, and add 20 mL of water, 1 mL of a 1:5 sulfuric acid solution, and 1 mL of a 1:25 solution of potassium permanganate. Cover the beaker with a watch glass, boil for a few seconds, and cool.

**Sample Preparation** Prepare as directed in the individual monograph.

**Procedure** Assemble the aerating apparatus as shown in Fig. 16, with bottles $c$ and $d$ empty and stopcock $b$ in the bypass position. Connect the apparatus to the absorption cell ($f$) in the instrument, and adjust the air or nitrogen flow rate so that in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the test solution. Obtain a baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the Standard Preparation as follows: destroy the excess permanganate by adding a 1:10 solution of hydroxylamine hydrochloride, dropwise, until the solution is colorless. Immediately wash the solution into bottle $c$ with water, and dilute with water to the 60-mL mark. Add 2 mL of 10% stannous chloride solution (prepared fresh each week by dissolving 10 g of SnCl$_2$·2H$_2$O in 20 mL of warm hydrochloric acid and diluting with 80 mL of water), and immediately reconnect bottle $c$ to the aerating apparatus. Turn stopcock $b$ from the bypass to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen has returned to the baseline. Disconnect bottle $c$ from the aerating apparatus, discard the Standard Preparation mixture, wash bottle $c$ with water, and repeat the foregoing procedure using the Sample Preparation; any absorbance produced by the Sample Preparation does not exceed that produced by the Standard Preparation.

**Method II**

**Dithizone Extraction Solution** Dissolve 30 mg of dithizone in 1000 mL of chloroform, add 5 mL of alcohol,
and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid. Discard the solution after 1 month.

**Diluted Dithizone Extraction Solution** Just before use, dilute 5 mL of *Dithizone Extraction Solution* with 25 mL of chloroform.

**Hydroxylamine Hydrochloride Solution** Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of thymol blue TS, and then add ammonium hydroxide until a yellow color develops. Add 10 mL of a 1:25 solution of sodium diethyldithiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not develop a yellow color when shaken with a dilute solution of cupric sulfate. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding one or two more drops of thymol blue TS, if necessary, then dilute with water to 100 mL, and mix.

**Mercury Stock Solution** Transfer 135.4 mg of mercuric chloride, accurately weighed, into a 100-mL volumetric flask, dissolve in and dilute with 1 N sulfuric acid to volume, and mix. Dilute 5.0 mL of this solution to 500.0 mL with 1 N sulfuric acid. Each milliliter contains the equivalent of 10 µg of mercury.

**Diluted Standard Mercury Solution** On the day of use, transfer 10.0 mL of *Mercury Stock Solution* into a 100-mL volumetric flask, dilute with 1 N sulfuric acid to volume, and mix. Each milliliter contains the equivalent of 1 µg of mercury.

**Sodium Citrate Solution** Dissolve 250 g of sodium citrate dihydrate in 1000 mL of water.

**Sample Solution** Dissolve 1 g of sample in 30 mL of 1.7 N nitric acid by heating on a steam bath. Cool to room temperature in an ice bath, stir, and pass through S and S No. 589, or equivalent, filter paper that has been previously washed with 1.7 N nitric acid, followed by water. Add 20 mL of *Sodium Citrate Solution* and 1 mL of *Hydroxylamine Hydrochloride Solution* to the filtrate.

**Procedure** [Note—Because mercuric dithizonate is light sensitive, perform this procedure in subdued light.] Prepare a control containing 3.0 mL of *Diluted Standard Mercury Solution* (3 µg Hg), 30 mL of 1.7 N nitric acid, 5 mL of *Sodium Citrate Solution*, and 1 mL of *Hydroxylamine Hydrochloride Solution*. Treat the control and the Sample Solution as follows: using a pH meter, adjust the pH of each solution to 1.8 with ammonium hydroxide, and transfer the solutions into different separators. Extract each with two 5-mL portions of *Dithizone Extraction Solution*, and then extract again with 5 mL of chloroform, discarding the aqueous solutions. Transfer the combined extracts from each separator into different separators, add 10 mL of 1:2 hydrochloric acid to each, shake well, and discard the chloroform layers. Extract the acid solutions with about 3 mL of chloroform, shake well, and discard the chloroform layers. Add 0.1 mL of 0.05 M disodium EDTA and 2 mL of 6 N acetic acid to each separator, mix, and then slowly add 5 mL of ammonium hydroxide. Stopper the separators, cool under a stream of cold water, and dry the outside of the separators. To avoid loss, carefully pour the solutions through the tops of the separators into separate beakers, and using a pH meter, adjust the pH of both solutions to 1.8 with 6 N ammonium hydroxide. Return the sample and control solutions to their original separators, add 5.0 mL of *Diluted Dithizone Extraction Solution*, and shake vigorously. Any color developed in the *Sample Solution* does not exceed that in the control.

**NICKEL LIMIT TEST**

[Note—Unless otherwise specified in the individual monograph, use *Method I*.]

Change to read:
Method I

**Atomic Absorption System Apparatus** Use a suitable atomic absorption spectrometer equipped with a nickel hollow-cathode lamp and an air–acetylene flame to measure the absorbance of the Blank Preparation, the Standard Preparations, and the Test Preparation as directed under Procedure.

**Test Preparation** Dissolve 20.0 g of sample in strong acetic acid TS, dilute acetic acid TS, and dilute with the same solvent to 150.0 mL. Add 2.0 mL of a saturated solution of ammonium pyrrolidinedithiocarbamate (about 10 g/L of water) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

**Blank Preparation** Prepare in the same manner as in the Test Preparation, but omit the sample.

**Standard Preparations** Prepare three Standard Preparations in the same manner as in the Test Preparation, but add 0.5 mL, 1.0 mL, and 1.5 mL, respectively, of 10 mg/kg nickel standard solution TS in addition to 20.0 g of sample.

**Procedure** Zero the instrument with the Blank Preparation. Concomitantly determine the absorbances of each of the Standard Preparations and of the Test Preparation at least three times each, and record the average of the steady readings for each. Between each measurement, aspirate the Blank Preparation, and ascertain that the reading returns to its initial blank value.

**Calculation** Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of nickel in the Test Preparation. Alternatively, plot on a graph the mean of the readings against the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the Test Preparation.

*Change to read:*

Method II

[Note—All glassware used must be soaked in 1% Nitric Acid for at least 2 h, and then rinsed with water.]

**1% Nitric Acid** Cautiously add 10 mL of nitric acid to a 1000-mL volumetric flask containing about 500 mL of water. Mix, and dilute with water to volume.

**Blank Solution** Use 1% Nitric Acid.

**Nickel Stock Standard Solution** Immediately before use, dilute an appropriate amount of nickel standard with 1% Nitric Acid to prepare a solution containing the equivalent of 10 µg of nickel per mL.

**Standard Solutions** Into three identical 100-mL volumetric flasks, introduce respectively 2.0 mL, 5.0 mL, and 10.0 mL of Nickel Stock Standard solution. Dilute with 1% Nitric Acid to volume and mix. These standards contain 0.2 µg, 0.5 µg, and 1.0 µg of nickel per mL.

**Test Solution** Weigh accurately a quantity of test specimen containing about 5 g of solids into a 100-mL volumetric flask. Dissolve in and dilute with 1% Nitric Acid to volume, and mix.

**Procedure** Concomitantly determine the absorbances of the Standard Solutions and the Test Solution at least three times each, at the wavelength of maximum absorbance at 352.0 nm with a suitable atomic absorption spectrophotometer equipped with an air–acetylene flame and a nickel hollow-cathode lamp using the Blank Solution to zero the instrument. Record the average of the steady readings for
each of the *Standard Solutions* and the *Test Solution*. Clear the nebulizer using the *Blank Solution* and aspirate each of the *Standard Solutions* and the *Test Solution* in turn. The standard chosen for reslope should be run every 4 to 5 samples. If there is a significant change in its response, reslope and repeat the previous samples. The standard deviation for the *Standard Solution* of 0.2 µg of nickel per mL must be less than 20%. Plot the absorbances of the *Standard Solutions* versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of nickel in the *Test Solution*. Calculate the quantity, in µg, of nickel in each g of test specimen taken:

\[
\text{Result} = 100C/W
\]

in which W is the weight, in g, of test specimen taken to prepare the *Test Solution*.

**PHOSPHORUS LIMIT TEST**

**Reagents**

*Ammonium Molybdate Solution (5%)*: Dissolve 50 g of ammonium molybdate tetrahydrate, \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot4\text{H}_2\text{O}\), in 900 mL of warm water, cool to room temperature, dilute with water to 1000 mL, and mix.

*Ammonium Vanadate Solution (0.25%)*: Dissolve 2.5 g of ammonium metavanadate, \(\text{NH}_4\text{VO}_3\), in 600 mL of boiling water, cool to 60° to 70°, and add 20 mL of nitric acid. Cool to room temperature, dilute with water to 1000 mL, and mix.

*Zinc Acetate Solution (10%)*: Dissolve 120 g of zinc acetate dihydrate, \(\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)\cdot2\text{H}_2\text{O}\), in 880 mL of water, and pass through Whatman No. 2V or equivalent filter paper before use.

*Nitric Acid Solution (29%)*: Add 300 mL of nitric acid (sp. gr. 1.42) to 600 mL of water, and mix.

*Standard Phosphorus Solution (100 µg P in 1 mL)*: Dissolve 438.7 mg of monobasic potassium phosphate, \(\text{KH}_2\text{PO}_4\), in water in a 1000-mL volumetric flask, dilute with water to volume, and mix.

**Standard Curve** Pipet 5.0, 10.0, and 15.0 mL of the *Standard Phosphorus Solution* into separate 100-mL volumetric flasks. To each of these flasks, and to a fourth, blank flask, add in the order stated 10 mL of *Nitric Acid Solution*, 10 mL of *Ammonium Vanadate Solution*, and 10 mL of *Ammonium Molybdate Solution*, mixing thoroughly after each addition. Dilute with water to volume, mix, and allow to stand for 10 min. Determine the absorbance of each standard solution in a 1-cm cell at 460 nm, with a suitable spectrophotometer, using the blank to set the instrument to zero. Prepare a standard curve by plotting the absorbance of each solution versus its concentration, in mg of phosphorus (P) per 100 mL.

**Treated Sample** Place 20 to 25 g of the starch sample in a 250-mL beaker, add 200 mL of a 7:3 methanol–water mixture, disperse the sample, and agitate mechanically for 15 min. Recover the starch by vacuum filtration in a 150-mL medium-porosity fritted-glass or Büchner funnel, and wash the wet cake with 200 mL of the methanol–water mixture. Reslurry the wet cake in the solvent, and wash it a second time in the same manner. Dry the filter cake in an air oven at a temperature below 50°, then grind the sample to 20-mesh or finer, and blend thoroughly. Determine the amount of dry substance by drying a 5-g portion in a vacuum oven, not exceeding 100 mm Hg, at 120° for 5 h.
[Note—The treatment outlined above is satisfactory for starch products that are insoluble in cold water. For pregelatinized starch and other water-soluble starches, prepare a 1% to 2% aqueous paste, place it in a cellophane tube, and dialyze against running distilled water for 30 h to 40 h. Precipitate the starch by pouring the solution into 4 volumes of acetone per volume of paste while stirring. Recover the starch by vacuum filtration in a medium-porosity fritted-glass or Büchner funnel, and wash the filter cake with absolute ethanol. Dry the filter cake, and determine the amount of dry substance as directed for water-insoluble starches.]

**Sample Preparation** Transfer about 10 g of the *Treated Sample*, calculated on the dry-substance basis and accurately weighed, into a Vycor dish, and add 10 mL of *Zinc Acetate Solution* in a fine stream, distributing the solution uniformly in the sample. Carefully evaporate to dryness on a hot plate, then increase the heat, and carbonize the sample on the hot plate or over a gas flame. Ignite in a muffle furnace at 550° until the ash is free from carbon (about 1 to 2 h), and cool. Wet the ash with 15 mL of water, and slowly wash down the sides of the dish with 5 mL of *Nitric Acid Solution*. Heat to boiling, cool, and quantitatively transfer the mixture into a 200-mL volumetric flask, rinsing the dish with three 20-mL portions of water and adding the rinsings to the flask. Dilute with water to volume, and mix. Transfer an accurately measured aliquot (V, in mL) of this solution, containing not more than 1.5 mg of phosphorus, into a 100-mL volumetric flask, and add 50 mL of water to a second flask to serve as a blank. To each flask add in the order stated 10 mL of *Nitric Acid Solution*, 10 mL of *Ammonium Vanadate Solution*, and 10 mL of *Ammonium Molybdate Solution*, mixing thoroughly after each addition. Dilute with water to volume, mix, and allow to stand for 10 min.

**Procedure** Determine the absorbance of the *Sample Preparation* in a 1-cm cell at 460 nm, with a suitable spectrophotometer, using the blank to set the instrument at zero. From the *Standard Curve*, determine the mg of phosphorus in the aliquot taken, recording this value as a. Calculate the amount, in mg/kg, of phosphorus (P) in the original sample by the equation:

\[
\text{mg/kg P} = \frac{(a \times 200 \times 1000)}{(V \times W)}
\]

in which W is the weight, in g, of the sample taken.

**SELENIUM LIMIT TEST**

**Reagents and Solutions**

*2,3-Diaminonaphthalene Solution:* On the day of use, dissolve 100 mg of 2,3-diaminonaphthalene (C₁₀H₁₀N₂) and 500 mg of hydroxylamine hydrochloride (NH₂OH·HCl) in sufficient 0.1 N hydrochloric acid to make 100 mL.

*Selenium Stock Solution:* Transfer 40.0 mg of powdered metallic selenium into a 1000-mL volumetric flask, and dissolve in 100 mL of 1:2 nitric acid, warming gently on a steam bath to effect solution. Cool, dilute with water to volume, and mix.

*Selenium Standard Solution:* Pipet 5.0 mL of *Selenium Stock Solution* into a 200-mL volumetric flask, dilute with water to volume, and mix. Each milliliter of this solution contains the equivalent of 1 µg of selenium (Se).

**Method I**

**Standard Preparation** Pipet 6.0 mL of *Selenium Standard Solution* into a 150-mL beaker, add 50 mL of
0.25 N nitric acid, and mix.

**Sample Preparation**  Using a 1000-mL combustion flask and 25 mL of 0.5 N nitric acid as the absorbing liquid, proceed as directed under *Oxygen Flask Combustion*, Appendix I, using the amount of sample specified in the individual monograph (and the magnesium oxide or other reagent, where specified).

[Note—If the sample contains water of hydration or more than 1% of moisture, dry it at 140°C for 2 h before combustion, unless otherwise directed.]

Upon completion of combustion, place a few milliliters of water in the cup or lip of the combustion flask, loosen the stopper of the flask, and rinse the stopper, sample holder, and sides of the flask with about 10 mL of water. Transfer the solution, with the aid of about 20 mL of water, into a 150-mL beaker, heat gently to boiling, boil for 10 min, and cool.

**Procedure**  Treat the *Sample Preparation*, the *Standard Preparation*, and 50 mL of 0.25 N nitric acid, to serve as the blank, similarly and in parallel as follows: add a 1:2 solution of ammonium hydroxide to adjust the pH of the solution to 2.0 ± 0.2. Dilute with water to 60.0 mL, and transfer to a low-actinic separator with the aid of 10.0 mL of water, adding the 10.0 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of 2,3-Diaminonaphthalene Solution, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 min. Add 5.0 mL of cyclohexane, shake vigorously for 2 min, and allow the layers to separate. Discard the aqueous phases, and centrifuge the cyclohexane extracts to remove any traces of water. Determine the absorbance of each extract in a 1-cm cell at the maximum at about 380 nm with a suitable spectrophotometer, using the extract from the blank to set the instrument. The absorbance of the extract from the *Sample Preparation* is not greater than that from the *Standard Preparation* when a 200-mg sample is tested, or not greater than one-half the absorbance of the extract from the *Standard Preparation* when a 100-mg sample is tested.

**Method II**

**Standard Preparation**  Pipet 6.0 mL of Selenium Standard Solution into a 150-mL beaker, add 50 mL of 2 N hydrochloric acid, and mix.

**Sample Preparation**  Transfer the amount of sample specified in the individual monograph into a 150-mL beaker, dissolve in 25 mL of 4 N hydrochloric acid, swirling if necessary to effect solution, heat gently to boiling, and digest on a steam bath for 15 min. Remove from heat, add 25 mL of water, and allow to cool to room temperature.

**Procedure**  Place the beakers containing the *Standard Preparation* and the *Sample Preparation* in a fume hood, and to a third beaker, add 50 mL of 2 N hydrochloric acid to serve as the blank. Cautiously add 5 mL of ammonium hydroxide to each beaker, mix, and allow the solution to cool. Treat each solution, similarly and in parallel, as directed under **Procedure** in **Method I**, beginning with "Add a 1:2 solution of ammonium hydroxide..."

**C. OTHERS**

**ALGINATES ASSAY**

In a suitable closed system, liberate the carbon dioxide from the uronic acid groups of about 250 mg of the test sample by heating with hydrochloric acid, and sweep the carbon dioxide, by means of an inert gas, into a
titration vessel containing excess standardized sodium hydroxide. Any suitable system may be used as long as it provides precautions against leakage and overheating of the reaction mixture, adequate sweeping time, avoidance of entrainment of hydrochloric acid, and meets the requirements of the System Suitability Test. One suitable system, with accompanying procedure, is given below.

**Apparatus** The apparatus is shown in Fig. 17. It consists essentially of a soda lime column, A, a mercury valve, B, connected through a side arm, C, to a reaction flask, D, by means of a rubber connection. Flask D is a 100-mL round-bottom, long-neck boiling flask, resting in a suitable heating mantle, E.

![FIGURE 17 Apparatus for Alginates Assay.](image)

The reaction flask is provided with a reflux condenser, F, to which is fitted a delivery tube, G, of 40-mL capacity, having a stopcock, H. The reflux condenser terminates in a trap, I, containing 25 g of 20-mesh zinc or tin, which can be connected with an absorption tower, J.

The absorption tower consists of a 45-cm tube fitted with a medium-porosity fritted glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-mL capacity, is blown above the fritted disk and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-mL Erlenmeyer flask, K, is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, L, which is connected to a suitable pump to provide vacuum and air supply, the choice of which is made by a three-way stopcock, M. The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, N.

All joints are a size 35/25 ground spherical type.

**Standard o-Glucurono-6,3-lactone** This chemical (C₆H₈O₆) is available as a reference standard with an assay of 100.0 ± 1.0% (24.99 ± 0.25% CO₂) from Aldrich Chemical Co.
System Suitability Test  Transfer about 250.0 mg of Standard d-Glucurono-6,3-lactone, accurately weighed, into the reaction flask, D, and carry out the Procedure described below. The system is considered suitable when the net titration results in a calculation of %CO$_2$ in a range of 24.73 to 25.26, which is equivalent to a range of 98.95% to 101.06% d-Glucurono-6,3-lactone.

Procedure  Transfer about 250 mg of sample, accurately weighed, into the reaction flask, D, add 25 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using syrupy phosphoric acid as a lubricant.

[Note—Stopcock grease may be used for the other connections.]

Check the system for air leaks by forcing mercury up into the inner tube of the mercury valve, B, to a height of about 5 cm. Turn off the pressure using the stopcock, M. If the mercury level does not fall appreciably after 1 to 2 min, the apparatus may be considered to be free from leaks. Draw carbon dioxide-free air through the apparatus at a rate of 3000 to 6000 mL/h. Raise the heating mantle, E, to the flask, heat the sample to boiling, and boil gently for 2 min. Turn off and lower the mantle, and allow the sample to cool for 15 min. Charge the delivery tube, G, with 23 mL of hydrochloric acid. Disconnect the absorption tower, J, rapidly transfer 25.0 mL of 0.25 N sodium hydroxide into the tower, add 5 drops of n-butanol, and reconnect the absorption tower. Draw carbon dioxide-free air through the apparatus at the rate of about 2000 mL/h, add the hydrochloric acid to the reaction flask through the delivery tube, K, using gentle air pressure, and then rinse down the absorption tower with three 15-mL portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 mL of a 10% solution of barium chloride (BaCl$_2$·2H$_2$O). Stopper the flask, shake gently for about 2 min, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid. Perform a blank determination (see General Provisions). Each milliliter of 0.25 N sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide (CO$_2$). Calculate the results on the dried basis.

α-AMINO NITROGEN (AN) DETERMINATION
Transfer 7 to 25 g of sample, accurately weighed, into a 500-mL volumetric flask with the aid of several 50-mL portions of warm, ammonia-free water, dilute with water to volume, and mix. Neutralize 20.0 mL of the solution with 0.2 N barium hydroxide or 0.2 N sodium hydroxide, using phenolphthalein TS as the indicator, and add 10 mL of freshly prepared phenolphthalein–formol solution (50 mL of 40% formaldehyde containing 1 mL of 0.05% phenolphthalein in 50% alcohol neutralized exactly to pH 7 with 0.2 N barium hydroxide or 0.2 N sodium hydroxide). Titrate with 0.2 N barium hydroxide or 0.2 N sodium hydroxide to a distinct red color, add a small, but accurately measured, volume of 0.2 N barium hydroxide or 0.2 N sodium hydroxide in excess, and back titrate to neutrality with 0.2 N hydrochloric acid. Conduct a blank titration using the same reagents, with 20 mL of water in place of the test solution. Each milliliter of 0.2 N barium hydroxide or 0.2 N sodium hydroxide is equivalent to 2.8 mg of α-amino nitrogen.

AMMONIA NITROGEN (NH$_3$-N) DETERMINATION
[CAUTION—Provide adequate ventilation.]
[Note—Use nitrogen-free reagents, where available, or reagents very low in nitrogen content.]
Transfer between 700 mg and 2.2 g of sample into a 500- to 800-mL Kjeldahl digestion flask of hard, moderately
thick, well-annealed glass. If desired, wrap the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer.

Add about 200 mL of water, and mix. Add a few granules of zinc to prevent bumping, tilt the flask, and cautiously pour sodium hydroxide pellets, or a 2:5 sodium hydroxide solution, down the inside of the flask so that it forms a layer under the solution, using a sufficient amount (usually about 25 g of solid sodium hydroxide) to make the mixture strongly alkaline. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser that has a delivery tube extending well beneath the surface of a measured excess of 0.5 N hydrochloric or sulfuric acid contained in a 500-mL flask. Add 5 to 7 drops of methyl red indicator (1 g of methyl red in 200 mL of alcohol) to the receiver flask. Rotate the Kjeldahl flask to mix its contents thoroughly, and heat until all of the ammonia has distilled, collecting at least 150 mL of distillate. Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate the excess acid with 0.5 N sodium hydroxide. Perform a blank determination (see General Provisions), substituting 2 g of sucrose for the sample, and make any necessary correction. Each milliliter of 0.5 N acid consumed is equivalent to 7.003 mg of ammonia nitrogen.

[Note—If it is known that the substance to be determined has a low nitrogen content, 0.1 N acid and alkali may be used, in which case each milliliter of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

Calculate the percent ammonia nitrogen by the formula:

\[ \text{Result} = \frac{(\text{NH}_3-N/S)}{\times 100} \]

in which \(\text{NH}_3-N\) is the weight, in milligrams, of ammonia nitrogen, and \(S\) is the weight, in milligrams, of sample.

**BENZENE (in Paraffinic Hydrocarbon Solvents)**

**Apparatus** (See Chromatography, Appendix IIA.) Use a suitable gas chromatograph, equipped with a column, or equivalent, that will elute \(n\)-decane before benzene under the conditions of the System Suitability Test. Column materials and conditions that have been found suitable for this method are listed in the accompanying tables. See Fig. 18 for a typical chromatogram obtained with column No. 5.
Reagents

Isooctane: 99 mole percent minimum containing less than 0.05 mole percent aromatic material.

Benzene: 99.5 mole percent minimum.

Internal Standard: n-Decane and either n-undecane or n-dodecane according to the requirement of the System Suitability Test.

Reference Solution A: Prepare a standard solution containing 0.5% by weight each of the Internal Standard and of benzene in isooctane.

Reference Solution B: Prepare a standard solution containing about 0.5% by weight each of n-decane, of Internal Standard, and of benzene in isooctane.

Calibration  Select the instrument conditions necessary to give the desired sensitivity. Inject a known volume of Reference Solution A, and change the attenuation, if necessary, so that the benzene peak is measured with a chart deflection of not less than 25% or more than 95% of full scale. When choosing the attenuation, consider all unresolved peaks to represent a single compound. There may be tailing of the nonaromatic peak, but do not use any conditions that lead to a depth of the valley ahead of the benzene peak (A) less than 50% of the weight of the benzene peak (B) as depicted in Fig. 19.

If there is tailing of the nonaromatic material, construct a baseline by drawing a line from the bottom of the valley ahead of the benzene peak to the point of tangency after the peak (see Fig. 20). Measure the areas of the benzene peak and the internal standard peak by any of the following means: triangulation, planimeter, paper cutout, or mechanical or electronic integrator. Do not use integrators on peaks without a constant baseline, unless the integrator has provision for making baseline corrections with accuracy at least as good as that of manual methods.

Calculate a response factor for benzene (R<sub>b</sub>) relative to the Internal Standard by the formula:
\[
\text{Result} = \frac{A_i}{W_i} \times \frac{W_b}{A_b}
\]

in which \(A_i\) is the area of the Internal Standard peak in arbitrary units corrected for attenuation; \(W_i\) is the weight percent of Internal Standard in Reference Solution \(A\); \(A_b\) is the area of the benzene peak in arbitrary units corrected for attenuation; and \(W_b\) is the weight percent of benzene in Reference Solution \(A\).

**Procedure**  Place approximately 0.1 mL of Internal Standard into a tared 25-mL volumetric flask, weigh on an analytical balance, dissolve in and dilute with the sample to be analyzed to volume.

Using the exact instrumental conditions that were used in the calibration, inject the same volume of sample containing the Internal Standard. Before measuring the area of the Internal Standard and benzene peaks, change the attenuation to ensure at least 25\% chart deflection.

Measure the area of the Internal Standard and benzene peaks in the same manner as was used for the calibration. Calculate the weight percent of benzene in the sample (\(W_b\)) by the formula:

\[
\text{Result} = \frac{A_b \times R_b \times W_i \times 100}{A_i \times S}
\]

in which \(A_b\) is the area of the benzene peak corrected for attenuation; \(R_b\) is the relative response factor for benzene; \(W_i\) is the weight, in grams, of Internal Standard added; \(A_i\) is the area of the Internal Standard peak corrected for attenuation; and \(S\) is the weight, in grams, of the sample taken.

**System Suitability Test**  Inject the same volume of Reference Solution \(B\) as in the Calibration and record the chromatogram. \(n\)-Decane must be eluted before benzene, and the ratio of \(A\) to \(B\) (Fig. 19) must be at least 0.5 where \(A\) is equal to the depth of the valley between the \(n\)-decane and benzene peaks and \(B\) is equal to the height of the benzene peak.

**Column Materials and Conditions for the Determination of Benzene in Hexanes**
<table>
<thead>
<tr>
<th>Column No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid phase</td>
<td>CEF</td>
<td>PEF 200</td>
<td>CEF</td>
<td>DEGS</td>
<td>TCEPE</td>
<td>TCEPE</td>
<td>DEGS</td>
</tr>
<tr>
<td>Length, ft</td>
<td>15</td>
<td>6</td>
<td>16</td>
<td>10</td>
<td>15</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>m</td>
<td>—</td>
<td>4.5</td>
<td>2</td>
<td>5</td>
<td>3.1</td>
<td>—</td>
<td>313.7</td>
</tr>
<tr>
<td>Diameter, in (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inside</td>
<td>0.07(1.8)</td>
<td>—</td>
<td>0.07</td>
<td>0.18(4.5)</td>
<td>0.06(1.5)</td>
<td>0.01(.254)</td>
<td></td>
</tr>
<tr>
<td>Outside</td>
<td>1/8(3.2)</td>
<td>¼(6.4)</td>
<td>1/8</td>
<td>—</td>
<td>—</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>Weight, percent</td>
<td>17</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Solid support</td>
<td>Chromosorb P</td>
<td>Chromosorb P</td>
<td>Chromosorb P</td>
<td>Chromosorb P</td>
<td>Capillary</td>
<td>Chromosorb P</td>
<td></td>
</tr>
<tr>
<td>Mesh</td>
<td>60–80</td>
<td>60–80</td>
<td>60–80</td>
<td>80–100</td>
<td>60–80</td>
<td>—</td>
<td>80–100</td>
</tr>
<tr>
<td>Treatment</td>
<td>AW</td>
<td>AW</td>
<td>AW</td>
<td>none</td>
<td>AW</td>
<td>none</td>
<td>AW Sil</td>
</tr>
<tr>
<td>Inlet, deg</td>
<td>200</td>
<td>210</td>
<td>250</td>
<td>260</td>
<td>250</td>
<td>275</td>
<td>260</td>
</tr>
<tr>
<td>Detector, deg</td>
<td>200</td>
<td>155</td>
<td>250</td>
<td>200</td>
<td>175</td>
<td>250</td>
<td>240</td>
</tr>
<tr>
<td>Column, deg</td>
<td>115</td>
<td>95</td>
<td>90</td>
<td>100</td>
<td>115</td>
<td>95</td>
<td>65</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>N₂</td>
<td>He</td>
<td>He</td>
<td>He</td>
<td>N₂</td>
<td>N₂</td>
<td>He</td>
</tr>
<tr>
<td>Flow rate, cm³/min</td>
<td>30</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>1</td>
<td>3</td>
<td>52</td>
</tr>
<tr>
<td>Detector</td>
<td>Fi</td>
<td>TC</td>
<td>Fi</td>
<td>Fi</td>
<td>Fi</td>
<td>Fi</td>
<td>Fi</td>
</tr>
<tr>
<td>Recorder, mV</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sample, 1</td>
<td>5</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>0.8</td>
<td>5</td>
</tr>
<tr>
<td>Split</td>
<td>9 + 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>100 + 1</td>
<td>100 – 1</td>
<td>—</td>
</tr>
<tr>
<td>Area</td>
<td>Tri</td>
<td>El</td>
<td>DI</td>
<td>Tri Plan</td>
<td>El</td>
<td>El</td>
<td>Tri</td>
</tr>
</tbody>
</table>

**Abbreviations Used in Table:**

AW—Acid washed; CEF—N,N-Bis(2-cyanoethyl)formamide; DEGS—Diethylene Glycol Succinate; DI—Disk integrator; EI—Electronic integrator; Fi—Flame ionization; Sil—Silanized; TC—Thermal conductivity; TCEPE—Tetracyanoethyalted Pentaerythritol; Tri—Triangulation.

**Retention Times in Minutes for Selected Hydrocarbons under the Conditions for the Determination of Benzene in Hexanes**

<table>
<thead>
<tr>
<th>Column No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>3.4</td>
<td>2.0</td>
<td>6.5</td>
<td>6.7</td>
<td>5.4</td>
<td>6.1</td>
<td>6.7</td>
</tr>
<tr>
<td>Toluene</td>
<td>4.4</td>
<td>3.2</td>
<td>9.0</td>
<td>10.3</td>
<td>7.8</td>
<td>7.0</td>
<td>10.3</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>5.4</td>
<td>5.2</td>
<td>11.5</td>
<td>14.8</td>
<td>10.8</td>
<td>8.0</td>
<td>14.8</td>
</tr>
<tr>
<td>p-m-Xylenes</td>
<td>5.8</td>
<td>—</td>
<td>12.5</td>
<td>—</td>
<td>11.4</td>
<td>8.5</td>
<td>—</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>7.5</td>
<td>6.8</td>
<td>17.0</td>
<td>16.1</td>
<td>14.5</td>
<td>10.0</td>
<td>—</td>
</tr>
<tr>
<td>n-Undecane</td>
<td>3.0</td>
<td>2.8</td>
<td>3.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>n-Dodecane</td>
<td>—</td>
<td>—</td>
<td>12.8</td>
<td>8.5</td>
<td>6.5</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Chromium

Standards

Standard Chromium Solution (1000 mg/kg): Transfer 2.829 g of K$_2$Cr$_2$O$_7$, accurately weighed [National Institute of Standards and Technology (NIST) No. 136] into a 1-L volumetric flask; dissolve in and dilute with water to volume.

Standard Colorant Solution: Transfer 62.5 g of colorant previously shown to be free of chromium to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Apparatus Use any suitable atomic absorption spectrophotometer equipped with a fast response recorder and capable of measuring the radiation absorbed at 357.9 nm.

Instrument Parameters Wavelength setting: 357.9 nm; optical passes: 5; lamp current: 8 mA; lamp voltage: 500 V; fuel: hydrogen; oxidant: air; recorder: 1 mv with a scale expansion of 5 or 10. Alternatively, follow the instructions supplied with the instrument.

Procedure Set the instrument at the optimum conditions for measuring chromium as directed by the manufacturer's instructions. Prepare a series of seven standard chromium solutions containing Cr at approximately 5, 10, 15, 20, 40, 50, and 60 mg/kg by appropriate dilutions of the Standard Chromium Solution into 100-mL volumetric flasks; add 80 mL of the Standard Colorant Solution, and dilute each flask with water to volume.

Transfer 5 g of the colorant to be analyzed to a 100-mL volumetric flask; dissolve in and dilute with water to volume. Prepare a calibration curve using the series of standards, and using this curve, determine the chromium content of the colorant samples.

Ether Extracts

[CAUTION—Isopropyl ether forms explosive peroxides. To ensure the absence of peroxides, perform the following test: prepare a colorless solution of ferrous thiocyanate by mixing equal volumes of 0.1 N ferrous sulfate and 0.1 N ammonium thiocyanate. Using titanous chloride, carefully discharge any red coloration due to ferric ions. Add 10 mL of ether to 50 mL of the solution, and shake vigorously for 2 to 3 min. A red color indicates the presence of peroxides. If redistillation is necessary, the usual precautions against peroxide detonation should be observed. Immediately before use, pass the ether through a 30-cm column of chromatography-grade aluminum oxide to remove peroxides and inhibitors. ]

Apparatus Use an upward displacement-type liquid–liquid extractor, as shown in Fig. 21, with a sintered-glass diffuser and a working capacity of 200 mL. Suspend a piece of bright copper wire through the condenser, and place a small coil of copper wire (about 0.5 g) in the distillation flask.
Alkaline Ether Extract  Transfer 5 g of the colorant to a beaker, and dissolve in 150 mL of water. Add 2 mL of 2.5 N NaOH solution, transfer the solution into the extractor, and dilute with water to approximately 200 mL. Add 200 mL of ether to the distillation flask, and extract for 2 h with a reflux rate of about 15 mL/min. Set the extracted colorant solution aside. Transfer the ether extract into a separatory funnel, and wash with two 25-mL portions of 0.1 N NaOH followed by two 25-mL portions of water. Reduce the volume of the ether extract to about 5 mL by distillation (in portions) from a tared flask containing a small piece of clean copper coil.

Acid Ether Extract  Add 5 mL of 3 N hydrochloric acid to the extracted colorant solution set aside in the alkaline ether extract procedure above, mix, and extract with ether as directed above. Wash the ether extract with two 25-mL portions of 0.1 N hydrochloric acid and water. Transfer the washed ether in portions to the flask containing the evaporated alkaline extract, and carefully remove all the ether by distillation. Dry the residue in an oven at 85° for 20 min. Then allow the flask to cool in a desiccator for 30 min, and weigh. Repeat drying and cooling until a constant weight is obtained. The increase in weight of the tared flask, expressed as a percentage of the sample weight, is the combined ether extract.

Leuco Base

Reagents and Solutions
  Cupric Chloride Solution: Transfer 10.0 g of CuCl₂·2H₂O to a 1-L volumetric flask; dissolve in and dilute with dimethylformamide (DMF) to volume.
  Sample Solution: Prepare as directed in the individual monograph.

Procedure
  Solution 1: Pipet 50 mL of DMF into a 250-mL volumetric flask, cover, and place in the dark.
  Solution 2: Pipet 10 mL of the Sample Solution into a 250-mL volumetric flask, add 50 mL of DMF, and place in the dark.
**Solution 3:** Pipet 50 mL of Cupric Chloride Solution into a 250-mL volumetric flask, and gently bubble air through the solution for 30 min.

**Solutions 4a and 4b:** Pipet 10 mL of the Sample Solution into each of two 250-mL volumetric flasks, add 50 mL of Cupric Chloride Solution to each, and bubble air gently through the solutions for 30 min.

Dilute all of the solutions with water nearly to volume; incubate for 5 to 10 min, but no longer, in a water bath cooled with tap water; and dilute to volume. Record the spectrum for each solution between 500 and 700 nm using an absorbance range of 0 to 1 and a 1-cm pathlength cell; record all spectra on the same spectrogram.

<table>
<thead>
<tr>
<th>Curve No.</th>
<th>Solution in Sample Cell</th>
<th>Solution in Reference Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IVa</td>
<td>3</td>
<td>4a</td>
</tr>
<tr>
<td>IVb</td>
<td>3</td>
<td>4b</td>
</tr>
</tbody>
</table>

**Calculation:**

\[
\text{% Leuco Base} = \frac{[(IV - III) - (II - I)] \times 2500}{a \times W \times r},
\]

in which the Roman numerals I through IV represent the absorbance readings for solutions of the corresponding Arabic numerals (above) at the wavelength maximum; \(a\) is the absorptivity (for Fast Green, \(a = 0.156\) at 625 nm; for Brilliant Blue, \(a = 0.164\) at 630 nm); \(W\) is the weight, in grams, of the sample taken; and \(r\) is the ratio of the molecular weights of colorant and leuco base (for Fast Green, \(r = 0.9712\); for Brilliant Blue, \(r = 0.9706\)).

### Mercury

**Apparatus** The apparatus used for the direct microdetermination of mercury is shown in Fig. 22. It consists of a quartz combustion tube designed to hold a porcelain combustion boat (60 × 10 × 8 mm) and a small piece of copper oxide wire. The combustion tube is placed in a heavy-duty hinged combustion tube furnace (Lindburg Type 70T, or equivalent), and it is connected by clamped ball-joints at one end to a source of nitrogen and connected to a series of three traps at the other. The traps are constructed of a linear array of 18- × 2-mm Pyrex tubes connected by clamped ball-joints and extend from the connection at the combustion tube. Trap I contains anhydrous calcium sulfate packed between quartz-wool plugs, trap II contains ascarite packed between cotton plugs, and trap III contains aluminum oxide packed between cotton plugs. The nitrogen flow forces the mercury through the combustion tube, the three traps, and a section of Tygon tube to a mercury vapor meter (Beckman model K-23, or equivalent). The mercury released from a sample during combustion is quantitated by comparing the recorder response with that given by a series of mercury standards.
Reagents and Equipment

Absorbent Cotton

Aluminum Oxide:  Anhydrous.

Calcium Sulfate:  Anhydrous, dehydrate, or equivalent.

Asbestos Pads (1 × 0.5 × 1 cm):  Preheated at 800° for 1 h.

Ascarite:  20- to 30-mesh.

Copper Oxide Wire:  Preheated at 850° for 2 h.

Nitrogen:  Purified grade.

Quartz Wool

Sodium Carbonate:  Anhydrous, fine granular.

Standard Solution:  Transfer approximately 1.35 g of reagent-grade mercurous chloride, accurately weighed, into a 1-L volumetric flask. Dissolve in and dilute with water to volume. When diluted 100-fold, the solution contains 0.01 µg Hg per microliter (Diluted Standard Solution).

Procedure  Preheat the furnace to 650°, and adjust the nitrogen flow to 1 L/min.

Blank Analysis:  Place a square piece of preheated asbestos pad in the combustion boat, and cover it with sodium carbonate. Stop the nitrogen flow, disconnect the ball-joint, quickly insert the boat into the combustion tube with large forceps, and reconnect the joint. Note the time, allow the boat to sit in the tube with no nitrogen flow for exactly 1 min, and then restart the flow of nitrogen. Mercury elutes almost immediately with the reinstated nitrogen flow; note the recorder response. Allow about 30 s between runs.
Calibration: Determine the recorder response after the application to the asbestos pad of 1, 2, and 3 µL of the Diluted Standard Solution.

Sample Analysis: Transfer 25 mg of colorant, accurately weighed, to the combustion boat, and cover the sample completely with sodium carbonate. Follow the procedure used for the Blank Analysis, and calculate the mercury content using the standard curve.

Trap Problems

1. Some colorants (e.g., Brillant Blue and Fast Green) may give a response that is symmetrically dissimilar to the Hg peak. If such a response “carries over” to the next sample, then the aluminum oxide trap may need to be changed.
2. If the recorder response is of inadequate sensitivity (peak height induced by 0.01 µg less than 0.5 cm), then the traps are packed too tightly. Remove or redistribute packing first in the aluminum oxide trap, then try the other traps.
3. The traps will need changing periodically as indicated by a change in the physical appearance of the trap material or by chart responses of different retention times or different symmetry from that of mercury standards.
4. If two or more standards are run in succession, a later sample might give an erroneous mercury response. Run blanks and then repeat the sample analysis to confirm the validity of the response.

Sodium Chloride

Dissolve approximately 2 g of colorant, accurately weighed, in 100 mL of water, and add 10 g of activated carbon that is free of chloride and sulfate. Boil gently for 2 to 3 min. Cool to room temperature, add 1 mL of 6 N nitric acid, and stir. Dilute with water to volume in a 200-mL volumetric flask, and then filter through dry paper. Repeat the treatment with 2-g portions of carbon until no color is adsorbed onto filter paper dipped into the filtrate.

Transfer 50 mL of filtrate to a 250-mL flask. Add 2 mL of 6 N nitric acid, 5 mL of nitrobenzene, and 10 mL of standardized 0.1 N silver nitrate solution. Shake the flask until the silver chloride coagulates. Prepare a saturated solution of ferric ammonium sulfate, and add just enough concentrated nitric acid to discharge the red color; add 1 mL of this solution to the 250-mL flask to serve as the indicator. Titrate with 0.1 N ammonium thiocyanate solution that has been standardized against the silver nitrate solution until the color persists after shaking for 1 min. Calculate the weight percent of sodium chloride, P, by the equation:

\[ P = \left( \frac{V \times N}{W} \right) \times 22.79 \]

in which V is the net volume, in milliliters, of silver nitrate solution required; N is the normality of the silver nitrate solution; and W is the weight, in grams, of the sample taken. The factor 22.79 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

Sodium Sulfate

Place 25 mL of the decolorized filtrate obtained from the Sodium Chloride test into a 125-mL Erlenmeyer flask, and add 1 drop of a 0.5% phenolphthalein solution in 50% ethanol. Add 0.05 N sodium hydroxide, dropwise, until the solution is alkaline to pH paper, and then add 0.002 N hydrochloric acid until the indicator is decolorized. Add 25 mL of ethanol and about 0.2 g of tetrahydroquinone sulfate indicator. Titrate with 0.03 N barium chloride solution to a red endpoint. Make a blank determination.

Calculate the weight percent, P, of sodium sulfate by the equation:
\[ P = [(V - B) \times N/W] \times 55.4 \]

in which \( V \) is the volume, in milliliters, of barium chloride solution required to titrate the sample; \( B \) is the volume, in milliliters, of barium chloride solution required for the blank; \( N \) is the normality of the barium chloride solution; and \( W \) is the weight, in grams, of the sample taken. The factor 55.4 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

**Change to read:**

### Total Color

**Method I (Spectrophotometric)**

Pipet 10.0 mL of the dissolved colorant into a 250-mL Erlenmeyer flask containing 90 mL of 0.04 N ammonium acetate, and mix well. Determine the net absorbance of the solution relative to water at the wavelength maximum given for each color. Calculate the percentage of colorant present using the following equation, which presumes a 1-cm pathlength cell:

\[ \% \text{total color} = \frac{A \times 100}{a \times W} \]

in which \( A \) is the absorbance; \( a \) is the absorptivity specified in the individual monograph (L/(mg·cm)); \( W \) is the weight, in grams, of the sample taken; \( C \) is the concentration of the sample in the final test solution (mg/L); and \( b \) is the cell pathlength (cm).

**Method II (Titration with Titanium Chloride)**

**Apparatus** The apparatus for determining total color by titration with titanium chloride (\( \text{TiCl}_3 \)) is shown in Fig. 23. It consists of a storage bottle, \( A \), of 0.1 N titanium chloride titrant maintained under hydrogen produced by a Kipp generator; an Erlenmeyer flask, \( B \), equipped with a source of \( \text{CO}_2 \) or \( \text{N}_2 \) to maintain an inert atmosphere in which the reaction takes place; a stirrer; and the buret, \( C \).
FIGURE 23 Titanous Chloride Titration Apparatus.

Reagents and Solutions

Titanium Chloride Solution (0.1 N): Transfer 73 mL of commercially prepared 20% TiCl₃ solution into a storage bottle, and carefully add 82 mL of concentrated HCl per L of final solution. Mix well, and bubble CO₂ or N₂ through the solution for 1 h. Before standardizing, maintain the solution under a hydrogen atmosphere for at least 16 h using a Kipp generator.

Potassium Dichromate Solution (0.1 N, primary standard): Transfer 4.9032 g of K₂Cr₂O₇ (NIST No. 136) to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Ammonium Thiocyanate (50%): Transfer 500 g of NH₄SCN, ACS certified, to a 1-L volumetric flask; dissolve in about 600 mL of water, warming if necessary; and dilute to volume.

Ferrous Ammonium Sulfate: Fe(NH₄)₂(SO₄)₂·6H₂O, ACS certified.

Sodium Bitartrate

Standardization of the Titanium Chloride Solution  Drain any standing titanium chloride (TiCl₃) from the feed lines and buret, and refill with fresh solution. Add 3.0 g of Ferrous Ammonium Sulfate to a wide-mouth Erlenmeyer flask followed by 200 mL of water, 25 mL of 50% sulfuric acid, 25 mL of 0.1 N Potassium Dichromate Solution (by pipet), and 2 or 3 boiling chips. Boil the solution vigorously on a hot plate for 30 s to
remove dissolved air, then quickly transfer the flask to the titration apparatus, securely connect the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Add the 0.1 N Titanium Chloride Solution at a fast, steady drip to within 1 mL of the estimated endpoint (about 20 mL). Reduce the carbon dioxide flow, remove the solid-glass rod from the stopper assembly, pipet 10 mL of Ammonium Thiocyanate (50%) into the flask, insert the glass rod, and increase the carbon dioxide flow. Continue titrating slowly until the endpoint: a color change from brown-red to light green is observed. Perform a blank determination using the same reagents and quantities, and calculate the normality, N, of the 0.1 N Titanium Chloride Solution on the basis of three titrations by the equation:

\[ N = \left( \frac{V_r \times N_r}{V_t - V_b} \right) \]

in which \( V_r \) is the volume, in milliliters, of 0.1 N Potassium Dichromate used; \( N_r \) is the normality of the 0.1 N Potassium Dichromate; \( V_t \) is the volume, in milliliters, of 0.1 N Titanium Chloride Solution used; and \( V_b \) is the volume, in milliliters, of titanium dichloride used in the blank titration.

**Procedure**  Transfer the quantity of colorant prescribed in the individual monograph into a 500-mL wide-mouth Erlenmeyer flask and add 21 to 22 g of Sodium Bitartrate (sodium citrate for Sunset Yellow), 275 mL of water, and two or three boiling chips. Boil the solution vigorously on a hot plate for 30 s to remove dissolved air, then quickly transfer the flask to the titration apparatus, securely connect the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Titrate the sample until the color lightens, wait 20 s, and then continue the addition with about 2 s between drops. When the color is almost completely bleached, wait 20 s, and then continue the addition with 5 s between drops. A complete color change indicates the endpoint. Perform a blank determination using the same reagents and quantities, and calculate the total color, T, in percent and on the basis of three titrations, by the equation:

\[ T = \left( \frac{(V_t - V_b)(W \times F_s)}{W_p} \right) \times 100 \times N \]

in which \( V_t \) is the volume of titrant used; \( V_b \) is the volume of titrant required to produce the endpoint in a blank; \( W \) is the weight, in grams, of the sample taken; \( F_s \) is a factor derived from the stoichiometry of the reaction characteristics of each colorant and is given in the individual monograph; and \( N \) is the normality of the titrant.

**Method III** (Gravimetric)
Transfer approximately 0.5 g of colorant, accurately weighed, to a 400-mL beaker, add 100 mL of water, and heat to boiling. Add 25 mL of 1:50 hydrochloric acid, and bring to a boil. Wash down the sides of the beaker with water, cover, and keep on a steam bath for several hours or overnight. Cool to room temperature, and quantitatively transfer the precipitate into a tared filtering crucible with 1:100 hydrochloric acid. Wash the precipitate with two 15-mL portions of water, and dry the crucible for 3 h at 135°C. Cool in a desiccator, and weigh. Calculate the total color, P, in weight percent, by the equation:

\[ P = \left( \frac{(W_p \times F)}{W_s} \right) \times 100 \]

in which \( W_p \) is the weight, in grams, of the precipitate; \( F \) is the gravimetric conversion factor given in the individual monograph; and \( W_s \) is the original weight, in grams, of the sample taken.

**Uncombined Intermediates and Products of Side Reactions**
Uncombined Intermediates and Products of Side Reactions

Method I

Sample Solution Transfer approximately 2 g of colorant to a 100-mL volumetric flask; dissolve in and dilute with water to volume.

Apparatus Pack a 2.5- × 45-cm glass column with approximately 20 g of cellulose (Whatman CF-11 grade, or equivalent) that has been slurried in the eluant and from which the fines have been removed by decantation. Equilibrate the column thoroughly with the eluant, 35% ammonium sulfate.

Procedure Pipet 5 mL of Sample Solution into a beaker containing 5 g of cellulose that has been slurried in eluant and from which the fines have been removed by decantation. Stir the mixture thoroughly, add 10 g of ammonium sulfate, and stir until uniformly mixed. Mix the slurry with 15 mL of eluant, and apply it to the column. Allow the fluid to enter the column, and wash the beaker with eluant until the sample is quantitatively transferred. Elute the column with approximately 500 mL of 35% ammonium sulfate, and collect a total of eight 60-mL fractions. Divide each collected fraction in half and add 0.5 mL of NH₄OH to one half and 0.5 mL of HCl to the other.

Calculation After identifying each intermediate and side product by comparing spectra of the fractions with commercial standards, calculate the concentration, C, of each using the equation:

\[ C = \frac{A}{a \times b} \]

in which A is the absorbance at the wavelength of maximal absorption; b is the cell pathlength, in centimeters; and a is the absorptivity given in the individual monograph.

Method II

Apparatus Use a suitable high-performance liquid chromatography system (see Chromatography, Appendix II/A) equipped with a dual wavelength detector system such that the effluent can be monitored serially at 254 nm and 325 to 385 nm (wide-band pass). Use a 1-m × 2.1-mm (id) column, or equivalent, packed with a strong anion-exchange resin (Dupont No. 830950405, or equivalent).

Operating Conditions The operating conditions required may vary depending on the system used. The following conditions have been shown to give suitable results for Allura Red, Tartrazine, and Sunset Yellow.

Allura Red

Primary Eluant: 0.01 M aqueous Na₂B₄O₇.
Secondary Eluant: 0.20 M NaClO₄ in aqueous 0.01 M Na₂B₄O₇.
Sample Size: 20 µL of a 0.25% solution.
Flow Rate: 0.60 mL/min.
Gradient: Linear, in two phases: 0% to 18% in 40 min, 18% to 62% in 8 min more, then hold for 18 min more at 62%.
Temperature: 50 °C.
Pressure: 1000 psi.
Order of Elution: (1) Cresidinesulfonic acid (CSA); (2) unknown; (3) Schaeffer’s salt (SS); (4) unknown; (5) 4,4'-diazocaminobis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA); (6) unknown; (7) Allura Red; (8) 6,6'-oxybis(2-naphthalenesulfonic acid) (DONS).

Tartrazine

Primary Eluant: 0.01 M aqueous Na₂B₄O₇.
Secondary Eluant: 0.10 M NaClO₄ in aqueous 0.01 M Na₂B₄O₇.
Sample Size: 50 µL of a 0.15% solution, prepared within 13 min of injection.
Flow Rate: 1.00 mL/min.
Gradient: Exponential at 4%/min: 0.95%.
Temperature: 50°C.
Pressure: 1000 psi.
Order of Elution: (1) Phenylhydrazine-p-sulfonic acid (PHSA); (2) sulfanilic acid (SA); (3) 1-(4-sulfophenyl)-3-ethylcarboxy-5-hydroxypyrazolone (PY-T); (4) 1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone (EEPT); (5) 4,4¢-(diazoamino)-dibenzenesulfonic acid (DAADBSA).

Sunset Yellow
Primary Eluant: 0.01 M aqueous Na₂B₄O₇.
Secondary Eluant: 0.20 M NaClO₄ in aqueous 0.01 M Na₂B₄O₇.
Sample Size: 5 µL of a 1% solution.
Flow Rate: 0.50 mL/min.
Gradient: Linear in four phases: 0% to 11% in 10 min; hold 25 min; 11% to 38% in 10 min; 38% to 42% in 10 min; 42% to 98% in 20 min; hold 20 min.
Temperature: 50°C.
Pressure: 1000 psi.
Order of Elution: (1) Sulfanilic acid (SA); (2) Schaeffer's salt (SS); (3) 4,4¢-(diazoamino)-dibenzenesulfonic acid (DAADBSA); (4) R-salt dye; (5) Sunset Yellow; (6) 6,6¢-oxybis(2-naphthalenesulfonic acid) (DON5).

Standard Solutions
Allura Red: Prepare a solution containing 0.25 g of colorant, 0.5 mg of CSA, 0.75 mg of SS, 0.25 mg of DMMA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na₂B₄O₇ to volume.
Tartrazine: Prepare a solution containing 0.15 g of colorant and 0.3 mg each of PHSA, SA, PY-T, EEPT, and DAADBSA in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na₂B₄O₇ to volume.
Sunset Yellow: Prepare a solution containing 0.25 g of colorant, 0.5 mg of SA, 0.75 mg of SS, 0.25 mg of DAADBSA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M Na₂B₄O₇ to volume.

Test Solutions Prepare at least four test solutions, each containing the colorant, and one impurity, accurately weighed, dissolved in 0.1 M Na₂B₄O₇, and diluted to volume in a 100-mL volumetric flask. The solutions should encompass the range of concentrations, evenly spaced, given below for each constituent:
Allura Red (250 mg): CSA (0.05 to 0.5 mg); SS (0.05 to 0.75 mg); DONS (0.5 to 2.5 mg); DMMA (0.025 to 0.25 mg). Inject 20 µL of each solution.
Tartrazine (150 mg): SA (7.5 to 300 µg); PY-T (7.5 to 300 µg); EEPT (7.5 to 300 µg); DAADBSA (7.5 to 300 µg). Inject 50 µL of each solution.
Sunset Yellow (250 mg): SA (0.05 to 0.5 mg); SS (0.05 to 0.75 mg); DONS (0.5 to 2.5 mg); DAADBSA (0.05 to 0.25 mg). Inject 20 µL of each solution.

System Suitability
Resolution: Elute the column, or equivalent, with the gradient specified under Operating Conditions until a smooth baseline is obtained. Inject an aliquot of the Standard Solution. The resolution of the eluted
components matches or exceeds that shown for the corresponding colorant (see Figs. 24, 25, and 26). After determining that the column, or equivalent, will give the required resolution, allow it to rest for 2 weeks before use.

FIGURE 24 Allura Red–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375 to 385 nm.

FIGURE 25 Tartrazine–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375 to 385 nm.
FIGURE 26 Sunset Yellow—Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375 to 385 nm.

**Calibration:** Inject the designated volume of each Test Solution onto a conditioned column, and prepare a standard curve corresponding to each unreacted intermediate and side reaction product. Determine the area, A, for each peak from the integrator if an automated system is used or by multiplying the peak height by the width at one-half the height. The peak height alone may be used for EEPT, PY-T, and DAADBSA. Calculate the concentration, $C_i$, of each intermediate or side product using the equation:

$$C_i = m A_i + b$$

in which $A_i$ is the area of its corresponding chromatographic peak. Calculate the slope, $m$, and intercept, $b$, using the following linear regression equations:

$$m = \frac{N \sum C_i A_i - \sum C_i \sum A_i}{N \sum A_i^2 - (\sum A_i)^2}$$

$$b = \bar{A}_i - m \bar{C}_i$$

in which $\bar{A}$ and $\bar{C}$ are the calculated averages of the peak areas and concentrations, respectively, used to construct the standard curve for one intermediate or side reaction product. Calculate the correlation coefficient, $r$, from the following equation:

$$r = \frac{\sum (C_i - \bar{C})(A_i - \bar{A})}{\sum (C_i - \bar{C})^2 \times \sum (A_i - \bar{A})^2}$$

Each time the system is calibrated, add the new data to those accumulated from previous analyses. The correlation coefficient must be between 0.95 and 1.00 for any single experiment or from accumulated data.

Recalibrate the system after every 10 determinations or 2 days, whichever occurs first.

**Sample Preparation** Prepare as directed in the individual monograph.

**Procedure** Inject the volume of Sample Preparation as designated in the monograph into the column.
Determine the concentration of intermediates and side reaction products from the peak areas using the slope, m, and intercept, b, calculated under *Calibration* by the equation:

\[ C_S = mA_S + b \]

in which \( C_S \) is the concentration of the unknown in the *Sample Preparation* and \( A_S \) its corresponding peak area.

**Loss on Drying (Volatile Matter)**
Transfer 1.5 to 2.5 g of colorant, accurately weighed, to a tared crucible. Heat in a vacuum oven at 135° for 12 to 15 h. Lower the pressure in the oven to -125 mm Hg, and continue heating for an additional 2 h. Cover the crucible, and allow to cool in a desiccator. Reweigh the crucible when cool. The loss of weight is defined as the volatile matter.

**Water-Insoluble Matter**
Transfer about 1 g of colorant, accurately weighed, to a 250-mL beaker, and add 200 mL of boiling water. Stir to facilitate dissolution of the color.

Tare a filtering crucible equipped with a glass fiber filter (Reeve Angel, No. 5270, or equivalent). Filter the solution with the aid of suction when it has cooled to ambient temperature. Rinse the beaker three times, pouring the rinsings through the crucible. Wash the filter with water until the filtrate is colorless.

Dry the crucible and filter in an oven at 135° for at least 3 h, cool them in a desiccator, and reweigh to the nearest 0.1 mg. Calculate the percent water-insoluble matter, I, by the equation:

\[ I = \frac{W_C}{W_S} \times 100 \]

in which \( W_C \) is the difference in crucible weight and \( W_S \) is the sample weight.

*Add the following:*

**ELEMENTAL IMPURITIES BY ICP**
Before the initial use of either of the procedures below, the analyst should ensure that the procedure is appropriate for the instrument and sample used. *Method I* can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–OES). *Method II* can be used for elemental impurities generally amenable to detection by inductively coupled plasma–mass spectrometry (ICP–MS). If no method is specified in the individual monograph, analysts are instructed to use *Method II* (ICP–MS).

**Method I: ICP–OES**

**Reagents** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.
Aqua Regia: Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%-5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

**Sample Preparation**  Use this sample preparation procedure unless otherwise specified in the individual monograph. [Note—Weights and volumes provided may be adjusted to meet the requirements of the microwave digestion apparatus used, if proportions remain constant.] Dehydrate and predigest 0.5 g of sample in 5 mL of freshly prepared Aqua Regia. Sulfuric acid may also be used as a last resort. [Note—Sulfuric acid should be used only when absolutely needed because addition of sulfuric acid may cause an extreme exothermic reaction and result in elements being lost and because the viscosity of sulfuric acid is higher than that of other acids, which affects the overall flow of solution.] Allow the sample to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of Aqua Regia and digest, using a closed vessel microwave technique. Microwave until digestion or extraction is complete. Repeat if necessary by adding an additional 5 mL of Aqua Regia. [Note—Follow the recommended procedures provided by the manufacturer of the closed vessel microwave digestion apparatus to ensure safe usage. In closed vessel microwave digestion, the use of concentrated hydrofluoric acid (HF) is not recommended; however, when its use is necessary, practice the utmost caution in the preparation of test articles, and review or establish local procedures for safe handling, safe disposal, and HF-tolerant instrumental configurations.]

**Sample Solution**  Allow the digestion vessel containing the Sample Preparation to cool (for mercury measurements, add an appropriate stabilizer, such as gold at about 0.1 ppm), and dilute with water to 50.0 mL.

**Calibration Solution 1** 2J of the element of interest in a matched matrix (acid concentrations similar to that of the Sample Solution), where J is the limit for the specific elemental impurity. [Note—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

**Calibration Solution 2** 0.1J of the element of interest in a matched matrix (acid concentrations similar to that of the Sample Solution), where J is the limit for the specific elemental impurity. [Note—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

**Check Standard Solution** 1 ppm of the element of interest in a matched matrix (acid concentrations similar to that of the Sample Solution). [Note—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

**Blank**  Matched matrix (acid concentrations similar to that of the Sample Solution)

**Elemental Spectrometric System** (See Plasma Spectrochemistry, Appendix IIc)
- **Mode:** ICP
- **Detector:** Optical emission spectroscopy
- **Rinse:** 5% Aqua Regia
- **Calibration:** Two-point, using Calibration Solution 1, Calibration Solution 2, and Blank

**System Suitability**
- **Sample:** Check Standard Solution
- **Suitability requirement:** The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [Note—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the Check Standard Solution.]
**Analysis** Analyze according to manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size.

**Calculation** Upon completion of the analysis, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (µg/mL) as follows:

\[ C = \frac{(A \times V_1)}{W} \times \left( \frac{V_2}{V_3} \right) \]

where \( C \) is the concentration of the analyte, µg/g; \( A \) is the instrument reading, µg/mL, \( V_1 \) is the volume of the initial test article preparation, mL; \( W \) is the weight of the test article preparation, g; \( V_2 \) is the total volume of any dilution performed, mL; and \( V_3 \) is the volume of the aliquot of initial test article preparation used in any dilution performed, mL.

Similarly, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (ng/mL) as follows:

\[ C = \frac{(A \times V_1)}{W} \times \left( 1 \mu g/1000 \text{ ng} \right) \left( \frac{V_2}{V_3} \right) \]

where \( A \) is the instrument reading, ng/mL; and the other factors are as defined above.

**Method II: ICP–MS**

**Reagents** All reagents used for the preparation of sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.

*Aqua Regia:* Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%–5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

**Sample Preparation** Proceed as directed under *Method I*.

**Sample Solution** Allow the digestion vessel containing the *Sample Preparation* to cool, and add appropriate internal standards at appropriate concentrations (for mercury measurements, gold should be one of the internal standards). Dilute with water to 50.0 mL.

**Calibration Solution 1** Proceed as directed under *Method I*.

**Calibration Solution 2** Proceed as directed under *Method I*.

**Blank** Matched matrix (acid concentrations similar to that of the *Sample Solution*)

**Elemental Spectrometric System** (see *Plasma Spectrochemistry*, Appendix IIC)

*Mode:* ICP. [Note—An instrument with a cooled spray chamber is recommended.]

*Detector:* Mass spectrometer

*Rinse:* 5% Aqua Regia

*Calibration:* Calibration Solution 1, Calibration Solution 2, and Blank

**System Suitability**

*Sample:* Calibration Solution 1

*Suitability requirement:* The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [Note—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the Check Standard Solution.]
Analysis  Analyze according to the manufacturer's suggestions for the program and m/z. Calculate and report results based on the original sample size. [Note—Arsenic is subject to interference from argon chloride. Appropriate measures, including a sample preparation without Aqua Regia, must be taken to correct for the interference, depending on instrumental capabilities.]

Calculation  Upon completion of the analysis, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (µg/mL) as follows:

\[ C = \frac{(A \times V_1/W) \times (V_2/V_3)}{} \]

where \( C \) is the concentration of the analyte, µg/g; \( A \) is the instrument reading, µg/mL; \( V_1 \) is the volume of the initial test article preparation, mL; \( W \) is the weight of the test article preparation, g; \( V_2 \) is the total volume of any dilution performed, mL; and \( V_3 \) is the volume of the aliquot of initial test article preparation used in any dilution performed, mL.

Similarly, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (ng/mL) as follows:

\[ C = \frac{(A \times V_1/W) \times (1 \, \mu g/1000 \, ng)(V_2/V_3)}{} \]

where \( A \) is the instrument reading, ng/mL; and the other factors are as defined above.

**GLUTAMIC ACID**

Apparatus  Use an ion-exchange amino acid analyzer, equipped with sulfonated polystyrene columns, in which the effluent from the sample is mixed with ninhydrin reagent and the absorbance of the resultant color is measured continuously and automatically at 570 and 440 nm by a recording photometer.

Standard Solution  Transfer 1250 ± 2 mg of reagent-grade glutamic acid, accurately weighed, into a 500-mL volumetric flask. Fill the flask half-full with water, add 5 mL of hydrochloric acid to help dissolve the amino acid, dilute with water to volume, and mix. Prepare the standard for analysis by diluting 1 mL of this solution with 4 mL of 0.2 N sodium citrate, pH 2.2, buffer. This Standard Solution contains 0.5 mg of glutamic acid per milliliter (\( C_S \)).

Sample Preparation  Dilute 5 mg of sample, accurately weighed, to exactly 5 mL with 0.2 N sodium citrate, pH 2.2, buffer. Remove any insoluble material by centrifugation or filtration.

Procedure  Using 2-mL aliquots of the Standard Solution and Sample Preparation, proceed according to the apparatus manufacturer's instructions. From the chromatograms thus obtained, match the retention times produced by the Standard Preparation with those produced by the Sample Solution, and identify the peak produced by glutamic acid. Record the area of the glutamic acid peak from the sample as \( A_U \), and that from the standards as \( A_S \).

Calculations  Calculate the concentration, \( C_A \), in milligrams per milliliter, of glutamic acid in the Sample Preparation by the formula:

\[ \text{Result} = A_U \times \frac{C_S}{A_S} \]
in which \( C_S \) is the concentration, in milligrams per milliliter, of glutamic acid in the Standard Solution.

Calculate the percent glutamic acid, on the basis of total protein, by the formula:

\[
\text{Result} = \frac{100 \times C_A}{6.25 \times N_T}
\]

in which 6.25 is the conversion factor for protein and amino acids, and \( N_T \) is the percent total nitrogen determined in the monograph Assay.

Calculate the percent glutamic acid in the sample by the formula:

\[
\text{Result} = 100 \times \frac{C_A}{S_W}
\]

in which \( S_W \) is the weight, in milligrams, of the sample taken.

**HYDROXYPROPOXYL DETERMINATION**

**Apparatus** The apparatus for hydroxypropoxyl group determination is shown in Fig. 27.

![FIGURE 27 Apparatus for Hydroxypropoxyl Determination.](image)

The boiling flask, \( D \), is fitted with an aluminum foil-covered Vigreaux column, \( E \), on the side arm and with a bleeder tube through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, \( B \), is attached to the bleeder tube through tube \( C \), and a condenser, \( F \), is attached to the Vigreaux column. The boiling flask and steam generator are immersed in an oil bath, \( A \), equipped with a thermoregulator such that a temperature of 155\(^\circ\) and the desired heating rate may be maintained. The distillate is collected in a 150-mL beaker, \( G \), or other suitable container.

**Procedure** Unless otherwise directed, transfer about 100 mg of the sample, previously dried at 105\(^\circ\) for 2 h and accurately weighed, into the boiling flask, and add 10 mL of chromium trioxide solution (60 g in 140 mL of water). Immerse the steam generator and the boiling flask in the oil bath (at room temperature) to the level of the top of the chromium trioxide solution. Start cooling water through the condenser, and pass nitrogen gas through the boiling flask at the rate of one bubble per second. Starting at room temperature, raise the temperature of the oil bath to 155\(^\circ\) over a period of not less than 30 min, and maintain this temperature until the end of the determination. Distill until 50 mL of distillate is collected. Detach the condenser from the Vigreaux column, and wash it with water, collecting the washings in the distillate container. Titrate the combined washings and distillate with 0.02 N sodium hydroxide to a pH of 7.0, using a pH meter set at the expanded scale.
Note—Phenolphthalein TS may be used for this titration if it is also used for all standards and blanks.

Record the volume, \( V_a \), of the 0.02 N sodium hydroxide used. Add 500 mg of sodium bicarbonate and 10 mL of 2 N sulfuric acid, and then after evolution of carbon dioxide has ceased, add 1 g of potassium iodide. Stopper the flask, shake the mixture, and allow it to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate to the sharp disappearance of the yellow color, confirming the endpoint by the addition of a few drops of starch TS. Record the volume of 0.02 N sodium thiosulfate required as \( Y_a \).

Make several reagent blank determinations, using only the chromium trioxide solution in the above procedure. The ratio of the sodium hydroxide titration (\( V_b \)) to the sodium thiosulfate titration (\( Y_b \)), corrected for variation in normalities, will give the acidity-to-oxidizing ratio, \( V_b/Y_b = K \), for the chromium trioxide carried over in the distillation. The factor \( K \) should be constant for all determinations.

Make a series of blank determinations using 100 mg of methylcellulose (containing no foreign material) in place of the sample, recording the average volume of 0.02 N sodium hydroxide required as \( V_m \) and the average volume of 0.02 N sodium thiosulfate required as \( Y_m \).

Calculate the hydroxypropoxyl content of the sample, in milligrams, by the formula:

\[
\text{Result} = 75.0 \times [N_1(V_a - V_m) - kN_2(Y_a - Y_m)]
\]

in which \( N_1 \) is the exact normality of the 0.02 N sodium hydroxide solution, \( N_2 \) is the exact normality of the 0.02 N sodium thiosulfate solution, and \( k = V_bN_1/Y_bN_2 \).

The percentage of substitution, by weight, of hydroxypropoxyl groups, determined as directed above, may be converted to molecular substitution per glucose unit by reference to Fig. 28.

\[\text{FIGURE 28 Chart for Converting Percentage of Substitution, by Weight, of Hydroxypropoxyl Groups to Molecular Substitution per Glucose Unit.}\]

**METHOXYL DETERMINATION**

**Apparatus** The apparatus for methoxyl determination, as shown in Fig. 29, consists of a boiling flask, \( A \), fitted with a capillary side arm to provide an inlet for carbon dioxide and connected to a column, \( B \), which separates aqueous hydriodic acid from the more volatile methyl iodide. After the methyl iodide passes through a suspension of aqueous red phosphorus in the scrubber trap, \( C \), it is absorbed in the bromine–acetic acid absorption tube, \( D \). The carbon dioxide is introduced from a device arranged to minimize pressure fluctuations and connected to the apparatus by a small capillary containing a small plug of cotton.
Reagents

*Acetic Potassium Acetate:* Dissolve 100 g of potassium acetate in 1000 mL of a mixture consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride.

*Bromine–Acetic Acid Solution:* On the day of use, dissolve 5 mL of bromine in 145 mL of the Acetic Potassium Acetate solution.

*Hydriodic Acid:* Use special-grade hydriodic acid suitable for alkoxy determination, or purify reagent grade as follows: distill over red phosphorus in an all-glass apparatus, passing a slow stream of carbon dioxide through the apparatus until the distillation is terminated and the receiving flask has completely cooled. [CAUTION—Use a safety shield, and conduct the distillation in a fume hood.]

Collect the colorless, or almost colorless, constant-boiling acid distilling between 126° and 127°. Store the acid in a cool, dark place in small, brown, glass-stoppered bottles previously flushed with carbon dioxide and finally sealed with paraffin.

**Procedure**  Fill trap C half-full with a suspension of about 60 mg of red phosphorus in 100 mL of water, introduced through the funnel on tube D and the side arm that connects with the trap at C. Rinse tube D and the side arm with water, collecting the rinsings in trap C, then charge absorption tube D with 7 mL of Bromine–Acetic Acid Solution. Place the sample, previously accurately weighed in a tared gelatin capsule, into the boiling flask A, along with a few glass beads or boiling stones, then add 6 mL of Hydriodic Acid. Connect the flask to the condenser, using a few drops of the acid to seal the junction, and begin passing the carbon dioxide through the apparatus at the rate of about two bubbles per second. Heat the flask in an oil bath at 150°, continue the reaction for 40 min, and drain the contents of absorption tube D into a 500-mL Erlenmeyer flask containing 10 mL of a 1:4 solution of sodium acetate. Rinse tube D with water, collecting the rinsings in the flask, and dilute with water to about 125 mL. Discharge the red-brown color of bromine by adding formic acid, dropwise with swirling, then add 3 drops in excess. Usually a total of 12 to 15 drops of formic acid is required. Allow the flask to stand for 3 min, add 15 mL of 2 N sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate, adding starch TS near the endpoint. Perform a blank determination with the same quantities of the same reagents, including the gelatin capsule, and in the same manner, and make any necessary correction. Each milliliter of 0.1 N sodium thiosulfate is equivalent to 0.517 mg (517 µg)
of methoxyl groups (–OCH₃).

**NITROGEN DETERMINATION (Kjeldahl Method)**

*Change to read:*

> [CAUTION—Provide adequate ventilation in the laboratory, and do not permit accumulation of exposed mercury.]

> [2S (FCC7)]

> [Note—All reagents should be nitrogen free, where available, or otherwise very low in nitrogen content.]

*Change to read:*

**Method I**

Use this method unless otherwise directed in the individual monograph. It is not applicable to certain nitrogen-containing compounds that do not yield their entire nitrogen upon digestion with sulfuric acid.

**Nitrites and Nitrates Absent**

Unless otherwise directed, transfer from 700 mg to 2.2 g of sample into a 500- to 800-mL Kjeldahl digestion flask of hard borosilicate, moderately thick, well-annealed glass, wrapping the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer if desired. Add 700 mg of mercuric oxide or 650 mg of metallic mercury, 15 g of powdered potassium sulfate or anhydrous sodium sulfate, and 25 mL of 93% to 98% sulfuric acid. (If a sample weight greater than 2.2 g is used, increase the sulfuric acid by 10 mL for each additional gram of sample.) Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Place the flask in an inclined position (about 45°) and heat gently keeping the temperature below the boiling point until frothing ceases, adding a small amount of paraffin, if necessary, to reduce frothing. [CAUTION—The digestion should be conducted in a fume hood, or the digestion apparatus should be equipped with a fume exhaust system.]

Boil increase the heat until the acid boils briskly and continue the heating process until the solution clears, and then continue boiling for 30 min longer (or for 2 h for samples containing organic material). Cool, add about 200 mL of water, mix, and then cool to below 25°. Add 25 mL of sulfide or thiosulfate solution (40 g of K₂S₂O₃, 40 g of Na₂S, or 80 g of Na₂S₂O₃·5H₂O in 1000 mL of water), and mix to precipitate the mercury. Add cautiously 100 mL of a 2:5 sodium hydroxide solution, in such a manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, to make the mixture strongly alkaline. Add a few granules of zinc to prevent bumping, tilt the flask, and cautiously pour sodium hydroxide pellets or a 2:5 solution of sodium hydroxide down the inside of the flask so that it forms a layer under the acid solution, using a sufficient amount (usually about 25 g of solid NaOH) to make the mixture strongly alkaline. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube of which extends well beneath the surface of a measured excess of 0.5 N hydrochloric or sulfuric acid contained in a 500-mL flask. Add from 5 to 7 drops of methyl red indicator (1 g of methyl red in 200 mL of alcohol) to the receiver flask.
Rotate 100 mL of a 1:25 boric acid solution contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Gently, rotate the Kjeldahl flask to mix its contents thoroughly, and then heat until all of the ammonia has distilled, collecting at least 150 mL of distillate (about 80% of the contents of the flask).

Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate the excess acid with 0.5 N sodium hydroxide with 0.5 N sulfuric acid, determining the endpoint potentiometrically.

Perform a blank determination, substituting 2 g of sucrose for the sample, and make any necessary correction (see General Provisions). Each milliliter of 0.5 N acid consumed is equivalent to 7.003 mg of nitrogen.

[Note—An indicator solution can also be used to determine the titration endpoint. For example, dissolve 0.2 g methyl red in 100 mL 95% ethanol, 1 g bromocresol green in 500 mL 95% ethanol, then combine 1 part of the methyl red solution and 5 parts of the bromocresol green solution. Add 3 mL methyl red/bromocresol green indicator solution per L of boric acid solution. Then, titrate the sample to the first trace of pink.]

[Note—if the substance to be determined is known to have a low nitrogen content, 0.1 N acid and alkali may be used, in which case each milliliter of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

[Note—Nitrogen recovery verification can be run to check for accuracy of the procedure and the equipment.]

1. Nitrogen loss Use 0.12 g ammonium sulfate and 0.85 g sucrose. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 99%.

2. Digestion efficiency Use 0.16 g lysine hydrochloride or 0.18 g tryptophan, with 0.67 g sucrose per flask. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 98%.

Nitrites and Nitrates Present
[Note—This procedure is not applicable to liquids or to materials having a high chlorine-to-nitrate ratio.]

Unless otherwise directed, transfer from 700 mg to 2.2 g of sample a quantity of sample, accurately weighed, corresponding to about 150 mg of nitrogen into a Kjeldahl flask, and add 40 mL of 93% to 98% sulfuric acid containing 2g of salicylic acid. Mix thoroughly by shaking, and then allow to stand for 30 min or more, with occasional frequent shaking. Add 5 g of Na₂S₂O₃·5H₂O or 2 g of zinc dust (as an impalpable powder, not granules or filings), shake, and allow to stand for 5 min. Heat over a low flame until frothing ceases, then remove the heat, add 700 mg of mercuric oxide (or 650 mg of metallic mercury) and 15 g of powdered potassium sulfate (or anhydrous sodium sulfate), and boil briskly until the solution clears. Continue boiling for 30 min longer (or for 2 h for samples containing organic material), and then continue as directed under Nitrates and Nitrates Absent, beginning with “Cool, add about 200 mL of water...” mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under Nitrates and Nitrites Absent, beginning with “Incline the flask at an angle of about 45°. When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

Method II (Semimicro)
[Note—Automated instruments may be used in place of this manual method, provided the automated equipment has been properly calibrated.]
Transfer an accurately weighed or measured quantity of sample, equivalent to about 2 or 3 mg of nitrogen, into the digestion flask of a semimicro Kjeldahl apparatus. Add 1 g of a 10:1 powdered mixture of potassium sulfate:cupric sulfate, using a fine jet of water to wash down any material adhering to the neck of the flask, and then pour 7 mL of sulfuric acid down the inside wall of the flask to rinse it. Cautiously add down the inside of the flask 1 mL of 30% hydrogen peroxide, swirling the flask during the addition.

[CAUTION— Do not add any peroxide during the digestion. ]

Heat over a free flame or an electric heater until the solution has attained a clear blue color and the walls of the flask are free from carbonized material. Cautiously add 20 mL of water, cool, then add through a funnel 30 mL of a 2:5 solution of sodium hydroxide, and rinse the funnel with 10 mL of water. Connect the flask to a steam distillation apparatus, and immediately begin the distillation with steam. Collect the distillate in 15 mL of a 1:25 solution of boric acid to which has been added 3 drops of methyl red–methylene blue TS and enough water to cover the end of the condensing tube. Continue passing the steam until 80 to 100 mL of distillate has been collected, then remove the absorption flask, rinse the end of the condenser tube with a small quantity of water, and titrate with 0.01 N sulfuric acid. Each milliliter of 0.01 N acid is equivalent to 140 µg of nitrogen.

When more than 2 or 3 mg of nitrogen is present in the measured quantity of the substance to be determined, 0.02 or 0.1 N sulfuric acid may be used in the titration if at least 15 mL of titrant is required. If the total dry weight of the material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide added before distillation.

SPECTROPHOTOMETRIC IDENTIFICATION TESTS

Spectrophotometric tests contribute meaningfully toward the identification of many compendial chemical substances. The test procedures that follow are applicable to substances that absorb IR and/or UV radiation. The IR absorption spectrum of a substance, compared with that obtained concomitantly for the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV absorption spectrum, on the other hand, does not exhibit a high degree of specificity. Conformance with both IR absorption and UV absorption test specifications leaves little doubt, if any, regarding the identity of the specimen under examination.

Infrared Spectra  This test is used for comparison of an IR spectrum for a sample specimen with a reference spectrum provided in the individual monograph.

Sample specimens should be prepared using the same technique as that used for the provided spectra. Unless otherwise noted in the individual monograph or spectrum caption, the spectra for liquid substances were obtained on neat liquids contained in fixed-volume sodium chloride cells or between salt plates. Spectra for solid substances were obtained on a potassium bromide pellet or a mineral oil (Nujol or equivalent) dispersion, as indicated in individual monographs or spectrum caption.

Infrared Absorption  This test is used for comparison of the IR spectrum of a sample specimen with that of a physical USP Reference Standard.

Sample and USP Reference Standard specimens should both be prepared for analysis using the same technique, as directed in the individual monographs, which use the below letter designations. Sample and USP Reference Standard specimens should be used as either dried or undried specimens as directed on the Reference Standard Label.

Record the spectra of the sample and USP Reference Standard specimens over the range of about 2.6 to 15
μm (3800 cm$^{-1}$ to 650 cm$^{-1}$) unless otherwise specified in the individual monograph.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Specimen Preparation Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Intimately in contact with an internal reflection element for attenuated total reflectance (ATR) analysis</td>
</tr>
<tr>
<td>E</td>
<td>Pressed as a thin sample against a suitable plate for IR microscopic analysis</td>
</tr>
<tr>
<td>F</td>
<td>Suspended neat between suitable (for example sodium chloride or potassium bromide) plates</td>
</tr>
<tr>
<td>K</td>
<td>Mixed intimately with potassium bromide and compressed into a translucent pellet</td>
</tr>
<tr>
<td>M</td>
<td>Finely ground and dispersed in mineral oil</td>
</tr>
<tr>
<td>S</td>
<td>A solution of designated concentration, prepared as directed in the individual monograph, and examined in 0.1-mm cells (unless otherwise specified in the individual monograph)</td>
</tr>
</tbody>
</table>

[Note—A and E techniques can be used as alternative methods for K, M, F, and S where testing is performed qualitatively and the Reference Standard spectra are similarly obtained.]

**Ultraviolet Absorption**  The test is used for comparison of the UV spectrum of a sample specimen with that of a physical USP Reference Standard.

Sample and USP Reference Standard specimens should be prepared using the solvents and concentrations specified in the individual monograph.

Record the spectra of the sample and USP Reference Standard solutions concomitantly using 1-cm cells over the spectral range of 200 to 400 nm, unless otherwise indicated in the individual monograph. Calculate absorptivities and/or absorbance ratios where these criteria are included in an individual monograph. Unless otherwise specified, absorbances indicated for these calculations are those measured at the maximum absorbance at the wavelength specified in the individual monograph. Where the absorbance is to be measured at a wavelength other than that of maximum absorbance, the abbreviations (min) and (sh) are used to indicate a minimum and shoulder, respectively, in an absorbance spectrum.

**SULFUR (by Oxidative Microcoulometry) (Based on ASTM D3120)**

[Note—All reagents used in this test should be reagent grade; water should be of high purity, and gases must be high-purity grade.]

**Apparatus**  Use the Dohrmann Microcoulometric Titrating System (MCTS-30), or equivalent (shown in Fig. 30), unless otherwise specified in an individual monograph. It consists of a constant rate injector, A, a pyrolysis furnace, B, a quartz pyrolysis tube, C, a granular-tin scrubber, D, a titration cell, E, and a microcoulometer with a digital readout, F.

![FIGURE 30 Microcoulometric Titrating System for the Determination of Sulfur in Hexanes.](image)

**Granular-Tin Scrubber:**  Place 5 g of 20- to 30-mesh granular reagent-grade tin between quartz-wool plugs in an elongated 18/9-12/5 standard-taper adaptor that connects the pyrolysis tube and the titration cell.

**Microcoulometer:**  Must have variable attenuation; gain control; and be capable of measuring the potential of
the sensing-reference electrode pair, and comparing this potential with a bias potential, amplifying the potential difference, and applying the amplified difference to the working-auxiliary electrode pair to generate a titrant. The microcoulometer output voltage signal must also be proportional to the generating current.

**Pyrolysis Furnace:** The sample should be pyrolyzed in an electric furnace having at least two separate and independently controlled temperature zones, the first being an inlet section that can maintain a temperature sufficient to volatilize all the organic sample. The second zone is a pyrolysis section that can maintain a temperature sufficient to pyrolyze the organic matrix and oxidize all the organically bound sulfur. A third outlet temperature zone is optional.

**Pyrolysis Tube:** Must be fabricated from quartz and constructed in such a way that a sample, which is vaporized completely in the inlet section, is swept into the pyrolysis zone by an inert gas where it mixes with oxygen and is burned. The inlet end of the tube shall hold a septum for syringe entry of the sample and side arms for the introduction of oxygen and inert gases. The center or pyrolysis section should be of sufficient volume to ensure complete pyrolysis of the sample.

**Sampling Syringe:** A microlitre syringe of 10-µL capacity capable of accurately delivering 1 to 10 µL of sample into the pyrolysis tube. Three-in × 24-gauge needles are recommended to reach the inlet zone of the pyrolysis furnace.

**Titration Cell:** Must contain a sensor-reference pair of electrodes to detect changes in triiodide ion concentration and a generator anode–cathode pair of electrodes to maintain constant triiodide ion concentration and an inlet for a gaseous sample from the pyrolysis tube. The sensor electrode shall be platinum foil and the reference electrode platinum wire in saturated triiodide half-cell. The generator anode and cathode half-cell shall also be platinum. The titration cell shall be placed on a suitable magnetic stirrer.

**Preparation of Apparatus:** Carefully insert the quartz pyrolysis tube into the furnace, attach the tin scrubber, and connect the reactant and carrier-gas lines. Add the Cell Electrolyte Solution (see below) to the titration cell, and flush the cell several times. Maintain an electrolyte level of 3.2 to 6.6 mm above the platinum electrodes. Place the titration cell on a magnetic stirrer, and connect the cell inlet to the tin scrubber outlet. Position the platinum-foil electrodes (mounted on the movable cell head) so that the gas-inlet flow is parallel to the electrodes with the generator anode adjacent to the generator cathode. Assemble and connect the coulometer in accordance with the manufacturer's instructions. Double-wrap the adaptor containing the tin scrubber with heating tape and turn the heating tape on. Adjust the flow of the gases, the pyrolysis furnace temperature, the titration cell, and the coulometer to the desired operating conditions. Typical operating conditions are as follows:
The tin scrubber must be conditioned to sulfur, nitrogen, and chlorine before quantitative analysis can be achieved. A solution containing 10 mg/kg butyl sulfide, 100 mg/kg pyridine, and 200 mg/kg chlorobenzene in isooctane has proven an effective conditioning agent. With a fresh scrubber installed and heated, two 30-µL samples of this conditioning agent injected at a flow rate of 0.5 µL/s produce a steadily increasing response, with final conditioning indicated by a constant reading from the offset during the second injection.

**Reagents**

*Argon or Helium* (argon preferred): High-purity grade, used as the carrier gas. Two-stage gas regulators must be used.

*Cell Electrolyte Solution:* Dissolve 0.5 g of potassium iodide and 0.6 g of sodium azide in 500 mL of high-purity water, add 5 mL of glacial acetic acid, and dilute to 1 L. Store in a dark bottle or in a dark place and prepare fresh at least every 3 months.

*Oxygen:* High-purity grade, used as the reactant gas.

*Iodine:* Resublimed, 20-mesh or less, for saturated reference electrode.

*Sulfur Standard* (approximately 100 mg/kg): Transfer 0.1569 g of n-butyl sulfide, accurately weighed, into a tared 500-mL volumetric flask. Dilute to the mark with isooctane, and reweigh. Calculate the sulfur concentration (S), in percent, by the formula:

\[ S = \frac{W_b}{W_s} \times 2.192 \times 10^5 \]

in which \( W_b \) is the weight of n-butyl sulfide and \( W_s \) is the weight of the solution.

**Calibration** Prepare a calibration standard (approximately 5 mg/kg) by pipetting 5 mL of *Sulfur Standard* into a 10-mL volumetric flask and diluting with isooctane to volume. Fill and clamp the syringe onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter in case of long-term drift in the automatic baseline zero circuitry. Switch \( S_1 \) automatically starts the stepper-motor syringe drive and initiates the analysis cycle. At 2.5 min (before the 3-min meter hold point) set the digital meter with the scan potentiometer to correspond to the sulfur content of the known standard to the nearest 0.01 mg/kg. At the 3-min point, the number displayed on the meter stops, the plunger drive block is retracted.
to its original position, as preset by switch $S_2$, and a baseline re-equilibration period equal to the injection period elapses before a ready light and a beeper indicate that a new sample may be injected. Repeat the Calibration step a total of at least four times.

**Procedure** Rinse the syringe several times with sample; then fill it, clamp it onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter. Turn on switch $S_1$ to start the stepper-motor syringe drive automatically and initiate the analysis cycle. After the 3-min hold point, the number displayed on the meter corresponds to the sulfur content of the injected sample.

![FIGURE 31 Raney Nickel Reduction Apparatus.](image)

---

1 If the sample fails the test because of known or suspected interference, another aliquot may be run on a 6-ft × 2-mm (id) column, or equivalent, of 0.2% Carbowax 1500 on Carbopak C, operating at 100°C isothermal, with 20 mL/min of helium carrier flow. Under these conditions, the 1,4-dioxane elutes in about 4 min.

2 If a sample solution was prepared initially to ensure sample homogeneity, this is the weight of the original sugar digested (not the weight of the solution).

3 Suitable nickel standards are available from e.g. Fisher Scientific, Fair Lawn, NJ (nickel, reference standard solution, 1000 ppm ± 1%, certified, application: for atomic absorption) or RICCA Chemical Company, Arlington, TX (nickel standard, 1000 ppm Ni, for atomic absorption).

4 To be used or sold for use to color food that is marketed in the United States, color additives must be from batches that have been certified by the U.S. Food and Drug Administration (FDA). If color additives are not from FDA-certified batches, they are not permitted color additives for food use in the United States, even if they are compositionally equivalent. The FD&C names can be applied only to FDA-certified batches of these color additives.

5 Adapted from ASTM D3120 Standard Test Method for Trace Quantities of Sulfur in Light Liquid Petroleum Hydrocarbons by Oxidative Microcoulometry. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9555, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.

Please check for your question in the FAQs before contacting USP.
<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Kristie Laurvick</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8356</td>
<td></td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 1144*

*FCC Seventh Edition Supplement 1 Page 1497*
Appendix VII: Fats and Related Substances, page 1532 of the First Supplement to FCC 7. On the basis of data and comments received for the proposed new monograph for ARA from Fungal (Mortierella alpina) Oil, it is proposed to add two new methods: Unsaponifiable Matter, Method II and Lovibond Color. The existing method for Unsaponifiable Matter will become Method I, and a Note is being added to indicate that Method I is to be used in the absence of other instructions in the individual monograph. The new Unsaponifiable Matter, Method II is based on AOCS Official Method Ca 6b-53 and is indicated for fats and oils containing higher levels of unsaponifiable matter than what is usually found in tallow and greases. The new Lovibond Color method is based on AOCS Official Method Cc 13e-92.

(FIEC: K. Laurvick)  C93058

APPENDIX VII: FATS AND RELATED SUBSTANCES

ACETYL VALUE

Change to read:
(Based on AOCS Method Cd 4-40

The acetyl value is defined as the number of mg of potassium hydroxide required to neutralize the acetic acid obtained by saponifying 1 g of the acetylated sample.

Acetylation  Boil 50 mL of the oil or melted fat with 50 mL of freshly distilled acetic anhydride for 2 h under a reflux condenser. Pour the mixture into a beaker containing 500 mL of water, and boil for 15 min, bubbling a stream of nitrogen or carbon dioxide through the mixture to prevent bumping. Cool slightly, remove the water, add another 500 mL of water, and boil again. Repeat for a third time with another 500-mL portion of water, and remove the wash water, which should be neutral to litmus. Transfer the acetylated fat to a separator, and wash with two 200-mL portions of warm water, separating as much as possible of the wash water each time. Transfer the washed sample to a beaker, add 5 g of anhydrous sodium sulfate, and let stand for 1 h, agitating occasionally to assist drying. Filter the oil through a dry filter paper, preferably in an oven at 100°–110°, and keep the filtered oil in the oven until it is completely dry. The acetylated product should be a clear, brilliant oil.

Saponification  Weigh accurately from 2–2.5 g each of the acetylated oil and of the original, untreated sample into separate 250-mL Erlenmeyer flasks. Add to each flask 25.0 mL of 0.5 N alcoholic potassium hydroxide, and continue as directed in the Procedure under Saponification Value, in this Appendix, beginning with “Connect an air condenser . . . .” Record the saponification value of the untreated sample as S, and that of the acetylated oil as S', then calculate the acetyl value of the sample by the formula:

\[
Result = \frac{(S' - S)}{(1.000 - 0.00075S)}
\]
ACID VALUE

Change to read:
(Based on AOCS Methods Te 1a-64\textsuperscript{1}FCC\textsuperscript{7} and Cd 3d-63\textsuperscript{1}FCC\textsuperscript{7})
The acid value is defined as the number of mg of potassium hydroxide required to neutralize the fatty acids in 1 g of the test substance.

Method I (Commercial Fatty Acids)
Unless otherwise directed, weigh accurately about 5 g of the sample into a 500-mL Erlenmeyer flask, and dissolve it in 75 to 100 mL of hot alcohol, previously boiled and neutralized to phenolphthalein TS with sodium hydroxide. Agitation and further heating may be necessary to effect complete solution of the sample. Add 0.5 mL of phenolphthalein TS, and titrate immediately, while shaking, with 0.5 N sodium hydroxide to the first pink color that persists for at least 30 s. Calculate the acid value by the formula:

\[
\text{Result} = 56.1V \times N/W
\]
in which V is the volume, in mL, and N is the normality, respectively, of the sodium hydroxide solution; and W is the weight, in g, of the sample taken.

Method II (Animal Fats and Vegetable and Marine Oils)
Prepare a solvent mixture consisting of equal parts, by volume, of isopropyl alcohol and toluene. Add 2 mL of a 1% solution of phenolphthalein in isopropyl alcohol to 125 mL of the mixture, and neutralize with alkali to a faint but permanent pink color. Weigh accurately the appropriate amount of well-mixed liquid sample indicated in the table below, dissolve it in the neutralized solvent mixture, warming if necessary, and shake vigorously while titrating with 0.1 N potassium hydroxide to the first permanent pink color of the same intensity as that of the neutralized solvent before mixing with the sample. Calculate the acid value by the formula:

\[
\text{Result} = 56.1V \times N/W
\]
in which V is the volume, in mL, and N is the normality, respectively, of the potassium hydroxide solution; and W is the weight, in g, of the sample taken.

<table>
<thead>
<tr>
<th>Acid Value</th>
<th>Sample Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1</td>
<td>20</td>
</tr>
<tr>
<td>1–4</td>
<td>10</td>
</tr>
<tr>
<td>4–15</td>
<td>2.5</td>
</tr>
<tr>
<td>15–75</td>
<td>0.5</td>
</tr>
<tr>
<td>75 and over</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Add the following:

\textbf{ANISIDINE VALUE}
The Anisidine Value is defined as 100 times the optical density measured in a 1-cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the method below. [Note—Carry out the operations as rapidly as possible, avoiding exposure to actinic light.]
Reagents and Solutions

*Test Solution A*   Dissolve 0.500 g of the substance to be examined in isooctane, and dilute with isooctane to 25.0 mL.

*Test Solution B*   To 5.0 mL of *Test Solution A* add 1.0 mL of a 2.5 g/L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.

*Standard Solution*   To 5.0 mL of isooctane add 1.0 mL of a 2.5 g/L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.

**Procedure**    Measure the absorbance of *Test Solution A* at 350 nm using isooctane as the blank. Measure the absorbance of *Test Solution B* at 350 nm exactly 10 min after its preparation, using the *Standard Solution* as the compensation liquid. Calculate the *Anisidine Value* using the formula:

\[
\text{Result} = 25 \times \frac{(1.2A_S - A_B)}{m}
\]

where \(A_S\) is the absorbance of *Test Solution B* at 350 nm; \(A_B\) is the absorbance of *Test Solution A* at 350 nm; and \(m\) is the weight, in g, of the substance to be examined in *Test Solution A*.

1S (FCC7)

**CHLOROPHYLL**

*Change to read:*

(Based on AOCS Method Cc 13d-55\(^1\)\(^A\)\(^{\text{FCC7}}\))

Use a reliable spectrophotometer with a sample holder equilibrated at 44° ± 3° to obtain absorbance values at 630, 670, and 710 nm. Calculate the concentration of chlorophyll (C) using the following equation:

\[
C = \frac{[A_{670} - (A_{630}/2) - (A_{710}/2)]/(K \times b)}{C}
\]

in which C is the concentration of chlorophyll, in mg/kg; A is the absorbance at the wavelength indicated by the subscript; K is the constant for the specific spectrophotometer being used and is equal to 0.1016 for the Beckman Model DU; and b is the optical pathlength through the sample, in cm.

**COLD TEST**

*Change to read:*

(Based on AOCS Method Cc 11-53\(^1\)\(^A\)\(^{\text{FCC7}}\))

Filter a sample (200–300 mL), and transfer to a clean, dry bottle. Fill the bottle completely, and insert a cork stopper. Seal with paraffin, and equilibrate at 25° in a water bath so that it is completely covered. Next, immerse the bottle in an ice and water bath so it is completely covered. Monitor the bath during the test and replenish the ice frequently to keep the bath at 0°.

After 5.5 h remove the bottle from the bath. The sample must be clear; fat crystals or cloudiness must be totally absent.
COLOR

Change to read:
(AOCS-Wesson) (Based on AOCS Method Cc 13b-45\textsuperscript{1} \textsuperscript{FOC7})

**Apparatus**  Use a Lovibond tintometer or the equivalent and a set of color comparison glasses that conform to the AOCS-Wesson Tintometer Color Scale (available from the National Institute of Standards and Technology). A minimum set of glasses consists of

<table>
<thead>
<tr>
<th>Red</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
<th>0.6</th>
<th>0.8</th>
<th>0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>2.0</td>
<td>2.5</td>
<td>3.0</td>
<td>3.5</td>
<td>4.0</td>
<td>5.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>7.6</td>
<td>8.0</td>
<td>9.0</td>
<td>10.0</td>
<td>11.0</td>
<td>12.0</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>5.0</td>
<td>10.0</td>
<td>15.0</td>
<td>20.0</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>70.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For making color comparisons, use color tubes of clear, colorless glass with a smooth, flat, polished bottom (length 154 mm; id 19 mm; od 22 mm), and marked to indicate liquid columns of 25.4 and 133.35 mm.

**Procedure**  Add 0.1 g of diatomaceous earth to a 60-g sample, agitate for 2.5 min at room temperature (or 10\degree to 15\degree above the melting point if the sample is not liquid), and filter. Adjust the temperature to 25\degree–35\degree (or not more than 100 above the melting point), and fill the color tube to the desired mark. Place the tube in the tintometer (in a dark booth or cabinet), and match the sample color as closely as possible with a standard glass.

*Add the following:*

**LOVIBOND COLOR**
(Based on AOCS method Cc 13e-92\textsuperscript{1})

**Apparatus**

- **Colorimeter**  A colorimeter such as the universal Lovibond Tintometer Model F/C and Model F (BS684)\textsuperscript{2}, or equivalent. The Lovibond Schofield Tintometer Wesson Colorimeter, and the AOCS Tintometer have been found not suitable for use in this method.

- **Color racks**  Use red, yellow, blue, and neutral racks with color readings as follows, and fitted with colorless compensating slides:
<table>
<thead>
<tr>
<th></th>
<th>0.1–0.9</th>
<th>1.0–9.0</th>
<th>10.0</th>
<th>70.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td>0.1–0.9</td>
<td>1.0–9.0</td>
<td>10.0–70.0</td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>0.1–0.9</td>
<td>1.0–9.0</td>
<td>10.0–40.0</td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>0.1–0.9</td>
<td>1.0–3.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Lighting cabinet*  Should consist of two 60-watt pearl (not coated) lamps, operated at the correct main voltage and each illuminating the sample and white reference field at 45°. The lamps shall be positioned at either side of the viewing tube (Tintometer AF 905/E, AF 900/C, Model E, or equivalent2). [Note—Lamps shall not be used for longer than 100 h. Change lamps in pairs.]

The viewing tube shall have a field of view subtending 2° at the eye and it shall contain a daylight correction filter. The lighting cabinet shall enable the samples and white reference field to be viewed at 90° to normal. Inspect at frequent intervals for dirt particles and aging of the paint. Repaint, when necessary, with a matte white paint when the color becomes darker than Munsell Notation 5Y 9/1 (also obtainable from Tintometer Ltd.2), or equivalent reference.

*Spillage tray*  Use where required.

*Glass cells*  Use cells made of optical glass with optical path lengths as follows: 1.6 mm (1/16 in), 3.2 mm (1/8 in), 6.4 mm (¼ in), 12.7 mm (½ in), 25.4 mm (1 in), 76.2 mm (3 in), 133.4 mm (5¼ in).

*Sample*  Use a sample that is completely liquid, clear, and bright. If the sample is not liquid at room temperature, heat it to a temperature of about 10° above the melting point. Avoid heating if it is likely to cause a color change.

*Procedure*  Carry out all determinations in subdued ambient light (i.e. not facing a window or in direct sunlight). Pour the prepared *Sample* into an appropriate *Glass cell* of sufficient optical path length to give color readings within the ranges required. The *Glass cell* should be thoroughly cleaned and dried before use and prewarmed, if necessary, to prevent solid matter from separating from the *Sample* during the determination.

Place the cell containing the sample in the *Lighting cabinet* and close the viewing tube. Immediately determine the color of the *Sample*, initially by using the color racks in the ratio of 10 yellow to 1 red and using the minimum number of blue or neutral units to obtain the match. Do not use more than 9.0 blue or 3.0 neutral. To ensure that the number of glass surfaces in both the *Sample* and color filter fields are the same, the racks holding the color filters should be fitted with compensating slides. Record the size of the cell used and the red, yellow, blue, or neutral readings forming the color match.

The test must be carried out by two trained operators. Because the onset of eye fatigue is rapid, operators should rest their eyes after each 30-s period of matching. If the requirements under *Repeatability* (*r*) in Table 1
are not satisfied, a third trained operator must carry out the test. The mean of the two closest readings (of three) should be taken.

**Results**  Compare the mean of the results obtained by the two trained operators with the requirements in *Table 1*. If the requirements of *Repeatability* are not met, the mean of the two closest readings (of three) should be taken. Express the results in terms of the number of red, yellow, and blue or neutral readings needed to obtain the match and the length of the cell used. Color measurements taken in one cell length should not be used to calculate the color values for another cell length.

**Repeatability**  The difference between two test results on the same material, in the same laboratory under the same conditions, should not exceed the repeatability value, \(r\).

**Reproducibility**  The difference between two test results on the same material, under the same conditions in different laboratories, should not exceed the reproducibility value, \(R\).

**Table 1: Repeatability and Reproducibility Limits.**

<table>
<thead>
<tr>
<th>Color Scale</th>
<th>Level</th>
<th>(r)</th>
<th>(R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red: 133.4 mm cell (5¾ in)</td>
<td>2</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Yellow: 133.4 mm cell (5¾ in)</td>
<td>20</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

**FATTY ACID COMPOSITION**

*Change to read:*

(Based on AOCS Methods Ce 1-62\(^1\)\(^{FCC7}\) and Ce 1b-89\(^1\)\(^{FCC7}\))

**Apparatus**  Use a suitable gas chromatograph (*see Appendix IIA*) equipped with a flame ionization detector (FID) and containing either a 3.05-m × 2- or 4-mm id glass column packed with preconditioned 10%, by weight, DEGS-PS on 100- to 120-mesh diatomaceous earth (Chromosorb WHP, or equivalent) or a 30-m × 0.20- to 0.35-mm id capillary fused silica column, or equivalent, containing a suitable stationary phase.

**Operating Conditions**  The operating conditions may vary with the instrument used, but a suitable chromatogram may be obtained using a temperature program 180°–215°; inlet temperature (injector), 300°; detector, 300°; and a suitable carrier gas flow.

**Standard Solutions**  Run through the chromatograph a commercially available standard containing a mixture of fatty-acid methyl esters. Fatty acids and methyl esters with a wide range of carbon numbers and double-bond configurations can be purchased. The calculated concentration should compare to that claimed within ±2 \(\sigma\), where \(\sigma\) is the standard deviation calculated from at least 10 replicate determinations, preferably made over a period of several days.

Determine that the system is functioning properly: inject into the chromatograph a suitable number of samples of the standard to ensure that the resolution factor, \(R\), defining the efficiency of the separation between methyl stearate and methyl oleate is 0.9 or greater. Calculate \(R\) by the equation:
\[ R = 2 \times \frac{(t_2 - t_1)}{(w_2 + w_1)} \]

in which \( t_2 \) and \( t_1 \) are the retention times of peak 2 and peak 1, respectively, and \( w_2 \) and \( w_1 \) are the corresponding widths of the bases of the peaks obtained by extrapolating relatively straight sides of the peaks to the baseline. Baseline separation of the various components in both the standard and the sample preparations is desirable.

**Change to read:**

**Sample Preparation** (for fats and oils) (Based on AOCS Method Ce 2-66\(^{1}\)FCC7)

Introduce 100–1000 mg of the fat into a 50- or 125-mL reaction flask. Add 4–10 mL of 0.5 N methanolic sodium hydroxide, and add a boiling chip. Attach a condenser, and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5–10 min. Add 5–12 mL of 12.5% boron fluoride–methanol reagent (this reagent contains 125 g of boron fluoride per L of methanol and is available commercially) through the condenser, and boil for 2 min. Add 2–5 mL of heptane through the condenser, and boil for 1 min longer. Remove from heat, remove condenser, and add about 15 mL of saturated sodium chloride solution. Stopper the flask, and shake vigorously for 15 s. Transfer about 1 mL of the heptane solution into a test tube and add a small amount of anhydrous sodium sulfate. The dry heptane solution may then be injected directly into a gas chromatograph.

The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screw-cap vial at \( 2^\circ \) for 24 h. For longer storage, they should be sealed in a glass ampule, subjected first to a vacuum and then backfilled with nitrogen and stored at \( -20^\circ \) (freezer).

**Procedure**

Inject an appropriate volume (0.1–1.0 µL) of the sample into the chromatograph. If an automated system is used, follow the manufacturer's instructions; if calculations are to be done manually, proceed as follows:

Calculate the area percentage of each component \( (C_N) \) by the equation:

\[ C_N = \frac{[A_N/T_S]}{100} \]

where \( A_N \) is the area of the peak corresponding to component \( C_N \) and \( T_S \) is the total area for all detected components \( [T_S = \Sigma A_N] \).

**Add the following:**

**FATTY ACID COMPOSITION (SATURATED, cis-MONOUNSATURATED, and cis-POLYUNSATURATED) IN OILS CONTAINING LONG CHAIN POLYUNSATURATED FATTY ACIDS**

(Based on AOCS Methods Ce 1i-07 and Ce 2-66\(^{1}\))

**Apparatus**

Use a gas chromatograph (see Appendix IIA) suitable for use with capillary columns, a temperature-controlled split/splitless injector operated in split mode, and a flame-ionization detector (FID). The capillary GC column should be of fused silica, 30-m × 0.25-mm, with a 0.25-µm coating of polyethylene glycol (PEG)\(^3\).
Operating Conditions  The carrier gas should be gas chromatography grade hydrogen or helium (99.99% or better purity) that has been dried, and from which the oxygen has been removed using suitable filters. Do not use nitrogen as a carrier gas for this method. The flame gases should be gas chromatography grade hydrogen and air and the make-up gas should be gas chromatography grade nitrogen or helium. Use a 78.5-mm × 4-mm (i.d.) × 6.3-mm (o.d.) base deactivated precision injection port split liner with glass wool. The injection port should be operated at 235°C. The detector should be operated at 325°C. The column (oven) temperature should be held at 170°C initially, with a 1°C/min ramp and a final temperature of 225°C. When a hydrogen carrier gas is used, the column head pressure is 77.9 kPa (11.3 psi) with a constant flow rate of 1.2 mL/min, a linear velocity of 43 cm/s, and a split ratio of 100:1. When helium is used as the carrier gas, the column head pressure is 226 kPa (32.77 psi) with a constant flow rate of 2.4 mL/min, a linear velocity of 53 cm/s, and a split ratio of 100:1. [Note—These conditions may not be appropriate for the determination of very long chain fatty acids (25:0 and greater).]

Reagents and Solutions

Internal Standard Solution  2.0 mg/mL of USP Triticosanoin RS in chloroform. [Note—Care must be taken to prevent the loss of chloroform during use and storage. This solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and, therefore, a change in the concentration of the solution. Store the solution in a refrigerator in a well-sealed amber bottle when not in use.]

System Suitability Preparation  USP Menhaden Oil RS

Standard Solution  Prepare a 20 mg/mL solution of USP FAME Standard Mixture RS in either n-heptane or n-hexanes as follows: Dilute 100 mg of USP FAME Standard Mixture RS in 5 mL of solvent, rinsing the ampule containing the standard with the solvent to ensure complete and homogeneous transfer of the mixture.

Sample Preparation (for fats and oils)  Transfer sufficient Internal Standard Solution into a 50- or 125-mL reaction flask so that the concentration in the final solution, after the oil is added, is 0.05–0.10 mg of internal standard per 1 mg of oil. Evaporate the chloroform (from the Internal Standard Solution) from the flask, then introduce 100–1000 mg of the oil to the reaction flask. Add 4–10 mL of 0.5 N methanolic sodium hydroxide, and add a boiling chip. Attach a condenser, and heat the mixture on a steam bath until the fat globules go into solution. This step should take 5–10 min. Add 5–12 mL of 12.5% boron fluoride–methanol reagent (this reagent contains 125 g of boron fluoride per L of methanol and is available commercially) through the condenser, and boil for 2 min. [Note—The addition of antioxidants such as pyrogallol or BHT at a level of 1 mg/mg of the sample may help protect highly unsaturated fatty acids from oxidation during methylation.]

Add 2–5 mL of heptane through the condenser, and boil for 1 min longer. Remove from heat, remove condenser, and add about 15 mL of saturated sodium chloride solution. Stopper the flask, and shake vigorously for 15 s. Dilute the fatty acid methyl ester (FAME) so obtained in n-heptane or n-hexanes to a concentration of approximately 15–20 mg/mL of FAME in solvent.

System Suitability  Proceed as directed under Sample Preparation using the System Suitability Preparation in place of the sample oil. Using a microsyringe suitable for gas chromatography (10-µL), inject 1 µL of the fatty acid methyl esters obtained from the preparation into the chromatograph and record the resulting chromatogram. Compare the chromatogram to the one obtained using commercially available authentic standards of fatty acid methyl esters, if needed, to identify the peaks. Baseline separation should be obtained between 23:0 (the internal standard) and 6c, 9c, 12c, 15c, 18c-21:5 (21:5n-3). Baseline separation should also be obtained between 24:0 and 4c, 7c, 10c, 13c, 16c, 19c-22:6 (DHA or 22:6n-3), which should be almost baseline resolved from the 24:1 isomers.
Theoretical and Empirical Correction Factors  Theoretical correction factors may be calculated as directed below or may be available from several reference sources. Empirical correction factors are determined from the analysis of the Standard Solution. Using the microsyringe described under System Suitability, inject 1 µL of the Standard Solution into the chromatograph and record the resulting chromatogram. Calculate the theoretical correction factors (TCF) for each fatty acid using the equation:

\[ TCF_X = \frac{MW_X}{(N_X - 1) \times (AWC)} \times (1.3344) \]

in which TCF_X is the theoretical flame ionization detector response factor for fatty acid X (as the methyl ester) with respect to 23:0 FAME internal standard; MW_X is the molecular weight of component X; N_X is the number of carbon atoms in the fatty acid methyl ester of component X; AWC is the atomic weight of carbon (12.011); and 1.3344 is the TCF for 23:0 FAME.

Empirical correction factors are required for long chain polyunsaturated fatty acid methyl esters of 20 carbons or more and three or more double bonds of which standards are readily available. Using the certificate of analysis for USP FAME Standard Mixture RS, which should list both the purity (P) and amount (Amt_FAME X) of each fatty acid methyl ester used to make up the standard, calculate the actual amount (AAmt_FAME X) of each fatty acid methyl ester using the equation:

\[ A\text{Amt}_{\text{FAME} X} = P \times A\text{mt}_{\text{FAME} X} \]

The response factor (RF) for each peak is determined using the equation:

\[ RF_{\text{FAME} X} = \frac{\text{Area}_{\text{FAME} X}}{A\text{Mt}_{\text{FAME} X}} \]

where Area_{FAME X} is the peak area of the fatty acid methyl ester obtained from the chromatogram.

Each RF is then made relative to the 23:0 RF using the equation:

\[ RRF_{\text{FAME} X} = \frac{RF_{\text{FAME} X}}{RF_{23:0}} \]

The empirical correction factor (ECF) for each FAME is then calculated by taking the inverse of the RRF using the equation:

\[ ECF_{\text{FAME} X} = \frac{1}{RRF_{\text{FAME} X}} \]

Procedure  Using the microsyringe described under System Suitability, inject 1 µL of the Sample Preparation into the chromatograph, and record the resulting chromatogram.

Calculate the amount, in g, of individual fatty acids, expressed as FAME (W_{FAME X}) and triacylglycerol (W_{TAG X}) equivalents using the equations:

\[ W_{\text{FAME} X} = \frac{(A_X \times W_{\text{TAG-IS}} \times F \times R_X)}{A_{IS}} \]

\[ W_{\text{TAG} X} = W_{\text{FAME} X} \times F_{\text{TAG} X} \]

in which A_X is the peak area count for fatty acid X from the chromatogram obtained; W_{TAG-IS} is the weight of 23:0 internal standard (in g) added to the oil; F is a factor for converting the weight of the internal standard
(which is 23:0 triacylglycerol) from the triacylglycerol form to its corresponding weight of the fatty acid methyl ester form (1.0037); \( R_X \) is the theoretical correction factor (TCF) or empirical correction factor (ECF) for the fatty acid methyl esters relative to 23:0 methyl ester internal standard determined; \( A_{IS} \) is the peak area count for the internal standard; and \( F_{\text{TAG}X} \) is the conversion factor for fatty acid methyl esters to triacyl glycerols for individual fatty acids (from the table below). The TCF should be applied to the analytical data for optimum accuracy and to minimize variation between laboratories because of differences in calculating response factors. TCFs are also used for fatty acids where standards are not available. ECFs are needed due to the large deviation from TCFs for long chain polyunsaturated fatty acids of 20 carbons or more and three or more double bonds.

**Calculation of Total Fat**  
Calculate the amount of total fat in the sample tested (sum of all fatty acids; expressed as triacylglycerols) using the equation:

\[
\text{Total fat (g/100 g portion of test sample)} = \left( \sum W_{\text{TAG}/W_{\text{TS}}} \right) \times 100
\]

where \( W_{\text{TS}} \) is the weight of the sample, in g.

**Calculation of Individual Fatty Acids**  
Calculate the weight, in g, of each individual fatty acid (\( W_X \)) using the equation:

\[
W_X \text{ (per g of sample)} = W_{\text{FAME}X} \times F_{\text{FA}X}
\]

where \( F_{\text{FA}X} \) is the factor for conversion of the fatty acid methyl ester to its corresponding fatty acid (from the table below).

**Calculation of Saturated Fats**  
Calculate the weight of saturated fats (sum of all saturated fatty acids) using the equation:

\[
\text{Saturated fat (g/100 g portion of test sample)} = \left( \sum \text{Saturated } W_X/W_{\text{TS}} \right) \times 100
\]

where \( \sum \text{Saturated } W_X \) is the sum of all saturated fatty acids (4:0; 5:0; 6:0; 7:0; 8:0; 9:0; 10:0; iso 10:0; 11:0; 12:0; anteiso and iso 12:0; 13:0; anteiso and iso 13:0; 14:0; anteiso and iso 14:0; 15:0; anteiso and iso 15:0; 2,6,10,14-tetramethyl 15:0; 16:0; anteiso and iso 16:0; 3,7,11,15-tetramethyl 16:0; 17:0; anteiso and iso 17:0; 18:0; anteiso and iso 18:0; 19:0; anteiso 19:0; 20:0; iso 20:0; 21:0; iso 21:0; 22:0; 24:0; 25:0; 26:0; 27:0; 28:0; 29:0; and 31:0).

**Calculation of \textit{cis}-Monounsaturated Fat**  
Calculate the weight of \textit{cis}-monounsaturated fat (fatty acids containing one double bond) using the equation:

\[
\text{cis}-\text{Monounsaturated fat (g/100 g portion of test sample)} = \left( \sum \text{cis-monounsaturated } W_X/W_{\text{TS}} \right) \times 100
\]

where \( \sum \text{cis-monounsaturated } W_X \) is the sum of all \textit{cis}-monounsaturated fatty acids.

**Calculation of \textit{cis}-Polyunsaturated Fat**  
Calculate the weight of \textit{cis}-polyunsaturated fat (fatty acids containing two or more double bonds) using the equation:

\[
\text{cis-Polyunsaturated fat (g/100 g portion of test sample)} = \left( \sum \text{cis-polyunsaturated } W_X/W_{\text{TS}} \right) \times 100
\]

where \( \sum \text{cis-polyunsaturated } W_X \) is the sum of all \textit{cis}-polyunsaturated fatty acids.

**Calculation of EPA and DHA**  
Calculate the weight of EPA and DHA using the equation:

\[
\text{EPA or DHA (g/100 g portion of test sample)} = \left( \sum \text{EPA or DHA } W_X/W_{\text{TS}} \right) \times 100
\]
Factors for Converting FAME to FA and TAG Equivalents
<table>
<thead>
<tr>
<th>Fatty Acid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( F_{\text{FAx}} )</th>
<th>( F_{\text{TAGx}} )</th>
<th>Fatty Acid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( F_{\text{FAx}} )</th>
<th>( F_{\text{TAGx}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:0</td>
<td>0.8626</td>
<td>0.9868</td>
<td>20:0</td>
<td>0.9570</td>
<td>0.9959</td>
</tr>
<tr>
<td>5:0</td>
<td>0.8792</td>
<td>0.9884</td>
<td>20:1</td>
<td>0.9568</td>
<td>0.9959</td>
</tr>
<tr>
<td>6:0</td>
<td>0.8922</td>
<td>0.9897</td>
<td>20:2</td>
<td>0.9565</td>
<td>0.9958</td>
</tr>
<tr>
<td>7:0</td>
<td>0.9027</td>
<td>0.9907</td>
<td>20:3</td>
<td>0.9562</td>
<td>0.9958</td>
</tr>
<tr>
<td>8:0</td>
<td>0.9114</td>
<td>0.9915</td>
<td>20:4</td>
<td>0.9560</td>
<td>0.9958</td>
</tr>
<tr>
<td>9:0</td>
<td>0.9186</td>
<td>0.9922</td>
<td>20:5</td>
<td>0.9557</td>
<td>0.9958</td>
</tr>
<tr>
<td>10:0</td>
<td>0.9247</td>
<td>0.9928</td>
<td>21:0</td>
<td>0.9588</td>
<td>0.9960</td>
</tr>
<tr>
<td>10:1</td>
<td>0.9239</td>
<td>0.9927</td>
<td>21:5</td>
<td>0.9576</td>
<td>0.9959</td>
</tr>
<tr>
<td>11:0</td>
<td>0.9300</td>
<td>0.9933</td>
<td>22:0</td>
<td>0.9604</td>
<td>0.9962</td>
</tr>
<tr>
<td>11:1</td>
<td>0.9293</td>
<td>0.9932</td>
<td>22:1</td>
<td>0.9602</td>
<td>0.9962</td>
</tr>
<tr>
<td>12:0</td>
<td>0.9346</td>
<td>0.9937</td>
<td>22:2</td>
<td>0.9600</td>
<td>0.9962</td>
</tr>
<tr>
<td>12:1</td>
<td>0.9339</td>
<td>0.9937</td>
<td>22:3</td>
<td>0.9598</td>
<td>0.9962</td>
</tr>
<tr>
<td>13:0</td>
<td>0.9386</td>
<td>0.9941</td>
<td>22:4</td>
<td>0.9595</td>
<td>0.9961</td>
</tr>
<tr>
<td>13:1</td>
<td>0.9380</td>
<td>0.9941</td>
<td>22:5</td>
<td>0.9593</td>
<td>0.9961</td>
</tr>
<tr>
<td>14:0</td>
<td>0.9421</td>
<td>0.9945</td>
<td>22:6</td>
<td>0.9590</td>
<td>0.9961</td>
</tr>
<tr>
<td>14:1</td>
<td>0.9416</td>
<td>0.9944</td>
<td>23:0 (IS)</td>
<td>0.9619</td>
<td>0.9964</td>
</tr>
<tr>
<td>15:0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9453</td>
<td>0.9948</td>
<td>23:5</td>
<td>0.9609</td>
<td>0.9963</td>
</tr>
<tr>
<td>Tetra Methyl 15:0</td>
<td>0.9551</td>
<td>0.9957</td>
<td>24:0</td>
<td>0.9633</td>
<td>0.9965</td>
</tr>
<tr>
<td>15:1</td>
<td>0.9449</td>
<td>0.9947</td>
<td>24:1</td>
<td>0.9631</td>
<td>0.9965</td>
</tr>
<tr>
<td>16:0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9481</td>
<td>0.9950</td>
<td>24:3</td>
<td>0.9628</td>
<td>0.9964</td>
</tr>
<tr>
<td>Tetra Methyl 16:0</td>
<td>0.9570</td>
<td>0.9959</td>
<td>24:4</td>
<td>0.9626</td>
<td>0.9964</td>
</tr>
<tr>
<td>16:1</td>
<td>0.9477</td>
<td>0.9950</td>
<td>24:5</td>
<td>0.9624</td>
<td>0.9964</td>
</tr>
<tr>
<td>16:2</td>
<td>0.9473</td>
<td>0.9950</td>
<td>24:6</td>
<td>0.9621</td>
<td>0.9964</td>
</tr>
<tr>
<td>16:3</td>
<td>0.9469</td>
<td>0.9949</td>
<td>25:0</td>
<td>0.9646</td>
<td>0.9966</td>
</tr>
<tr>
<td>16:4</td>
<td>0.9465</td>
<td>0.9949</td>
<td>26:0</td>
<td>0.9658</td>
<td>0.9967</td>
</tr>
<tr>
<td>17:0</td>
<td>0.9507</td>
<td>0.9953</td>
<td>26:5</td>
<td>0.9650</td>
<td>0.9966</td>
</tr>
<tr>
<td>17:1</td>
<td>0.9503</td>
<td>0.9952</td>
<td>26:6</td>
<td>0.9648</td>
<td>0.9966</td>
</tr>
<tr>
<td>18:0</td>
<td>0.9530</td>
<td>0.9955</td>
<td>27:0</td>
<td>0.9670</td>
<td>0.9968</td>
</tr>
<tr>
<td>18:1</td>
<td>0.9527</td>
<td>0.9955</td>
<td>28:0</td>
<td>0.9680</td>
<td>0.9969</td>
</tr>
<tr>
<td>18:2</td>
<td>0.9524</td>
<td>0.9954</td>
<td>28:7</td>
<td>0.9670</td>
<td>0.9968</td>
</tr>
<tr>
<td>18:3</td>
<td>0.9520</td>
<td>0.9954</td>
<td>28:8</td>
<td>0.9668</td>
<td>0.9968</td>
</tr>
<tr>
<td>18:4</td>
<td>0.9517</td>
<td>0.9954</td>
<td>29:0</td>
<td>0.9690</td>
<td>0.9970</td>
</tr>
<tr>
<td>18:5</td>
<td>0.9514</td>
<td>0.9953</td>
<td>30:0</td>
<td>0.9700</td>
<td>0.9971</td>
</tr>
<tr>
<td>19:0</td>
<td>0.9551</td>
<td>0.9957</td>
<td>31:0</td>
<td>0.9708</td>
<td>0.9972</td>
</tr>
<tr>
<td>19:1</td>
<td>0.9548</td>
<td>0.9957</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Only one factor is given for all positional and geometric isomers and for branched-chain FAME, as the factors are dependent only on the content of carbon to which hydrogen is bonded.

<sup>b</sup> 3,7,11-Trimethylundecanoic acid (TMDD) has equivalent \( F_{\text{FAx}} \) and \( F_{\text{TAGx}} \) to 15:0.

<sup>c</sup> 4,8,12-Trimethyltridecanoic acid (TMTD) has equivalent \( F_{\text{FAx}} \) and \( F_{\text{TAGx}} \) to 16:0.
FREE FATTY ACIDS

Change to read:
(Based on AOCS Method Ca 5a-40)

Unless otherwise directed, accurately weigh the appropriate amount of the sample, indicated in the table below, into a 250-mL Erlenmeyer flask or other suitable container. Add 2 mL of phenolphthalein TS to the specified amount of hot alcohol, neutralize with alkali to the first faint, but permanent, pink color, and then add the hot, neutralized alcohol to the sample container. Titrate with the appropriate normality of sodium hydroxide, shaking vigorously, to the first permanent pink color of the same intensity as that of the neutralized alcohol. The color must persist for at least 30 s. Calculate the percentage of free fatty acids (FFA) in the sample by the formula:

\[
\text{Result} = \frac{VNe}{W}
\]

in which V is the volume and N is the normality of the sodium hydroxide used; W is the weight of the sample, in g; and e is the equivalence factor given in the monograph.

<table>
<thead>
<tr>
<th>FFA Range (%)</th>
<th>Grams of Sample</th>
<th>Milliliters of Alcohol</th>
<th>Strength of NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00–0.2</td>
<td>56.4 ± 0.2</td>
<td>50</td>
<td>0.1 N</td>
</tr>
<tr>
<td>0.2–1.0</td>
<td>28.2 ± 0.2</td>
<td>50</td>
<td>0.1 N</td>
</tr>
<tr>
<td>1.0–30.0</td>
<td>7.05 ± 0.05</td>
<td>75</td>
<td>0.25 N</td>
</tr>
<tr>
<td>30.0–50.0</td>
<td>7.05 ± 0.05</td>
<td>100</td>
<td>0.25–1.0 N</td>
</tr>
<tr>
<td>50.0–100</td>
<td>3.525 ± 0.001</td>
<td>100</td>
<td>1.0 N</td>
</tr>
</tbody>
</table>

FREE GLYCERIN OR PROPYLENE GLYCOL

Change to read:
(Based on AOCS Method Ca 14-56)

Reagents and Solutions Use the Periodic Acid Solution, Potassium Iodide Solution, and Chloroform as described under 1-Monoglycerides, in this Appendix.

Procedure To the combined aqueous extracts obtained as directed under 1-Monoglycerides, add 50.0 mL of Periodic Acid Solution. Run two blanks by adding 50.0 mL of this reagent solution to two 500-mL glass-stoppered Erlenmeyer flasks, each containing 75 mL of water. Continue as directed in the Procedure under 1-Monoglycerides, beginning with “... and allow to stand for at least 30 min but no longer than 90 min.”

Calculation Calculate the percentage of free glycerin in the original sample by the formula:

\[
\text{Result} = (b - S) \times N \times 2.30/W
\]

or calculate the percentage of free propylene glycol by the formula:

\[
\text{Result} = (b - S) \times N \times 3.81/W
\]
in which \( b \) is the number of mL of sodium thiosulfate consumed in the blank determination; \( S \) is the number of mL required in the titration of the aqueous extracts from the sample; \( N \) is the exact normality of the sodium thiosulfate; \( W \) is the weight, in g, of the original sample taken; 2.30 is the molecular weight of glycerin divided by 40; and 3.81 is the molecular weight of propylene glycol divided by 20.

[Note—If the aqueous extract contains more than 20 mg of glycerin or more than 30 mg of propylene glycol, dilute the extract in a volumetric flask and transfer a suitable aliquot into a 500-mL glass-stoppered Erlenmeyer flask before proceeding with the test. The weight of the sample should be corrected in the calculation.]

HEXANE-INSOLUBLE MATTER

If the sample is plastic or semisolid, soften a portion by warming it at a temperature not exceeding 60\(^\circ\)C, and then mix it thoroughly. Transfer 100 g of well-mixed sample into a 1500-mL wide-mouth Erlenmeyer flask, add 1000 mL of solvent hexane, and shake until the sample is dissolved. Pass the resulting solution through a 600-mL Corning “C” porosity, or equivalent, filtering funnel that previously has been dried at 105\(^\circ\)C for 1 h, cooled in a desiccator, and weighed. Wash the flask with two successive 250-mL portions of solvent hexane, and pass the washings through the filter. Dry the funnel at 105\(^\circ\)C for 1 h, cool to room temperature in a desiccator, and weigh. From the gain in weight of the funnel, calculate the percentage of the hexane-insoluble matter in the sample.

HYDROXYL VALUE

Change to read:

(Based on AOCS Methods Cd 4-40\(^{1}\)\(^{\text{FCC7}}\) and Cd 13-60\(^{1}\)\(^{\text{FCC7}}\))

The hydroxyl value is defined as the number of mg of potassium hydroxide equivalent to the hydroxyl content of 1 g of the unacetylated sample.

**Method I**

Proceed as directed under **Acetyl Value**, in this Appendix, but calculate the hydroxyl value by the formula:

\[
\text{Result} = \left( S' - S \right) / \left( 1.000 - 0.00075S' \right)
\]

**Method II**

Unless otherwise directed, accurately weigh the appropriate amount of the sample indicated in the table below, transfer it into a 250-mL glass-stoppered Erlenmeyer flask, and add 5.0 mL of pyridine–acetic anhydride reagent (mix 3 volumes of freshly distilled pyridine with 1 volume of freshly distilled acetic anhydride).
<table>
<thead>
<tr>
<th>Hydroxyl Value</th>
<th>Sample Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–20</td>
<td>10</td>
</tr>
<tr>
<td>20–50</td>
<td>5</td>
</tr>
<tr>
<td>50–100</td>
<td>3</td>
</tr>
<tr>
<td>100–150</td>
<td>2</td>
</tr>
<tr>
<td>150–200</td>
<td>1.50</td>
</tr>
<tr>
<td>200–250</td>
<td>1.25</td>
</tr>
<tr>
<td>250–300</td>
<td>1</td>
</tr>
<tr>
<td>300–350</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Pipet 5 mL of the pyridine–acetic anhydride reagent into a second 250-mL flask for the reagent blank. Heat the flasks for 1 h on a steam bath under reflux condensers, then add 10 mL of water through each condenser, heat for 10 min longer, and allow the flasks to cool to room temperature. Add 15 mL of n-butyl alcohol, previously neutralized to phenolphthalein TS with 0.5 N alcoholic potassium hydroxide, through the condenser, then remove the condensers, and wash the sides of the flasks with 10 mL of n-butyl alcohol. To each flask add 1 mL of phenolphthalein TS, and titrate to a faint pink endpoint with 0.5 N alcoholic potassium hydroxide, recording the mL required for the sample as S and that for the blank as B. To correct for free acid, mix about 10 g of the sample, accurately weighed, with 10 mL of freshly distilled pyridine, previously neutralized to phenolphthalein, add 1 mL of phenolphthalein TS, and titrate to a faint endpoint with 0.5 N alcoholic potassium hydroxide, recording the mL required as A. Calculate the hydroxyl value by the formula:

\[
\text{Result} = [B + (WA/C) - S] \times 56.1N/W
\]

in which W and C are the weights, in g, of the samples taken for acetylation and for the free acid determination, respectively; and N is the exact normality of the alcoholic potassium hydroxide.

**IODINE VALUE**

*Change to read:*

(Based on AOCS Method Cd 1d-92¹ ▲FCC7)

The iodine value is a measure of unsaturation and is expressed as the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the test substance.

**Modified Wijs Method** (Acetic Acid/Cyclohexane Method)

**Wijs Solution**  Dissolve 13 g of resublimed iodine in 1000 mL of glacial acetic acid. Pipet 10.0 mL of this solution into a 250-mL flask, add 20 mL of potassium iodide TS and 100 mL of water, and titrate with 0.1 N sodium thiosulfate, adding starch TS near the endpoint. Record the volume required as A. Set aside about 100 mL of the iodine–acetic acid solution for future use. Pass chlorine gas, washed and dried with sulfuric acid, through the remainder of the solution until a 10.0-mL portion requires not quite twice the volume of 0.1 N sodium thiosulfate consumed in the titration of the original iodine solution. A characteristic color change occurs when the desired amount of chlorine has been added. Alternatively, *Wijs Solution* may be prepared by dissolving 16.5 g of iodine monochloride, ICl, in 1000 mL of glacial acetic acid. Store the solution in amber bottles sealed with paraffin until ready for use, and use within 30 days.
Total Halogen Content  Pipet 10.0 mL of *Wijs Solution* into a 500-mL Erlenmeyer flask containing 150 mL of recently boiled and cooled water and 15 mL of potassium iodide TS. Titrate immediately with 0.1 N sodium thiosulfate, recording the volume required as B.  

**Halogen Ratio**  Calculate the I/Cl ratio by the formula:

\[ \text{Result} = \frac{A}{B - A} \]

The halogen ratio must be between 1.0 and 1.2. If the ratio is not within this range, the halogen content can be adjusted by adding the original solution or by passing more chlorine through the solution.  
[Note—*Wijs Solution* is commercially available.]  

Procedure  The appropriate weight of the sample, in g, is calculated by dividing the number 25 by the expected iodine value. Melt the sample, if necessary, and pass it through a dry filter paper. Transfer the accurately weighed quantity of sample into a clean, dry, 500-mL glass-stoppered bottle or flask containing 20 mL of glacial acetic acid/cyclohexane, 1:1, v/v, and pipet 25.0 mL of *Wijs Solution* into the flask. The excess of iodine should be between 50% and 60% of the quantity added, that is, between 100% and 150% of the quantity absorbed. Swirl, and let stand in the dark for 1.0 h where the iodine value is <150 and for 2.0 h where the iodine value is \( \geq 150 \). Add 20 mL of potassium iodide TS and 100 mL of recently boiled and cooled water, and titrate the excess iodine with 0.1 N sodium thiosulfate, adding the titrant gradually and shaking constantly until the yellow color of the solution almost disappears. Add starch TS, and continue the titration until the blue color disappears entirely. Toward the end of the titration, stopper the container and shake it violently so that any iodine remaining in solution in the glacial acetic acid/cyclohexane, 1:1, solution may be taken up by the potassium iodide solution. Concomitantly, conduct two determinations on blanks in the same manner and at the same temperature. Calculate the iodine value by the formula:

\[ \text{Result} = (B - S) \times 12.69N/W \]

in which B - S represents the difference between the volumes of sodium thiosulfate required for the blank and for the sample, respectively; N is the normality of the sodium thiosulfate; and W is the weight, in g, of the sample taken.

**MELTING RANGE**

Fats of animal and vegetable origin do not exhibit a sharp melting point. For the purpose of this test, melting range is defined as the range of temperature in which the sample becomes a perfectly clear liquid after first passing through a stage of gradual softening, during which it may become opalescent.

Apparatus  Use any suitable commercial or other apparatus. Use melting-point capillary tubes—id, 1 mm; od, 2 mm; length, 50–80 mm; and open at both ends.

Change to read:

Procedure  

**Capillary Method**  (Based on AOCS Method Cc 1-25\(^1\)) Melt the sample and pass it through filter paper; the sample must be absolutely dry. Dip three capillary tubes in the liquid sample so that the oil stands approximately 10 mm high in the tubes, and fuse the end of the tube containing the sample without burning it. Place the tubes containing the liquid sample in a beaker, and equilibrate them at least 16 h at 4°–10° in a
refrigerator. Determine the melting range, using a temperature increase of 0.5° per min when within 10° of the anticipated melting point. The melting ranges of the three samples should be no more than 0.5° apart.

**1-MONOGLYCERIDES**

*Change to read:*

(Based on AOCS Method Cd 11-57\(^{\text{FCC7}}\))

### Reagents and Solutions

**Periodic Acid Solution**  Dissolve 5.4 g of periodic acid, \(\text{H}_2\text{IO}_6\), in 100 mL of water, add 1900 mL of glacial acetic acid, and mix. Store in a light-resistant, glass-stoppered bottle or in a clear, glass-stoppered bottle protected from light.

**Chloroform**  Use chloroform meeting the following test: To each of three 500-mL flasks add 50.0 mL of Periodic Acid Solution, then add 50 mL of chloroform and 10 mL of water to two of the flasks and 50 mL of water to the third. To each flask add 20 mL of potassium iodide TS, mix gently, and continue as directed in the Procedure, beginning with “. . . allow to stand at least 1 min. . . .” The difference between the volume of 0.1 N sodium thiosulfate required in the titrations with and without the chloroform is not greater than 0.5 mL.

### Procedure

Melt the sample, if not liquid, at a temperature not higher than 10° above its melting point, and mix thoroughly. Transfer an accurately weighed portion of the sample, equivalent to about 150 mg of 1-monoalkylglycerides, into a 100-mL beaker (or weigh a sample equivalent to 20 mg of glycerin or 30 mg of propylene glycol if only Free Glycerin or Propylene Glycol is to be determined), and dissolve in 25 mL of chloroform. Transfer the solution, with the aid of an additional 25 mL of chloroform, into a separator, wash the beaker with 25 mL of water, and add the washing to the separator. Stopper the separator tightly, shake vigorously for 30–60 s, and allow the layers to separate. (Add 1–2 mL of glacial acetic acid to break emulsions formed due to the presence of soap.) Collect the aqueous layer in a 500-mL glass-stoppered Erlenmeyer flask, and extract the chloroform solution again using two 25-mL portions of water. Retain the combined aqueous extracts for the determination of Free Glycerin or Propylene Glycol (in this Appendix). Transfer the chloroform to a 500-mL glass-stoppered Erlenmeyer flask, and add 50.0 mL of Periodic Acid Solution to this flask and to each of two blank flasks containing 50 mL of chloroform and 10 mL of water. Swirl the flasks during the addition of the reagent, and allow to stand for at least 30 min, but no longer than 90 min. To each flask, add 20 mL of potassium iodide TS, and allow to stand at least 1 min, but no longer than 5 min, before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate, using a magnetic stirrer to keep the solution thoroughly mixed, to the disappearance of the brown iodine color, then add 2 mL of starch TS and continue the titration to the disappearance of the blue color. Calculate the percentage of 1-monoglycerides\(^5\) in the sample by the formula:

\[
\text{Result} = (B - S) \times N \times 17.927/W
\]

in which \(B\) is the number of mL of sodium thiosulfate consumed in the blank determination; \(S\) is the number of mL required in the titration of the sample; \(N\) is the exact normality of the sodium thiosulfate; \(W\) is the weight, in g, of the sample taken; and 17.927 is the molecular weight of glycercyl monostearate divided by 20.
TOTAL MONOGLYCERIDES

Preparation of Silica Gel  Place about 10 g of 100- to 200-mesh silica gel of a grade suitable for chromatographic work in a tared weighing bottle, cap immediately, and weigh accurately. Remove the cap, dry at 200° for 2 h, cap immediately, and cool for 30 min. Raise the cap momentarily to equalize the pressure, then weigh again, reheat for 5 min at 200°, cool, and reweigh. Repeat this 5-min drying cycle until two consecutive weights agree within 10 mg. Calculate the percentage of water in the original silica gel (A) by the formula:

\[
\text{Result} = \frac{(\text{loss in wt/sample wt}) \times 100}{100}
\]

then calculate the amount of water required to adjust the water content to 5% by the formula:

\[
\text{Result} = W \times \frac{(5 - A)}{95}
\]

in which W is the weight, in g, of the undried sample to be used.

Accurately weigh the appropriate amount of the undried silica gel to be used in the determination, transfer to a suitable blender or mixer, and add the calculated amount of water to give a final water content of 5% ± 0.1%. Blend for 1 h to ensure complete water distribution, and store in a sealed container. Determine the water content of the adjusted silica gel as directed above, and readjust if necessary.

[Note—Each new lot of silica gel should be checked for suitability by the analysis of a monoglyceride of known composition.]

Sample Preparation   [CAUTION—To avoid rearrangement of partial glycerides, use extreme caution in applying heat to the samples, and do not heat above 50°.]  

Samples Melting Below 50°  Melt the sample, if necessary, by warming for short periods below 50°, not exceeding a total of 30 min.

Samples Melting Above 50°  Grind about 10 g in a mortar and pestle, chilling solid samples, if necessary, in carbon dioxide.

Weigh accurately about 1 g of the prepared sample into a 100-mL beaker, add 15 mL of chloroform, and warm, if necessary, to effect solution. Use only minimal heat, and do not heat above 40°.

Preparation of Chromatographic Column   Connect a 19- × 290-mm chromatographic tube, equipped with an outer 19/22 standard-taper joint at the top and a coarse, fritted-glass disk and inner 19/22 standard-taper joint at the bottom, with an adapter consisting of an outer 19/22 joint connected to a Teflon stopcock. Do not grease the joints. Weigh 30 g of the prepared silica gel into a 150-mL beaker, add 50–60 mL of petroleum ether, and stir slowly with a glass rod until all air bubbles are expelled. Transfer the slurry to the column through a powder funnel, and open the stopcock, allowing the liquid level to drop to about 2 cm above the silica gel. Transfer any silica gel slurry remaining in the beaker into the column with a minimum amount of petroleum ether, then rinse the funnel and sides of the column. Drain the solvent through the stopcock until the level drops to 2 cm above the silica gel, and remove the powder funnel.

Procedure   Carefully add the Sample Preparation to the prepared column. Open the stopcock, and adjust the flow rate to about 2 mL/min, discarding the eluate. Rinse the sample beaker with 5 mL of chloroform, and add the rinsing to the column when the level drops to 2 cm above the silica gel. Never allow the column to become dry on top, and maintain a flow rate of 2 mL/min throughout the elution. Avoid interruptions during elution as
they may cause pressure buildup and result in leakage through the stopcock or cracks in the silica gel packing.

Attach a 250-mL reservoir separator, provided with a Teflon stopcock and a 19/22 standard-taper drip tip inner joint, to the column. Add 200 mL of benzene, elute, and discard the eluate, which contains the triglycerides fraction. When the level of benzene drops to 2 cm above the silica gel, add 200 mL of a 1:10 mixture of ether in benzene, elute, and discard the eluate, which contains the diglycerides and the free fatty acid fraction. When all of the ether–benzene solvent has been added from the separator and the level in the column drops to 2 cm above the silica gel, add from 250–300 mL of ether, and collect the monoglyceride fraction in a tared flask. Rinse the tip of the column into the flask with a few mL of ether, and evaporate to dryness on a steam bath under a stream of nitrogen or dry air. Cool for at least 15 min, weigh, then reheat on the steam bath for 5 min in the same manner. Cool, reweigh, and repeat the 5-min evaporation, cooling, and reweighing procedures until two consecutive weights agree within 2 mg. The weight of the residue represents the total monoglycerides in the sample taken.

**OXYETHYLENE DETERMINATION**

**Apparatus**  The apparatus for oxyethylene group determination is shown in Fig. 35. It consists of a boiling flask, A, fitted with a capillary side tube to provide an inlet for carbon dioxide and connected by a condenser with trap B, which contains an aqueous suspension of red phosphorus. The first absorption tube, C, contains a silver nitrate solution to absorb ethyl iodide. Absorption tube D is fitted with a 1.75-mm spiral rod (23 turns, 8.5-mm rise per turn), which is required to provide a longer contact of the evolved ethylene with the bromine solution. A standard-taper adapter and stopcock are connected to tube D to permit the transfer of the bromine solution into a titration flask without loss. A final trap, E, containing a potassium iodide solution, collects any bromine swept out by the flow of carbon dioxide.
Dimensions of the apparatus not readily determined from Fig. 35 are as follows: carbon dioxide inlet capillary, 1-mm id; flask A, 28-mm diameter, 12/18 standard-taper joint; condenser, 9-mm id; inlet to trap B, 2-mm id; inlet to trap C, 7/15 standard-taper joint, 2-mm id; trap C, 14-mm id; trap D, inner tube, 8-mm od, 2-mm opening at bottom of spiral; outer tube, approximately 12.5-mm id; side arm 7 cm from top of inserted spiral, 3.5-mm id, 2-mm opening at bottom.

Reagents

Hydriodic Acid  Use special-grade hydriodic acid suitable for alkoxyl determinations, or purify reagent-grade as follows: Distill over red phosphorus in an all-glass apparatus, passing a slow stream of carbon dioxide through the apparatus until the distillation is terminated and the receiving flask has completely cooled.

[CAUTION—Use a safety shield, and conduct the distillation in a hood.]

Silver Nitrate Solution  Dissolve 15 g of silver nitrate in 50 mL of water, mix with 400 mL of alcohol, and add a few drops of nitric acid.

Bromine–Bromide Solution  Add 1 mL of bromine to 300 mL of glacial acetic acid saturated with dry potassium iodide (about 5 g). Fifteen mL of this solution requires about 40 mL of 0.05 N sodium thiosulfate. Store in a brown bottle in a dark place, and standardize at least once a day during use.

Procedure  Fill trap B with enough of a suspension of 60 mg of red phosphorus in 100 mL of water to cover the inlet tube. Pipet 10 mL of the Silver Nitrate Solution into tube C and 15 mL of the Bromine–Bromide Solution into tube D, and place 10 mL of a 1:10 solution of potassium iodide in trap E. Transfer an accurately weighed quantity of the sample specified in the monograph into the reaction flask, A, and add 10 mL of Hydriodic Acid along with a few glass beads or boiling stones. Connect the flask to the condenser, and begin passing carbon dioxide through the apparatus at the rate of about one bubble per s. Heat the flask in an oil bath at 140°–145°, and continue the reaction at this temperature for at least 40 min. Heating should be continued until the cloudy reflux in the condenser becomes clear and until the supernatant liquid in the silver nitrate tube, C, is almost completely clarified. Five min before the reaction is terminated, heat the Silver Nitrate Solution in tube C in a hot water bath at 50°–60° to expel any dissolved olefin. At the completion of the decomposition, cautiously disconnect tubes D and C in the order named, then disconnect the carbon dioxide source and remove the oil bath. Connect tube D to a 500-mL iodine flask containing 150 mL of water and 10 mL of a 1:10 solution of potassium iodide, run the Bromine–Bromide Solution into the flask, and rinse the tube and spiral with water. Add the potassium iodide solution from trap E to the flask, rinsing the side arm and tube with a few mL of water, stopper the flask, and allow to stand for 5 min. Add 5 mL of 2 N sulfuric acid, and titrate immediately with 0.05 N sodium thiosulfate, using 2 mL of starch TS for the endpoint. Transfer the silver nitrate solution from tube C into a flask, rinsing the tube with water, dilute to 150 mL with water, and heat to boiling. Cool, and titrate with 0.05 N ammonium thiocyanate, using 3 mL of ferric ammonium sulfate TS as the indicator. Perform a blank determination. Calculate the percentage of oxyethylene groups (—CH₂CH₂O—), as ethylene, by the formula:

\[ \text{Result} = (B - S) \times N \times 2.203/W \]

in which \( B - S \) represents the difference between the volumes of sodium thiosulfate required for the blank and the sample solution, respectively; \( N \) is the normality of the sodium thiosulfate; \( W \) is the weight, in g, of the sample taken; and 2.203 is an equivalence factor for oxyethylene. Calculate the percentage of oxyethylene groups, as ethyl iodide, by the formula:

\[ \text{Result} = (B' - S') \times N' \times 4.405/W \]
in which $B' - S'$ represents the difference between the volumes of ammonium thiocyanate required for the blank and the sample solution, respectively; $N'$ is the normality of the ammonium thiocyanate; and 4.405 is an equivalence factor for oxyethylene. The sum of the values so obtained represents the percentage of oxyethylene groups in the sample taken.

**PEROXIDE VALUE**

Unless otherwise indicated in the monograph, use Method I: Acetic Acid–Isooctane Method.

The peroxide value is defined as the number of milliequivalents of peroxide per 1000 g of sample that oxidizes potassium iodide under the given test conditions.

**Change to read:**

**Method 1: Acetic Acid–Isooctane Method**  
(Based on AOCS Method Cd 8b-90\(^1\)\(^{FCC7}\))

**Solutions**

*Acetic Acid–Isooctane Solution*  
Mix 3 volumes of glacial acetic acid with 2 volumes of isooctane. [Note — Use a fume hood at all times, and avoid inhalation, ingestion, and skin contact.]

*Saturated Potassium Iodide Solution*  
Dissolve an excess of potassium iodide in recently boiled water. Prepare fresh daily, and make certain the solution remains saturated during use.

*Starch Indicator Solution*  
Make a paste with 1 g of starch and a small amount of cold water, and add it, while stirring to 200 mL of boiling water. Remove from heat within a few seconds, and cool. If desired, add salicylic acid (1.25 g/L) as a preservative. The solution may be kept refrigerated at 4°–10° for not more than 3 weeks. Test the solution for sensitivity before use by placing 5 mL of the solution in 100 mL of water and adding 0.05 mL of 0.1 N potassium iodide solution. The deep blue color produced must be discharged by 0.05 mL of 0.1 N sodium thiosulfate. If the solution fails the test, prepare a fresh starch solution.

**Procedure**  
Transfer 5.00 ± 0.05 g of sample, accurately weighed, into a 250-mL Erlenmeyer flask fitted with a glass stopper, and add 50 mL of Acetic Acid–Isooctane Solution. Swirl to dissolve the sample, and add 0.5 mL of Saturated Potassium Iodide Solution. Allow the sample solution to stand, agitating it occasionally, for exactly 1 min, and immediately add 30 mL of water. Titrate with 0.1 N sodium thiosulfate solution, adding the solution gradually while constantly agitating until the yellow iodine color has almost disappeared. Add 0.5 mL of a 10% sodium lauryl sulfate solution, and then add approximately 0.5 mL of Starch Indicator Solution. Continue the titration while constantly agitating, especially near the endpoint to liberate all of the iodine from the solvent layer. Add 0.1 N thiosulfate solution dropwise until the blue color just disappears. If the titration is less than 0.5 mL using 0.1 N sodium thiosulfate, repeat the determination using 0.01 N sodium thiosulfate. Conduct a blank determination (see General Provisions), and make any necessary correction. Calculate the peroxide value by the formula:

\[
\text{Result} = \frac{[(S - B) \times N \times 1000]}{W}
\]

in which $S$ is the volume, in milliliters, of 0.1 N sodium thiosulfate consumed by the sample; $B$ is the volume, in milliliters, of 0.1 N sodium thiosulfate consumed by the blank; $N$ is the normality of the sodium thiosulfate solution; and $W$ is the weight, in grams, of the sample taken.

**Change to read:**
Method II  (Alternatively, follow the AOCS Method Cd 8-53\(^1\)\(^{\text{FCC7}}\)) [Note—To make the solutions referenced below, please see the Fifth Edition, pages 974 (0.1 N sodium thiosulfate solution, dilute to 1:1 v:v) and 969 (starch TS).]

Accurately weigh about 10 g of sample, add 30 mL of a 3:2 mixture of glacial acetic acid:chloroform, and mix. Add 1 mL of a saturated solution of potassium iodide, mix, and allow it to stand for 10 min. Add 100 mL of water, begin titrating with 0.05 N sodium thiosulfate, adding starch TS as the endpoint is approached, and continue the titration until the blue starch color has just disappeared. Perform a blank determination (see General Provisions), and make any necessary correction. Calculate the peroxide value, as milliequivalents of peroxide per kilogram of the sample, by the formula:

\[
\text{Result} = S \times N \times 1000/W
\]

in which S is the net volume, in milliliters, of sodium thiosulfate solution required for the sample; N is the exact normality of the sodium thiosulfate solution; and W is the weight, in grams, of the sample taken.

REICHERT-MEISSSL VALUE

Change to read:

(Based on AOCS Method Cd 5-40\(^1\)\(^{\text{FCC7}}\))

The Reichert-Meissl value is a measure of soluble volatile fatty acids (chiefly butyric and caproic). It is expressed in terms of the number of mL of 0.1 N sodium hydroxide required to neutralize the fatty acids obtained from a 5-g sample under the specified conditions of the method.

Apparatus  Use a glass distillation apparatus of the same dimensions and construction as that shown in Fig. 36.

![FIGURE 36 Reichert-Meissl Distillation Apparatus. [Note—A suitable heating mantle may be substituted for the burner.]](image-url)
Reagents

*Sodium Hydroxide Solution*  Prepare a solution containing 50.0% by weight of NaOH, and protect from contact with carbon dioxide. Allow the solution to settle, and use only the clear liquid.

*Glycerin–Sodium Hydroxide Mixture*  Add 20 mL of the *Sodium Hydroxide Solution* to 180 mL of glycerin.

Procedure  Unless otherwise directed, accurately weigh about 5 g of the sample, previously melted if necessary, into the 300-mL distillation flask. Add 20.0 mL of the *Glycerin–Sodium Hydroxide Mixture*, and heat until the sample is completely saponified, as indicated by the mixture becoming perfectly clear. Shake the flask gently if any foaming occurs. Add 135 mL of recently boiled and cooled water, dropwise at first to prevent foaming, then add 6 mL of 1:5 sulfuric acid and a few pieces of pumice stone or silicon carbide. Rest the flask on a piece of heat-proof board having a center hole 5 cm in diameter, and begin the distillation, regulating the flame so as to collect 110 mL of distillate in 30 ± 2 min (measure time from the passage of the first drop of distillate from the condenser to the receiving flask), letting the distillate drip into the flask at a temperature not higher than 20°C.

When 110 mL has distilled, disconnect the receiving flask, and remove the flame. Mix the contents of the flask with gentle shaking, and immerse almost completely for 15 min in water cooled to 15°C. Filter the distillate through dry, 9-cm, moderately retentive paper (S & S No. 589 White Ribbon, or equivalent), add phenolphthalein TS, and titrate 100 mL of the filtrate with 0.1 N sodium hydroxide to the first pink color that remains unchanged for 2–3 min. Perform a blank determination using the same quantities of the same reagents, and calculate the Reichert-Meissl value by the formula:

\[
\text{Result} = 1.1 \times (S - B)
\]

in which S is the volume of 0.1 N sodium hydroxide required for the sample, and B is the volume required for the blank.

Saponification Value

*Change to read:*

(Based on AOCS Methods Tl 1a-64\(^{\text{FCC7}}\) and Cd 3-25\(^{\text{FCC7}}\))

The saponification value is defined as the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters in 1 g of the test substance.

Procedure  Melt the sample, if necessary, and pass it through a dry filter paper to remove any traces of moisture. Unless otherwise directed, weigh accurately into a 250-mL flask a sample of such size that the titration of the sample solution after saponification will require between 45% and 55% of the volume of 0.5 N hydrochloric acid required for the blank, and add to the flask 50.0 mL of 0.5 N alcoholic potassium hydroxide. Connect an air condenser, at least 65 cm in length, to the flask, and reflux gently until the sample is completely saponified (usually 30 min to 1 h). Cool slightly, wash the condenser with a few mL of water, add 1 mL of phenolphthalein TS, and titrate the excess potassium hydroxide with 0.5 N hydrochloric acid. Heat the contents of the flask to boiling, again titrate to the disappearance of any pink color that may have developed, and record the total volume of acid required. Perform a blank determination using the same amount of 0.5 N alcoholic potassium hydroxide. Calculate the saponification value by the formula:

\[
\text{Result} = 56.1(B - S) \times N/W
\]
in which \( B - S \) represents the difference between the volumes of 0.5 N hydrochloric acid required for the blank and the sample, respectively; \( N \) is the normality of the hydrochloric acid; and \( W \) is the weight, in g, of the sample taken.

[Note—A "masked phenolphthalein indicator" may be used with off-color materials. Prepare the indicator by dissolving 1.6 g of phenolphthalein and 2.7 g of methylene blue in 500 mL of alcohol, and adjust the pH with alcoholic alkali solution so that the greenish blue color is faintly tinged with purple. The color change, when going from acid to alkali, is from green to purple.]

**SOAP**

Prepare a solvent mixture consisting of equal parts, by volume, of benzene and methanol, add bromophenol blue TS, and neutralize with 0.5 N hydrochloric acid, or use neutralized acetone as the solvent. Accurately weigh the amount of sample specified in the individual monograph, dissolve it in 100 mL of the neutralized solvent mixture, and titrate with 0.5 N hydrochloric acid to a definite yellow endpoint. Calculate the percentage of soap in the sample by the formula:

\[
\text{Result} = \frac{VN_e}{W}
\]

in which \( V \) and \( N \) are the volume and normality, respectively, of the hydrochloric acid; \( W \) is the weight of the sample, in g; and \( e \) is the equivalence factor given in the monograph.

**SPECIFIC GRAVITY**

The specific gravity of a fat or oil is determined at 25°, except when the substance is a solid at that temperature, in which case the specific gravity is determined at the temperature specified in the monograph, and is referred to water at 25°.

Clean a suitable pycnometer by filling it with a saturated solution of chromic acid (CrO₃) in sulfuric acid and allowing it to stand for at least 4 h. Empty the pycnometer, rinse it thoroughly, then fill it with recently boiled water, previously cooled to about 20°, and place in a constant-temperature bath at 25°. After 30 min, adjust the level of water to the proper point on the pycnometer, and stopper. Remove the pycnometer from the bath, wipe dry with a clean cloth free from lint, and weigh. Empty the pycnometer, rinse several times with alcohol and then with ether, allow to dry completely, remove any ether vapor, and weigh. Determine the weight of the contained water at 25° by subtracting the weight of the pycnometer from its weight when full.

Pass the oil or melted sample through filter paper to remove any impurities and the last traces of moisture, and cool to a few degrees below the temperature at which the determination is to be made. Fill the clean, dry pycnometer with the sample, and place it in the constant-temperature bath at the specified temperature. After 30 min, adjust the level of the oil to the mark on the pycnometer, insert the stopper, wipe dry, and weigh. Subtract the weight of the empty pycnometer from its weight when filled with the sample, and divide the difference by the weight of the water contained at 25°. The quotient is the specific gravity at the temperature of observation, referred to water at 25°.

**STABILITY**
Delete the following:
▲(Active Oxygen Method)▲FCC7

Add the following:
▲Unless otherwise indicated in the monograph, use Method I: Active Oxygen Method▲FCC7

Change to read:

▲Method I: Active Oxygen Method (Based on AOCS Method Cd 12-57)▲FCC7

Fat stability is the time, in h, required for a sample of fat or oil to attain a peroxide value of 100. This period of time is determined by interpolation between two measurements and is assumed to be an index of resistance to rancidity. [CAUTION—All equipment must be scrupulously clean. Do not use chromic acid or other acidic cleaning agents. All receptacles in the heater must be calibrated for temperature under the exact conditions of the test. During the test, the temperature must be monitored in a sample tube containing the recommended quantity of oil.]

Apparatus Use a suitable heating block and aeration apparatus, such as shown in JAOS 33 (1956), pp. 628–630.

Sampling Remove the samples from large containers or processing equipment with sampling devices only of stainless steel, aluminum, nickel, or glass. Solid fat samples should be taken at least 5 cm from the walls of large containers and 2.5 cm from the walls of small containers. If liquid oil is to be poured from a container, clean the spout or lip with an acetone-moistened cloth. Under no circumstances should samples be taken from containers equipped with plastic or enameled tops or paper or wax liners.

Procedure Unless already completely liquid, the sample should be melted at a temperature not more than 10° above its melting point. Pour 20 mL into each of two or more sample tubes ensuring that the sample does not contact the tube where the stopper will later fit. Insert the aeration tube assembly so that the end of the air delivery tube is 5 cm below the surface of the sample. Place the sample tube in a container of vigorously boiling water for 5 min (during this time adjust the air flow rate from the manifold). Remove the tube, wipe dry, and transfer immediately to the constant-temperature heater, maintained at 97.8 ± 0.2°, and connect the aeration tube to the manifold. Determine to the nearest h the time required for the sample to attain a Peroxide Value (in this appendix) of 100 milliequivalents (meq) as follows: With 1-g samples determine when the peroxide value is approximately 75 and 125 meq, then perform the test on four 5-g samples determining the peroxide value in duplicate at the times corresponding to 75 and 125 meq. Make a second determination on two 5-g samples exactly 1 h after the first pair. Plot these values against aeration time; the AOM stability value in h is given where the line crosses 100 meq.

Add the following:

▲Method II: Oil Stability Index (Based on AOCS Method Cd 12b-92)

The oil stability index (OSI) is an indication of the resistance of a fat or oil to oxidation. It is measured as the length of time required, under specific accelerated oxidation conditions, before a rapid acceleration of oxidation occurs for a test oil or fat, also called the “induction period”▲FCC7

Add the following:
Add the following:

**Sampling** The samples should be kept cool and in the dark. Remove samples from large containers with clean sampling devices only of stainless steel, aluminum, nickel, or glass. Solid fat samples should be taken at least 5 cm from walls of large containers and 2.5 cm from walls of small containers. If liquid oil is to be poured, clean the spout with a clean, acetone-moistened cloth. Samples should be protected from heat, light, and air before analysis.

**Procedure** Fill the conductivity tubes with 50 mL of water (deionized or distilled with conductivity <5 µS-cm\(^{-1}\)) and attach the probes. Verify that the water conductivity in the tube is 25 µS-cm\(^{-1}\) or less and that the conductivity is constant. Unless already completely liquid, the sample should be melted at a temperature not more than 10\(^{\circ}\) above its melting point. Carefully place a 5.0 ± 0.2 g sample (Oxidative Stability and Rancimat instruments without an insert), or 2.5 ± 0.2 g sample (Rancimat instruments fitted with disposable inserts) into the bottom of the reaction tube, avoiding coating the sides of the tubes and contamination of samples. Connect tubing from the air manifold to the conductivity measurement tube, adjusting aeration tubes to within 5 mm of bottom of both the reaction and the conductivity tubes. Adjust the airflow to 2.5 ± 0.2 mL/s unless otherwise indicated in the individual monograph. Set the instrument to the temperature indicated in the individual monograph.

Use a computer or multichannel strip chart recorder to monitor the conductivity of each probe. Plot water conductivity versus time in h. Calculate the OSI value of the sample as the time in h which corresponds to the inflection point of the conductivity versus time curve. This time-based end point can be determined either by a micro-processor-computed slope/change algorithm or a maximum of the second derivative, or by the tangential method. [Note—Improper temperature control is the most likely source of error for this procedure. Temperature calibration of the instrument should be performed before sample analysis by checking the actual temperature of the sample in the bath. Temperatures during analysis should be maintained within at least ± 0.1 \(^{\circ}\). For temperature calibration of the Rancimat instrument, a NIST traceable calibrated platinum resistance (RTD) digital thermometer can be used.\(^9\)]

[Note—Water in the effluent trap must not exceed 25\(^{\circ}\) to minimize loss of formic acid.]
[Note—Trace-metal contamination of glassware will cause accelerated oxidation; rinse water should be tested; chromate cleaning solutions should not be used; only detergents without surface-active agents should be used for cleaning.]
[Note—Reaction tube cleaning procedures for instruments that do not utilize disposable glassware, or for precautionary cleaning of disposable glassware are described elsewhere.\(^8\)]

**TOCOPHEROLS**
(Based on AOCS Method Ce 8-89\(^6\))
[Note—All solutions containing tocopherols should be stored in low-actinic glassware, or suitably protected from light.]

**Standard Stock Solutions** Prepare individual standard stock solutions of α-, β-, γ-, and δ-tocopherol, unless otherwise directed in the monograph, according to the following general procedure.

Transfer 10 mg of suitable tocopherol standard into a 100-mL volumetric flask, and dilute with hexane to volume. Pipet 10 mL of this solution into an amber glass-round-bottom flask, and remove all hexane using a suitable rotary evaporator at a temperature not higher than 40°C. Restore atmospheric pressure with nitrogen and remove the flask from the evaporator as soon as all solvent has been removed. Dissolve remaining tocopherol residue with 10 mL of methanol. Measure the absorbance (A) of this solution using a suitable spectrophotometer at the wavelength specified in table below for the individual tocopherol being measured. Calculate the concentration (µg/mL) of the individual tocopherol in the solution by the following formula:

\[
\text{Result} = \frac{A}{a \times b}
\]

in which A is the absorbance of the solution at the measured wavelength; a is the absorptivity of the individual tocopherol from the table below; b is the cell path length (cm).

<table>
<thead>
<tr>
<th>Tocopherol</th>
<th>Measurement Wavelength (nm)</th>
<th>Absorptivity (mL ⋅ µg⁻¹ ⋅ cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>292</td>
<td>0.0076</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>296</td>
<td>0.0089</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>298</td>
<td>0.0091</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>298</td>
<td>0.0087</td>
</tr>
</tbody>
</table>

[Note—The Standard Stock Solutions can be stored for up to one week under refrigerated conditions in low-actinic glassware or suitably protected from light.]

**Standard Solution** Prepare a mixed tocopherol standard solution, unless otherwise directed in the monograph, as follows. Mix appropriate volumes of the individual tocopherol Standard Stock Solutions and dilute with hexane to give a solution containing between 1 and 5 µg/mL of each tocopherol. [Note—A more concentrated Standard Solution may be necessary if UV detection is used.] [Note—The Standard Solution should be prepared fresh daily and stored under refrigerated conditions in low-actinic glassware or suitably protected from light.]

**Test Solution** Accurately weigh 2 g of the sample into a 25-mL volumetric flask. Add hexane to dissolve the Test Sample, and dilute with hexane to volume. [Note—If a fluorescence detector is used, it may be necessary to further dilute this test solution before analysis.] [Note—The Test Solution should be prepared and analyzed on the same day, and stored in low-actinic glassware or suitably protected from light.]

**Apparatus** Use a suitable high-performance liquid chromatography system (see Chromatography, Appendix IIA) equipped with either a UV (292 nm) or fluorescence (290 nm excitation and 330 nm emission) detector, or as directed in the monograph. Use a 250 × 4 mm analytical column packed with a 5-µm microparticulate silica.

**Operating Conditions** [Note—New columns should be washed and conditioned for 10 min with methanol, then dichloromethane, followed by hexane at 1 mL/min. All columns should be equilibrated, if necessary with
mobile phase for 30 min before analysis.]

**Mobile phase**  Isopropanol in hexane (0.5:99.5, v/v)

**Flow rate**  About 1 mL/min

**Injection volume**  About 20 µL

**System Suitability**  Inject the *Standard Solution* into the chromatograph and measure the peak responses on the resulting chromatograms. The retention time for α-tocopherol is not less than 5 min. The resolution, R, between β- and γ-tocopherol is not less than 1.0. The relative standard deviation for the α-tocopherol peak area for replicate injections is no more than 5%.

**Procedure**  Inject the *Test Solution* into the chromatograph and measure the peak responses on the resulting chromatograms. The relative retention times for α-, β-, γ-, and δ-tocopherol are approximately 1.0, 1.6, 3.0, and 1.7, respectively. Separately calculate the concentration, in mg/kg, of each tocopherol in the portion of the sample taken by the following formula:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right)
\]

where \(r_U\) is the peak response for the tocopherol from the *Test Solution*; \(r_S\) is the peak response for the tocopherol from the *Standard Solution*; \(C_S\) is the concentration of tocopherol in the *Standard Solution* (µg/mL); and \(C_U\) is the concentration of sample in the *Test Solution* (g/mL).

**UNSAPONIFIABLE MATTER**

**Change to read:**

(Based on AOCS Method Ca 6a-40) *(Based on AOCS Methods Ca 6a-40 and Ca 6b-53, respectively)*

**Change to read:**

Unless indicated otherwise in the individual monograph, use *Method I*. *(FCC7)*

**Change to read:**

**Method I** *(FCC7)*

This procedure determines those substances frequently found dissolved in fatty materials that cannot be saponified by alkali hydroxides but that are soluble in the ordinary fat solvents.

**Procedure**  Accurately weigh 5.0 g of the sample into a 250-mL flask, add a solution of 2 g of potassium hydroxide in 40 mL of alcohol, and boil gently under a reflux condenser for 1 h or until saponification is complete. Transfer the contents of the flask to a glass-stoppered extraction cylinder (approximately 30 cm in length, 3.5 cm in diameter, and graduated at 40, 80, and 130 mL). Wash the flask with sufficient alcohol to make a volume of 40 mL in the cylinder, and complete the transfer with warm and then cold water until the total volume is 80 mL. Finally, wash the flask with a few mL of petroleum ether, add the washings to the
cylinder, cool the contents of the cylinder to room temperature, and add 50 mL of petroleum ether.

Insert the stopper, shake the cylinder vigorously for at least 1 min, and allow both layers to become clear. Siphon the upper layer as completely as possible without removing any of the lower layer, collecting the ether fraction in a 500-mL separator. Repeat the extraction and siphoning at least six times with 50-mL portions of petroleum ether, shaking vigorously each time. Wash the combined extracts, with vigorous shaking, with 25-mL portions of 10% alcohol until the wash water is neutral to phenolphthalein, and discard the washings. Transfer the ether extract to a tared beaker, and rinse the separator with 10 mL of ether, adding the rinsings to the beaker. Evaporate the ether on a steam bath just to dryness, and dry the residue to constant weight, preferably at 75°–80° under a vacuum of not more than 200 mm Hg, or at 100° for 30 min. Cool in a desiccator, and weigh to obtain the uncorrected weight of unsaponifiable matter.

Determine the quantity of fatty acids in the residue as follows: Dissolve the residue in 50 mL of warm alcohol (containing phenolphthalein TS and previously neutralized with sodium hydroxide to a faint pink color), and titrate with 0.02 N sodium hydroxide to the same color. Each mL of 0.02 N sodium hydroxide is equivalent to 5.659 mg of fatty acids, calculated as oleic acid.

Subtract the calculated weight of fatty acids from the weight of the residue to obtain the corrected weight of unsaponifiable matter in the sample.

Add the following:

**Method II**

This procedure determines those substances frequently found dissolved in fats and oils that cannot be saponified by the usual caustic treatment, but are soluble in ordinary fat and oil solvents. Included in this group of compounds are higher aliphatic alcohols, sterols, pigments, and hydrocarbons. This method is applicable to fats and oils containing higher levels of unsaponifiable matter than usually found in normal tallows and greases. It is especially suited for marine oils.

**Procedure** Accurately weigh 2.0–2.5 g of the well-mixed sample into a 250-mL conical flask with a ground-glass joint. Add 25 mL of alcohol and 1.5 mL of a 50% (w/w) aqueous solution of potassium hydroxide. Boil the contents of the flask gently but steadily under reflux with occasional swirling for 30 min or until saponification is complete. The material must be completely saponified before proceeding. No loss of alcohol should occur during saponification. Transfer the warm solution to a graduated, glass-stoppered extraction cylinder with a capacity of about 200 mL, using a total of 50 mL of water.

[CAUTION— Alcohol is flammable.] Wash the conical flask with 50 mL of diethyl ether and add the washing to the extraction cylinder. Cool the contents of the cylinder to room temperature.

Insert the stopper into the extraction cylinder and shake vigorously for at least 1 min. Allow contents to settle until both layers are clear and use a glass siphon to remove the upper layer as completely as possible without including any of the lower portion. [Note—A 500-mL separatory funnel may be used in place of the extraction cylinder and siphon. If this substitution is made, draw off the lower aqueous layer into a second separatory funnel, retaining the diethyl ether extract in the first funnel. Repeat the diethyl ether extraction of the aqueous phase, as noted above, combining all of the diethyl ether extracts in the first separatory funnel.] Transfer the diethyl ether layer to a 250-mL separatory funnel. Repeat this extraction two more times, using 50-mL portions of diethyl ether each time and shaking vigorously with each extraction. If necessary, continue extracting with diethyl ether until no more unsaponifiable material remains. Rotate the combined diethyl ether extracts gently with 20 mL of water. Violent agitation at this step may result in emulsions that are difficult to break. Allow the layers to separate completely and draw off the lower aqueous layer. Wash the diethyl ether layer two more
times, using 20 mL of water each time and shaking gently each time, discarding the lower aqueous layer after separation.

Wash the combined extracts in the separatory funnel three times, using 20-mL portions of 0.5 N potassium hydroxide, and shaking vigorously. Follow each alkali washing by washing with 20 mL of water. If an emulsion forms during this washing procedure, allow to separate as much as possible, discard the clear aqueous layer and proceed with the next step, leaving any emulsion in the separatory funnel with the diethyl ether layer. After the third washing with 0.5 N potassium hydroxide, wash the diethyl ether with successive 20-mL portions of water until the washings are no longer alkaline to phenolphthalein.

Transfer the diethyl ether extract to a tared beaker, rinsing the separatory funnel and its pouring edge with diethyl ether and adding the rinsings to the solution in the beaker. Evaporate to dryness in a water bath, using a gentle stream of clean, dry nitrogen. When almost all of the diethyl ether has been evaporated, add 2–3 mL of acetone and remove all traces of solvent with the aid of a stream of nitrogen. Complete the drying to constant weight in a vacuum oven at 75°–80° and an internal pressure of NMT 200 mm of mercury. Cool in a desiccator and weigh to obtain the mass of the residue, A, in g.

After weighing the residue, add to it 2 mL of diethyl ether and then 10 mL of alcohol which contains phenolphthalein indicator. The alcohol used should be previously neutralized to the phenolphthalein endpoint. Titrate the solution with 0.02 N sodium hydroxide to the same final color. Correct the weight of the residue for free fatty acid content, B, in g, using the following relationship: 1 mL of 0.02 N sodium hydroxide is equivalent to 0.0056 g of oleic acid. A reagent blank correction should also be determined as, C, in g.

Calculate the percent of unsaponifiable matter in the sample:

\[
\text{Result} = \frac{A - (B + C)}{W} \times 100
\]

in which W is the mass, in g, of the sample used.\(^{3S (FCC7)}\)

\section*{VOLATILE ACIDITY}

\subsection*{Modified Hortvet-Sellier Method}

\textbf{Apparatus} Assemble a modified Hortvet-Sellier distillation apparatus as shown in Fig. 37, using a sufficiently large (approximately 38- × 203-mm) inner Sellier tube and large distillation trap.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{FCC7_Fig37.png}
\caption{Modified Hortvet-Sellier Distillation Apparatus.}
\end{figure}

\textbf{Procedure} Transfer the amount of sample, accurately weighed, specified in the monograph into the inner tube of the assembly, and insert the tube in the outer flask containing about 300 mL of recently boiled hot water. To the sample add 10 mL of approximately 4 N perchloric acid [35 mL (60 g) of 70% perchloric acid in 100 mL of water], and connect the inner tube to a water-cooled condenser through the distillation trap. Distill by heating the outer flask so that 100 mL of distillate is collected within 20–25 min. Collect the distillate in 100-mL
portions, add phenolphthalein TS to each portion, and titrate with 0.5 N sodium hydroxide. Continue the distillation until a 100-mL portion of the distillate requires no more than 0.5 mL of 0.5 N sodium hydroxide for neutralization.

[CAUTION—Do not distill to dryness.]

Calculate the weight, in mg, of volatile acids in the sample taken by the formula:

\[ \text{Result} = V \times e \]

in which \( V \) is the total volume, in mL, of 0.5 N sodium hydroxide consumed in the series of titrations; and \( e \) is the equivalence factor given in the monograph.

---

2. Available from The Tintometer Limited, Waterloo Road, Salisbury, Wiltshire, SP1 2JY, UK. Tel: +44 1722 327242; Fax: +44 1722 412322.
4. Restek part no. 21022-211.5, SGE part no. 092002, or equivalent.
5. The monoglyceride may be calculated to some monoester other than glyceryl monostearate by dividing the molecular weight of the monoglyceride by 20 and substituting the value so obtained for 17.927 in the formula, using 17.80, for example, in calculating to the monooleate.
6. Omnim Inc., Rockland, MA, USA (manufactured under license from Archer Daniel Midland Co., Decatur, IL, USA).
7. Brinkmann Instruments, Inc., Westbury NY, USA (subsidiary of Sybron Corporation).
8. Suitable alternative equipment is described in AOCS Official Method Cd 12b-92. Official Methods and Recommended Practices of the American Oil Chemists’ Society, American Oil Chemists’ Society, 5th Edn, Champaign, IL.
9. Omega Engineering, Inc. (Stanford, CT) provides such a thermometer. A custom 3 wire 2-mm × 7-mm sensor probe is required for calibration of sample temperature with air flowing through the sample.
10. LiChrosorb Si 60, Spherisorb S5W, or equivalent.

Please check for your question in the FAQs before contacting USP.
BRIEFING

Appendix VIII: Oleoresins, FCC 7 page 1232. In order to add modern methods of analysis that reflect what is currently practiced in the industry, and on the basis of comments received, it is proposed to add a new method of analysis for the determination of capsicum oleoresins (Total Capsaicinoids Content) and to move the method for Scoville Heat Units to the General Information chapter. The proposed new method is based on the Assay of the monograph for Capsicum Oleoresin, published in USP 32–NF 27. The proposed conversion to Scoville heat units is based on the AOAC Official Method 995.03. Comments by interested parties are encouraged.

(FIEC: C. Mejia) C89498

APPENDIX VIII: OLEORESINS

COLOR VALUE

Sample Preparation  Transfer 70 to 100 mg of the sample, previously mixed well by shaking and accurately weighed, into a 100-mL volumetric flask, dissolve in acetone, dilute to volume with acetone, and mix. Allow the solution to stand for 2 min, then pipet 10 mL into a second 100-mL volumetric flask, dilute to volume with acetone, and mix.

Procedure  Determine the absorbance of the Sample Preparation with a suitable spectrophotometer in a 1-cm cell at 460 nm, using acetone as the blank. Record the value obtained as AS. In the same manner, determine the absorbance of a National Institute of Standards and Technology Standard Glass Filter 930, and record the value obtained as AF.

[Note—The recommended range for absorbance values is between 0.30 and 0.70. Solutions having absorbances greater than 0.70 should be diluted with acetone to one-half the original concentration, and those having absorbances less than 0.30 should be discarded and the Sample Preparation prepared with a larger sample. Appropriate adjustments should be made in the sample weight (W) used in the Calculation below.]

Calculation  Determine the instrument correction factor, F, by the formula:

\[
\text{Result} = \frac{A_N}{A_F}
\]

in which AN is the absorbance of the filter as stated by the National Institute of Standards and Technology. Calculate the color value of the sample by the formula:

\[
\text{Result} = \left(AS \times 164 \times F\right)/W
\]

in which W is the weight, in g, of sample taken.

CURCUMIN CONTENT
Sample Preparation  Transfer about 500 mg of sample, accurately weighed, into a 100-mL volumetric flask, and record the weight, in milligrams, as W. Dissolve the sample in about 75 mL of acetone, dilute to volume with acetone, and mix. Pipet a 5-mL portion of this solution into a second 100-mL volumetric flask, dilute to volume with acetone, and mix. Finally, pipet a 1-mL portion of the last solution into a 50-mL volumetric flask, dilute to volume with acetone, and mix.

[Note—Protect all solutions from light by using active glassware or by covering the glassware with aluminum foil. Make the absorbance readings as soon as possible after the solutions are prepared.]

Procedure  Determine the absorbance of the Sample Preparation in a 1-cm cell at the wavelength of maximum absorption between 420 and 425 nm with a suitable spectrophotometer, using acetone as the blank. Calculate the percent curcumin in the sample by the formula:

\[ \text{Result} = (A \times 100)(165 \times b \times c) \]

in which A is the absorbance of the Sample Preparation; 100 is the conversion to percent; 165 is the absorptivity factor, in liters per gram-centimeter, for curcumin; b is the path length of the cell; and c is the concentration, in grams per liter, of the solution presented to the spectrophotometer.

Calculate c by the formula:

\[ \text{Result} = W \times 5 \times 10^6 \]

in which W is the starting weight, in milligrams, of the sample, and 5 \times 10^6 is the conversion factor for the dilution schedule.

PIPERINE CONTENT

Stock Standard Solution  Purify piperine by repeated crystallization from isopropanol until a product having a melting range of 129° to 130° is obtained. Transfer 100.0 mg of the crystals, accurately weighed, into a 100-mL volumetric flask, dissolve in ethylene dichloride, dilute to volume with ethylene dichloride, and mix. Pipet 10.0 mL of this solution into a second 100-mL volumetric flask, dilute to volume with ethylene dichloride, and mix.

Standard Dilutions  Pipet 1.0, 3.0, 5.0, and 10.0 mL of the Stock Standard Solution (corresponding to 0.1, 0.3, 0.5, and 1.0 mg of piperine, respectively) into separate 100-mL volumetric flasks, dilute each flask to volume with ethylene dichloride, and mix. Determine the absorbance of each dilution at once, as directed in the Procedure.

Sample Preparation  Heat a portion of the sample to 100° on a steam bath or in an oven (but not on a hot plate), mix with a glass stirring rod, and transfer 100 mg, accurately weighed, into a 100-mL volumetric flask. Dissolve in ethylene dichloride, dilute to volume with ethylene dichloride, and mix. Pipet 1.0 mL of this solution into a second 100-mL volumetric flask, dilute to volume with ethylene dichloride, and mix. Determine the absorbance of the solution at once, as directed in the Procedure.

Procedure  Determine the absorbance of the Sample Preparation and of each of the Standard Dilutions in 1-cm cells at the wavelength of maximum absorption at about 342 nm with a suitable spectrophotometer, using ethylene dichloride as the blank. Prepare a standard curve of concentration, in mg per 100 mL, versus absorbance for the four Standard Dilutions, including the absorbance at zero concentration obtained with the blank. From the standard curve, determine the concentration of piperine in the Sample Preparation, and record the value as C, in mg per 100 mL. Calculate the percentage of piperine in the sample by the formula:
100 × (100C/W) in which W is the weight, in mg, of sample taken.

RESIDUAL SOLVENT
This procedure is for the determination of acetone, ethylene dichloride, hexane, isopropanol, methanol, methylene chloride, and trichloroethylene residues.

Distilling Head Use a Clevenger trap designed for use with oils heavier than water. A suitable design is shown in Fig. 38a.

Toluene The toluene used for this analysis should not contain any of the solvents determined by this method. The purity may be determined by gas chromatographic analysis, using one of the following columns or their equivalent: (1) 17% by weight of Ucon 75-H-90,000 on 35/80-mesh Chromosorb W; (2) 20% Ucon LB-135 on 35/80-mesh Chromosorb W; (3) 15% Ucon LB-1715 on 60/80-mesh Chromosorb W; or (4) Porapak Q 50/60 mesh. Follow the conditions described under Procedure, and inject the same amount of toluene as will be injected in the analysis of the solvents. If impurities interfering with the test are present, they will appear as peaks occurring before the toluene peak and should be removed by fractional distillation.

Benzene The benzene used for this analysis should be free from interfering impurities. The purity may be determined as described under Toluene.

Detergent and Antifoam Any such products that are free from volatile compounds may be used. If volatile compounds are present, they may be removed by prolonged boiling of the aqueous solutions of the products.

Reference Solution A Prepare a solution in Toluene containing 2500 ppm of benzene. If the toluene available contains benzene as the only impurity, the benzene level can be determined by gas chromatography and sufficient benzene added to bring the level to 2500 ppm.

Reference Solution B Prepare a solution containing 0.63% v/w of acetone in water.

Sample Preparation A (all solvents except methanol) Place 50.0 g of the sample, 1.00 mL of Reference Solution A, 10 g of anhydrous sodium sulfate, 50 mL of water, and a small amount each of Detergent and
Antifoam in a 250-mL round-bottom flask with a 24/40 ground-glass neck. Attach the Distilling Head, a 400-mm water-cooled condenser, and a receiver, and collect approximately 15 mL of distillate. Add 15 g of anhydrous potassium carbonate to the distillate, cool while shaking, and allow the phases to separate. All of the solvents except methanol will be present in the toluene layer, which is used in the Procedure. Draw off the aqueous layer for use in Sample Preparation B.

**Sample Preparation B** (methanol only) Place the aqueous layer obtained from Sample Preparation A in a 50-mL round-bottom distilling flask with a 24/40 ground-glass neck, add a few boiling chips and 1.00 mL of Reference Solution B, and collect approximately 1 mL of distillate, which will contain any methanol from the sample, together with acetone as the internal standard. The distillate is used in the Procedure.

**Procedure** Use a gas chromatograph equipped with a hot-wire detector and a suitable sample-injection system or on-column injection. Under typical conditions, the instrument contains a 1/4-in. (od) × 6- to 8-ft column, or equivalent, maintained isothermally at 70° to 80°. The flow rate of dry carrier gas is 50 to 80 mL/min, and the sample size is 15 to 20 µL (for the hot-wire detector). The column selected for use in the chromatograph depends on the components to be analyzed and, to a certain extent, on the preference of the analyst. The columns 1, 2, 3, and 4, as described under Toluene, may be used as follows: (1) This column separates acetone and methanol from their aqueous solution. It may be used for the separation and analysis of hexane, acetone, and trichloroethylene in the toluene layer from Sample Preparation A. The elution order is acetone, methanol, and water, or hexane, acetone, isopropanol plus methylene chloride, benzene, trichloroethylene, and ethylene dichloride plus toluene. (2) This column separates methylene chloride and isopropanol, and ethylene dichloride. The elution order is hexane plus acetone, methylene chloride, isopropanol, benzene, ethylene dichloride, trichloroethylene, and toluene. (3) This is the best general-purpose column, except for the determination of methanol. The elution order is hexane, acetone, benzene, ethylene dichloride, and toluene. (4) This column is used for the determination of methanol, which elutes just after the large water peak.

**Calibration** Determine the response of the detector for known ratios of solvents by injecting known mixtures of solvents and benzene in toluene. The levels of the solvents and benzene in toluene should be of the same magnitude as they will be present in the sample under analysis.

Calculate the areas of the solvents with respect to benzene, and then calculate the calibration factor, F, as follows:

\[ F \text{ (solvent)} = (\text{wt} \% \text{ solvent}/\text{wt} \% \text{ benzene}) \times (\text{area of benzene}/\text{area of solvent}) \]

The recovery of the various solvents from the oleoresin sample, with respect to the recovery of benzene, is as follows: hexane, 52%; acetone, 85%; isopropanol, 100%; methylene chloride, 87.5%; trichloroethylene, 113%; ethylene dichloride, 102%; and methanol, 87%.

**Calculation** Calculate the ppm of residual solvent (except methanol) by the equation:

\[ \text{Res. solv.} = \{(43.4 \times F \text{ (solvent)} \times 100)/[\% \text{ recovery of solvent}]) \times (\text{area of solvent}/\text{area of benzene}) \]

in which 43.4 is the ppm of benzene internal standard, related to the 50-g oleoresin sample taken for analysis. Calculate the ppm of residual methanol by the equation:

\[ \text{Methanol} = \{(100 \times F \text{ (methanol)})/0.87\} \times (\text{area of methanol}/\text{area of benzene}) \]

in which 100 is the ppm of acetone internal standard, related to the 50-g oleoresin sample taken for analysis.

*Delete the following:*
**SCOVILLE HEAT UNITS**

**Sample Preparation**  Transfer 200 mg of the sample into a 50-mL volumetric flask, dilute to volume with alcohol, and mix thoroughly by shaking. Allow the insolubles to settle before use.

**Sucrose Solution**  Prepare a suitable volume of a 10% w/v solution of sucrose in water.

**Standard Solution**  Add 0.15 mL of the Sample Preparation to 140 mL of the Sucrose Solution, and mix. This solution contains the equivalent of 240,000 Scoville Heat Units.

**Test Solutions**  If the oleoresin sample is claimed to contain more than 240,000 Scoville Heat Units, prepare one or more dilutions according to the following table:

<table>
<thead>
<tr>
<th>Scoville Heat Units</th>
<th>Standard Solution (mL)</th>
<th>Sucrose Solution (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>360,000</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>480,000</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>600,000</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>720,000</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>840,000</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td>960,000</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>1,080,000</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>1,200,000</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>1,320,000</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>1,440,000</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>1,560,000</td>
<td>20</td>
<td>110</td>
</tr>
<tr>
<td>1,680,000</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>1,800,000</td>
<td>20</td>
<td>130</td>
</tr>
<tr>
<td>1,920,000</td>
<td>20</td>
<td>140</td>
</tr>
<tr>
<td>2,040,000</td>
<td>20</td>
<td>150</td>
</tr>
</tbody>
</table>

If the oleoresin sample is claimed to contain less than 240,000 Scoville Heat Units, prepare one or more dilutions according to the following table:

<table>
<thead>
<tr>
<th>Scoville Heat Units</th>
<th>Sample Preparation (mL)</th>
<th>Sucrose Solution (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>0.15</td>
<td>60</td>
</tr>
<tr>
<td>117,500</td>
<td>0.15</td>
<td>70</td>
</tr>
<tr>
<td>170,000</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>205,000</td>
<td>0.15</td>
<td>120</td>
</tr>
</tbody>
</table>

**Procedure**  Select five panel members who are thoroughly experienced with this method. Instruct the panelists to swallow 5 mL of the solution corresponding to the claimed content of Scoville Heat Units. The sample passes the test if three of the five panel members perceive a pungent or stinging sensation in the throat.

3S (FCC7)

*Add the following:*
**TOTAL CAPSAICINOIDS CONTENT**

**Standard Solution** 0.5 mg/mL of USP Capsaicin RS in methanol

**Test Solution** Transfer the equivalent to about 1000 mg of capsicum oleoresin, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume, and mix.

**Apparatus** Use a suitable high-performance liquid chromatographic system (see Chromatography, Appendix II A) equipped with a UV detector (280 nm). Use an online 0.45-µm filter, and a 30-cm × 3.9-cm (id) stainless steel column or equivalent, packed with octadecylsilane (C18) chemically bonded to porous silica or ceramic micro-particles of 1.5–10 µm particle size.¹

**Mobile Phase** Mixture of methanol and 2% acetic acid (56:44, v/v), filtered through a 0.5-µm or finer porosity filter, and degassed

**Suitability Requirements** Allow the chromatographic system to equilibrate at a flow rate of 2 mL/min, then inject 10 µL of the Standard Solution. The relative retention times for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin are about 0.9, 1.0, and 1.6, respectively. The resolution between the norhydrocapsaicin peak and the capsaicin peak is NLT 1.2. The tailing factor is NMT 2.0, and the relative standard deviation for replicate injections is NMT 2.0%.

**Procedure** Separately inject equal volumes (about 10 µL) of the Standard Solution and the Test Solution into the chromatograph, record the chromatograms, and measure the responses for the three major peaks. Use peak areas where responses are indicated. Calculate the percentage of total capsaicins in the portion of capsicum oleoresin taken by the formula:

\[
\frac{C/W \times (r_U/r_S)}{C/W \times (r_U/r_S)}
\]

in which C is the concentration of USP Capsaicin RS in the Standard Solution corrected for purity based on the USP label claim (mg/mL); W is the weight of capsicum oleoresin in the sample used to prepare the Test Solution (mg); r_U is the sum of the peak responses for nordihydrocapsaicin, capsaicin, and dihydrocapsaicin obtained from the sample; and r_S is the peak response obtained from the capsaicin in the Standard Solution.

[Note—1.0 µg total capsaicinoids/g = ca 15 Scoville Heat Units (SHU)]

☑️3S (FCC7)

---

**VOLATILE OIL CONTENT**

Weigh accurately an amount of sample sufficient to yield 2 to 5 mL of volatile oil, and transfer with the aid of water into a 1000- or 2000-mL round-bottom shortneck flask with a 24/40 ground-glass neck. Add a magnetic stirring bar and about 500 mL of water, and connect a Clevenger trap of the proper type (see Figs. 38a and 38b) and a 400-mm water-cooled condenser. Heat the flask with stirring, and distill at a rate of 1 to 1.5 drops per s until two consecutive readings taken at 1-h intervals show no change of oil volume in the trap. Cool to room temperature, allow to stand until the oil layer is clear, and read the volume of oil collected, estimating to the nearest 0.02 mL. Calculate the percentage (v/w) of volatile oil in the sample by the formula:

\[
\text{Result} = \frac{100(V/W)}{}
\]

in which V is the volume, in mL, of oil collected; and W is the weight, in g, of sample taken.

¹ µBondapack C18, Water Corp., or equivalent.
Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D.</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8571</td>
<td></td>
</tr>
</tbody>
</table>
Appendix XIV: Markers for Authenticity Testing. It is proposed to add a new Appendix to the FCC containing analytical methods for markers of authenticity. Tests contained within this Appendix are not monograph requirements, but rather are provided as informational methods for FCC users.

The first method proposed for this Appendix is for determining the biobased content of 1,3-propanediol (see the Briefing under 1,3-Propanediol). The method is based on ASTM D6866 Method B and uses radiocarbon analysis with accelerator mass spectrometry to determine the content of biobased carbon in a sample (renewable organic carbon such as that derived from recent plant and animal sources) as a percent of the weight (mass) of the total organic carbon in the sample. Besides quantifying biobased content, results from this method can be used to distinguish 1,3-propanediol produced synthetically using fossil-based (petroleum-derived) raw materials from 1,3-propanediol produced using biobased (plant-derived) raw materials. Comments are encouraged on other food ingredients that this method could be expanded to include.

It is anticipated that additional methods will be proposed for inclusion in this Appendix as they become available. FCC stakeholders are encouraged to comment on this proposed method and to submit additional methods for consideration.

(FIEC: J. Moore)  C95370

Add the following:

Appendix XIV: MARKERS FOR AUTHENTICITY TESTING
Tests contained within this Appendix are not monograph requirements, but rather are provided as informational methods for FCC users.

BIOBASED CONTENT OF 1,3-PROPANEDIOL (BASED ON ASTM D6866 METHOD B)

Principle: Biobased content is the amount of biobased carbon (renewable organic carbon such as that derived from recent plant and animal sources) in the material or sample as a percent of the weight (mass) of the total organic carbon in the sample. It is measured by comparing the radiocarbon signature of a sample to that of a modern reference standard derived from plant sources of a known age. Radiocarbon signatures of samples and modern reference standards are analyzed by combustion to convert carbon to CO\(_2\) and using accelerator mass spectrometry to relate the signatures between the two. A biobased content value of 100% indicates that all carbon originated from modern plants or animals. A value of 0% indicates all carbon originated from petrochemicals. A value between 0% and 100% indicates the relative amount of carbon derived from recent plants and animals versus petrochemicals.

The basic principle behind using radiocarbon as a bio-source-marker lies in the nature of its formation and decay. Radiocarbon is formed continually in the atmosphere from nitrogen-14 in the air reacting with thermal neutrons from cosmic radiation. Radiocarbon undergoes radioactive beta-decay to form nitrogen-14, with a half-life of 5730 years. Living plant and animal feedstocks will have radiocarbon in them in an amount defined as 100%. Petrochemicals will not have any radiocarbon (i.e., 0%) because it has long since decayed away. By
comparing the radiocarbon content of 1,3-propanediol to modern plants, it can be assigned a biobased value representing the amount of plant carbon versus petrochemically-sourced carbon in the material.

**Vacuum and gas transfer manifold system:** The vacuum manifold system should be capable of air and non-condensable gas evacuation, sample introduction, water distillation, cryogenic gas transfer, and temperature and pressure monitoring. Required equipment includes:

- Manifold tubing that is composed of clean stainless steel;
- Vacuum pump(s) capable of achieving a vacuum of 101 Pa or less within the vacuum region;
- Calibrated pressure transducer with coupled or integrated signal response controllers;
- Calibrated sample collection volume with associated temperature readout; and
- Clean quartz tubing for sample combustion and subsequent gas transfer, quantification, and storage.

See *Figure 1* for a picture of an example setup. Adapt a “tube cracker” to the system. The “tube cracker” is comprised of parts made from stainless steel and compression fittings with appropriate welds. Pictures and descriptions of suitable configurations for this “tube cracker” are shown in *Figure 2* and *Figure 3*.

![Figure 1. Vacuum and Gas Transfer Manifold System.](image1)

![Figure 2. Flexible Glass Tube Cracker.](image2) Close up of a portion of the manifold in *Figure 1*, showing hardware and glassware used in transferring carbon dioxide from combustion tubes to a manifold suitable to “scrub” the gas free of non-carbon-dioxide gaseous species. Visible are ports, valves, pressure transducer, glass storage vessels, and a flexible (and expandable) stainless steel “tube cracker” suitable for cracking open sealed quartz
or pyrex tubes of less than 9.5-mm diameter.

Figure 3. Flexible Glass Tube Cracker with Three-Way Valve. Close up of the “tube cracker” assembly in Figure 2, showing the standard Swagelock fittings typically used in a line configuration.

Combustion and trapping system: A temperature controlled furnace or suitable alternative equipment (e.g.,
continuous flow interfaces and associated CO₂ trapping system) capable of quantitative CO₂ recovery.

**System suitability:** Setup conditions should be effective in combusting all organic carbon bearing species to CO₂. A suitable procedure for determining recovery is to combust a material of known organic carbon content, and use calibrated pressure and volume variables to calculate percent recovery. Available standardized materials are NIST SRM 8541 (USGS graphite, pure carbon) or NIST SRM 8542 (ANU Sucrose, C₁₂H₂₂O₁₁). The system should be tested with a broad range of materials including those with very high water content and with highly volatile components.

**Accelerator mass spectrometry (AMS) system:** AMS facilities are equipped to make direct measurement of isotopes with mass separation of only one neutron. Although methods of analysis are published, they are not standardized and each facility will have its own standard operating procedures. As such, it is recommended to obtain quality assurance methods, staffing expertise, and any accreditations (e.g., ISO-17025); and to validate reference standard results with sample results. AMS facilities will typically validate reliability in detection by measuring known radiocarbon content standards such as TIRI-wood (known radiocarbon content 0.571 ± 0.3 pMC) available through the Department of Statistics, University of Glasgow (Scotland); NIST SRM 4990c (known radiocarbon content 1.341 ± 0.6 pMC); or the ratio of NIST SRM 4990c to 4990b (1.2993 non-normalized; 1.3407 normalized).

**Isotope ratio mass spectrometry (IRMS) system:** Stable carbon isotopic ratios (¹³C/¹²C) are utilized by the AMS facility to ensure systematic isotopic fractionation between reference materials and unknowns. Some AMS facilities are capable of measuring the ratio directly in the AMS and making a total fractionation correction, some AMS facilities make assumptions as to systematic fraction between all reference standards and samples, and some AMS facilities will make a separate ¹³C/¹²C in an IRMS. AMS facilities do not generally report the ratio because it is one of many variables used in the calculation of final radiocarbon results. However, because it is a commonly used variable in the food industry, if a ¹³C/¹²C is obtained from the AMS laboratory, it is important to obtain the methodology from which it was derived. Values reported by IRMS measurement on the sample material will include natural and any manufacturing-induced fractionation effects. Values reported from within an AMS detector will include the same, plus laboratory- and AMS-induced fractionation effects, which can be large. Because confusion is possible, it is recommended to use only IRMS values directly on the sample material for interpretation of ¹³C/¹²C within the sample.

**Sample:** Use a sample size with mass proportional to generate approximately 1 to 4 cm³ of CO₂ (0.5–2 mg of carbon) for the AMS facility, and suitable for volume capacity of the combustion and trapping system.

**¹⁴C Standard:** Any one of the following ¹⁴C standards is suitable for use. Use enough standard to generate approximately 1 to 4 cm³ of CO₂ (0.5–2 mg of carbon) for the AMS facility, and enough suitable for volume capacity of the combustion and trapping system.

- NIST SRM 4990c, Oxalic Acid, C₂H₂O₄, 27% carbon;
- NIST SRM 4990b, Oxalic Acid, C₂H₂O₄, 27% carbon; or
- NIST SRM 8542, Sucrose, C₁₂H₂₂O₁₁, 42% carbon

**Analysis:** Analyze the Sample and ¹⁴C Standard as follows:

[Note—This standard applies specifically to organic carbon components; inorganic carbon components are factored out of the final result (per the definition of “biobased”). The correction is made by (1) isolating the organic component and measuring it independent of inorganic components; (2) factoring out the carbonate radiocarbon contribution using mass-fraction determinations and radiocarbon content analysis on total]
carbon versus inorganic carbon; or (3) in extreme cases, measuring the components individually rather than combined in the final sample.

Preparation: Place an approximately 2-cm length of CuO into a suitable quartz sample tube (9.5-mm OD and ~20 cm long) that has been previously cleaned furnace-baked at 200° for NLT 2 h and torch sealed at the bottom end. Fill the tube with nitrogen gas. Place liquid nitrogen around the tube to a level slightly above the CuO, and allow to equilibrate. Insert a tiny drop or glass rod piece wetted with the Sample or 14C Standard into the tube. [Note—1,3-Propanediol will freeze upon contact with the CuO.] With the tube adapted to the Vacuum manifold system, evacuate the nitrogen to a pressure of NMT 101 Pa. Flame seal the glass tube at the top end, and remove the liquid nitrogen.

Combustion: Combust the evacuated sample tube at 900° for 2 to 4 h using the Combustion and trapping system. [Note—Combustion is indicated by the appearance of elemental copper in the tube upon cooling. The remaining presence of CuO will indicate excess was available and complete combustion was achieved. If all CuO has been reduced to elemental copper, incomplete combustion occurred and re-combustion should be performed using a larger quantity of CuO.] [Note—“Sealed tube” combustion can lead to pressure explosion either during or after heating if too much 1,3-propanediol is used.] After combustion, score the quartz sample tube to facilitate a clean break within the flexible hose portion of the “tube cracker” assembly adapted to the manifold. With the manifold closed to the vacuum pump, crack the quartz tubing and immediately cryogenically transfer the liberated sample CO2 to a transfer manifold, and distill the gas to remove residual water using a slurry of dry ice and alcohol maintained at −76°. Simultaneously release the sample CO2 gas, and immediately condense in a calibrated volume. Close the calibrated volume, and allow the CO2 to equilibrate to room temperature. Record the volume (cm3) of CO2 recovered from the combustion.

Carbon isotope analysis: Transfer the Sample and 14C Standard preparations to borosilicate break seal tubes for analysis of 14C/13C (or 14C/12C) using the AMS system and if appropriate, 13C/12C using the IRMS system. [Note—AMS analysis can be done using either 14C/13C (or 14C/12C) ratios, but the same ratio must be determined for both the Sample and 14C Standard.]

Calculate the percent modern carbon (pMC) in the Sample taken:

Result = (A/A_O) × 100

in which A is the 14C/13C (or 14C/12C) of the Sample preparation, and A_O is the the 14C/13C (or 14C/12C) of the 14C Standard preparation.

Calculate the pMC in the Sample taken:

Result = (pMC × C)

in which pMC is the percent modern carbon relative to NIST SRM 4990c, NIST SRM 4990b, or NIST SRM 8542, calculated above; and C is the correction factor for anthropogenic effects associated with thermo-nuclear weapons testing, 0.95, as of ASTM-D6866-10, August 6, 2010. [Note—The most up-to-date factor should be used here, and can be obtained from review of the latest publication revision of ASTM-D6866.]

Explanation of calculation and assumptions: The analysis is performed by comparing the 14C/13C (or 14C/12C) ratio within the Sample to the NIST standard. The analytical result from the comparison is termed “percent modern carbon (pMC)”. A value of 100 pMC would indicate the ratio of the Sample was identical to the
The percent biobased content is calculated from the pMC value by multiplying the pMC value by a constant. This constant represents an assumed correction factor for anthropogenic effects associated with thermo-nuclear weapons testing. As mentioned previously, radiocarbon is naturally created in the atmosphere by reactions of nitrogen-14 with neutrons produced by cosmic rays. This same reaction occurred on a very large scale when thermo-nuclear weapons were tested in the atmosphere in the 1950s–1960s. The explosions created “excess” radiocarbon in the atmosphere of non-natural origin, leading to a factor of 1.93 times “natural” by 1963 when the atmospheric nuclear weapons testing was halted with the signing of the joint non-proliferation treaty. Since that time, the excess has been fixed within the biosphere, taken up by oceans, and diluted with combustion of fossil fuels. As of the writing of this Appendix, ASTM-D6866-10 cites the correction factor to be 0.95 (1/1.053 factor). This indicates the atmosphere still contains 5.3% excess radiocarbon. As a consequence, the analytical pMC measure is multiplied by 0.95 to derive the percent biobased equivalent. This correction factor predicatably will continue to change at a rate of 0.3%–0.5% per year and will be accounted for appropriately within future revisions to the ASTM-D6866 standard.

Two important assumptions are made in the calculation of the final biobased content result:

1. All the organic carbon within the Sample was derived from plant bodies (or animal) that were actively respiring CO₂ either within the last 10 years or more than 40,000 years ago. With this criteria met, a biobased content value of 100% indicates that the Sample is entirely from recent biomass (or animal), a value of 0% means the sample is entirely from petrochemicals (or other fossil source), and a value in between represents the percentage carbon from each.

2. The organic carbon component within the Sample can be effectively and precisely isolated from inorganic carbon components.

Performance characteristics: Typical precision is ±0.1%–0.5% RSD on the analytical measure. Uncertainties exist within the reported result beyond the analytical errors. Absolute error on the biobased content result is ±3%. Typical indeterminant errors are local variation in the atmospheric correction factor, heterogeneity in the Sample, and the effectiveness in the laboratory to extract all carbon species as CO₂.


Please check for your question in the FAQs before contacting USP.
BRIEFING

Test Solutions (TS) and Other Reagents, FCC 7 page 1257. Three revisions are proposed to this section:

1. Add a new test solution, Acetic Periodic Acid TS, to support a proposed new monograph for Citric and Fatty Acid Esters of Glycerol (see the Briefing under Citric and Fatty Acid Esters of Glycerol).
2. Add a new test solution, Ferroin TS, to support a proposed new monograph for Ferrous Ammonium Phosphate (see the Briefing under Ferrous Ammonium Phosphate).
3. The instructions for preparing Starch TS are being revised on the basis of comments received. The mercuric iodide preservative is being removed and analysts are instructed to prepare the solution fresh. The sensitivity test is also being revised because of problems encountered in visually determining the color change.

(FIEC: K. Laurvick) C97351

TEST SOLUTIONS (TS) AND OTHER REAGENTS

Certain of the following test solutions are intended for use as acid–base indicators in volumetric analyses. Such solutions should be adjusted so that when 0.15 mL of the indicator solution is added to 25 mL of carbon dioxide-free water, 0.25 mL of 0.02 N acid or alkali, respectively, will produce the characteristic color change.

In general, the directive to prepare a solution “fresh” indicates that the solution is of limited stability and must be prepared on the day of use.

**Acetic Acid** (approximately 17.5 N) Use ACS reagent-grade Acetic Acid, Glacial (99.7% of CH₃COOH).

**Acetic Acid TS, Diluted** (1 N) A solution containing about 6% (w/v) of CH₃COOH. Prepare by diluting 60.0 mL of glacial acetic acid, or 166.6 mL of 36% acetic acid (6 N), with sufficient water to make 1000 mL.

**Acetic Acid TS, Strong** (5 N) A solution containing 30% (v/v) of CH₃COOH. Prepare by diluting 300.0 mL of glacial acetic acid with sufficient water to make 1000 mL.

**Add the following:**

- **Acetic Periodic Acid TS** Dissolve 2.7 g of periodic acid (H₅IO₆) in 50 mL of water, add 950 mL of glacial acetic acid, and mix thoroughly. [CAUTION—This solution is an oxidizing agent and is dangerous in contact with organic materials. Do not use cork or rubber stoppers on storage bottles.] [Note—Store this solution protected from light.] See (FCC7)

**Alcohol** (Ethanol; Ethyl Alcohol; C₂H₅OH) Use ACS reagent-grade Ethyl Alcohol (not less than 95.0%, by volume, of C₂H₅OH). [Note—for use in assays and tests involving ultraviolet spectrophotometry, use ACS reagent-grade Ethyl Alcohol Suitable for Use in Ultraviolet Spectrophotometry.]

**Alcohol, Absolute** (Anhydrous Alcohol; Dehydrated Alcohol) Use ACS reagent-grade Ethyl Alcohol, Absolute (not less than 99.5%, by volume, of C₂H₅OH).

**Alcohol, Diluted** A solution containing 41.0% to 42.0%, by weight, corresponding to 48.4% to 49.5%, by
volume, at 15.56°, of C₂H₅OH.

Alcohol, 70% (at 15.56°) A 38.6:15 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.884 at 25°. To prepare 100 mL, dilute 73.7 mL of alcohol to 100 mL with water at 25°.

Alcohol, 80% (at 15.56°) A 45.5:9.5 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.857 at 25°. To prepare 100 mL, dilute 84.3 mL of alcohol to 100 mL with water at 25°.

Alcohol, 90% (at 15.56°) A 51:3 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.827 at 25°. To prepare 100 mL, dilute 94.8 mL of alcohol to 100 mL with water at 25°.

Alcohol, Aldehyde-Free Dissolve 2.5 g of lead acetate in 5 mL of water, add the solution to 1000 mL of alcohol contained in a glass-stoppered bottle, and mix. Dissolve 5 g of potassium hydroxide in 25 mL of warm alcohol, cool, and add slowly, without stirring, to the alcoholic solution of lead acetate. Allow to stand for 1 h, then shake the mixture vigorously, allow to stand overnight, decant the clear liquid, and recover the alcohol by distillation. Ethyl Alcohol FCC, Alcohol USP, or USSD #3A or #30 may be used. If the titration of a 250-mL sample of the alcohol by Hydroxylamine Hydrochloride TS does not exceed 0.25 mL of 0.5 N alcoholic potassium hydroxide, the above treatment may be omitted.

Alcoholic Potassium Hydroxide TS See Potassium Hydroxide TS, Alcoholic.

Alkaline Cupric Tartrate TS (Fehling’s Solution) See Cupric Tartrate TS, Alkaline.

Alkaline Mercuric Potassium Iodide TS (Nessler’s Reagent) See Mercuric–Potassium Iodide TS, Alkaline.

Ammonia–Ammonium Chloride Buffer TS (approximately pH 10) Dissolve 67.5 g of ammonium chloride (NH₄Cl) in water, add 570 mL of ammonium hydroxide (28%), and dilute with water to 1000 mL.

Ammonia TS (6 N in NH₃) A solution containing between 9.5% and 10.5% of NH₃. Prepare by diluting 400 mL of ammonium hydroxide (28%) with sufficient water to make 1000 mL.

Ammonia TS, Stronger (15.2 N in NH₃) (Ammonium Hydroxide; Stronger Ammonia Water) Use ACS reagent-grade Ammonium Hydroxide, which is a practically saturated solution of ammonia in water, containing between 28% and 30% of NH₃.

Ammoniacal Silver Nitrate TS Add 6 N ammonium hydroxide, dropwise, to a 1:20 solution of silver nitrate until the precipitate that first forms is almost, but not entirely, dissolved. Filter the solution, and place in a dark bottle. [CAUTION—Ammoniacal Silver Nitrate TS forms explosive compounds on standing. Do not store this solution, but prepare a fresh quantity for each series of determinations. Neutralize the excess reagent and rinse all glassware with hydrochloric acid immediately after completing a test.]

Ammonium Acetate TS Dissolve 10 g of ammonium acetate (NH₄C₂H₃O₂) in sufficient water to make 100 mL.

Ammonium Carbonate TS Dissolve 20 g of ammonium carbonate and 20 mL of Ammonia TS in sufficient water to make 100 mL.

Ammonium Chloride TS Dissolve 10.5 g of ammonium chloride (NH₄Cl) in sufficient water to make 100 mL.

Ammonium Molybdate TS Dissolve 6.5 g of finely powdered molybdic acid (85%) in a mixture of 14 mL of water and 14.5 mL of ammonium hydroxide. Cool the solution, and add it slowly, with stirring, to a well-cooled
mixture of 32 mL of nitric acid and 40 mL of water. Allow to stand for 48 h, and pass through a fine-porosity, sintered-glass crucible lined at the bottom with a layer of glass wool. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 mL of Sodium Phosphate TS to 5 mL of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage, use only the clear, supernatant solution.

**Ammonium Oxalate TS**  Dissolve 3.5 g of ammonium oxalate \((\text{NH}_4)_2\text{C}_2\text{O}_4\cdot\text{H}_2\text{O}\) in sufficient water to make 100 mL.

**Ammonium Sulfanilate TS**  To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide, and mix. Add, with stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves, adjust the pH of the solution to about 4.5 with 2.7 N hydrochloric acid, using Bromocresol Green TS as an outside indicator, and dilute to 25 mL.

**Ammonium Sulfide TS**  Saturate 6 N ammonium hydroxide with hydrogen sulfide (H\(_2\)S), and add two-thirds of its volume of 6 N ammonium hydroxide. Residue upon ignition: not more than 0.05%. The solution is not rendered turbid either by Magnesium Sulfate TS or by Calcium Chloride TS (carbonate). This solution is unsuitable for use if an abundant precipitate of sulfur is present. Store it in small, well-filled, dark amber-colored bottles in a cold, dark place.

**Ammonium Thiocyanate TS** (1 N)  Dissolve 8 g of ammonium thiocyanate (NH\(_4\)SCN) in sufficient water to make 100 mL.

**Anthrone TS**  Carefully dissolve about 0.1 g of anthrone in 100 g of sulfuric acid. Use a freshly prepared solution.

**Antimony Trichloride TS**  Dissolve 20 g of antimony trichloride (SbCl\(_3\)) in chloroform to make 100 mL. Filter if necessary.

**Barium Chloride TS**  Dissolve 12 g of barium chloride (BaCl\(_2\cdot2\text{H}_2\text{O}\)) in sufficient water to make 100 mL.

**Barium Diphenylamine Sulfonate TS**  Dissolve 300 mg of \(p\)-diphenylamine sulfonic acid barium salt in 100 mL of water.

**Barium Hydroxide TS**  Use a saturated solution of barium hydroxide in recently boiled water. Use a freshly prepared solution.

**Benedict’s Qualitative Reagent**  See Cupric Citrate TS, Alkaline.

**Benzidine TS**  Dissolve 50 mg of benzidine in 10 mL of glacial acetic acid, dilute with water to 100 mL, and mix.

**Bismuth Nitrate TS**  Reflux 5 g of bismuth nitrate \([\text{Bi(NO}_3\text{)}_3\cdot5\text{H}_2\text{O}]\) with 7.5 mL of nitric acid and 10 mL of water until dissolved, cool, filter, and dilute with water to 250 mL.

**Bromine TS** (Bromine Water)  Prepare a saturated solution of bromine by agitating 2 to 3 mL of bromine (Br\(_2\)) with 100 mL of cold water in a glass-stoppered bottle, the stopper of which should be lubricated with petrolatum. Store it in a cold place protected from light.

**Bromocresol Blue TS**  Use Bromocresol Green TS.

**Bromocresol Green TS**  Dissolve 50 mg of bromocresol green in 100 mL of alcohol, and filter if necessary.

**Bromocresol Purple TS**  Dissolve 250 mg of bromocresol purple in 20 mL of 0.05 N sodium hydroxide, and
dilute with water to 250 mL.

**Bromophenol Blue TS** Dissolve 100 mg of bromophenol blue in 100 mL of 1:2 alcohol, and filter if necessary.

**Bromothymol Blue TS** Dissolve 100 mg of bromothymol blue in 100 mL of 1:2 alcohol, and filter if necessary.

**Calcium Chloride TS** Dissolve 7.5 g of calcium chloride (CaCl$_2$·2H$_2$O) in sufficient water to make 100 mL.

**Calcium Hydroxide TS** A solution containing approximately 140 mg of Ca(OH)$_2$ in each 100 mL. To prepare, add 3 g of calcium hydroxide [Ca(OH)$_2$] to 1000 mL of water, and agitate the mixture vigorously and repeatedly for 1 h. Allow the excess calcium hydroxide to settle, and decant or draw off the clear, supernatant liquid.

**Calcium Sulfate TS** A saturated solution of calcium sulfate in water.

**Carr-Price Reagent** See Antimony Trichloride TS.

**Ceric Ammonium Nitrate TS** Dissolve 6.25 g of ceric ammonium nitrate [(NH$_4$)$_2$Ce(NO$_3$)$_6$] in 100 mL of 0.25 N nitric acid. Prepare the solution fresh every third day.

**Chlorine TS** (Chlorine Water) A saturated solution of chlorine in water. Place the solution in small, completely filled, light-resistant containers. Chlorine TS, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

**Chromotropic Acid TS** Dissolve 50 mg of chromotropic acid or its sodium salt in 100 mL of 75% sulfuric acid (made by cautiously adding 75 mL of 95% to 98% sulfuric acid to 33.3 mL of water).

**Cobaltous Chloride TS** Dissolve 2 g of cobaltous chloride (CoCl$_2$·6H$_2$O) in 1 mL of hydrochloric acid and sufficient water to make 100 mL.

**Cobalt–Uranyl Acetate TS** Dissolve, with warming, 40 g of uranyl acetate [UO$_2$(C$_2$H$_3$O$_2$)$_2$·2H$_2$O] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Similarly, prepare a solution containing 200 g of cobaltous acetate [Co(C$_2$H$_3$O$_2$)$_2$·4H$_2$O] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 h to separate the excess salts from solution, and then pass through a dry filter.

**Congo Red TS** Dissolve 500 mg of congo red in a mixture of 10 mL of alcohol and 90 mL of water.

**Copper Sulfate TS** Dissolve 12.5 g of cupric sulfate in sufficient water to make 100 mL.

**Cresol Red TS** Triturate 100 mg of cresol red in a mortar with 26.2 mL of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 mL.

**Cresol Red–Thymol Blue TS** Add 15 mL of Thymol Blue TS to 5 mL of Cresol Red TS, and mix.

**Crystal Violet TS** Dissolve 100 mg of crystal violet in 10 mL of glacial acetic acid.

**Cupric Citrate TS, Alkaline (Benedict's Qualitative Reagent)** With the aid of heat, dissolve 173 g of sodium citrate (C$_6$H$_5$Na$_3$O$_7$·2H$_2$O) and 117 g of sodium carbonate (Na$_2$CO$_3$·H$_2$O) in about 700 mL of water, and filter through paper, if necessary. In a separate container, dissolve 17.3 g of cupric sulfate (CuSO$_4$·5H$_2$O) in about 100 mL of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, dilute to 1000 mL, and mix.

**Cupric Nitrate TS** Dissolve 2.4 g of cupric nitrate [Cu- (NO$_3$)$_2$·3H$_2$O] in sufficient water to make 100 mL.
Cupric Sulfate TS  Dissolve 12.5 g of cupric sulfate (CuSO₄·5H₂O) in sufficient water to make 100 mL, and mix.

Cupric Tartrate TS, Alkaline (Fehling's Solution) The Copper Solution (A): Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate, CuSO₄·5H₂O, showing no trace of efflorescence or of adhering moisture, in sufficient water to make 500 mL. Store this solution in small, tight containers. The Alkaline Tartrate Solution (B): Dissolve 173 g of crystallized potassium sodium tartrate (KNaC₄H₄O₆·4H₂O) and 50 g of sodium hydroxide (NaOH) in sufficient water to make 500 mL. Store this solution in small, alkali-resistant containers. For use, mix exactly equal volumes of solutions A and B at the time required.

Cyanogen Bromide TS  Dissolve 5 g of cyanogen bromide in water to make 50 mL.
[CAUTION— Prepare this solution in a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

Denigès' Reagent  See Mercuric Sulfate TS.

Dichlorophenol–Indophenol TS  Warm 100 mg of 2,6-dichlorophenol–indophenol sodium with 100 mL of water. Filter and use within 3 days.

2,7-Dihydroxynaphthalene TS  Dissolve 100 mg of 2,7-dihydroxynaphthalene in 1000 mL of sulfuric acid, and allow the solution to stand until the initial color disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately 1 month if stored in a dark bottle.

Diphenylamine TS  Dissolve 1 g of diphenylamine in 100 mL of sulfuric acid. The solution should be colorless.

Diphenylcarbazone TS  Dissolve about 1 g of diphenylcarbazone (C₁₃H₁₂N₄O) in sufficient alcohol to make 100 mL. Store this solution in a brown bottle.

α,α'-Dipyridyl TS  Dissolve 100 mg of α,α'-dipyridyl (C₁₀H₈N₂) in 50 mL of absolute alcohol.

Dithizone TS  Dissolve 25.6 mg of dithizone in 100 mL of alcohol.

Eosin Y TS (adsorption indicator)  Dissolve 50 mg of eosin Y in 10 mL of water.

Eriochrome Black TS  Dissolve 200 mg of eriochrome black T and 2 g of hydroxylamine hydrochloride (NH₂OH·HCl) in sufficient methanol to make 50 mL, and filter. Store the solution in a light-resistant container and use within 2 weeks.

p-Ethoxychrysoidin TS  Dissolve 50 mg of p-ethoxychrysoidin monohydrochloride in a mixture of 25 mL of water and 25 mL of alcohol, add 3 drops of hydrochloric acid, stir vigorously, and filter if necessary to obtain a clear solution.

Fehling's Solution  See Cupric Tartrate TS, Alkaline.

Ferric Ammonium Sulfate TS  Dissolve 8 g of ferric ammonium sulfate [FeNH₄(SO₄)₂·12H₂O] in sufficient water to make 100 mL.

Ferric Chloride TS  Dissolve 9 g of ferric chloride (FeCl₃·6H₂O) in sufficient water to make 100 mL.

Ferric Chloride TS, Alcoholic  Dissolve 100 mg of ferric chloride (FeCl₃·6H₂O) in 50 mL of absolute alcohol. Prepare this solution fresh.
**Ferric Sulfate TS, Acid**  Add 7.5 mL of sulfuric acid to 100 mL of water, and dissolve 80 g of ferrous sulfate in the mixture with the aid of heat. Mix 7.5 mL of nitric acid and 20 mL of water, warm, and add to this the ferrous sulfate solution. Concentrate the mixture until, upon the sudden disengagement of ruddy vapors, the black color of the liquid changes to red. Test for the absence of ferrous iron, and, if necessary, add a few drops of nitric acid and heat again. When the solution is cold, add sufficient water to make 110 mL.

**Add the following:**
- **Ferroin TS**  Dissolve 0.7 g of ferrous sulfate and 1.76 g of phenanthroline hydrochloride in 70 mL of water. Transfer the solution to a 100-mL volumetric flask, and dilute with water to volume. Test the sensitivity of Ferroin TS by adding 0.1 mL of Ferroin TS and 0.15 mL of osmium tetroxide solution (2.5 g/L of osmium tetroxide in 0.05 M sulfuric acid) to 50 mL of 1 M sulfuric acid. Add 0.1 mL of a 0.1 M ammonium cerium (IV) nitrate solution; a color change from red to light blue should be observed.

**Ferrous Sulfate TS**  Dissolve 8 g of clear crystals of ferrous sulfate (FeSO₄·7H₂O) in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

**Formaldehyde TS**  A solution containing approximately 37.0% (w/v) of HCHO. It may contain methanol to prevent polymerization.

**Fuchsin-Sulfurous Acid TS**  Dissolve 200 mg of basic fuchsin in 120 mL of hot water, and allow the solution to cool. Add a solution of 2 g of anhydrous sodium sulfite in 20 mL of water, and then add 2 mL of hydrochloric acid. Dilute the solution with water to 200 mL, and allow to stand for at least 1 h. Prepare this solution fresh.

**Hydrochloric Acid**  (approximately 12 N)  Use ACS reagent-grade Hydrochloric Acid (36.5% to 38.0% of HCl).

**Hydrochloric Acid TS, Diluted**  (2.7 N)  A solution containing 10% (w/v) of HCl. Prepare by diluting 226 mL of hydrochloric acid (36%) with sufficient water to make 1000 mL.

**Hydrogen Peroxide TS**  A solution containing between 2.5 and 3.5 g of H₂O₂ in each 100 mL. It may contain suitable preservatives, totaling not more than 0.05%.

**Hydrogen Sulfide TS**  A saturated solution of hydrogen sulfide made by passing H₂S into cold water. Store it in small, dark, amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H₂S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of Ferric Chloride TS. Store in a cold, dark place.

**Hydroxylamine Hydrochloride TS**  Dissolve 3.5 g of hydroxylamine hydrochloride (NH₂OH·HCl) in 95 mL of 60% alcohol, and add 0.5 mL of a 1:1000 solution of bromophenol blue and 0.5 N alcoholic potassium hydroxide until a green tint develops in the solution. Then add sufficient 60% alcohol to make 100 mL.

**8-Hydroxyquinoline TS**  Dissolve 5 g of 8-hydroxyquinoline (oxine) in sufficient alcohol to make 100 mL.

**Indigo Carmine TS**  *(Sodium Indigotindisulfonate TS)*  Dissolve a quantity of sodium indigotindisulfonate, equivalent to 180 mg of C₁₆H₁₀N₂·O₂(SO₃Na)₂, in sufficient water to make 100 mL. Use within 60 days.

**Iodine TS**  Dissolve 14 g of iodine (I₂) in a solution of 36 g of potassium iodide (KI) in 100 mL of water, add 3 drops of hydrochloric acid, dilute with water to 1000 mL, and mix.

**Iodinated Zinc Chloride**  Dissolve 10 g of potassium iodide, KI, and 0.15 g of iodine, I, in 10 mL of water. Add this solution to 100 mL of a 60% solution of zinc chloride, ZnCl₂, in water (sp. gr. 1.8). Keep a few crystals of iodine in the solution.
Isopropanol \([\text{Isopropyl Alcohol; 2-Propanol; } (\text{CH}_3)_2\text{CHOH}]\) Use ACS reagent-grade Isopropyl Alcohol. [Note —For use in assays and tests involving ultraviolet spectrophotometry, use ACS reagent-grade Isopropyl Alcohol Suitable for Use in Ultraviolet Spectrophotometry.]

Isopropanol, Anhydrous (Dehydrated Isopropanol) Use isopropanol that has been previously dried by shaking with anhydrous calcium chloride, followed by filtering.

Lead Acetate TS Dissolve 9.5 g of clear, transparent crystals of lead acetate \([\text{Pb(C}_2\text{H}_3\text{O}_2)_2\cdot3\text{H}_2\text{O}]\) in sufficient recently boiled water to make 100 mL. Store in well-stopped bottles.

Lead Subacetate TS Triturate 14 g of lead monoxide \((\text{PbO})\) to a smooth paste with 10 mL of water, and transfer the mixture to a bottle, using an additional 10 mL of water for rinsing. Dissolve 22 g of lead acetate \([\text{Pb(C}_2\text{H}_3\text{O}_2)_2\cdot3\text{H}_2\text{O}]\) in 70 mL of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 min, then set it aside, shaking it frequently during 7 days. Finally, filter, and add enough recently boiled water through the filter to make 100 mL.

Lead Subacetate TS, Diluted Dilute 3.25 mL of Lead Subacetate TS with sufficient water, recently boiled and cooled, to make 100 mL. Store in small, well-fitted, tight containers.

Litmus TS Digest 25 g of powdered litmus with three successive 100-mL portions of boiling alcohol, continuing each extraction for about 1 h. Filter, wash with alcohol, and discard the alcohol filtrate. Macerate the residue with about 25 mL of cold water for 4 h, filter, and discard the filtrate. Finally, digest the residue with 125 mL of boiling water for 1 h, cool, and filter.

Magnesia Mixture TS Dissolve 5.5 g of magnesium chloride \((\text{MgCl}_2\cdot6\text{H}_2\text{O})\) and 7 g of ammonium chloride \((\text{NH}_4\text{Cl})\) in 65 mL of water, add 35 mL of 6 N ammonium hydroxide, set the mixture aside for a few days in a well-stoppered bottle, and filter. If the solution is not perfectly clear, filter it before using.

Magnesium Sulfate TS Dissolve 12 g of crystals of magnesium sulfate \((\text{MgSO}_4\cdot7\text{H}_2\text{O})\), selected for freedom from efflorescence, in water to make 100 mL.

Malachite Green TS Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

Mayer's Reagent See Mercuric–Potassium Iodide TS.

Mercuric Acetate TS Dissolve 6 g of mercuric acetate \([\text{Hg(C}_2\text{H}_3\text{O}_2)_2]\) in sufficient glacial acetic acid to make 100 mL. Store in tight containers protected from direct sunlight.

Mercuric Chloride TS Dissolve 6.5 g of mercuric chloride \((\text{HgCl}_2)\) in water to make 100 mL.

Mercuric–Potassium Iodide TS (Mayer's Reagent) Dissolve 1.358 g of mercuric chloride \((\text{HgCl}_2)\) in 60 mL of water. Dissolve 5 g of potassium iodide \((\text{KI})\) in 10 mL of water. Mix the two solutions, and add water to make 100 mL.

Mercuric–Potassium Iodide TS, Alkaline (Nessler's Reagent) Dissolve 10 g of potassium iodide \((\text{KI})\) in 10 mL of water, and add slowly, with stirring, a saturated solution of mercuric chloride until a slight red precipitate remains undissolved. To this mixture add an ice-cold solution of 30 g of potassium hydroxide \((\text{KOH})\) in 60 mL of water, then add 1 mL more of the saturated solution of mercuric chloride. Dilute with water to 200 mL. Allow the precipitate to settle, and draw off the clear liquid. A 2-mL portion of this reagent, when added to 100 mL of a 1:300,000 solution of ammonium chloride in ammonia-free water, instantly produces a yellow-brown color.

Mercuric Sulfate TS (Denigès’ Reagent) Mix 5 g of yellow mercuric oxide \((\text{HgO})\) with 40 mL of water, and
while stirring, slowly add 20 mL of sulfuric acid, then add another 40 mL of water, and stir until completely dissolved.

**Mercurous Nitrate TS**  Dissolve 15 g of mercurous nitrate in a mixture of 90 mL of water and 10 mL of 2 N nitric acid. Store in dark, amber-colored bottles in which a small globule of mercury has been placed.

**Methanol (Methyl Alcohol)**  Use ACS reagent-grade Methanol.

**Methanol, Anhydrous (Dehydrated Methanol)**  Use Methanol.

**p-Methylaminophenol Sulfate TS**  Dissolve 2 g of p-methylaminophenol sulfate \([(\text{HOC}_6\text{H}_4\text{NHCH}_3)_2\text{H}_2\text{SO}_4]\) in 100 mL of water. To 10 mL of this solution add 90 mL of water and 20 g of sodium bisulfite. Confirm the suitability of this solution by the following test: Add 1 mL of the solution to each of four tubes containing 25 mL of 0.5 N sulfuric acid and 1 mL of Ammonium Molybdate TS. Add 5 µg of phosphate (PO$_4$) to one tube, 10 µg to a second, and 20 µg to a third, using 0.5, 1.0, and 2.0 mL, respectively, of Phosphate Standard Solution, and allow to stand for 2 h. The solutions in the three tubes should show readily perceptible differences in blue color corresponding to the relative amounts of phosphate added, and the one to which 5 µg of phosphate was added should be perceptibly bluer than the blank.

**Methylene Blue TS**  Dissolve 125 mg of methylene blue in 100 mL of alcohol, and dilute with alcohol to 250 mL.

**Methyl Orange TS**  Dissolve 100 mg of methyl orange in 100 mL of water, and filter if necessary.

**Methyl Red TS**  Dissolve 100 mg of methyl red in 100 mL of alcohol, and filter if necessary.

**Methyl Red–Methylene Blue TS**  Add 10 mL of Methyl Red TS to 10 mL of Methylene Blue TS, and mix.

**Methylrosaniline Chloride TS**  See Crystal Violet TS.

**Methyl Violet TS**  See Crystal Violet TS.

**Millon's Reagent**  To 2 mL of mercury in an Erlenmeyer flask add 20 mL of nitric acid. Shake the flask in a hood to break the mercury into small globules. After about 10 min add 35 mL of water, and if a precipitate or crystals appear, add sufficient 1:5 nitric acid (prepared from nitric acid from which the oxides have been removed by blowing air through it until it is colorless) to dissolve the separated solid. Add a 1:10 solution of sodium hydroxide, dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 mL more of the dilute nitric acid, and mix well. Prepare this solution fresh.

**α-Naphtholbenzein TS**  Dissolve 0.2 g of α-naphtholbenzein in glacial acetic acid to make 100 mL.

*Sensitivity*: Add 100 mL of freshly boiled and cooled water to 0.2 mL of a 1:1000 solution of α-naphtholbenzein in ethanol, and add 0.1 mL of 0.1 N sodium hydroxide: a green color develops. Add subsequently 0.2 mL of 0.1 N hydrochloric acid: the color of the solution changes to yellow-red.

**Naphthol Green TS**  Dissolve 500 mg of naphthol green B in water to make 1000 mL.

**Nessler's Reagent**  See Alkaline Mercuric–Potassium Iodide TS.

**Neutral Red TS**  Dissolve 100 mg of neutral red in 100 mL of 50% alcohol.

**Nickel Standard Solution TS** (10 mg/kg)  Prepare a 0.40% (w/v) solution of analytical reagent-grade nickel chloride (NiCl$_2$·6H$_2$O) with water. Pipet 1.0 mL of the solution into a 100-mL volumetric flask, and dilute with water to volume.
**Ninhydrin TS**  See *Triketohydrindene Hydrate TS*.

**Nitric Acid** (approximately 15.7 N)  Use ACS reagent-grade *Nitric Acid* (69.0% to 71.0% of HNO₃).

**Nitric Acid TS, Diluted** (1.7 N)  A solution containing about 10% (w/v) of HNO₃. Prepare by diluting 105 mL of nitric acid (70%) with water to make 1000 mL.

**Orthophenanthroline TS**  Dissolve 150 mg of orthophenanthroline (C₁₂H₈N₂·H₂O) in 10 mL of a solution of ferrous sulfate, prepared by dissolving 700 mg of clear crystals of ferrous sulfate (FeSO₄·7H₂O) in 100 mL of water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

**Oxalic Acid TS**  Dissolve 6.3 g of oxalic acid (H₂C₂O₄·2H₂O) in water to make 100 mL.

**Phenol Red TS (Phenolsulfonphthalein TS)**  Dissolve 100 mg of phenolsulfonphthalein in 100 mL of alcohol, and filter if necessary.

**Phenolphthalein TS**  Dissolve 1 g of phenolphthalein in 100 mL of alcohol.

**Phenolsulfonphthalein TS**  See *Phenol Red TS*.

**p-Phenylphenol TS**  On the day of use, dissolve 750 mg of p-phenylphenol in 50 mL of Sodium Hydroxide TS.

**Phosphoric Acid**  Use ACS reagent-grade *Phosphoric Acid* (not less than 85.0% of H₃PO₄).

**Phosphotungstic Acid TS**  Dissolve 1 g of phosphotungstic acid (approximately 24WO₃·2H₃PO₄·48H₂O) in water to make 100 mL.

**Picric Acid TS**  See *Trinitrophenol TS*.

**Potassium Acetate TS**  Dissolve 10 g of potassium acetate (KC₂H₃O₂) in water to make 100 mL.

**Potassium Chromate TS**  Dissolve 10 g of potassium chromate (K₂CrO₄) in water to make 100 mL.

**Potassium Dichromate TS**  Dissolve 7.5 g of potassium dichromate (K₂Cr₂O₇) in water to make 100 mL.

**Potassium Ferricyanide TS (10%)**  Dissolve 1 g of potassium ferricyanide [K₃Fe(CN)₆] in 10 mL of water. Prepare this solution fresh.

**Potassium Ferrocyanide TS**  Dissolve 1 g of potassium ferrocyanide [K₄Fe(CN)₆·3H₂O] in 10 mL of water. Prepare this solution fresh.

**Potassium Hydroxide TS (1 N)**  Dissolve 6.5 g of potassium hydroxide (KOH) in water to make 100 mL.

**Potassium Hydroxide TS, Alcoholic**  Use 0.5 N *Alcoholic Potassium Hydroxide* (see Volumetric Solutions in this section).

**Potassium Iodide TS**  Dissolve 16.5 g of potassium iodide (KI) in water to make 100 mL. Store in light-resistant containers.

**Potassium Permanganate TS**  Use 0.1 N *Potassium Permanganate* (see Volumetric Solutions in this section).

**Potassium Pyroantimonate TS**  Dissolve 2 g of potassium pyroantimonate in 95 mL of hot water. Cool quickly, and add a solution containing 2.5 g of potassium hydroxide in 50 mL of water and 1 mL of an 8.5:100
solution of sodium hydroxide. Allow to stand for 24 h, filter, and dilute with water to 150 mL.

**Potassium Sulfate TS**  
Dissolve 1 g of potassium sulfate (K₂SO₄) in sufficient water to make 100 mL.

**Quimociac TS**  
Dissolve 70 g of sodium molybdate (Na₂MoO₄·2H₂O) in 150 mL of water (Solution A).  
Dissolve 60 g of citric acid in a mixture of 85 mL of nitric acid and 150 mL of water, and cool (Solution B).  
Gradually add Solution A to Solution B, with stirring, to produce Solution C.  
Dissolve 5.0 mL of natural or synthetic quinoline in a mixture of 35 mL of nitric acid and 100 mL of water (Solution D).  
Gradually add Solution D to Solution C, mix well, and allow to stand overnight.  
Filter the mixture, add 280 mL of acetone to the filtrate, dilute with water to 1000 mL, and mix.  
Store in a polyethylene bottle.  
[CAUTION—This reagent contains acetone. Do not use it near an open flame. Operations involving heating or boiling should be conducted in a well-ventilated hood.]

**Quinaldine Red TS**  
Dissolve 100 mg of quinaldine red in 100 mL of glacial acetic acid.

**Schiff's Reagent, Modified**  
Dissolve 200 mg of rosaniline hydrochloride (C₂₀H₂₀ClN₃) in 120 mL of hot water.  
Cool, add 2 g of sodium bisulfite (NaHSO₃) followed by 2 mL of hydrochloric acid, and dilute with water to 200 mL.  
Store in a brown bottle at 15°C or lower.

**Silver Nitrate TS**  
Use 0.1 N Silver Nitrate (see Volumetric Solutions in this section).

**Sodium Bisulfite TS**  
Dissolve 10 g of sodium bisulfite (NaHSO₃) in water to make 30 mL.  
Prepare this solution fresh.

**Sodium Bitartrate TS**  
Dissolve 1 g of sodium bitartrate (NaHC₄H₄O₆·H₂O) in water to make 10 mL.  
Prepare this solution fresh.

**Sodium Borate TS**  
Dissolve 2 g of sodium borate (Na₂B₄O₇·10H₂O) in water to make 100 mL.

**Sodium Carbonate TS**  
Dissolve 10.6 g of anhydrous sodium carbonate (Na₂CO₃) in water to make 100 mL.

**Sodium Cobaltinitrite TS**  
Dissolve 10 g of sodium cobaltinitrite [Na₃Co(NO₂)₆] in water to make 50 mL, and filter if necessary.

**Sodium Fluoride TS**  
Dry about 500 mg of sodium fluoride (NaF) at 200°C for 4 h.  
Weigh accurately 222 mg of the dried sodium fluoride, and dissolve it in sufficient water to make exactly 100 mL.  
Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.  
Each mL of this final solution corresponds to 10 µg of fluorine (F).

**Sodium Hydroxide TS**  
(1 N)  
Dissolve 4.3 g of sodium hydroxide (NaOH) in water to make 100 mL.

**Sodium Indigotindisulfonate TS**  
See Indigo Carmine TS.

**Sodium Nitroferricyanide TS**  
Dissolve 1 g of sodium nitroferricyanide [Na₂Fe(NO)(CN)₅·2H₂O] in water to make 20 mL.  
Prepare this solution fresh.

**Sodium Phosphate TS**  
Dissolve 12 g of clear crystals of dibasic sodium phosphate (Na₂HPO₄·7H₂O) in water to make 100 mL.

**Sodium Sulfide TS**  
Dissolve 1 g of sodium sulfide (Na₂S·9H₂O) in water to make 10 mL.  
Prepare this solution fresh.

**Sodium Tetraphenylborate TS**  
Dissolve 1.2 g of sodium tetraphenylborate in water to make 200 mL.  
If
necessary, stir for 5 min with 1 g of freshly prepared hydrous aluminum oxide, and filter to clarify.

**Sodium Thiosulfate TS** Use 0.1 N Sodium Thiosulfate (see Volumetric Solutions in this section).

**Stannous Chloride TS** Dissolve 40 g of reagent-grade stannous chloride dihydrate (SnCl₂·2H₂O) in 100 mL of hydrochloric acid.

*Change to read:*

**Starch TS** Mix 1 g of a suitable starch with 10 mg of red mercuric oxide\(\text{(FCC7)}\) and sufficient cold water to make a thin paste. Add 20 mL of boiling water, boil for 1 min with continuous stirring, and cool. Use only the clear solution. Test the sensitivity of the Starch TS by adding one drop of a 50 mg/kg chlorine solution made by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution in 1000 mL of water to 1 mL of Starch TS. Test the sensitivity of the Starch TS by adding 5 mL of it to 100 mL of water. Add 0.05 mL of freshly prepared 0.1 N potassium iodide solution and 1 drop of 50 mg/kg chlorine solution, made by diluting 1 mL of a commercial 5% sodium hypochlorite, NaOCl, solution in 1000 mL of water. The deep blue color produced is discharged by 0.05 mL of 0.1 N sodium thiosulfate, as follows: Prepare a solution of 50 mg/kg chlorine by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution in 1000 mL of water. Combine 5 mL of Starch TS with 100 mL of water and add 0.5 mL of 0.1 N potassium iodide. Addition of one drop of the 50 mg/kg chlorine solution should give a swirl of color where the drop hits. Addition of 1 mL of 50 mg/kg chlorine solution should give a deep blue color throughout the solution. The deep blue color produced is discharged by addition of 0.05 mL of 0.1 N sodium thiosulfate. Prepare fresh solution when Starch TS no longer passes the sensitivity test.\(\text{(FCC7)}\)

**Starch Iodide Paste TS** Heat 100 mL of water in a 250-mL beaker to boiling, add a solution of 750 mg of potassium iodide (KI) in 5 mL of water, then add 2 g of zinc chloride (ZnCl₂) dissolved in 10 mL of water, and while the solution is boiling, add with stirring a smooth suspension of 5 g of potato starch in 30 mL of cold water. Continue to boil for 2 min, then cool. Store in well-closed containers in a cool place. This mixture must show a definite blue streak when a glass rod dipped in a mixture of 1 mL of 0.1 M sodium nitrite, 500 mL of water, and 10 mL of hydrochloric acid is streaked on a smear of the paste.

**Sulfanilic Acid TS** Dissolve 800 mg of sulfanilic acid (\(\text{p-}\text{NH}_2\text{C}_6\text{H}_4\text{SO}_3\text{H·H}_2\text{O}\)) in 100 mL of acetic acid. Store in tight containers.

**Sulfuric Acid (approximately 36 N)** Use ACS reagent-grade Sulfuric Acid (95.0% to 98.0% of H₂SO₄).

**Sulfuric Acid TS (95%)** Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to between 94.5% and 95.5% of H₂SO₄. Because the acid concentration may change upon standing or upon intermittent use, the concentration should be checked frequently and solutions assaying more than 95.5% or less than 94.5% discarded or adjusted by adding either diluted or fuming sulfuric acid, as required.

**Sulfuric Acid TS, Diluted (2 N)** A solution containing 10% (w/v) of H₂SO₄. Prepare by cautiously adding 57 mL of sulfuric acid (95% to 98%) or Sulfuric Acid TS to about 100 mL of water, then cool to room temperature, and dilute with water to 1000 mL.

**Tannic Acid TS** Dissolve 1 g of tannic acid (tannin) in 1 mL of alcohol, and add water to make 10 mL. Prepare this solution fresh.

**Thymol Blue TS** Dissolve 100 mg of thymol blue in 100 mL of alcohol, and filter if necessary.

**Thymolphthalein TS** Dissolve 100 mg of thymolphthalein in 100 mL of alcohol, and filter if necessary.
Triketohydrindene Hydrate TS (Ninhydrin TS)  Dissolve 200 mg of triketohydrindene hydrate (C₉H₄O₃·H₂O) in water to make 100 mL. Prepare this solution fresh.

Trinitrophenol TS (Picric Acid TS)  Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 mL of hot water. Cool the solution, and filter if necessary.

Xylenol Orange TS  Dissolve 100 mg of xylene orange in 100 mL of alcohol.