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\textsuperscript{FCC 6}

if slated for FCC 6; and

\textsuperscript{1S (FCC 6)}

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 \textsuperscript{111} or \textsuperscript{222}, 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, \textsuperscript{1S (FCC 6)} indicates that the proposed revision is slated for the First Supplement to FCC 6, and \textsuperscript{FCC 6} indicates that the revisions are proposed for FCC 6.
**BRIEFING**

Amaranth. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Amaranth 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. To measure *Total Color* the JECFA monograph uses a titrimetric procedure while the EU specifies use of a spectrophotometric procedure. The proposed FCC monograph averages the results from both procedures, consistent with other color additive monographs in FCC such as *Fast Green*.

3. 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.

4. A test procedure and specification for *Uncombined Intermediates and Products of Side Reactions* 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.

5. 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 IIIC for the analysis of colors.

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

7. 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.

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

---

*Add the following:*

**Amaranth**

CI Food Red 9

Naphthol Rot S

CI No. 16185

Class: Mono-Azo

Trisodium 3-hydroxy-4-(4-sulfonato-1-naphthylazo)-2,7-naphthalenedisulfonate
Amaranth occurs as reddish brown to dark reddish brown powder or granules. It is principally the trisodium salt of 3-hydroxy-4-(4-sulfonato-1-naphthylazo)-2,7-naphthalenedisulfonate and subsidiary coloring matters together with sodium chloride and/or sodium sulfate as the principal uncolored components. It is soluble in water and sparingly soluble in ethanol.

**Function:** Color

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

**IDENTIFICATION**

**Visible Absorption Spectrum**

**Sample solution:** Dissolve a sample in water, and dilute appropriately.

**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 520 nm.

**ASSAY**

**Total Color, Colors, Methods I and II, Appendix III C:** Both methods must be used.

**Method I:** (Spectrophotometric)

**Sample solution:** 10 mg/mL

**Analysis:** Determine as directed at 520 nm using 0.044 L/(mg·cm) for the absorptivity (a) for Amaranth.

**Method II:** (TiCl₃ Titration)

**Sample:** 0.7–0.8 g

**Analysis:** Determine as directed, except in the *Procedure* use 10 g of sodium citrate instead of 21–22 g of Sodium Bitartrate and use 150 mL of water instead of 275 mL. For the calculation, use 6.618 as the stoichiometric factor (Fₛ) for the trisodium salt of Amaranth.

**Acceptance criteria:** The average of results obtained from *Method I* and *Method II* is NLT 85% 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

• UNCOMBINED INTERMEDIATES AND PRODUCTS OF SIDE REACTIONS

Solution A: 0.2 N ammonium acetate
Solution B: Methanol
Mobile phase: See the gradient table below.

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<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
<th>Comments</th>
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<tr>
<td>44.5</td>
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<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Sample solution: 5 mg/mL in 0.02 M ammonium acetate
Standard solution: 25 µg/mL 4-amino-1-naphthalenesulfonic acid, 25 µg/mL 7-hydroxy-1,3-naphthalenesulfonic acid, 25 µg/mL 3-hydroxy-7-naphthalenesulfonic acid, 25 µg/mL 6-hydroxy-2-naphthalenesulfonic acid, 25 µg/mL 7-hydroxy-1,3,6-naphthalenetrisulfonic acid in 0.02 M ammonium acetate

Chromatographic system, Appendix IIA
Mode: High-performance liquid chromatography
Detector: UV-Vis
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 percentages of all impurities (4-amino-1-naphthalenesulfonic acid, 7-hydroxy-1,3-naphthalenesulfonic acid, 3-hydroxy-7-naphthalenesulfonic acid, 6-hydroxy-2-naphthalenesulfonic acid, and 7-hydroxy-1,3,6-naphthalenetrisulfonic acid) in the sample taken:

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

\( r_U \) = peak area for analyte in the Sample solution
\( r_S \) = peak area for analyte in the Standard solution
\( C_S \) = concentration of analyte in the Standard solution (µg/mL)
\( C_U \) = concentration of sample in the Sample solution (mg/mL)
\( F \) = mg-to-µg conversion factor, 1000

Acceptance criteria: NMT 0.5% for all five impurities combined

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 15%, 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 Amaranth 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 Amaranth (520 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 color 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 paper. Mark out the chromatography paper as shown in Figure 3.
Chromatographic solvent: Prepare a mixture of 2-butanone, acetone, and water (7:3:3). 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: 0.3 mg/mL sample prepared by diluting the Sample solution

Application volume: 0.10 mL

Analysis: No less than 2 h before analysis, arrange the filter-paper drapes in the glass tank, and pour sufficient 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 to separate chromatography sheets 0.1-mL aliquots of the Sample solution and Standard solution, 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 sheets, together with two plain sheets to act as blanks on the supporting frame. [Note—If required, several dried sheets may be developed simultaneously.]

Pour sufficient 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 solvent front to ascend approximately 17 cm above baseline, and then allow for 1 h of further development. Remove the supporting frame and transfer it to a drying cabinet at 50°–60° for 10–15 min. Remove the sheets from the frame.

For the Sample solution sheets, 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. For the Standard solution sheet, cut the entire band from the sheet, and cut an equivalent strip from the corresponding position of the plain (blank) sheet. Place each strip, subdivided into a suitable number of approximately equal portions, in a separate test tube. Add 5.0 mL of a mixture 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 520 nm using a suitable spectrophotometer with 40-mm closed cells against a filtered mixture of 5.0 mL of a mixture 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 520 nm, and correct the absorbances of the colored extracts with the blank values. Calculate the percent subsidiary coloring matter in the portion of the sample taken:

\[
\text{Result} = 0.03 \times D \times \left[\frac{(A_a + A_b + A_c + ... A_n)}{A_s}\right] \times 100
\]
0.03 = dilution factor for the Standard solution
D = total coloring matter content of the sample, determined from the Total Color test above and expressed as a decimal

\[ A_s = \text{absorbance from the Standard solution} \]

\[ A_a + A_b + A_c \ldots A_n = \text{sum of the absorbances of the subsidiary coloring matters from the Sample solution, corrected for the blank values} \]

**Acceptance criteria:** NMT 3%

**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 with water to 100 mL, 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 with 1 N hydrochloric acid to 100 mL.

**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:
Result = \( \frac{W_A}{W} \times 100 \)

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

\( W \) = 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%

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1 Amaranth 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>
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<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
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</table>
BRIEFING

Azorubine. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed, based on the Azorubine 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 United States but is approved in other countries.

1. The proposed 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. To measure Total Color, the JECFA monograph uses a titrimetric procedure, whereas the EU specifies the use of a spectrophotometric procedure. The proposed FCC monograph averages the results from both procedures, consistent with other color additive monographs in FCC such as Fast Green.

3. 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 B. Stakeholders are encouraged to submit a more modern lead procedure validated for this ingredient.

4. A test procedure and a specification for Uncombined Intermediates and Products of Side Reactions are proposed, which are based on those in JECFA. Stakeholders are encouraged to submit information about a supplier and appropriate concentrations for reference standards used in this test procedure.

5. Specifications proposed in the tests for Loss on Drying, Chloride, and Sulfates in the Combined Tests section are based on those in JECFA, but they use different test procedures—ones already existing in Appendix III B for the analysis of colors.

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

7. Specifications and test procedures proposed for Subsidiary Coloring Matters and Unsulfonated Primary Aromatic Amines are based on those in JEFCA. Stakeholders are encouraged to submit more modern test procedures, especially for Subsidiary Coloring Matters, for consideration in this monograph.

8. The proposed specification for Water-Insoluble Matter is based on that in JECFA, using a similar test procedure already in FCC Appendix III C.

(FI: J. Moore) C100463

Add the following:

Azorubine

Carmoisine

CI Food Red 3

CI No. 14720

Class: Mono-Azo

Disodium 4-hydroxy-3-(4-sulfonato-1-naphthylazo)-1-naphthalenesulfonate
Azorubine occurs as red powder or granules. It is principally the disodium salt of 4-hydroxy-3-(4-sulfonato-1-naphthylazo)-1-naphthalenesulfonate and subsidiary coloring matters, with sodium chloride and/or sodium sulfate as the principal uncolored components. It is soluble in water and sparingly soluble in ethanol.

**Function:** Color

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

### IDENTIFICATION

- **Visible Absorption Spectrum**
  - **Sample solution:** Dissolve a sample in water, and dilute appropriately.
  - **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 516 nm.

### ASSAY

- **Total Color, Colors, Methods I and II, Appendix IIIC:** Both methods must be used.
  - **Method I:** (Spectrophotometric)
    - **Sample solution:** 10 mg/mL
    - **Analysis:** Determine as directed at 516 nm, using 0.051 L/(mg·cm) for the absorptivity \( (a) \) for Azorubine.
  - **Method II:** (TiCl\(_3\) Titration)
    - **Sample:** 0.5–0.6 g
    - **Analysis:** Determine as directed, except under Procedure, use 15 g of Sodium Bitartrate instead of 21–22 g, and use 150 mL of water instead of 275 mL. For the calculation, use 7.962 as the stoichiometric factor \( (F_S) \) for the disodium salt of Azorubine.
    - **Acceptance criteria:** The average of the results obtained from Method I and Method II is NLT 85% 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

- **Uncalculated Intermediates and Products of Side Reactions**
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, followed by 6 min of 100% B to wash the column, and (0% A and 100% B) to (99% A and 1% B) in 14 min. to return to the initial gradient composition and equilibrate column.
Standard solution: 25 µg/mL of 4-amino-1-naphthalenesulfonic acid and 25 µg/mL of 4-hydroxy-1-naphthalenesulfonic acid in 0.02 M ammonium acetate
Sample solution: 5 mg/mL in 0.02 M ammonium acetate

Chromatographic system, Appendix IIA
Mode: High-performance liquid chromatography
Detector: UV
Column
Guard column: 15-mm × 4.6-mm 5-µm C18 column
Analytical column: 25-cm × 4.6-mm 5-µm C18 column
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 percentage of both impurities (4-amino-1-naphthalenesulfonic acid and 4-hydroxy-1-naphthalenesulfonic acid) in the portion of the sample taken:

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

\(r_U\) = peak area for analyte in the Sample solution
\(r_S\) = peak area for analyte in the Standard solution
\(C_S\) = concentration of analyte in the Standard solution (µg/mL)
\(C_U\) = concentration of sample in the Sample solution (mg/mL)
\(F\) = mg-to-µg conversion factor, 1000

Acceptance criteria: 4-amino-1-naphthalenesulfonic acid and 4-hydroxy-1-naphthalenesulfonic acid: NMT 0.5% combined

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 15%, 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 Azorubine 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 Azorubine (516 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 composed of a glass tank (A) and cover (B); frame to support chromatography paper (C); solvent tray (D); wire secondary frame (E) for supporting "drapes" of the filter paper; and 20-cm x 20-cm chromatography grade paper. Mark out the chromatography paper as shown in Figure 3.

**Chromatographic solvent:** Prepare a mixture of 2-butanone, acetone, water, and saturated aqueous solution of ammonium hydroxide (specific gravity of 0.880), (700:300:300:2). Shake for 2 min, allow the layers to separate, and use the upper layer.

**Sample solution:** 10 mg/mL sample
Standard solution: 0.1 mg/mL sample, prepared by diluting the Sample solution

Application volume: 0.10 mL

Analysis: NLT 2 h before analysis, arrange the filter-paper drapes in the glass tank, and pour sufficient 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 to separate chromatography sheets 0.1-mL aliquots of the Sample solution and Standard solution, 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°C in a drying cabinet for 5 min followed by 15 min at room temperature. Mount the dried sheets, together with two plain sheets to act as blanks on the supporting frame. [Note—If required, several dried sheets may be developed simultaneously.]

Pour sufficient 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 solvent front to ascend approximately 17 cm above base line, then remove the supporting frame and transfer it to a drying cabinet at 50–60°C for 10–15 min. Remove the sheets from the frame.

For the Sample solution sheets, cut each subsidiary band from each chromatography sheet as a strip, and cut an equivalent strip from the corresponding position of the plain (blank) sheet. For the Standard solution sheet, cut the entire band from the sheet, and cut an equivalent strip from the corresponding position of the plain (blank) sheet. Place each strip, subdivided into a suitable number of approximately equal portions, in a separate test tube. Add 5.0 mL of a mixture of water and acetone (1:1 by volume) 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 the blanks through 9-cm coarse-porosity filter papers into clean test tubes, and determine the absorbances of the colored extracts at 516 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 0.05 N sodium hydrogen carbonate solution. Measure the absorbances of the extracts of the blank strips at 516 nm, and correct the absorbances of the colored extracts with the blank values.

Calculate the percentage of 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 and expressed as a decimal
A_s = the absorbance from the Standard solution
\(A_a + A_b + A_c \ldots A_n\) = the sum of the absorbances of the subsidiary coloring matters from the Sample solution, corrected for the blank values

Acceptance criteria: NMT 1%

• 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, reextracted into acid, then determined spectrophotometrically after diazotization and coupling.]

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 with water at room temperature to the mark. 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 with 1 N hydrochloric acid to 100 mL.

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

**Sample solution:** Add 2.0 g of the sample to 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 2.0 mL of R salt solution, 10 mL of Sodium carbonate solution, and 10.0 mL of the Sample solution, and dilute with water to volume.

**Analysis:** Pipet 10-mL aliquots of each of the Standard solutions and the Sample solution into separate clean, dry test tubes. Cool the tubes for 10 min by immersion in a beaker of ice water, and 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 the R salt solution and 10 mL of the 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 with water to the mark, 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 percentage of 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%

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1 Azorubine 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.

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<td></td>
<td>1-301-816-8288</td>
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</tbody>
</table>
BRIEFING

BHA, *FCC 7* page 101. On the basis of comments and data received it is proposed to replace the outdated packed column GC Assay method with a modernized HPLC method. It is also proposed to add HPLC peak retention times from the Assay as a second Identification test. The HPLC method is based on one recently added to the *USP–NF* monograph for Butylated Hydroxyanisole appearing in *USP 34–NF 29*. It is also proposed to revise the Packaging and Storage statement for this monograph to include protection from light and heat exposure on the basis of data received.

(FI: J. Moore) C103928

BHA

Butylated Hydroxyanisole

```
CH_3
\( \text{H}_3\text{C} \quad \text{CH}_3 \quad \text{OH} \)
\( \text{H}_3\text{CO} \)
```

C\(_{11}\)H\(_{16}\)O\(_2\)

Formula wt 180.25

INS: 320

CAS: [25013-16-5]

DESCRIPTION

BHA occurs as a white or slightly yellow, waxy solid. It is predominantly 3-tert-butyl-4-hydroxyanisole (3-BHA), with varying amounts of 2-tert-butyl-4-hydroxyanisole (2-BHA). It melts between 48\(^\circ\) and 63\(^\circ\). It is freely soluble in alcohol and in propylene glycol, and insoluble in water.

Function: Antioxidant

*Change to read:*

Packaging and Storage: Store in well-closed containers protected from light and heat.\(\text{FCC8}\)

IDENTIFICATION

*Change to read:*

- Procedure\(\text{FCC8}\)

Sample solution: 100 µg/mL in 72% alcohol

Analysis: Add 2 mL of sodium borate TS and 1 mL of a 100 µg/mL solution of 2,6-dichloroquinone chlorimide in absolute alcohol to 5 mL of the Sample solution and mix.

Acceptance criteria: A blue color appears.
**Add the following:**

- **A. Procedure**

**Acceptance criteria:** The retention times of 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole from the Sample solution correspond to those from the Standard solution, as obtained in the Assay.\(^{\text{FCC8}}\)

**ASSAY**

**Change to read:**

- **Procedure**

  **Internal standard solution:** 5 mg/mL of 4-tert-butylphenol in acetone

  **Mixed standard solution:** 9 mg/mL of USP 3-tert-Butyl-4-hydroxyanisole RS and 1 mg/mL of USP 2-tert-Butyl-4-hydroxyanisole RS in Internal standard solution

  **Sample:** 100 mg

  **Sample solution:** Transfer the Sample into a 10-mL volumetric flask, dissolve in and dilute to volume with Internal standard solution, and mix.

  **Chromatographic system,** Appendix II-A

  - **Mode:** Gas chromatography
  - **Detector type:** Flame-ionization
  - **Column:** 1.8 m × 2 mm (id) stainless-steel column, or equivalent, packed with 10% silicone GE XE-60, or equivalent
  - **Column temperature:** Isothermal, between 175° and 185°
  - **Carrier gas:** Helium
  - **Flow rate:** 30 mL/min
  - **Injection volume:** About 5 µL

  **System suitability**

  - **Sample:** Mixed standard solution

  **Suitability requirements**

  - **Resolution:** NLT 1.3 between the 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole isomers
  - **Relative standard deviation:** NMT 2.0% for the 3-tert-butyl-4-hydroxyanisole isomer and NMT 6.0% for the 2-tert-butyl-4-hydroxyanisole isomer, for replicate injections
  - **Tailing factor:** NMT 2.0

  **Analysis:** Inject the Mixed standard solution and separately inject the Sample solution. Measure the areas under the peaks for each isomer and the Internal standard (4-tert-butylphenol) in each chromatogram and calculate the quantity, I (mg), of each isomer in the Sample taken by the equation:

  \[
  I = 10 \times C_S \times \left( R_U / R_S \right)
  \]

  - \( C_S \) = concentration (mg/mL) of the isomer in the Mixed standard solution
  - \( R_U \) = ratio of the area of the isomer to that of the Internal standard in the chromatogram from the Sample solution
  - \( R_S \) = ratio of the area of the isomer to that of the Internal standard in the chromatogram from the Mixed standard solution

  Calculate the weight (mg) of \( C_{11}H_{16}O_2 \) in the sample taken by adding the quantities of the two isomers.

**Solution A:** 5% Acetic acid
**Mobile phase:** Acetonitrile and Solution A (45:55)

**Standard solution:** 90 µg/mL of USP 3-tert-Butyl-4-hydroxyanisole RS and 10 µg/mL of USP 2-tert-Butyl-4-hydroxyanisole RS in Mobile phase

**Sample solution:** 100 µg/mL in Mobile phase

**Chromatographic system,** Appendix IIA

- **Mode:** HPLC
- **Detector:** UV 290 nm
- **Column:** 4.6-mm × 75-mm; packed with 3.5-µm octadecylsilane chemically bonded to porous silica or ceramic micro-particles packing

**Column temperature:** 30°C

**Flow rate:** 1.2 mL/min

**Injection size:** 20 µL

**System suitability**

**Sample:** Standard solution

[Note—The retention times of 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole are about 4.2 and 4.6 min, respectively.]

**Suitability requirements**

- **Resolution:** NLT 1.5 between the 3-tert-butyl-4-hydroxyanisole isomer and 2-tert-butyl-4-hydroxyanisole isomer peaks
- **Tailing factor:** NMT 1.5
- **Relative standard deviation:** NMT 2.0% for the 3-tert-butyl-4-hydroxyanisole isomer and 2-tert-butyl-4-hydroxyanisole isomer 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.

Calculate the percentage of each isomer (3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole) 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 area response for the analyte in the Sample solution
- \(r_S\) = peak area response for the analyte in the Standard solution
- \(C_S\) = concentration of the analyte in the Standard solution (µg/mL)
- \(C_U\) = concentration of the sample in the Sample solution (µg/mL)

Calculate the percentage of \(C_{11}H_{16}O_2\) in the sample taken by adding the percentages of the two isomers.

**Acceptance criteria:** NLT 98.5% \(C_{11}H_{16}O_2\)

**SPECIFIC TESTS**

- **Residue on Ignition (Sulfated Ash), Method I, Appendix IIC**
  - **Sample:** 10 g
  - **Acceptance criteria:** NMT 0.05%

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

*FCC Seventh Edition Page 101*
**BRIEFING**

**Cassia Oil, FCC 7 page 200.** On the basis of comments received, it is proposed to revise the *Rosin or Rosin Oils* test under *Specific Tests*. Comments received indicate that the current method does not specify that the hexane layer is the layer of interest in this analysis and that this should therefore be made clear to the user. Comments are encouraged.

(Fi: C. Mejia) C103877

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**Cassia Oil**

Cinnamon Oil

FEMA: 2258
CAS: [8007-80-5]

**DESCRIPTION**

Cassia Oil occurs as a yellow or brown liquid having the characteristic odor and taste of cassia cinnamon. It is the volatile oil obtained by steam distillation from the leaves and twigs of *Cinnamomum cassia* Blume (Fam. Lauraceae), rectified by distillation, and consisting mainly of cinnamic aldehyde. Upon aging or exposure to air it darkens and thickens. It is soluble in glacial acetic acid and in alcohol.

**Function:** Flavoring agent

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

**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.

  ![Spectrogram](image)

  **Cassia Oil**

**ASSAY**

- **ALDEHYDES, Aldehydes and Ketones, Neutral Sulfite Method, Appendix VI**
  
  **Acceptance criteria:** NLT 80.0%, by volume, of total aldehydes

**SPECIFIC TESTS**

- **ANGULAR ROTATION, Optical (Specific) Rotation, Appendix IIIB:** Use a 100-mm tube.
  
  **Acceptance criteria:** Between –1° and +1°

- **CHLORINATED COMPOUNDS, Appendix VI**
  
  **Acceptance criteria:** Passes test

- **REFRACTIVE INDEX, Appendix IIIB**
[Note—Use an Abbé or other refractometer of equal or greater accuracy.]

**Acceptance criteria:** 1.602–1.614 at 20°

**Change to read:**

- **Rosin or Rosin Oils**
  - **Sample:** 2 mL
  - **Analysis:** Shake the Sample in a test tube with 5–10 mL of solvent hexane, and allow the liquids to separate. Decant the hexane layer, which is just slightly colored, into another test tube, shake it with an equal volume of 1:1000 cupric acetate solution, and allow the phases to separate.
  - **Acceptance criteria:** The mixture hexane layer does not turn green.

- **Solubility in Alcohol, Appendix VI**
  - **Acceptance criteria:** One mL of sample dissolves in 2 mL of 70% alcohol.

- **Specific Gravity:** Determine by any reliable method (see General Provisions).
  - **Acceptance criteria:** 1.045–1.063

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

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<td>(Fl2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>

*FCC Seventh Edition Page 200*
Cyclamic Acid. Because there is currently no FCC monograph for this food ingredient, a new monograph is proposed based on European Commission Directive 95/31/EC (July 5, 1995) and on the Cyclohexylsulfamic Acid monograph from the 46th Session (1996) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) with subsequent updates from the 57th Session (2001). 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, 2011). Impurities for selenium, arsenic, and heavy metals are included in the EC Directive 95/31/EC. These limits are not included in the proposed FCC monograph because the rationale for them is unclear and they are absent in the JECFA monograph. Also, it is the policy of FCC to list specific elemental impurities and not generic limits such as heavy metals. Comments on the absence of selenium and arsenic limits are encouraged. Also encouraged are more modern methods of analysis as they may be used in industry.

(C: K. Laurvick)  C100275

Add the following:

Cyclamic Acid

Cyclohexanesulfamic Acid

Cyclohexylsulfamic Acid

C₆H₁₃NO₃S

Formula wt 179.24

INS: 952

CAS: [100-88-9]

DESCRIPTION

Cyclamic Acid occurs as a practically colorless, white crystalline powder. It is soluble in water and in ethanol.

Function: Sweetener

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION

A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix III

Reference standard: USP Cyclamic 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.

B. PROCEDURE
Sample solution: 20 mg/mL

Analysis: Acidify the Sample solution with hydrochloric acid. Add 1 mL of barium chloride TS to the acidified solution, then filter if any turbidity or precipitate forms. When a clear solution is obtained, add 1 mL of 10% sodium nitrite solution.

Acceptance criteria: A white precipitate forms.

ASSAY

Phenolphthalein solution: Dissolve 0.2 g of phenolphthalein in 60 mL of 90% ethanol, and dilute with water to 100 mL.

Sample: 350 mg

Analysis: Transfer the Sample to a 250-mL flask, and dissolve it in 50 mL of water. Titrate the solution with 0.1 N sodium hydroxide, using Phenolphthalein solution as the indicator. Each mL of 0.1 N sodium hydroxide is equivalent to 17.82 mg of C₆H₁₃NO₃S.

Acceptance criteria: 98.0%–102.0% of C₆H₁₃NO₃S, 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: 0.02 µL/mL of tetradeclane 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-cm × 0.32-mm (i.d.) fused-silica column with poly(dimethyl)(dipheynl)siloxane containing 95% of methyl groups and 5% of phenyl groups¹ as stationary phase (film thickness 0.51 µm)

Carrier gas: Helium

Flow rate: 1.8 mL/min

Temperature

Injection port: 250°C

Detector: 270°C
Column: See the temperature program in the table below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Temperature (°)</th>
</tr>
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<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>
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</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 min, 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: 105° for 1 h
  Acceptance criteria: NMT 1%

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

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1 DB-5 available from J&W Scientific, SE-52 available from Restek Corp., or equivalent.
BRIEFING

DHA from Algal (Cryptothecodinium) Oil, page 1452 of the First Supplement to FCC 7. On the basis of comments received, a revision to the minimum content of docosahexaenoic acid (DHA) content under Identification and the Assay is proposed. This revision is intended to harmonize with international regulatory opinions and approvals in China, Russia, and Australia. Batch data supporting the revision is specifically requested.

(FI: K. Laurvick) C99752

DHA from Algal (Cryptothecodinium) Oil

**DESCRIPTION**

DHA from Algal (Cryptothecodinium) Oil occurs as a light yellow to orange colored oil providing a source of docosahexaenoic acid (DHA, C_{22}H_{32}O_{2}) (C22:6 n-3), an omega-3 long-chain polyunsaturated fatty acid. It is obtained from fermentation of the species of microalgae Cryptothecodinium cohnii, usually 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. Docosahexaenoic acid is the only significant polyunsaturated fatty acid present; DHA content may be standardized with other oils. Suitable antioxidants may be added.

**Function:** Source of DHA

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

**IDENTIFICATION**

**Change to read:**

- **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 time of the peak of the docosahexaenoic acid methyl ester from the Sample Preparation corresponds to that from the Standard Solution. The area percentage for the methyl esters of the fatty acids from the Sample Preparation 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>Linoleic acid</td>
<td>18:2 n-6</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dihomo-gamma-linolenic acid</td>
<td>20:3 n-6</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Eicosapentanoic acid</td>
<td>20:5 n-3</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Docosapentaenoic acid</td>
<td>22:5 n-6</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>22:6 n-3</td>
<td>▲40.0 ▲35.0 ▲FCC 8</td>
<td>47.0</td>
</tr>
</tbody>
</table>

**ASSAY**

**Change to read:**

- **DHA**, Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids, Appendix VII
Acceptance criteria: NLT 40.0% FCC 35.0% docosahexaenoic acid (DHA)

IMPURITIES
Inorganic Impurities
- ARSENIC

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 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. This method was developed using a Perkin-Elmer Model 5100, HGA-600 furnace, and an AS-60 autosampler with Zeeman effect 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°
- 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 solution of water and nitric acid (4:1). [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 µg/L, 5.0 µg/L, 10.0 µg/L, 25.0 µg/L, 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°C using a 1-s ramp, a 65-s hold, and a 300-mL/min argon flow.
2. Char the sample at 1000°C 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°C set temperature and a 300-mL/min argon flow.
4. Atomize at 2400°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, 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:

Result = (C × DF × V)/W
C = concentration of arsenic 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

• Lead

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: 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 µg/L, 5.0 µg/L, 10.0 µg/L, 25.0 µg/L, 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 (above).

[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:

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

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

- \( C \) = 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 (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:** 5–6 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 solution of water and nitric acid (4:1). [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 NIST spectrometric standard solutions).

Calibration standard solutions: 20 ng, 60 ng, 100 ng, 200 ng, 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 (above).

[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: 5–6 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 in 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 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 nanograms of mercury in the aliquot analyzed.

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

\[ \text{Result} = \left( \frac{A \times DF}{W \times 1000} \right) \]

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

[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

SPECIFIC TESTS
- Anisidine Value, Appendix VII
Acceptance criteria: NMT 20.0

- **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 0.4%

- **PEROXIDE VALUE, Appendix VII**
  Acceptance criteria: NMT 5.0 mEq/kg

- **TOTAL OXIDATION VALUE**
  Analysis: Calculate by the formula:

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

  \[ \text{PV} = \text{peroxide value, determined above} \]
  \[ \text{AV} = \text{anisidine value, determined above} \]
  Acceptance criteria: NMT 26

- **UNSATURABLE MATTER, Appendix VII**
  Acceptance criteria: NMT 3.5%

**OTHER REQUIREMENTS**

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

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

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

DHA from Algal (Ulkenia) Oil. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received. The test method for Mercury is consistent with other FCC oil monographs, and the test methods for Arsenic, Cadmium, and Lead are consistent with newer FCC oil monographs using ICP technology.

(Fl: K. Laurvick) C95149

Add the following:

DHA from Algal (Ulkenia) Oil

Ulkenia DHA Oil

DESCRIPTION

DHA from Algal (Ulkenia) Oil occurs as a slightly waxy to liquid, light yellow to orange colored oil providing a source of docosahexaenoic acid (DHA, C$_{22}$H$_{32}$O$_{2}$) (C22:6 n-3), an omega-3 long-chain polyunsaturated fatty acid. It is obtained from fermentation of a thraustochytrid microalgae, Ulkenia sp., followed by extraction and refining. Extraction can be pure pressing or supported by solvents approved for food processing. Solvents, if used, are subsequently removed by vacuum distillation. The oil may be degummed, deacidified, winterized, bleached, and deodorized to substantially remove free fatty acids, phospholipids, odor and flavor components, and other material. Docosahexaenoic acid is the main polyunsaturated fatty acid present; DHA content may be standardized with other oils. Suitable antioxidants may be added.

Function: Source of DHA

Packaging and Storage: Store in tight, light-resistant containers, under inert gas and below 5°. 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 time of the peak of the docosahexaenoic acid methyl ester from the Sample Preparation corresponds to that from the Standard Solution. The percentage of the fatty acids (calculated using the results from the corresponding methyl esters) from the Sample Preparation, meets the requirements for each fatty acid as indicated in the table below.

<table>
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<tr>
<th>Fatty Acid Shorthand Notation</th>
<th>Lower Limit (area %)</th>
<th>Upper Limit (area %)</th>
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<tr>
<td>Myristic acid 14:0</td>
<td>1.5</td>
<td>4.5</td>
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<tr>
<td>Stearic acid 18:0</td>
<td>0.5</td>
<td>2.0</td>
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<tr>
<td>Eicosapentaenoic acid 20:5 n-3</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Docosapentaenoic acid 22:5 n-6</td>
<td>8.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Docosapentaenoic acid 22:5 n-3</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Docosahexaenoic acid 22:6 n-3</td>
<td>40.0</td>
<td>55.0</td>
</tr>
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</table>

ASSAY

• **DHA**, Fatty Acid Composition (Saturated, cis-Monounsaturated, and cis-Polyunsaturated) in Oils Containing Long Chain Polyunsaturated Fatty Acids, Appendix VII
Acceptance criteria: NLT 40.0% docosahexaenoic acid (DHA), as the percentage of total fatty acids (w/w)

**IMPURITIES**

Inorganic Impurities
- **Arsenic**, *Elemental Impurities by ICP*, Appendix IIIC
  - Acceptance criteria: NMT 0.1 mg/kg
- **Cadmium**, *Elemental Impurities by ICP*, Appendix IIIC
  - Acceptance criteria: NMT 0.1 mg/kg
- **Lead**, *Elemental Impurities by ICP*, Appendix IIIC
  - 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**: 5–6 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 solution of water and nitric acid (4:1). [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 ng, 60 ng, 100 ng, 200 ng, 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
- [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 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:** 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:** 5–6 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 vs. 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) to 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 = 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 0.1 mg/kg

**SPECIFIC TESTS**
- **Acid Value, Appendix VII**
  **Acceptance criteria:** NMT 0.5
- **Peroxide Value, Appendix VII**
  **Acceptance criteria:** NMT 5.0 mEq/kg
- **Unsaponifiable Matter, Appendix VII**
  **Acceptance criteria:** NMT 4.5%
OTHER REQUIREMENTS

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

1. CEM Model MDS-2100, or equivalent.

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

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

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</table>
| Monograph      | Kristie Laurvick  
Scientific Liaison  
1-301-816-8356 | (Fl2010) Monographs - Food Ingredients |
BRIEFING

Ferrous Citrate, FCC 7 page 396. On the basis of comments and data received, it is proposed to revise the Acceptance criteria in the Assay to represent food-grade materials currently in commerce. Comments from interested parties are encouraged.

(FI: C. Mejia) C71286

Ferrous Citrate
FeC₆H₆O₇

DESIGNATION

Ferrous Citrate occurs as a slightly gray-green powder or as white crystals.

Function: Nutrient

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION

- Citrate, Appendix IIIA
  Sample solution: 100 mg/mL
  Acceptance criteria: Passes test

- Iron (Ferrous Salts), Appendix IIIA
  Sample solution: 100 mg/mL
  Acceptance criteria: Passes tests

ASSAY

Change to read:

- Ferrous Iron Content
  Sample: 400 mg
  Analysis: Dissolve the Sample in 20 mL of 16:100 sulfuric acid, add 5 mL of 85% phosphoric acid, dilute with approximately 50 mL of water, and immediately titrate with 0.1 N ceric sulfate, using orthophenanthroline TS as the indicator. Perform a blank determination (see General Provisions), and make any necessary correction. Each mL of 0.1 N ceric sulfate is equivalent to 5.585 mg of Fe.
  Acceptance criteria: NLT 22.0% NLT 20.0% \( ^{\text{FCC}}_{8} \) of ferrous Fe

IMPURITIES

Inorganic Impurities

- Chloride, Chloride and Sulfate Limit Tests, Chloride Limit Test, Appendix IIIB
  Sample solution: Heat 100 mg of sample in a mixture of 25 mL of water and 2 mL of nitric acid until it dissolves. Cool, dilute with water to 100 mL, and mix. Take 10 mL of this solution and dilute to 30–40 mL with water.
  Control: 20 µg chloride (2 mL of Standard Chloride Solution)
  Analysis: Proceed as directed in the Procedure, beginning with “add 1 mL of silver nitrate TS…”
  Acceptance criteria: Any turbidity produced by the Sample solution does not exceed that shown in the Control. (NMT 0.2%)

- Ferric Iron
Sample: 2 g
Analysis: Dissolve the Sample in a mixture of 100 mL of water and 10 mL of hydrochloric acid contained in a 250-mL glass-stoppered flask, add 3 g of potassium iodide, shake well, and allow the mixture to stand in the dark for 5 min. Titrate any liberated iodine with 0.1 N sodium thiosulfate, using starch TS as an indicator. Perform a blank determination (see General Provisions), and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 5.585 mg of ferric iron.

Acceptance criteria: NMT 3.0%

- **Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**
  - Sample: 1 g
  - Acceptance criteria: NMT 2 mg/kg

- **Sulfate, Chloride and Sulfate Limit Tests, Chloride Limit Test, Appendix IIIB**
  - Sample solution: Dissolve 500 mg of sample in 1 mL of 2.7 N hydrochloric acid, and dilute to 30-40 mL with water.
  - Control: 300 µg sulfate (30 mL of Standard Sulfate Solution)
  - Analysis: Proceed as directed in the Procedure, beginning with “add 3 mL of barium chloride TS…”
  - Acceptance criteria: Any turbidity produced by the Sample solution does not exceed that shown in the Control. (NMT 0.06%)

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

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<tr>
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<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(Fl2010) Monographs - Food Ingredients</td>
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</table>

FCC Seventh Edition Page 396
BRIEFING

Gellan Gum, FCC 7 page 425. On the basis of comments received, it is proposed to add a new Additional Information section to describe standardization practices common for materials of commerce and their proper labeling. It is also proposed to update the monograph Description to reflect the taxonomic reclassification of the microorganism producing Gellan Gum to Sphingomonas elodea.

(FL: J. Moore) C101671

Gellan Gum
INS: 418

CAS: [71010-52-1]

DESCRIPTION

Change to read:

Gellan Gum occurs as an off-white powder. It is a high-molecular-weight polysaccharide gum produced by fermentation of a carbohydrate with a pure culture of *Pseudomonas elodea* ▲Sphingomonas elodea (previously identified as *Pseudomonas elodea*, but later reclassified), purified by recovery with isopropyl alcohol, dried, and milled. It is a heteropolysaccharide comprising a tetrasaccharide-repeating unit of one rhamnose, one glucuronic acid, and two glucose units. The glucuronic acid is neutralized to mixed potassium, sodium, calcium, and magnesium salts. It may contain acyl (glyceryl and acetyl) groups as the O-glycosidically linked esters. It is soluble in hot or cold deionized water.

Function: Stabilizer; thickener

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION

- **A. Procedure**

  **Sample solution:** Prepare a 1% solution by dissolving 1 g of sample in 99 mL of deionized water. Using a motorized stirrer and a propeller-type stirring blade, stir the mixture for about 2 h. [Note—Save part of this solution for Identification test B.]

  **Analysis:** Draw a small amount of the Sample solution into a wide-bore pipet, and transfer it into a solution of 10% calcium chloride.

  **Acceptance criteria:** A tough, worm-like gel forms instantly.

- **B. Procedure**

  **Sample solution:** Use the Sample solution from Identification test A.

  **Analysis:** Add 0.5 g of sodium chloride to the Sample solution, heat the solution to 80°, stirring constantly, and hold the temperature at 80° for 1 min. Stop heating and stirring the solution, and allow it to cool to room temperature.

  **Acceptance criteria:** A firm gel forms.

ASSAY

- **Alginates Assay, Appendix IIIC**

  **Sample:** 1.2 g, undried

  **Acceptance criteria:** A sample yields NLT 3.3% and NMT 6.8% of carbon dioxide (CO₂), calculated on the dried basis.
IMPURITIES

Inorganic Impurities
- **Lead, Lead Limit Test, Appendix IIIB**
  - **Sample solution**: Prepare as directed for organic compounds using 2 g of sample.
  - **Control**: 4 µg Pb (4 mL of Diluted Standard Lead Solution)
  - **Acceptance criteria**: NMT 2 mg/kg

Organic Impurities
- **Isopropyl Alcohol**
  - **IPA standard solution**: 1 mg/mL of isopropyl alcohol (chromatography grade) in water
  - **TBA standard solution**: 1 mg/mL of tert-butyl alcohol (chromatography grade) in water
  - **Mixed standard solution**: Pipet 4 mL each of the IPA standard solution and the TBA standard solution into a 125-mL graduated Erlenmeyer flask, dilute to about 100 mL with water, and mix. The solution contains about 40 µg/mL each of isopropyl alcohol and tert-butyl alcohol.
  - **Sample**: 5 g
  - **Sample solution**: Disperse 1 mL of a suitable antifoam emulsion, such as Dow-Corning G-10, or equivalent, in 200 mL of water contained in a 1000-mL 24/40 round-bottom distilling flask. Add the Sample, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill about 100 mL, adjusting the heat so that foam does not enter the column. Add 4.0 mL of TBA standard solution to the distillate to obtain the Sample solution.

Chromatographic system, Appendix IIA
- **Mode**: Gas chromatography
- **Detector type**: Flame-ionization
- **Column**: 1.8-m × 3.2-mm (id) stainless steel, or equivalent, packed with 80- to 100-mesh Porapak QS, or equivalent
- **Temperature**
  - **Column**: 165°C
  - **Injection port**: 200°C
  - **Detector**: 200°C
  - **Carrier gas**: Helium
  - **Flow rate**: 80 mL/min
  - **Injection volume**: About 5 µL

**Analysis**: Inject the Mixed standard solution and separately inject the Sample solution. From the chromatogram of the Mixed standard solution, determine the areas of the isopropyl alcohol and tert-butyl alcohol peaks and calculate the response factor, F, from the formula:

\[
F = \frac{A_{IPA}}{A_{TBA}}
\]

- \(A_{IPA}\) = area of the isopropyl alcohol peak
- \(A_{TBA}\) = area of the tert-butyl alcohol peak

[Note—The retention times of isopropyl alcohol and tert-butyl alcohol are about 2 min and 3 min.]

From the chromatogram of the Sample solution, calculate the isopropyl alcohol content, in mg/kg, in the portion of the sample taken by the formula:

\[
\text{Result} = \frac{(S_{IPA} \times 4000)}{(F \times S_{TBA} \times W)}
\]
\[ S_{IPA} = \text{area of the isopropyl alcohol peak in the Sample preparation chromatogram} \]

\[ S_{TBA} = \text{area of the tert-butyl alcohol peak in the Sample preparation chromatogram} \]

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

**Acceptance criteria:** NMT 0.075%

**SPECIFIC TESTS**

- **Loss on Drying, Appendix IIIC:** 105° for 2.5 h
  
  **Acceptance criteria:** NMT 15.0%

**ADDITIONAL INFORMATION**

*Add the following:*

Materials of commerce are often comprised of FCC Gellan Gum standardized with significant amounts of FCC Sucrose or other suitable sugars to create a material suitable for applications requiring specific functionality at low levels of gellan gum, the ratio of which will be determined based on the natural variation of the gellan gum. Such standardized materials should be identified as the standardized form (gellan gum standardized with sugar) to indicate the presence and type of any added FCC-grade or other suitable sugars, and cannot be identified as pure FCC Gellan Gum unless the material meets the monograph requirements.

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

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<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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FCC Seventh Edition Page 425
BRIEFING

(+)-Limonene, page 1476 of the First Supplement to FCC 7. On the basis of comments and data received, a revision to the Acceptance criteria for Angular Rotation is proposed. According to comments received, the food-grade materials of commerce are of a higher enantiomeric purity than the material described by the existing Acceptance criteria. It is therefore proposed to adjust the range of the Angular rotation specifications to reflect materials currently available. Comments of interested parties are encouraged.

(F: C. Mejia) C104150

(+)-Limonene 1S (FCC7)

d-Limonene 1S (FCC7)

d-p-Mentha-1,8-diene

Cinene

\[
\text{CH}_3
\]

\[
\text{H}_2\text{C} = \text{CH}_3
\]

\[\text{C}_{10}\text{H}_{16}\]

DESCRIPTION

Change to read:

(+)-Limonene 1S (FCC7) occurs as a colorless liquid. It may contain a suitable antioxidant.

Odor: Mildly citrus, free from camphoraceous and terpene notes
Solubility: Slightly soluble in glycerin; miscible in alcohol, most fixed oils; insoluble or practically insoluble in propylene glycol, water

Boiling Point: ~177°
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.

\[ (+)-\text{Limonene} \]

ASSAY

• **Procedure:** Proceed as directed under M-1a, Appendix XI.
  **Acceptance criteria:** NLT 93.0% of C₁₀H₁₆

SPECIFIC TESTS

• **Refractive Index**, Appendix II: At 20°
  **Acceptance criteria:** 1.471–1.474

• **Specific Gravity:** Determine at 25° by any reliable method (see General Provisions).
  **Acceptance criteria:** 0.838–0.843

OTHER REQUIREMENTS

Change to read:
• **Angular Rotation**, Optical (Specific) Rotation, Appendix IIB: Use a 100-mm tube.
  **Acceptance criteria:** Between +96° and +104°; +104° and +125°

• **Peroxide Value**, M-11, Appendix XI
  **Acceptance criteria:** NMT 5.0

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

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<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**

**Neohesperidine Dihydrochalcone.** Because there is currently no FCC monograph for this food ingredient, a new monograph is proposed based on the Neohesperidin-Dihydrochalcone monograph in the *European Pharmacopoeia*, version 6.8. Specifications in this monograph are also based on Commission Directive 95/31/EC (July 5, 1995), which includes criteria for the purity of this ingredient as a sweetener in foodstuffs. Specifications for *Organic Impurities, Related Compounds* are not calculated on the dried basis, which is consistent with the *European Pharmacopoeia* monograph. Data and comments related to these specifications are encouraged.

(FI: K. Laurick)  C99744

**Add the following:**

▲Neohesperidine Dihydrochalcone

1-{4-{[2-O-(6-Deoxy-α-L-mannopyranosyl)-β-d-glucopyranosyl]oxy}-2,6-dihydroxyphenyl}-3-(3-hydroxy-4-methoxyphenyl)propan-1-one

Hesperetin dihydrochalcone-4'-'β-neohesperidoside

NHDC

Neohesperidin-Dihydrochalcone

Neohesperidine DC

\[
\begin{align*}
\text{C}_{28}\text{H}_{36}\text{O}_{15} & & \text{Formula wt 612.6} \\
\text{INS: 959} & & \text{CAS: [20702-77-6]} \\
& & \text{FEMA: 3811}
\end{align*}
\]

**DESCRIPTION**

Neohesperidine Dihydrochalcone occurs as a white to off-white or yellowish-white powder. It is a flavonoid dihydrochalcone, and is practically insoluble in water and in methylene chloride, soluble in methanol, and freely soluble in dimethyl sulfoxide.

**Function:** Sweetener, flavor enhancer

**Packaging and Storage:** Store in well-closed containers protected from light.
IDENTIFICATION

• **A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix III**
  
  **Reference standard:** USP Neohesperidine Dihydrochalcone 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 for the principal peak in the chromatogram of the *Sample solution* corresponds to that in the chromatogram of the *Standard solution*, as obtained in the Assay.

ASSAY

• **PROCEDURE**
  
  **Solution A:** 0.5% (v/v) glacial acetic acid in water
  
  **Mobile phase:** Solution A and acetonitrile (20:80)
  
  **Sample solution:** 1.0 mg/mL in dimethyl sulfoxide
  
  **System suitability solution:** Suspend 100 mg of Neohesperidine Dihydrochalcone in 10.0 mL of a 100-g/L solution of concentrated sulfuric acid in water. Heat the suspension for 5 min on a water bath. Immediately dilute 1.0 mL of the resulting solution with dimethyl sulfoxide to 50.0 mL. [Note—Related compounds F\textsuperscript{1} and G\textsuperscript{2} are prepared in situ in this solution.]
  
  **Standard solution:** 1.0 mg/mL of USP Neohesperidine Dihydrochalcone RS in dimethyl sulfoxide
  
  **Chromatographic system,** Appendix IIA
  
  **Mode:** High-performance liquid chromatography
  
  **Detector:** UV 282 nm
  
  **Column:** 15-cm × 3.9-mm column packed with octadecylsilane chemically bonded to spherical silica particles, 3–10 µm in diameter, with a carbon loading of 7%
  
  **Flow rate:** 1.0 mL/min
  
  **Injection size:** 10 µL
  
  **System suitability**
  
  **Samples:** System suitability solution and Standard solution
  
  **Suitability requirement 1:** The resolution, R, between the peaks for neohesperidine dihydrochalcone (the first peak) and related compound F (the second peak) is NLT 2.5 for the System suitability solution.
  
  **Suitability requirement 2:** The relative standard deviation is NMT 2.0% for the peak response of the main peak in the chromatogram of the Standard solution.
  
  **Analysis:** Separately inject the Standard solution and the Sample solution into the chromatograph, record the chromatograms, and measure the peak responses for neohesperidine dihydrochalcone in each chromatogram.
  
  [Note—The approximate relative retention times are 0.4 for related compound B, 0.7 for related compound D, 1.0 for neohesperidine dihydrochalcone, 1.2 for related compound F, and 3.7 for related compound G. The run time for the experiment should be 5 times the retention time of neohesperidine dihydrochalcone. The chromatogram obtained from the Standard solution should be similar to the chromatogram provided with USP Neohesperidine Dihydrochalcone RS.]

  Calculate the percentage of Neohesperidine Dihydrochalcone in the sample taken:
  
  \[
  \text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100
  \]
\[ r_U = \text{peak response for neohesperidine dihydrochalcone from the chromatogram of the Sample solution} \]
\[ r_S = \text{peak response for neohesperidine dihydrochalcone from the chromatogram of the Standard solution} \]
\[ C_S = \text{concentration of Neohesperidine Dihydrochalcone in the Standard solution (mg/mL)} \]
\[ C_U = \text{concentration of the Sample solution (mg/mL)} \]

**Acceptance criteria:** NLT 96.0% of C\textsubscript{29}H\textsubscript{36}O\textsubscript{15}, calculated on the dried basis

**IMPURITIES**

**Inorganic Impurities**

- **Arsenic**, *Elemental Impurities by ICP, Method I, Appendix IIIIC*
  
  **Acceptance criteria:** NMT 3 mg/kg, calculated on the dried basis

- **Lead**, *Elemental Impurities by ICP, Method I, Appendix IIIIC*
  
  **Acceptance criteria:** NMT 2 mg/kg, calculated on the dried basis

**Organic Impurities**

- **Related Compounds**
  
  **Solution A** and **Mobile phase:** Prepare as directed in the Assay.
  
  **Sample solution:** 2.0 mg/mL in dimethyl sulfoxide
  
  **Standard solution A:** Use the **Standard solution**, as prepared in the Assay.
  
  **Standard solution B:** 40 µg/mL of USP Neohesperidine Dihydrochalcone Related Compound B RS\textsuperscript{3} in dimethyl sulfoxide
  
  **Standard solution C:** Dilute 1.0 mL of **Standard solution A** with dimethyl sulfoxide to 100.0 mL. (Contains 10 µg/mL of USP Neohesperidine Dihydrochalcone RS.)
  
  **Standard solution D:** Use the **System suitability solution**, as prepared in the Assay.

**Chromatographic system**, Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** UV 282 nm
- **Column:** 15-cm × 3.9-mm column packed with octadecylsilane chemically bonded to spherical silica particles, 3–10 µm in diameter, with a carbon loading of 7%
- **Flow rate:** 1.0 mL/min
- **Injection size:** 10 µL

**System suitability**

- **Sample:** **Standard solution D**
- **Suitability requirement:** The resolution, R, between the peaks for neohesperidine dihydrochalcone (the first peak) and related compound F (the second peak) is NLT 2.5.

**Analysis:** Separately inject each of the **Standard solutions** and the **Sample solution** into the chromatograph, record the chromatograms, and measure the peak areas for neohesperidine dihydrochalcone in each chromatogram. Compare the peak areas obtained for each related compound to the relevant standard, as described in the **Acceptance criteria**, disregarding all peaks with a peak area of less than 0.05 times the area of the principal peak in the chromatogram obtained from **Standard solution C**. [Note—The approximate relative retention times are 0.4 for related compound B, 0.7 for related compound D, 1.0 for neohesperidine dihydrochalcone, 1.2 for related compound F, and 3.7 for related compound G. The run time for the experiment should be 5 times the retention time of neohesperidine dihydrochalcone. The chromatogram obtained from **Standard solution A** should be similar to the chromatogram provided with USP Neohesperidine Dihydrochalcone RS.]

**Acceptance criteria**
Related compound B: The peak area for neohesperidine dihydrochalcone related compound B is NMT the area of the principal peak in the chromatogram obtained from Standard solution B (NMT 2%).

Related compound D: The peak area for related compound D is NMT twice the area of the principal peak in the chromatogram obtained from Standard solution C (NMT 2%).

Any other single related compound: The peak area for any other single related compound is NMT 0.5 times the area of the principal peak in the chromatogram obtained from Standard solution C (NMT 0.5%).

Total of all related compounds (apart from related compound B): The total of the peak areas for all related compounds, apart from neohesperidine dihydrochalcone related compound B, is NMT 2.5 times the area of the principal peak in the chromatogram obtained from Standard solution C (NMT 2.5%).

SPECIFIC TESTS

• Loss on Drying, Appendix II C: 105° for 3 h
  Acceptance criteria: NMT 11%

• Residue on Ignition (Sulfated Ash), Appendix II C
  Sample: 1.0 g
  Acceptance criteria: NMT 0.2%, calculated on the dried basis

1 Related compound F is: 1-[4-{β-d-glucopyranosyloxy}-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperetin-dihydrochalcone dihydrochalcone 7'-glucoside).

2 Related compound G is: 3-(3-hydroxy-4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (hesperetin-dihydrochalcone).

3 Related compound B is: 7-{[2-O-(6-deoxy-α-L-mannopyranosyl)]β-d-glucopyranosyl[oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one (neodiosmin).

4 Related compound D is: 1-[4-{[2-O-(6-deoxy-α-L-mannopyranosyl)]β-d-glucopyranosyl[oxy]-2,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)propan-1-one (naringin-dihydrochalcone).

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| Monograph     | Kristie Laurvick  
Scientific Liaison  
1-301-816-8356 | (FI2010) Monographs - Food Ingredients |
BRIEFING

Ponceau 4R. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed, based on the Ponceau 4R 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 United States 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. To measure Total Color, the JECFA monograph uses a titrimetric procedure, whereas the EU specifies the use of a spectrophotometric procedure. The proposed FCC monograph averages the results from both procedures, consistent with other color additive monographs in FCC such as Fast Green.

3. 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 IIIIB. Stakeholders are encouraged to submit a more modern lead procedure validated for this ingredient.

4. A proposed test procedure and a specification for Uncombined Intermediates and Products of Side Reactions are based on those in JECFA. Stakeholders are encouraged to submit information about a supplier and appropriate concentrations for reference standards used in this test procedure.

5. Proposed specifications in the tests for Loss on Drying, Chloride, and Sulfates in the Combined Tests section are based on those in JECFA, but they use different test procedures—ones already existing in FCC Appendix IIIC for the analysis of colors.

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

7. 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.

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

(FI: J. Moore) C100610

Add the following:

▲Ponceau 4R!

CI Food Red 7

Cochineal Red A

New Coccine

CI No. 16255

Class: Mono-Azo
Trisodium-2-hydroxy-1-(4-sulfonato-1-naphthylazo)-6,8-naphthalenedisulfonate

C_{20}H_{11}N_{2}Na_{3}O_{10}S_{3}·1.5 H_{2}O

INS: 124

CAS: [2611-82-7]

DESCRIPTION
Ponceau 4R occurs as reddish powder or granules. It is principally the trisodium salt of 2-hydroxy-1-(4-sulfonato-1-naphthylazo)-6,8-naphthalenedisulfonate and subsidiary coloring matters, with sodium chloride and/or sodium sulfate as the principal uncolored components. It is soluble in water and sparingly soluble in ethanol.

Function: Color

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION
• VISIBLE ABSORPTION SPECTRUM
  Sample solution: Dissolve a sample in water and dilute appropriately.
  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 505 nm.

ASSAY
• TOTAL COLOR, Colors, Methods I and II, Appendix III C: Both methods must be used.
  Method I: (Spectrophotometric)
    Sample solution: 10 mg/mL
    Analysis: Determine as directed at 505 nm, using 0.043 L/(mg·cm) for the absorptivity (a) for Ponceau 4R.
  Method II: (TiCl_{3} Titration)
    Sample: 0.7–0.8 g
    Analysis: Determine as directed, except under Procedure, use 15 g of Sodium Bitartrate instead of 21–22 g, and use 150 mL of water instead of 275 mL. For the calculation, use 6.337 as the stoichiometric factor (F_{S}) for the disodium salt of Ponceau 4R.
    Acceptance criteria: The average of results obtained from Method I and Method II is NLT 85% total coloring matters.

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

Organic Impurities
• UNCOMBINED INTERMEDIATES AND PRODUCTS OF SIDE REACTIONS
**Solution A:** 0.2 N ammonium acetate  
**Solution B:** Methanol  
**Mobile phase:** See Table 1.

### Table 1

<table>
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<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
<th>Comments</th>
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<tr>
<td>0</td>
<td>98</td>
<td>2</td>
<td>Analysis</td>
</tr>
<tr>
<td>49</td>
<td>0</td>
<td>100</td>
<td>Wash</td>
</tr>
<tr>
<td>55</td>
<td>0</td>
<td>100</td>
<td>Return to initial gradient and column equilibration</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>0</td>
<td></td>
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</table>

**Standard solution:** 25 µg/mL of 4-amino-1-naphthalenesulfonic acid, 25 µg/mL of 7-hydroxy-1,3-naphthalenedisulfonic acid, 25 µg/mL of 3-hydroxy-7-naphthalenesulfonic acid, 25 µg/mL of 6-hydroxy-2-naphthalenesulfonic acid, and 25 µg/mL of 7-hydroxy-1,3,6-naphthalenetrisulfonic acid in 0.02 M ammonium acetate  
**Sample solution:** 5 mg/mL in 0.02 M ammonium acetate

**Chromatographic system,** Appendix IIA  
**Mode:** High-performance liquid chromatography  
**Detector:** UV-Vis  
**Column**  
- **Guard column:** 4.6-mm × 15-mm 5-µm C18 column  
- **Analytical column:** 4.6-mm × 25-cm 5-µm C18 column  
**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 percentage of all impurities (4-amino-1-naphthalenesulfonic acid, 7-hydroxy-1,3-naphthalenedisulfonic acid, 3-hydroxy-7-naphthalenesulfonic acid, 6-hydroxy-2-naphthalenesulfonic acid, and 7-hydroxy-1,3,6-naphthalenetrisulfonic acid) in the portion of the sample taken:

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

- \( r_U \) = peak area for analyte in the Sample solution  
- \( r_S \) = peak area for analyte in the Standard solution  
- \( C_S \) = concentration of analyte in the Standard solution (µg/mL)  
- \( C_U \) = concentration of sample in the Sample solution (mg/mL)  
- \( F \) = mg-to-µg conversion factor, 1000

**Acceptance criteria:** NMT 0.5% for all five impurities combined

### SPECIFIC TESTS

**Combined Tests**

**Tests**  
- Loss on Drying (Volatile Matter), Colors, Appendix III C  
- Chloride, Sodium Chloride, Colors, Appendix III C  
- Sulfates (as sodium salts), Sodium Sulfate, Colors, Appendix III C

**Acceptance criteria:** NMT 20%, 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 Ponceau 4R 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 Ponceau 4R (505 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 composed of a glass tank (A) and cover (B); frame to support chromatography paper (C); solvent tray (D); wire 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.*
Chromatographic solvent: Prepare a mixture of 2-butanone, acetone, and water (7:3:3). Shake for 2 min, allow the layers to separate, and use the upper layer.

Sample solution: 10 mg/mL sample

Standard solution: 0.1 mg/mL sample prepared by diluting the Sample solution

Application volume: 0.10 mL

Analysis: No less than 2 h before analysis, arrange the filter-paper drapes in the glass tank, and pour sufficient 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 to separate chromatography sheets 0.1 mL aliquots of Sample solution and Standard solution, as uniformly as possible within the confines of the 7-mm × 18-cm 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°C in a drying cabinet for 5 min followed by 15 min at room temperature. Mount the dried sheets, together with two plain sheets to act as blanks on the supporting frame. [Note—if required, several dried sheets may be developed simultaneously.]

Pour sufficient 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 solvent front to ascend approximately 17 cm above base line, then allow for 1 hr of further development. Remove the supporting frame and transfer it to a drying cabinet at 50–60°C for 10–15 min. Remove the sheets from the frame.

For the Sample solution sheets, 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. For the Standard solution sheet, cut the entire band from the sheet, and cut an equivalent strip from the corresponding position of the plain (blank) sheet. Place each strip, subdivided into a suitable number of approximately equal portions, in a separate test tube. Add 5.0 mL of a mixture of water and acetone (1:1 by volume) 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 505 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 505 nm and correct the absorbances of the colored extracts with the blank values.

Calculate the percentage of 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 and expressed as a decimal
A_s = the absorbance from the Standard solution
A_b + A_c + ... + A_n = the sum of the absorbances of the subsidiary coloring matters from the Sample solution, corrected for the blank values

Acceptance criteria: NMT 1%

• **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, reextracted into acid, 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 with water at room temperature to the mark. Dilute 10.0 mL of this solution with water to 100 mL, 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, 2.0 mL of the R salt solution, and 10.0 mL of the Sodium carbonate solution, and dilute with water to volume.

  **Sample solution:** Add 2.0 g of the sample to 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 2.0 mL of the R salt solution, 10 mL of the Sodium carbonate solution, and 10.0 mL of the Sample solution, and dilute with water to volume.

  **Analysis:** Pipet 10-mL aliquots of each of the Standard solutions and the Sample solution into separate clean, dry test tubes. Cool the tubes for 10 min by immersion in a beaker of ice water, and 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 the R salt solution and 10 mL of the 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 with water to the mark, 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 percentage of unsulfonated primary aromatic amine (as aniline) in the portion of the sample taken:
Result = \(\frac{W_A}{W} \times 100\)

\(W_A\) = weight of aniline in the Sample solution calculated from the standard curve (g/100 mL)
\(W\) = 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%

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1 Ponceau 4R is approved for use in some countries but banned in others, such as the United States.

2 Whatman No 1, or equivalent

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BRIEFING

Sodium Molybdate Dihydrate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Sodium Molybdate Dihydrate monograph prepared by the British and European Pharmacopoeias (7th ed.), and on comments and data received. Interested parties are encouraged to submit comments.

1. Impurities proposed are consistent with those specified in the British and European Pharmacopoeias. Comments pertaining to alternative methods of analysis for these impurities and to their relevance in current articles of commerce are especially requested.

2. The method and limits proposed for Arsenic, Cadmium, Lead, and Mercury are based on comments and data received, as well as on the existing method in the FCC, because the method referenced by the British and European Pharmacopoeias includes insufficient detail. Interested parties are encouraged to submit comments and data pertinent to these methods, their limits, and relevance in current articles of commerce.

(FIEC: C. Mejia) C101746

Add the following:

Sodium Molybdate Dihydrate
Molybdic Acid Disodium Salt, Dihydrate
Disodium Molybdate Dihydrate
Sodium Molybdate (VI)

\[ \text{Formula wt } 241.9 \]
\[ \text{CAS: [10102-40-6]} \]

DESCRIPTION

Sodium Molybdate Dihydrate occurs as a white or almost white powder, or colorless crystals. It is freely soluble in water.

Function: Nutrient

Packaging and Storage: Store in a tightly sealed container in a cool, dry place, away from direct light and moisture.

IDENTIFICATION

- Sodium, Appendix IIIA
  
  Sample solution: 75 mg/mL
  Acceptance criteria: A dense white precipitate is formed.

- Molybdenum
  
  Sample: 0.2 g
  Analysis: Dissolve the Sample in 5 mL of a mixture of equal volumes of nitric acid and water, and add 0.1 g of ammonium chloride. Add 0.3 mL of a 9% w/v solution of dibasic sodium phosphate, and heat slowly at
50°–60°. A yellow precipitate is formed.

Acceptance criteria: Passes test

- **INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix III C**
- Reference standard: USP Sodium Molybdate Dihydrate 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*.

**ASSAY**

- **PROCEDURE**
  - Sample: 0.100 g
  - Analysis: Dissolve the dry *Sample* in 30 mL of water, add 0.5 g of hexamethylenetetramine and 0.1 mL of a 250 g/L solution of nitric acid. Heat until the mixture reaches 60°, and titrate with 0.05 M lead nitrate using 4-(2-pyridylazo) resorcinol monosodium salt as indicator. Each mL of 0.05 M lead nitrate is equivalent to 10.30 mg of Na$_2$MoO$_4$.
  - Acceptance criteria: 99.0%–101.0% on the dried basis

**IMPURITIES**

Inorganic Impurities

- **Ammonium**

  - Ammonium standard solution: Transfer 0.741 g of ammonium chloride to a 1000-mL volumetric flask, and dilute with water to volume. Transfer 10 mL of this solution to a 1000-mL volumetric flask, and dilute again with water to volume. Transfer 400 mL of this solution to a 1000-mL volumetric flask, and dilute further with water to volume. Each mL contains 1 ppm of NH$_4^+$.
    - Note—Prepare this solution immediately before use.

  - Silver manganese paper: Immerse strips of slow filter paper into a solution containing 8.5 g/L of manganese sulfate and 8.5 g/L of silver nitrate. Maintain strips in the solution for a few minutes, remove, and allow to dry over diphosphorus pentoxide protected from acid and alkaline vapors.
  - Sample: 0.1 g
  - Analysis: Transfer the *Sample* to a 25-mL container fitted with a cap, dissolve in 1 mL of water, and add 0.30 g of magnesium oxide. Place a 5-mm square of Silver manganese paper wetted with a few drops of water under the polyethylene cap, and close immediately. Swirl, avoiding projections of liquid, and allow to stand at 40° for 30 min. The silver manganese paper shows a grey color that should not be more intense than that of a standard prepared using 1 mL of *Ammonium standard solution*, 1 mL of water, and 0.30 g of magnesium oxide.
  - Acceptance criteria: NMT 10 mg/kg

- **Chloride, Chloride and Sulfate Limit Tests, Chloride Limit Test, Appendix IIIB**
  - Sample: 400 mg
  - Control: 20 µg of chloride (2 mL of *Standard Chloride Solution*).
  - Note—Prepare this solution immediately before use.
  - Acceptance criteria: Any turbidity produced by the *Sample* does not exceed that produced by the *Control* (NMT 50 mg/kg).

- **Phosphates**

  - Phosphate standard solution: Transfer 0.286 g of monobasic potassium phosphate to a 1000-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains 200 ppm of PO$_4^{3-}$.
[Note—Prepare this solution immediately before use.]

**Sample:** 2 g

**Analysis:** Mix the Sample with 13 mL of water, and heat. Then dissolve 8.0 g of ammonium nitrate in the still hot solution. Add this solution to 27 mL of a mixture of equal volumes of nitric acid and water. Prepare the standard solution at the same time and in the same manner by dissolving 1.0 g of ammonium nitrate in 12 mL of water and adding 1 mL of the Phosphate standard solution. Any yellow color or opalescence in the sample solution should not be more intense within 3 h than the prepared standard solution.

**Acceptance criteria:** NMT 200 mg/kg of PO$_4^{3-}$

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

- **Cadmium**, *Elemental Impurities by ICP, Method I*, Appendix IIIC
  **Acceptance criteria:** NMT 5 mg/kg

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

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

**SPECIFIC TESTS**

- **Loss on Drying**, Appendix IIIC: 140° for 3h
  **Acceptance criteria:** 14.0%–16.0%

▲FCC8

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

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<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

**Sodium Phosphate, Dibasic, FCC 7 page 954.** On the basis of comments and data received, to add modern methods of analysis that reflect current practices in the industry, and to account for the lack of specificity of the current Assay, it is proposed to add a new test to determine pyrophosphate content and acceptance criteria. Comments by interested parties are encouraged.

(FI: C. Mejia) C101601

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**Sodium Phosphate, Dibasic**

Disodium Monohydrogen Phosphate

Disodium Phosphate

\[ \text{Na}_2\text{HPO}_4 \]

Formula wt, anhydrous 141.96

\[ \text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} \]

Formula wt, dihydrate 177.99

INS: 339(ii)

CAS: anhydrous [7558-79-4]

dihydrate [10028-24-7]

---

**DESCRIPTION**

Sodium Phosphate, Dibasic occurs as a white, crystalline powder or as granules. It may be anhydrous or contain two molecules of water of hydration. The anhydrous form is hygroscopic. Both forms are freely soluble in water and insoluble in alcohol.

**Function:** Emulsifier; texturizer; buffer; nutrient

**Packaging and Storage:** Store in tightly closed containers.

---

**IDENTIFICATION**

- **P**HOSPHA**T**E, Appendix IIIA
  - **Sample solution:** 50 mg/mL
  - **Acceptance criteria:** Passes test

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

---

**ASSAY**

- **PROCEDURE**
  - **Sample:** 6.5 g, previously dried at 105° for 4 h
  - **Analysis:** Transfer the Sample into a 250-mL beaker. Add 50.0 mL of 1 N hydrochloric acid and 50.0 mL of water, and stir until the sample is completely dissolved. Place the electrodes of a suitable pH meter in the solution, and titrate the excess acid with 1 N sodium hydroxide to the inflection point occurring at about pH 4. Record the buret reading, and calculate the volume (A) of 1 N hydrochloric acid consumed by the sample:
A = 50 − x

x = volume of 1 N sodium hydroxide used in the titration (mL)

Continue the titration with 1 N sodium hydroxide until the inflection point occurring at about pH 8.8 is reached, record the buret reading, and calculate the volume (B) of 1 N sodium hydroxide required in the titration between the two inflection points (pH 4 to pH 8.8). When A is equal to or less than B, each mL of the volume A of 1 N hydrochloric acid is equivalent to 142.0 mg of Na₂HPO₄. When A is greater than B, each mL of the volume 2B − A of 1 N sodium hydroxide is equivalent to 142.0 mg of Na₂HPO₄.

Acceptance criteria: NLT 98.0% of Na₂HPO₄, on the dried basis

IMPURITIES

Inorganic Impurities

- Arsenic, Arsenic Limit Test, Appendix IIIB
  Sample solution: 1 g in 35 mL of water
  Acceptance criteria: NMT 3 mg/kg
- Fluoride, Fluoride Limit Test, Method IV, Appendix IIIB
  Sample: 2 g
  Acceptance criteria: NMT 0.005%
- Lead, Lead Limit Test, APDC Extraction Method, Appendix IIIB
  Acceptance criteria: NMT 4 mg/kg

SPECIFIC TESTS

Add the following:

- Pyrophosphate
  Mobile phase: 20 mM to 70 mM KOH linear gradient, electrochemically generated, from 0 to 20 min
  Standard stock solution: In a 100-mL volumetric flask, dissolve 0.256 g of tetra sodium pyrophosphate decahydrate (> 99% Na₄P₂O₇·10H₂O) in water and dilute to volume. The resulting solution contains 1000 µg/mL of pyrophosphate anion (P₂O₇⁴⁻).
  Standard solutions: 0.5 µg/mL, 2.0 µg/mL, and 10 µg/mL of pyrophosphate anion in water, from the Standard stock solution
  System suitability solutions: Add commercial orthophosphate ion chromatographic standard (1000 µg/mL) to the 10 µg/mL Standard solution for a final solution of 10 µg/mL orthophosphate (PO₄³⁻).
  Sample solution: 100 µg/mL sample in water
  Chromatographic system, Appendix II A
    Mode: High-performance liquid chromatography, ion chromatography¹
    Detector: Electrolytic conductivity detector with eluent suppression
    Column: 2-mm × 25-cm, anion-exchange analytical column², and 2-mm × 50-mm anion–exchange guard column³
    Flow rate: About 0.25 mL/min
    Injection size: 10 µL
  System suitability
    Samples: System suitability solutions and 0.5 µg/mL Standard solution
    Suitability requirements
Suitability requirement 1: Resolution of NLT 5 between 10 µg/mL orthophosphate (PO$_4^{3-}$) and pyrophosphate (P$_2$O$_7^{4-}$) anions (from the System suitability solutions)

Suitability requirement 2: Signal-to-noise ratio is NLT 10 for an injection of 0.5 µg/mL pyrophosphate Standard solution, where peak height is expressed in µs, and baseline noise is the maximum deflection of the baseline (µs) in a blank at the retention time of pyrophosphate over the same baseline peak width in min.

[Note—Conditions may be adjusted for other analytical conditions or brands of equipment.]

Analysis: Establish an eluent flow through the columns until a stable baseline is obtained. 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 calibration curve by plotting the peak areas versus concentrations of the Standard solutions in µg/mL pyrophosphate ion. From the calibration curve, determine the concentration ($C_U$) of pyrophosphate in the Sample solution in µg/mL. Calculate the percentage of pyrophosphate in the sample:

$$\text{Result} = \frac{C_U}{C_{smp}} \times 100$$

$C_U$ = concentration of pyrophosphate in the Sample solution determined from the standard curve (µg/mL)

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

To determine % tetra sodium pyrophosphate (Na$_4$P$_2$O$_7$) multiply the Result by 1.53.

Acceptance criteria: NMT 2% pyrophosphate calculated as Na$_4$P$_2$O$_7$\textsuperscript{FCC8}

- Loss on Drying, Appendix IIC: 120° for 4 h

Acceptance criteria

Anhydrous: NMT 5.0%

Dihydrate: 18.0%–22.0%

- Insoluble Substances

Sample: 10 g

Analysis: Dissolve the Sample in 100 mL of hot water, and pass through a tared filtering crucible (not glass).

Wash the insoluble residue with hot water, dry at 105° for 2 h, cool, and weigh.

Acceptance criteria: NMT 0.2%

---

1. Ion exchange chromatograph AG11 ICS-2000, ICS-3000 Dionex Corporation (Sunnyvale, CA), or equivalent.

2. IonPac AS11 (Dionex Corporation, Sunnyvale, CA), or equivalent.

3. IonPac (Dionex Corporation, Sunnyvale, CA), or equivalent.

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

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(F2010) Monographs - Food Ingredients

FCC Seventh Edition Page 954
**Stearyl Alcohol.** Because there is currently no *FCC* monograph for this food ingredient, a new monograph is proposed based on the *USP 32–NF 27* monograph for *Stearyl Alcohol*. Specifications for *Copper* and *Zinc* are included because of their potential use as catalysts in the manufacture of this ingredient. Limited data has been received to support these limits. Comments including data are encouraged, particularly in reference to *Copper* and *Zinc* specifications and modernization of the Assay method.

(FL: K. Laurvick) C100473

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**Add the following:**

▲Stearyl Alcohol

1-Octadecanol

Octadecyl Alcohol

\[ \text{C}_{18}\text{H}_{38}\text{O} \]

Formula wt 270.5

CAS: [112-92-5]

**DESCRIPTION**

Stearyl Alcohol occurs as colorless to white granules or flakes. It is manufactured by the transesterification and distillation of unrefined coconut or palm oil using a zinc catalyst and in the presence of methanol. The resulting methyl esters are then hydrogenated in the presence of a copper catalyst. It is purified and catalysts are removed through fractional distillation. Stearyl Alcohol is soluble in alcohol, in ether, in benzene, and in acetone, and is insoluble in water.

*Function:* Texturizer

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

**IDENTIFICATION**

- **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 *System suitability solution*, as obtained in the Assay.

**ASSAY**

- **PROCEDURE**

  **System suitability solution:** Prepare a solution containing 9 mg/mL of USP Stearyl Alcohol RS and 1 mg/mL of USP Cetyl Alcohol RS in dehydrated alcohol.

  **Sample solution:** 10 mg/mL in dehydrated alcohol

  **Chromatographic system,** Appendix II A

  **Mode:** Gas chromatography

  **Detector:** Flame ionization

  **Column:** 2-m × 3-mm column packed with 10% liquid phase dimethylpolysiloxane gum on a support of siliceous earth for gas chromatography that has been flux-calcined by mixing diatomite Na\(_2\)CO\(_3\) flux
and calcining above 900°. The silaceous earth may be silanized.

**Carrier gas:** Helium

**Temperature**
- **Injection port:** 275°
- **Column:** 205°
- **Detector:** 250°

**Injection volume:** 2 µL

**System suitability**
- **Sample:** System suitability solution

**Suitability requirement 1:** The resolution, R, between cetyl alcohol and stearyl alcohol is NLT 4.0.

**Suitability requirement 2:** The relative standard deviation for replicate injections, calculated with the area ratio of stearyl alcohol to cetyl alcohol, is NMT 1.5%.

**Analysis:** Inject the *Sample solution* into the chromatograph, record the chromatogram, and measure the areas for the major peaks. Calculate the percentage of $C_{18}H_{38}O$ in the portion of the sample taken:

$$\text{Result} = \left( \frac{r_U}{r_S} \right) \times 100$$

$r_U$ = peak area for stearyl alcohol obtained from the chromatogram of the *Sample solution*

$r_S$ = sum of the areas of all of the peaks (excluding the solvent peak) obtained from the chromatogram of the *Sample solution*

**Acceptance criteria:** NLT 90.0%

**IMPURITIES**

**Inorganic Impurities**
- **Copper,** *Elemental Impurities by ICP,* Appendix IIIC
  - **Acceptance criteria:** NMT 1 mg/kg
- **Lead,** *Elemental Impurities by ICP,* Appendix IIIC
  - **Acceptance criteria:** NMT 1 mg/kg
- **Zinc,** *Elemental Impurities by ICP,* Appendix IIIC
  - **Acceptance criteria:** NMT 1 mg/kg

**SPECIFIC TESTS**
- **Acid Value,** Appendix VII
  - **Acceptance criteria:** NMT 2
- **Hydroxyl Value**
  - **Sample:** 2 g

**Analysis:** Transfer the *Sample* to a dry, glass-stoppered, 250-mL flask, and add 2 mL of pyridine, followed by 10 mL of toluene. To the mixture add 10.0 mL of a solution of acetyl chloride in toluene (10:90 v/v). Insert the stopper into the flask, and immerse in a water bath heated at 60°–65° for 20 min. Add 25 mL of water, again insert the stopper into the flask, and shake vigorously for several minutes to decompose the excess acetyl chloride. Add 0.5 mL of phenolphthalein TS, and titrate to a permanent pink endpoint with 1 N sodium hydroxide, shaking the flask vigorously toward the end of the titration to maintain the contents in an emulsified condition. Perform a blank titration (see *General Provisions*). Calculate the hydroxyl value of the sample taken:
Result = \((V_B - V_S) \times 56.1 \times N \times 1/W\)

- \(V_B\) = volume of titrant consumed in the blank determination (mL)
- \(V_S\) = volume of titrant consumed in the Sample determination (mL)
- \(N\) = exact normality of the sodium hydroxide used in the titration (mol/L)
- \(W\) = weight of Sample used (g)

Acceptance criteria: Between 195 and 220

- **Iodine Value**, Appendix VII
  - Acceptance criteria: NMT 2
- **Melting Range or Temperature**, Appendix IIB
  - Acceptance criteria: 55°–60°

---

1 OV-1 on SULFECOPORT (available at www.sigma-aldrich.com), or equivalent.

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<td>1-301-816-8356</td>
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BRIEFING

Xanthan Gum, FCC 7 page 1091. On the basis of comments and data received, it is proposed to remove the Pyruvic Acid test and specification from the monograph. Pyruvic acid has historically been used to aid in substantiating the identity of this ingredient based on its presence above 1.5%, but is no longer a suitable for this purpose as new fermentation technologies have been developed to minimize the content of this constituent in “reduced pyruvate” xanthan gum products. The removal of this test and specification is consistent with a change made in the xanthan gum monograph in Japan’s Specifications and Standards for Food Additives, 8th Edition. FCC is also seeking a validated modern instrumental method for determining the pyruvate content of xanthan gum to include in the FCC as an optional method for interested users. Such methods can be submitted to Jeff Moore, Ph.D. at JM@usp.org.

(F: J. Moore) C101672

Xanthan Gum
INS: 415
CAS: [11138-66-2]

DESCRIPTION

Change to read:
Xanthan Gum occurs as a cream-colored powder. It is a high-molecular-weight polysaccharide gum produced by a pure culture fermentation of a carbohydrate with Xanthomonas campestris, purified by recovery with isopropyl alcohol or ethanol, dried, and milled. It contains d-glucose and d-mannose as the dominant hexose units, along with d-glucuronic acid and pyruvic acid, and it is prepared as the sodium, potassium, or calcium salt. The pyruvic acid content of this ingredient is variable depending on the fermentation process used, and in the case of “reduced-pyruvate” materials may be less than 1.5%. It is readily soluble in hot or cold water, but it is insoluble in alcohol. Its solutions are neutral.

Function: Stabilizer; thickener; emulsifier; suspending agent; bodying agent; foam enhancer

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION

• Procedure
  Sample solution: Transfer 300 mL of water, previously heated to 80°, into a 400-mL beaker and stir rapidly with a mechanical stirrer. At the point of maximum agitation, add a dry blend of 1.5 g of sample and 1.5 g of locust bean gum. Stir until the gums dissolve, and then continue stirring for 30 min longer. Do not allow the water temperature to drop below 60°. Discontinue stirring, and allow the solution to cool at room temperature for at least 2 h.
  Control solution: Prepare as described above for the Sample solution, but using a 1% solution of sample and omitting the locust bean gum.
  Acceptance criteria: For the Sample solution, a firm, rubbery gel forms after the temperature of the mixture drops below 40°. No such gel forms in the Control solution.

ASSAY

• Alginites Assay, Appendix IIIC
  Sample: 1.2 g
  Acceptance criteria: 4.2%–5.4% of carbon dioxide (corresponds to 91.0%–117.0% of xanthan gum),
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

**Change to read:**

**Organic Impurities**

- **ETHANOL AND ISOPROPYL ALCOHOL**
  
  - **EtOH standard solution:** 1 mg/mL of ethanol (chromatography grade) in water
  - **IPA standard solution:** 1 mg/mL of isopropyl alcohol (chromatography grade) in water
  - **TBA standard solution:** 1 mg/mL of tert-butyl alcohol (chromatography grade) in water
  - **Mixed standard solution:** Pipet 4 mL each of the EtOH standard solution, the IPA standard solution, and the TBA standard solution into a 125-mL graduated conical flask, dilute to about 100 mL with water, and mix. The solution contains about 40 µg/mL each of ethanol, isopropyl alcohol, and tert-butyl alcohol.
  
  - **Sample:** 5 g
  - **Sample solution:** Disperse 1 mL of a suitable antifoam emulsion, such as Dow-Corning G-10, or equivalent, in 200 mL of water contained in a 1000-mL 24/40 round-bottom distilling flask. Add the Sample and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill about 100 mL, adjusting the heat so that foam does not enter the column. Add 4.0 mL of TBA standard solution to the distillate to obtain the Sample solution.

**Chromatographic system, Appendix IIA**

- **Mode:** Gas chromatography
- **Detector type:** Flame-ionization
- **Column:** 1.8-m × 3.2-mm (id) stainless steel, or equivalent, packed with 80- to 100-mesh Porapak QS, or equivalent
- **Temperature**
  - **Column:** 165°
  - **Injection port:** 200°
  - **Detector:** 200°
- **Carrier gas:** Helium
- **Flow rate:** 80 mL/min
- **Injection volume:** About 5 µL

**Analysis:** Inject the Mixed standard solution and separately inject the Sample solution. From the chromatogram of the Mixed standard solution, calculate the response factors for ethanol and isopropyl alcohol:

\[ R_{\text{EtOH}} = \frac{A_{\text{EtOH}}}{A_{\text{TBA}}} \]

\[ R_{\text{IPA}} = \frac{A_{\text{IPA}}}{A_{\text{TBA}}} \]
From the chromatogram of the Sample solution, calculate the concentrations of ethanol and isopropyl alcohol in the Sample taken:

\[
\text{EtOH (mg/kg)} = \frac{(S_{\text{EtOH}} \times 4000)}{(R_{\text{EtOH}} \times S_{\text{TBA}} \times W)}
\]

\[
\text{IPA (mg/kg)} = \frac{(S_{\text{IPA}} \times 4000)}{(R_{\text{IPA}} \times S_{\text{TBA}} \times W)}
\]

- **Pyruvic Acid**

**Sample solution:** Dissolve 600.0 mg of sample in sufficient water to make 100 mL. Transfer 10.0 mL of this solution into a 50 mL glass-stoppered flask. Pipet 20 mL of 1 N hydrochloric acid into the flask, weigh the flask, and reflux for 3 h, taking precautions to prevent loss of vapors. Cool to room temperature, and add water to make up for any weight loss during refluxing. Pipet 1.0 mL of a 1:200 solution of 2,4-dinitrophenylhydrazine prepared in 2 N hydrochloric acid into a 30 mL separatory funnel; add 2.0 mL of the refluxed solution, mix, and allow the mixture to stand at room temperature for 5 min. Extract the mixture with 5 mL of ethyl acetate, and discard the aqueous layer. Extract the hydrazone from the ethyl acetate with three 5 mL portions of sodium carbonate TS, collecting the extracts in a 50 mL volumetric flask. Dilute to volume with sodium carbonate TS, and mix.

**Standard solution:** Transfer 45.0 mg of pyruvic acid into a 500 mL volumetric flask, dilute with water and mix. Transfer 10.0 mL of the solution into a 50 mL glass-stoppered flask and continue as directed under Sample solution, beginning with "Pipet 20 mL of 1 N hydrochloric acid into the flask..."

**Analysis:** Determine the absorbance at 375 nm of each solution with a suitable spectrophotometer using 1-cm cells. Use sodium carbonate TS as the blank.

**Acceptance criteria:** The absorbance of the Sample solution is equal to or greater than that of the Standard solution (NLT 1.5%).

SPECIFIC TESTS

- **Loss on Drying**, Appendix IIC: 105° for 2.5 h
  - **Acceptance criteria:** NMT 15.0%
- **Viscosity**, Viscosity of Cellulose Gum, Appendix IIB
  - **Sample solution:** 10 mg/mL of xanthan gum and 10 mg/mL of potassium chloride
  - **Analysis:** Prepare a pair of identical Sample solutions and stir each for 2 h. Determine the viscosity (V₁) of one solution at 23.9°, using a No. 3 spindle rotating at 60 rpm (Brookfield, or equivalent). Determine the
viscosity (V₂) of the other solution in the same manner, but maintain the temperature at 65.6°C.

**Acceptance criteria**

\( V₁ \): NLT 600 cp  
\( (V₁/V₂) \): 1.02–1.45

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| Monograph      | Jeffrey Moore, Ph.D.  
Scientific Liaison  
1-301-816-8288 | (FI2010) Monographs - Food Ingredients |

*FCC Seventh Edition Page 1091*
BRIEFING

Appendix III: Chemical Tests and Determinations, page 1611 of the Second Supplement to FCC 7 and FCC Forum [December 2010]. On the basis of comments received, two revisions are proposed under B. Limit Tests. Comments are encouraged.

1. A revision is proposed to the Procedure in Method I for Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method. Comments received indicate that the sensitivity parameters are instrument specific and could vary from the range 0.14-0.25 abs-sec. Also, the normal linearity is instrument specific. Thus, the instrument's sensitivity and normal linearity determination should be done according to the instrument manufacturer's specifications. Additionally, to clarify that only water should be used to dilute the sample during Sample Digestion, it is proposed to add the phrase "with water" to this step.

2. Because of the toxic nature of powdered metallic selenium, its partial insolubility in a nitric acid solution, and based on comments and data received, a revision is proposed to Reagents and Solutions under Selenium Limit Test to add the alternative of a commercially available selenium stock solution for the preparation of the Selenium Standard Solution.

(FI: C. Mejia) C103872; C103874

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 mg 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.
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 mg 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-mL, 2.0-mL, 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 µg/mL, 0.02 µg/mL, 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:

\[ \text{Result} = \frac{C_A}{C_S} \]

in which \( C_A \) 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 Figure 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 Figures 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,
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 mL, 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 mL 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°) 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 nm 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 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 535–540 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 mL contains 12.5 µg of cadmium.

Sample Solution Transfer 10 g of the 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 the 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 mL, 2.00 mL, 3.00 mL, 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 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 1.5 µg/mL, 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 µg/mL. 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 µg, of cadmium in each mL of the Test Solution containing 0 mL of the Standard Preparation. Calculate the quantity, in mg/kg, 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 (SO₄) 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 mL 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 mL of the final solution contains 10 µg of sulfate (SO₄).

**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 Figure 15.

![Figure 15. Closed-System Vacuum Distillation Apparatus for 1,4-Dioxane.](image-url)
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 30–40 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 2–4 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°–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 15–20 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/mL) 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 5.0–5.5 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 the 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 mL (prepared by dissolving 22.2 mg of sodium fluoride, previously dried at 200°C 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 mg/kg, 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 µg/mL, 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/mL)**  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 mL, 2.0 mL, 3.0 mL, 5.0 mL, 10.0 mL, 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.\(^1\)\(^S\) (FCC7) with µg of F per 100 mL solution on the logarithmic scale.

**Procedure**  Transfer 1.00 g of the 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 µg, of the sample from the **Calibration Curve**.\(^1\)\(^S\) Determine the percentage of fluoride in the sample taken:

\[
\text{Result} = \left( \frac{C}{W_S} \right) \times 0.000001 \times 100\%
\]

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 µg to grams.\(^1\)\(^S\) (FCC7)

**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 mL.

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.
**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 the 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°–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 mL 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
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 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 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 the 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°–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 [Pb(NO₃)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 µg/mL, 1.0 µg/mL, 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 µg/mL, 5.0 µg/mL, 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 µg/mL, 10.0 µg/mL, 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°C, 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 µg/mL. Determine the lead concentration in the Sample Preparation by reference to the calibration curve. Calculate the quantity of lead, in mg/kg, in the sample taken:

\[
\text{Result} = 10C/W_S
\]

in which \(C\) is the concentration, in µg/mL, of lead from the standard curve; and \(W_S\) is the weight, in grams, of the sample taken.

Change to read:

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 B008-7600 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 5–10 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 ng/mL, 50.0 ng/mL, 25.0 ng/mL, 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 the 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 the 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 90°–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 (20–30 min). Cool. Add 0.5 mL of 50% hydrogen peroxide dropwise, heat at 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide, dropwise, and heat at 90°–100° for 5 to 10 min until clear. Cool, and dilute with water 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 according to manufacturer's specifications 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:

\[
m₀ = \frac{(0.0044 \text{ abs-sec})(25 \text{ pg/µL})(20 \text{ µL})}{(\text{measured} \ 25 \text{ pg/µL abs-sec})}
\]

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 and determine the instrument linearity according to manufacturer's instructions. 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 7–10 replicates of the Sample Preparation Blank and calculated as follows:

\[
DL = (3)(s.d. \text{ blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})/(\text{abs-sec } 10 \text{ ng/mL std})
\]

\[
QL = (10)(s.d. \text{ blank abs-sec})(10 \text{ pg/µL})(20 \text{ µL})/(\text{abs-sec } 10 \text{ ng/mL std})
\]

During method development, detection limits were typically 10–14 pg, corresponding to 0.5–0.7 ng/mL for 20 µL. This corresponds to a method detection limit of 3.3–4.7 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 beyond the linearity range 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 ng/mL).

Calculation of Lead Content: Calculate the lead level in the original sample as follows:

\[
Pb (\text{ng/g}) = (\text{blank-corrected Pb ng/mL})(\text{DF})(\text{sample vol (10 mL)})/\text{(sample wt (approx. 1.5 g))}^2
\]

Quality Assurance To ensure analytical accuracy, National Institute of Standards and Technology (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 (31.8–38.8 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 12–15 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 kg, 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 mL of this solution contains the equivalent of 10 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 mL 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 mL, 0.5 mL, 1 mL, 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 µg, 0.05 µg, 0.1 µg, and 0.2 µg of lead per mL. (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 the 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 µg/mL, 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 µg/mL, of the Sample Solution.
Calculate the quantity, in mg/kg, of lead in the sample:

\[
\text{Result} = 10C/W
\]

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 mL.
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 mL.
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 mL.
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 mL.

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 1.0–1.5 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 Figure 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.
[Note—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\(_3\))·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 mL 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 Figure 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 with 1 N sulfuric acid to 500.0 mL. Each mL 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 mL 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 the 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 the sample in strong acetic acid TS\(^3\) dilute acetic acid TS\(^3\) (FCC7) 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 the 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\(^3\) 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\(^3\) 232.0 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, \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}\), 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°–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, \(\text{Zn(C}_2\text{H}_3\text{O}_2\text{)}_2 \cdot 2\text{H}_2\text{O}\), 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 mL, 10.0 mL, 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 20–25 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%–2% aqueous paste, place it in a cellophane tube, and dialyze against running distilled water for 30–40 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:

\[
\text{mg/kg P} = \frac{a \times 200 \times 1000}{V \times W}
\]

in which W is the weight, in g, of the sample taken.

**SELENIUM LIMIT TEST**

*Change to read:*

**Reagents and Solutions**

2,3-Diaminonaphthalene Solution: On the day of use, dissolve 100 mg of 2,3-diaminonaphthalene (C_{10}H_{10}N_2) and 500 mg of hydroxylamine hydrochloride (NH_2OH·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 mL of this solution contains the equivalent of 1 µg of selenium (Se). ▲ Alternatively, the solution may be prepared using a commercially available stock solution diluted to 1 µg/mL. ▲FCC8
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° for 2 h before combustion, unless otherwise directed.]

Upon completion of combustion, place a few mL 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 the 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 Figure 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 d-Glucurono-6,3-lactone**  This chemical (C_{6}H_{8}O_{6}) is available as a reference standard with an assay of 100.0 ± 1.0% (24.99 ± 0.25% CO_{2}) 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 the 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, raise the heating mantle, and heat the reaction mixture to boiling. After 2 h, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, 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 mL 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 the 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 mL 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**
Transfer between 700 mg and 2.2 g of the 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 mL of 0.5 N acid consumed is equivalent to 7.003 mg of ammonia nitrogen.

Calculate the percent ammonia nitrogen:

\[
\text{Result} = \left( \frac{\text{NH}_3-N}{S} \right) \times 100
\]

in which \( \text{NH}_3-N \) is the weight, in mg, of ammonia nitrogen, and \( S \) is the weight, in mg, of the 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 Figure 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 Figure 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 Figure 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 ($R_b$) relative to the Internal Standard:

$$\text{Result} = \frac{A_i \times W_i}{A_b \times W_b}$$

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; $W_b$ is the weight percent of benzene in Reference Solution A; and $A_b$ is the area of the benzene peak in arbitrary units corrected for attenuation.

**Procedure** Place approximately 0.1 mL of Internal Standard into a tared 25-mL volumetric flask, weigh on an analytical balance, and 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 the 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 percentage of benzene in the sample ($W_B$):

$$\text{Result} = \frac{A_b \times R_b \times W_i}{A_i \times S} \times 100$$

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 $B$ (Figure 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>
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<tr>
<td>Diameter, in (mm)</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>1/4(6.4)</td>
<td>1/8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1/8</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>Chromosorb P</td>
<td>Capillary</td>
<td>Chromosorb P</td>
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<td>Mesh</td>
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<td>60–80</td>
<td>80–100</td>
<td>60–80</td>
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<td>80–100</td>
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<td>Treatment</td>
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<td>AW</td>
<td>AW</td>
<td>none</td>
<td>AW</td>
<td>none</td>
<td>AW Sil</td>
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<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>
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<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>
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<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; El—Electronic integrator; FI—Flame ionization; Sil—Silanized; TC—Thermal conductivity; TCEPE—Tetracyanoethylated 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>—</td>
<td>12.8</td>
<td>8.5</td>
<td>6.5</td>
<td>—</td>
</tr>
</tbody>
</table>

**COLORS**
Chromium

Standards

Standard Chromium Solution (1000 mg/kg): Transfer 2.829 g of K$_2$Cr$_2$O$_7$, accurately weighed (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 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 40 mg/kg, 50 mg/kg, 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 2–3 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 Figure 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 nm 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 Brillant 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 Brillant Blue, \( r = 0.9706 \)).

Mercury

Apparatus: The apparatus used for the direct microdetermination of mercury is shown in Figure 22. It consists of a quartz combustion tube designed to hold a porcelain combustion boat (60 \( \times \) 10 \( \times \) 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-\( \times \) 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°C for 1 h.

**Ascarite:** 20- to 30-mesh.

**Copper Oxide Wire:** Preheated at 850°C 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°C, 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 µL, 2 µL, 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., Brilliant 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 2–3 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:

\[ P = \frac{(V \times N)}{W} \times 22.79 \]

in which V is the net volume, in mL, 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:
\[ P = \left[ (V - B) \times \frac{N}{W} \right] 	imes 55.4 \]

in which \( V \) is the volume, in mL, of barium chloride solution required to titrate the sample; \( B \) is the volume, in mL, 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:

\[ \% \text{total color} = \frac{A}{(a \times C \times b)} \times 100 \]

in which \( A \) is the absorbance; \( a \) is the absorptivity specified in the individual monograph (L/(mg·cm)); \( C \) 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 (TiCl\(_3\)) is shown in Figure 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 CO\(_2\) or 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:

\[ N = \frac{V_r N_r}{V_t - V_b} \]

in which \( V_r \) is the volume, in mL, 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 mL, of 0.1 N *Titanium Chloride Solution* used; and \( V_b \) is the volume, in mL, 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:

\[ T = \left( \frac{V_t - V_b}{W \times F_s} \right) \times 100 \times N \]

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:

\[ P = \left( \frac{W_p \times F}{W_s} \right) \times 100 \]

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**
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 the 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; a is the absorptivity given in the individual monograph; and b is the cell pathlength, in cm.

Method II

**Apparatus**  Use a suitable high-performance liquid chromatography system (see Chromatography, Appendix IIA) 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'-diazobenzanis(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°.
Pressure: 1000 psi.
Order of Elution: (1) Phenylhydrazine-p-sulfonic acid (PHSA); (2) sulfanilic acid (SA); (3) 1-(4-sulfophenyl)-3-ethylcarboxy-5-hydroxyopyrazolone (PY-T); (4) 1-(4-sulfophenyl)-3-carboxy-5-hydroxyopyrazolone (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°.
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) (DONS).

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 Figures 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.
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:

$$C_i = mA_i + b$$

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:

$$m = \frac{\sum C_i A_i - \sum C_i \sum A_i}{\sum A_i^2 - (\sum A_i)^2}$$

$$b = \overline{A}_i - m\overline{C}_i$$

in which $\overline{A}$ and $\overline{C}$ 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:

$$r = \frac{\sum (C_i - \overline{C})(A_i - \overline{A})}{\sum (C_i - \overline{C})^2 \times \sum (A_i - \overline{A})^2}$$

Each time the system is calibrated, add the new data to those accumulated from previous analyses. The correlation coefficient must be 0.95–1.00 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:

\[
C_s = mA_s + b
\]

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 1.5–2.5 g of colorant, accurately weighed, to a tared crucible. Heat in a vacuum oven at 135° for 12–15 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, \( I \):

\[
I = \left( \frac{W_c}{W_s} \right) \times 100
\]

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 the 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:

\[
C = \frac{(A \times V_1)/W}{V_2/V_3}
\]

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:

\[
C = \frac{(A \times V_1)/W}{(1 \, \mu g/1000 \, ng)(V_2/V_3)}
\]

where \(A\) is the instrument reading, ng/mL; and the other factors are as defined above.

**Method II: ICP–MS**

**Reagents** All reagents used for the preparation of the 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:

\[
C = \left(\frac{A \times V_1}{W}\right) \times \left(\frac{V_2}{V_3}\right)
\]

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:

\[
C = \left(\frac{A \times V_1}{W}\right) \times \left(\frac{1 \mu g/1000 \text{ ng}}{V_2/V_3}\right)
\]

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 mL (C_S).

Sample Preparation  Dilute 5 mg of the 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 mg/mL, of glutamic acid in the Sample Preparation:

\[
\text{Result} = A_U \times \frac{C_S}{A_S}
\]

in which C_S is the concentration, in mg/mL, of glutamic acid in the Standard Solution.
Calculate the percent glutamic acid, on the basis of total protein:

\[
\text{Result} = \frac{100 \times C_A}{6.25 \times N_T}
\]

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:

\[
\text{Result} = 100 \times \frac{C_A}{S_W}
\]

in which \(S_W\) is the weight, in mg, of the sample taken.

**HYDROXYPROPOXYL DETERMINATION**

**Apparatus** The apparatus for hydroxypropoxyl group determination is shown in Figure 27.

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 thermostat such that a temperature of 155\(^\circ\)C 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\(^\circ\)C 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\(^\circ\)C 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 mg:

\[
\text{Result} = 75.0 \times \left[ N_1 (V_a - V_m) - kN_2 (Y_a - Y_m) \right]
\]

in which \( N_1 \) is the exact normality of the 0.02 N sodium hydroxide solution, \( k = V_bN_1/Y_bN_2 \), and \( N_2 \) is the exact normality of the 0.02 N sodium thiosulfate solution.

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 Figure 28.

![Figure 28](image)

**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 Figure 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 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 fume hood.]**

Collect the colorless, or almost colorless, constant-boiling acid distilling between 126°–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 mL 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:

| 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 1 g 2S (FCC7) of the sample into a 500- to 800-mL Kjeldahl digestion flask of hard borosilicate, 2S (FCC7) moderately thick, well-annealed glass, wrapping the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer if desired. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid 2S (FCC7)

Place the flask in an inclined position (about 45°), 2S (FCC7) and heat gently, 2S (FCC7) keeping the temperature below the boiling point 2S (FCC7) 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.]

Increase the heat until the acid boils 2S (FCC7) briskly, 2S (FCC7) and continue the heating process 2S (FCC7) until the solution clears, and then continue boiling for 30 min longer (or for 2 h for samples containing organic material). Cool, add about 150 2S (FCC7) mL of water, mix, and then cool to below 25°. 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. 2S (FCC7) Add a few granules of zinc to prevent bumping, 2S (FCC7) and immediately, 2S (FCC7) 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 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 2S (FCC7) 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 2S (FCC7) (about 80% of the contents of the flask). 2S (FCC7) Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate 2S (FCC7) with 0.5 N sulfuric acid, determining the endpoint potentiometrically. 2S (FCC7) Perform a blank determination, substituting 2 g of sucrose for the sample, and make any necessary correction (see General Provisions). Each mL of 0.5 N acid 2S (FCC7) 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. Also, a bromocresol green–methyl red solution can be used as an alternative. To make the solution, dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 180 mL of alcohol, and dilute with water to a final volume of 200 mL.]

[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 mL of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

[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 a quantity of sample, accurately weighed, corresponding to about 150 mg of nitrogen into a Kjeldahl flask, and add 25 mL of 93%–98% sulfuric acid containing 1 g of salicylic acid. Mix thoroughly by shaking, and then allow to stand for 30 min or more, with frequent shaking. Add 5 g of Na2S2O3·5H2O (as an impalpable powder, not granules or filings), 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 the 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.

[CACaution—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 mL 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 2.6–15
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 200–400 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 Figure 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.

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 the
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:

<table>
<thead>
<tr>
<th>Reactant gas flow (oxygen), cm³/min</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrier-gas flow (Ar, He), cm³/min</td>
<td>40</td>
</tr>
<tr>
<td>Furnace temperature, ºC</td>
<td>700 (maximum)</td>
</tr>
<tr>
<td>Inlet zone</td>
<td>800 to 1000</td>
</tr>
<tr>
<td>Pyrolysis zone</td>
<td>800 (maximum)</td>
</tr>
<tr>
<td>Outlet zone</td>
<td>200</td>
</tr>
<tr>
<td>Tin-scrubber temperature, ºC</td>
<td></td>
</tr>
<tr>
<td><strong>Titration cell</strong></td>
<td>Stirrer speed set to produce slight vortex</td>
</tr>
<tr>
<td><strong>Coulometer</strong></td>
<td></td>
</tr>
<tr>
<td>Bias voltage, mV</td>
<td>160</td>
</tr>
<tr>
<td>Gain</td>
<td>50</td>
</tr>
<tr>
<td>Constant Rate Injector, µL/s</td>
<td>0.25</td>
</tr>
</tbody>
</table>

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 isoctane, and reweigh. Calculate the sulfur concentration (S), in percent:

\[ S = \frac{W_b}{W_s} \times 2.192 \times 10^5 \]

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 isoctane 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° isothermal, with 20 nL/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: 610-832-9585, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.