4.8.15

AOAC Official Method 996.16
Selenium in Feeds and Premixes
First Action 1996
Final Action 1997

[Applicable to determination of total Se in range of 0.2–5500 μg/g or greater in all types of feed ingredients, finished feeds, and premixes.]

**Caution:** See Appendix B, safety notes on safe handling of acids—nitric and perchloric acids. 2,3-Diaminonaphthalene is highly toxic and carcinogenic. It causes eye and skin irritation and is irritating to mucous membranes and upper respiratory tract; may be fatal if swallowed. When handling powder form, wear gloves and protective clothing and appropriate NIOSH/MSHA approved respirator, and carry out the operations in a fume hood. Wash hands thoroughly after handling. Keep 2,3-diaminonaphthalene tightly closed in cool, dry storage area. In case of contact, immediately flush eyes or skin with copious amounts of water and discard contaminated clothing. If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen. If swallowed, wash out mouth with water provided person is conscious and call physician. Sodium borohydride is a flammable, toxic solid which reacts with water or acid to release flammable H. Avoid contact with skin and store in cool dry place. Destroy reagent by acidifying in hood.

**Fluorometric Method**

Results of Interlaboratory Study:
See Tables 996.16A and B for the results of the interlaboratory study supporting acceptance of the method.

Accuracy of method was substantiated by in-house analyses of NIST Standard Reference Materials (SRM). Results of analyzing 5 replicates of 3 different SRMs were as follows (numbers in parenthesis are the certified values):

- NIST-1567-Wheat Flour
  - 0.964 ± 0.011 (1.1 ± 0.2)
- NIST-1577a-Bovine Liver
  - 0.692 ± 0.020 (0.71 ± 0.07)
- NIST-1643c-Trace Elements in Water
  - 0.0120 ± 0.0002 (0.0127 ± 0.0007)

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of labs</th>
<th>No. of outlier labs</th>
<th>Mean, μg/g</th>
<th>s_r</th>
<th>RSD_r, %</th>
<th>s_R</th>
<th>RSD_R, %</th>
<th>r^d</th>
<th>R^e</th>
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<td>56</td>
<td>33</td>
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<td>160</td>
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<td>760</td>
<td>13</td>
<td>990</td>
<td>2100</td>
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</tbody>
</table>

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* Different Se concentration ranges are separated by line. In ascending order they are: 0.2–2.0 μg/g; 2.0–10 μg/g; 10–100 μg/g; and 100–5500 μg/g.
* Outliers determined by Cochran’s/Grubbs’ test (1% level).
* No. of test samples.
* r = 2.8 · s_r.
* R = 2.8 · s_r.
A. Principle
All forms of Se are converted by oxidative digestion to inorganic form, presumably Se⁴⁺ or Se⁶⁺. HClO₄ in oxidation mixture prevents loss of Se. Selenium must be in Se⁴⁺ form to react with 2,3-diaminonaphthalene. All forms of Se in digestion mixture are converted to Se⁴⁺ by reduction with HCl. Digestion reduction procedure is also suitable for quantitation of Se by continuous hydride generation atomic absorption (HGAA) method. Matrices containing high amounts of soluble Fe (≥1 mg Fe/10 mL) may cause difficulties with fluorometric procedure, but problem can usually be detected by the appearance of a black precipitate during the derivatization procedure, E(d)(3), and corrected by dilution or by increasing amount of chelating agent.

B. Apparatus
(a) Fluorometer.—With excitation at 375 nm and emission at 450 nm. If possible, adjust fluorometer to 1 scale unit = 1 ng.
(b) Fume hood.—Suitable for handling HClO₄.
(c) Digestion system.—21 × 26 × 7.4 cm aluminum block with 80 holes (22 mm diameter) set on 30 × 30 cm hot plate. (Any commercially available heating block is suitable, if tests and standard solutions can be heated simultaneously.) Alternatively, microKjeldahl digestion system capable of holding 30 mL flasks or straight-walled tubes may be used.
(d) Digestion vessels.—For digestion system. Screw capped (Teflon lined) 20 × 150 mm test tubes; microKjeldahl flasks, 30 mL, or straight-walled tubes are acceptable.
(e) Extractor mechanized rotation unit.—Maintaining 60–70 rpm/min. Hand-held container allowing mixing of rack (4 rows of 10 tubes) of tubes is suitable.
(f) Pipettor.—Delivering 5 mL ± 1%. 
(g) H₂O baths.—(1) Maintaining 60°±2°C, and (2) boiling H₂O.
(h) Vortex mixer.
(i) Volumetric flasks.—100 and 1000 mL.
(j) Erlenmeyer flasks.—250–1000 mL and 2 L.
(k) Filter paper.—Qualitative paper, 11 μm retention.

C. Reagents
All reagents should be at least analytical grade. Use deionized H₂O distilled in glass for preparation of solutions and dilutions.
(a) Cyclohexane.
(b) Hydrochloric acid solution.—0.1M. Pipet 8.3 mL concentrated HCl into 1 L volumetric flask and dilute to volume with H₂O. Proportionate amounts for any convenient volume may be used.
(c) Nitric acid.—70%.
(d) Perchloric acid.—70%.
(e) 2,3-Diaminonaphthalene (DAN) reagent.—Weigh 1.0 g DAN powder (97% purity) and transfer to 2 L Erlenmeyer flask. Add 500 mL 0.1M HCl and warm 15 min in 60°C water bath. Stir to help dissolve powder. Dilute to 1 L with 0.1M HCl. Extract solution 3–5 min with 40–50 mL cyclohexane and discard cyclohexane layer. Repeat extraction 3×. Filter DAN reagent through filter paper pre-wet with 0.1M HCl. DAN reagent is stable at least 2 weeks, when protected from light in refrigerator.

(f) Ethylenedinitrilo)tetaacetic acid (EDTA) standard solutions.—(1) EDTA standard stock solution.—0.1M. Place 37.2 g (ethylenedinitrilo)tetaacetic acid, disodium salt, into 1 L volumetric flask and dilute to volume with H₂O. (2) EDTA working standard solution.—0.01M. Depending of number of tubes to be analyzed, dilute sufficient volume EDTA standard stock solution (1 + 9) with H₂O to provide 15 mL/tube (e.g., 600 mL for 40 tubes).

(g) Selenite standard solutions.—(1) Selenite standard stock solution.—0.4 μg Se/mL. Pipet 1.00 mL selenite standard solution (1000 μg Se/mL in 1% HNO₃; commercially available atomic absorption standard solution is suitable) into 1 L volumetric flask and dilute to volume with 0.1M HCl. From this solution, pipet 40 mL into 100 mL volumetric flask and dilute to volume with 0.1M HCl. (Note: As alternative, dissolve 0.400 g Se in HNO₃ in 1 L volumetric flask and dilute to volume with 0.1M HCl; dilute 10.0 mL of this solution to 1 L with 0.1M HCl in volumetric flask. Finally dilute 10 mL of this solution to 100 mL with 0.1M HCl in volumetric flask and use directly.) (2) Selenite calibration standard solutions.—Pipet 0.00 (reagent blank), 0.200, 0.500, 1.00, and 1.50 mL selenite standard stock solution into separate digestion vessels to obtain 0.00, 0.08, 0.200, 0.400, and 0.600 μg Se/vessel.

(h) Sodium selenite standard solution.—0.4 μg Se/mL. Transfer 0.1915 g anhydrous Na₂SeO₄ into 1 L volumetric flask, dilute to volume with H₂O. Mix well. From this solution, pipet 5.00 mL into 1 L volumetric flask and dilute to volume with H₂O.

D. Quality Assurance
Starting with digestion, with each set of test solutions run 2 reagent blanks and at least 4 selenite standard solutions, C(g)(2), (e.g., 0.080, 0.200, 0.400, and 0.600 μg Se/vessel); and one tube containing 0.500 mL sodium selenate solution, C(h), (0.2 μg Se/vessel) to check adequacy of reduction step, since selenate does not react with DAN. Recoveries of 95–105% are expected. Otherwise, reanalyze the entire set.

Appropriate NIST Standard Reference Materials (SRMs) can be included in analysis, e.g., NIST 1643c, trace elements in water (most convenient to use), NIST 1567a, wheat flour; and NIST 1577b, bovine liver. Predigestion steps for SRMs may be omitted. Transfer or weigh appropriate amounts of SRMs directly into digestion tubes.

E. Determination
(a) Pre-digestion.—Weigh ca 10 g premix or feed into 250–1000 mL Erlenmeyer flask and record weight to the nearest 10 mg (W). (Use the largest flask feasible to minimize foaming problems.) Add slowly and with care 75 mL HNO₃ and boiling chip (or several glass beads). (Caution: Matrices with large amounts of limestone or easily oxidizable materials may cause foaming when HNO₃ is added.) Heat on hot plate until as much of material is in solution as possible and nitric oxide fumes subside (usually 15 min are adequate).
Cool solution and dilute quantitatively with H₂O so that Se content falls between 0.04–0.60 μg/mL. Record final volume of diluted predigest solution to the nearest mL (V₁).

(b) Digestion.—If block digestion system is not available, perform digestion and reduction procedures in microKjeldahl flasks using alternative digestion and reduction procedure, G. Otherwise, proceed as follows:

1. Mix thoroughly predigest solution from (a) to suspend all undissolved materials. Pipet 1.00 mL aliquots into test tubes (digestion vessels). If Se content of predigest solution is low (<0.02 μg/mL), aliquot up to 10 mL can be used. Record volume to nearest 0.01 mL (V₂).

2. Add porous boiling head to each tube, including blanks, selenite calibrating standard solutions, and Na₂SeO₄ standard solution. If glass beads are used, add 2–3 beads.

3. Add 4 mL HNO₃ and 1 mL HClO₄ [or 5 mL HClO₄–HNO₃ mixture (1 + 4, v/v)] to each tube.

4. Place tubes in aluminum heating block. Raise temperature slowly to 210°C (ca 2 h). White fumes of HClO₄ should be visible in tubes at completion of digestion. After reaching white fume stage, heat additional 15 min.

5. Remove tubes from heating block. Cool tubes to room temperature and heating block to 110°–150°C.

(c) Reduction.—Add 0.5 mL concentrated HCl to tubes from (e)(5). Place tubes again in heating block and heat 30 min. Ensure that temperature is maintained between 110°–150°C for the entire period.

(d) Derivatization and quantitation.—(1) Remove tubes from heating block and let cool. It is critical that at this step tubes are at room temperature. (Note: Procedure may be interrupted at any time, up to and including this step.)

2. Add 15 mL EDTA working standard solution, C(f)(2), and 2 mL DAN reagent, C(e), to test tube. (Note: Both solutions may be added simultaneously; however, they should not be mixed together more than 10 min immediately before use or precipitate will form.) Mix each tube well on Vortex mixer, taking Vortex to the bottom of tube at least twice.

3. Place rack of tubes in 60°C H₂O bath and maintain 30 min. Ensure that H₂O level is above level of reaction mixture.

4. Remove rack from H₂O bath and cool tubes 5 min in running tap H₂O.

5. Add 5 mL cyclohexane to each tube. Cap tubes with Teflon lined cap. Extract 5–10 min in rotating extraction unit (60–70 rpm/min). [Note: Extraction can be performed manually by shaking (inverting) rack of tubes for period of time that gives maximum extraction.]

6. Transfer cyclohexane layer into fluorometer cuvettes. Ensure that solution is free of any suspended H₂O droplets that might adhere to wall of cuvette in light path.

7. Set excitation wavelength of fluorometer at 375 nm and emission at 525 nm. Zero fluorometer with cyclohexane and read blank to judge quality of DAN reagent. If reading is greater than 2–3 fluorescence units, DAN reagent should be extracted again with cyclohexane. Zero fluorometer against blank.

8. Determine fluorescence (F) of selenite calibrating standard solutions and calculate regression equation for standard curve. Use slope (k) in calculating Se concentrations in test solutions. Depending on equipment available, this may be automatically done by built-in calibration procedure. [Note: Fluorescence response is linear when using selenite calibrating standard solutions at concentrations described in (g)(2). Standards containing as high as 2 μg Se/vessel may maintain linear relationship.]


F. Calculations

Depending on support software of fluorometer used, calibration data, dilution factors, and test portion weights may be stored in computer and final content of Se [μg/g (ppm)] may be printed out. Report μg Se/g to 3 significant digits.

When using manual system, calculate Se content in test sample as follows:

$$\mu g \text{ Se/g} = \frac{F}{k} \times \frac{V_1}{V_2} \times \frac{1}{W_v}$$

where F = fluorescence reading; k = slope of regression line obtained from plotting μg Sе (x-axis) vs fluorescence reading (y-axis) (k = y/x); W_v = weight of test portion, g; V₁ = total volume of diluted predigest solution, mL; V₂ = aliquot of predigest solution used in analysis, mL.

G. Alternative Digestion and Reduction Procedure

When block digestion system is not available, perform digestion and reduction procedures in microKjeldahl flasks as follows:

1. Pipet 1.00 mL predigest solution from (a) into 30 mL microKjeldahl flask. If Se content of predigest solution is low (<0.02 μg/mL), aliquot up to 10 mL can be used. Record volume to nearest 0.01 mL (V₂).

2. Add boiling head to each flask. Add 4 mL HNO₃ and 1 mL HClO₄ into each flask [or 5 mL HClO₄–HNO₃ mixture (1 + 4, v/v)], including blanks, selenite calibrating standard solutions, and Na₂SeO₄ standard solution.

3. Place flasks on microKjeldahl digestion rack with fume collecting manifold connected to aspiration system that collects most of the fumes from flasks. (Note: Manifold and aspiration system may be omitted if perchloric acid hood is available.) Adjust heaters to gently boil contents of flasks.

4. Heat flasks until HNO₃ has evaporated and white fumes of HClO₄ are clearly visible in neck of flask. Heat flasks 15 min after white fumes of HClO₄ are observed.

5. Remove flasks from digestion rack, cool to room temperature, and add 0.5 mL concentrated HCl.

6. Place flasks in boiling H₂O bath and maintain 30 min. Remove flasks from H₂O bath and cool to room temperature.

7. Quantitatively transfer digest to test tubes fitted with Teflon lined caps using 15 mL EDTA working standard solution, C(f)(2). Add 2 mL DAN reagent, C(e).

8. Proceed to (d), Derivatization and quantitation.

Continuous Hydride Generation Atomic Absorption (HGAA) Method

Results of Interlaboratory Study:

See Tables 996.16C and D for the results of the interlaboratory study supporting acceptance of the method.
Accuracy of method was substantiated by in-house analyses of NIST Standard Reference Materials (SRM). Results of analyzing 5 replicates of 3 different SRMs were as follows (numbers in parenthesis are the certified values):

- NIST-1567-Wheat Flour: 0.955 ± 0.027 (1.1 ± 0.2)
- NIST-1577a-Bovine Liver: 0.704 ± 0.022 (0.71 ± 0.07)
- NIST-1643c-Trace Elements in Water: 0.0121 ± 0.0009 (0.0127 ± 0.0007)

H. Principle

Wet digestion procedure is identical to that used in fluorometric method. Digestion-oxidation procedure converts all forms of Se to inorganic form, presumably Se^{4+} or Se^{6+}. Presence of HClO_4 in oxidation mixture prevents loss of Se. Selenium has to be in Se^{4+} form to be converted to volatile hydride. All forms of Se in digestion mixture are converted to Se^{4+} by reduction with HCl.

I. Apparatus

(a) Atomic absorption (AA) spectrometer.—Double beam, with deuterium lamp background corrector, Se hollow cathode lamp, and continuous hydride generation accessory. Operating conditions: warm up time, ≥20 min; acetylene-air flame: acetylene, 12 psi; air, 60 psi; wavelength, 196 nm; lamp current, 10 mA; slit width, 1 nm; equilibration time, 30 s; integration time, 5 s; replicates, 3. Continuous hydride generation accessory: N gas purge, 50 psi; flow rate of test solution, 8 mL/min; flow rate of 0.6% NaBH_4 + 0.5% NaOH, 1.1 mL/min; flow rate of 10M HCl, 1.1 mL/min.

(b) Fume hood.—See B(b).

(c) Digestion system.—See B(c).

(d) Digestion vessels.—See B(d). Calibrate test tubes to 20 mL.

(e) Vortex mixer.

(f) Dispensing pipet.—To deliver 19.0 mL. Equivalent pipet is suitable.

(g) Volumetric flasks.—100 and 1000 mL.

(h) Erlenmeyer flask.—250–1000 mL.

J. Reagents

All reagents should be at least analytical grade. Deionized water distilled in glass should be used for preparation of solutions and dilutions.

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Table 996.16C Results of interlaboratory study for determination of selenium in feeds and premixes by HGAA method

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<th>Sample</th>
<th>No. of labs</th>
<th>No. of outlier labs</th>
<th>N</th>
<th>Mean, µg/g</th>
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<th>RSD_r, %</th>
<th>s_R</th>
<th>RSD_R, %</th>
<th>r^d</th>
<th>R^e</th>
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<tbody>
<tr>
<td>Dry dog food</td>
<td>8</td>
<td>0</td>
<td>16</td>
<td>0.249</td>
<td>0.031</td>
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<td>0.051</td>
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<td>13</td>
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<td>47</td>
<td>26</td>
<td>58</td>
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<td>69</td>
<td>11</td>
<td>110</td>
<td>18</td>
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<td>300</td>
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<td>7</td>
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<td>14</td>
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<td>540</td>
<td>10</td>
<td>540</td>
<td>10</td>
<td>1520</td>
<td>1520</td>
</tr>
</tbody>
</table>

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a Different Se concentration ranges are separated by line. In ascending order they are: 0.2–2.0 µg/g; 2.0–10 µg/g; 10–100 µg/g; and 100–5500 µg/g.
b Outliers determined by Cochran’s/Grubbs’ test (1% level).
c No. of test samples.
d r = 2.8 × s_r.
e R = 2.8 × s_R.
Table 996.16D Summary of results of interlaboratory study for determination of selenium in feeds and premixes by HGAA

<table>
<thead>
<tr>
<th>Selenium, µg/g</th>
<th>s₁</th>
<th>RSD₁, %</th>
<th>sₙ</th>
<th>RSDₙ, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2–2.0</td>
<td>0.031–0.14</td>
<td>9.3–13</td>
<td>0.051–0.41</td>
<td>21–36</td>
</tr>
<tr>
<td>2.0–10.0</td>
<td>0.18–1.1</td>
<td>4.4–18</td>
<td>0.26–1.5</td>
<td>10–19</td>
</tr>
<tr>
<td>10.0–100</td>
<td>1.3–6.1</td>
<td>2.8–10</td>
<td>1.3–10</td>
<td>4.0–13</td>
</tr>
<tr>
<td>100–6000</td>
<td>20–540</td>
<td>3.8–11</td>
<td>47–540</td>
<td>6.1–26</td>
</tr>
</tbody>
</table>

(a) Hydrochloric acid solutions.—(1) 1 + 1 (v/v).—For dilution. Dilute concentrated HCl with equal volume of H₂O and place in dispensing pipet, I(f). (2) 10M.—For hydride generation. Transfer 400 mL concentrated HCl into 500 mL volumetric flask and dilute to volume with H₂O.

(b) Nitric acid.—70%.

(c) Perchloric acid.—70%.

(d) Sodium hydroxide.

(e) Sodium borohydride solution.—For hydride generation. Dissolve 2.5 g NaOH and 3.0 g NaBH₄ in 500 mL H₂O. (Caution: Sodium borohydride is a flammable, toxic solid which reacts with water or acid to release flammable H gas. Avoid contact with skin and store in cool dry place. If necessary, the reagent may be destroyed by acidifying under a hood.)

(f) Selenite standard solutions.—(1) Selenite standard stock solution.—1 µg/mL. Pipet 1.00 mL Se standard solution (1000 µg Se/mL in 1% HNO₃; commercially available AA standard solution is suitable) into 1 L volumetric flask and dilute to volume with 0.1M HCl. (Note: As alternative, dissolve 1.000 g Se in HNO₃ in 1 L volumetric flask and dilute to volume with 0.1M HCl. Dilute 10.0 mL of this solution to 1 L with 0.1M HCl in volumetric flask; finally dilute 10.0 mL of this solution with 0.1M HCl to 100 mL in volumetric flask.) (2) Selenite calibrating standard solutions.—Pipet 0.500, 1.00, and 2.00 mL selenite standard stock solution into separate 100 mL volumetric flasks and dilute to volume with HCl solution (1 + 1, v/v), J(a)(f), to obtain Se concentration of 5.00, 10.0, and 20.0 ng/mL.

(g) Sodium selenate (Na₂SeO₄) standard solution.—0.4 µg Se/mL. See C(h).

(h) Quality control (QC) standard solution.—8.00 ng/mL. Pipet 40 mL selenite standard stock solution, (f)(f), into 100 mL volumetric flask and dilute to volume with 0.1M HCl. From this solution, pipet 2.00 mL into 100 mL volumetric flask and dilute to volume with HCl solution (1 + 1, v/v).

K. Quality Assurance

Starting with digestion, with each set of test solutions run 2 reagent blanks, and 1 tube containing 0.500 mL Na₂SeO₄ solution, J(g), (0.2 µg Se/vessel) to check adequacy of reduction step, since selenate is not reduced to Se hydride by NaBH₄. Recoveries of 95–105% are expected. Otherwise, reanalyze the entire set.

Appropriate SRMs can be included in analysis, e.g., NIST 1643c, trace elements in water (most convenient to use), NIST 1567a, wheat flour; and NIST 1577b, bovine liver. Predigestion steps for SRMs may be omitted. Transfer or weigh appropriate amounts of SRMs directly into digestion tubes.

L. Determination

(a) Predigestion.—See E(a).

(b) Digestion.—See E(b). (Note: If block digestion is not available, perform digestion and reduction procedures in microKjeldahl flasks using alternate digestion and reduction procedure, N.)

(c) Reduction.—After tubes from (b) have cooled to room temperature, add 0.5 mL concentrated HCl. Place tubes again in heating block and heat 30 min. Ensure that temperature is maintained between 110°–150°C for the entire period. Remove tubes from heating block and let cool to room temperature. Dilute to 20 mL with HCl solution (1 + 1, v/v), J(a)(f), and mix well using Vortex mixer or cap and mix by inverting several times.

(d) HGAA determination.—(1) Calibrate instrument using HCl solution (1 + 1, v/v) as reagent blank and selenate calibrating standard solutions, J(f)(f). At the beginning of analysis, calibration procedure should be repeated until absorbance values (particularly for 5 ng/mL Se working standard solution) remain constant. AA spectrometer response is not necessarily linear for range of Se standard working solutions used in analysis. Concentration of unknowns must be determined from standard curve.

(2) Read QC standard solution. Concentration of 8.0 ± 0.4 ng Se/mL should be obtained. Otherwise, recalibrate instrument.

(3) Determine content of Se (C; ng/mL) in test solution.

(4) Check Se content in QC standard solution every 10–12 tests. If Se content falls outside acceptable range (i.e., 8.0 ± 0.4 ng/mL), “reslope” calibration curve or recalibrate instrument.

(5) Read each test twice, but not consecutively. Average the 2 values. Readings should agree within 10%. Greater deviation indicates mixing or standardization problem and series should be re-analyzed.

If test reading is outside of calibration curve, dilute portion of test solution with HCl solution (1 + 1, v/v) and repeat analysis. Use appropriate dilution factor in calculations.

M. Calculations

It is assumed that all test solutions are diluted to 20 mL (final dilution) and that AA spectrometer is calibrated in ng Se/mL. Calculate Se content, µg/mL, as follows:

\[
\text{µg Se/mL} = \frac{C \times 20 \times \frac{V}{V_a} \times 1}{W_s}
\]

where C = Se content in each tube determined by HGAA, ng/mL; \(V_i\) = total volume of diluted predigest solution, mL; \(V_a\) = aliquot of predigest solution analyzed, mL; \(W_s\) = weight of test portion, g.

N. Alternative Digestion and Reduction Procedure

If block digestion system is not available, perform digestion and reduction procedures in microKjeldahl flasks as follows:

(1) Pipet 1.00 mL predigest solution from L(a) into 30 mL microKjeldahl flask. If Se content of predigest solution is low (µg/mL), aliquot up to 10 mL can be used. Record volume to nearest 0.01 mL (\(V_a\)).

(2) Perform steps G(2)–(6).

(3) After flasks have cooled to room temperature, dilute to 20 mL with HCl solution (1 + 1, v/v) and mix thoroughly. Transfer diluted digest to test tubes.

(4) Proceed to L(d), HGAA determination.

Reference: J. AOAC Int. 80, 469 (1997).

CAS-771-97-1 (2,3-diaminonaphthalene)

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