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The method for the “Determination of Vitamins D₂ and D₃ in Infant Formula and Adult Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS)” was adopted as AOAC Official First Action during the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting” held June 29, 2011. During the meeting, an Expert Review Panel (ERP) evaluated the available validation information against standard method performance requirements (SMPRs) articulated by stakeholders. The method, approved by the ERP, is applicable for the determination of vitamin D (total vitamins D₂ and D₃). A range of products had been tested during a single-laboratory validation study. The products included butter, National Institute of Standards and Technology SRM 1849, eggs, cheese, yogurt, ready-to-eat cereal, bread, mushrooms, and tuna. The testing of the method established linearity in the range of 0.005–50 µg/mL. The recovery range was 93.4–100.9% for vitamin D₂ and 102.4–106.2% for vitamin D₃. The LOD and LOQ for vitamin D₂ were reported as 0.20 and 0.61 µg/100 g, respectively; for vitamin D₃, the reported values were 0.47 and 1.44 µg/100 g, respectively. The method met the SMPRs set by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). It was, therefore, decided that the method was appropriate for Official First Action Method status.

The alternative pathway, approved by the AOAC Board of Directors on March 28, 2011, allows selected methods to enter the review process to obtain AOAC Official First Action Method status after being reviewed by Expert Review Panels (ERP) and meeting the criteria required by the standard method performance requirements established by the stakeholder panel. The approved methods will retain First Action status for approximately 2 years to allow the ERPs to monitor the method and laboratories to use the method and submit comments. After this 2-year period, the ERPs will review the method again to determine if it is acceptable for recommendation as Final Action to the Official Methods Board (1).

Vitamins D₂ and D₃

Vitamin D₂, ergocalciferol, and vitamin D₃, cholecalciferol, are two forms of vitamin D found in the body. Vitamin D is essential to the development of bone and mineralization through calcium homeostasis as well as being involved in many other bodily functions (2). Because vitamin D is not readily available in many foods, the main source remains fortified foods (3). Because of the importance of the vitamin D levels in foods, methods that produce accurate results in a timely manner are needed. The method “Determination of Vitamins D₂ and D₃ in Infant Formula and Adult Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry” was submitted for consideration using the alternative pathway. The method was reviewed by an ERP and adopted as an AOAC Official First Action Method. The method is applicable to a wide variety of food products.
A ground, homogenized sample is mixed with an internal standard solution containing isotopically labeled vitamins D$_2$ and D$_3$, with ethanol and with potassium hydroxide. The mixture is heated to ~65°C and refluxed under nitrogen. After saponification and mixing, the samples are diluted with water and cooled to room temperature. Samples are quantitatively transferred to a separatory funnel using 40% ethanol in water, and cooled to room temperature. Samples are quantitatively saponification and mixing, the samples are diluted with water, transferred to a separatory funnel using 40% ethanol in water, and cooled to room temperature. Samples are quantitatively saponification and mixing, the samples are diluted with water, and reconstituted in methanol, and the samples assayed using a rotary vacuum system. The sample is reconstituted in methylene chloride and isopropyl alcohol and interferences removed by elution through a disposable SPE silica gel column.

A. Principle

B. Apparatus

Note: Equivalents can be used for all equipment listed below.

Table 2011.12B. Inlet method vitamin D

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>Column</td>
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<tr>
<td>Column temperature</td>
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<tr>
<td>Sample temperature</td>
<td>8°C</td>
</tr>
<tr>
<td>Sample loop</td>
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<tr>
<td>Flow rate</td>
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<tr>
<td>Injection volume</td>
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<tr>
<td>Seal wash</td>
<td>1.0 min</td>
</tr>
<tr>
<td>Run time</td>
<td>6.0 min</td>
</tr>
<tr>
<td>Wash solvents</td>
<td>Weak (600 μL); strong (600 μL)</td>
</tr>
</tbody>
</table>

C. Reagents

Note: Equivalents can be used for all reagents listed below.

Table 2011.12C. UPLC gradient

<table>
<thead>
<tr>
<th>Time, min</th>
<th>% A1, 2 mM NH$_4$COOH</th>
<th>% B1, 2 mM NH$_4$COOH:MeOH</th>
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<td>—</td>
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<td>100</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>95</td>
<td>6</td>
</tr>
</tbody>
</table>
Phillipsburg, NJ).

(b) 

**Ethanol.**—200 proof, HPLC grade; No. 459828 (Sigma-Aldrich).

(c) 

**Ammonium formate Fluka.**—No. 70221-25g-f (Sigma-Aldrich).

(d) 

**Pyrogallic acid.**—No. JT0288-01 (J.T. Baker).

(e) 

**Ascorbic acid.**—U.S. Pharmacopeia (USP) grade; Mallinckrodt No. MKS82903 (Phillipsburg, NJ).

(f) 

**Methylene chloride.**—HPLC grade; EMD Omnislolv No. PX1834-6 (Gibbstown, NJ).

(g) 

**Isopropanol.**—HPLC grade; EMD Omnislolv No. PX1834-6.

(h) 

**2,6-Di-tert-butyl-4-methylphenol (BHT).**—Sigma-Aldrich No. B1378.

(i) 

**Chromasolv LC/MS grade; Sigma-Aldrich No. 34966.**

(j) 

**Potassium hydroxide.**—50% (w/v); No. BDH3622-1 (BDH, VWR International LLC).

(k) 

**Sodium sulfate anhydrous.**—Granulat 12-60 mesh; J.T. Baker No. JT3375-5.

(l) 

**Polyethylene glycol 400.**—200 proof, HPLC grade; No. 459828 (Sigma-Aldrich).

(m) 

**Vitamin D2 (cholecalciferol).**—Cat. No. 1131009, purity ≥98% (USP, Rockville, MD).

(n) 

**Vitamin D2 (ergocalciferol).**—Cat. No. 1239005, purity ≥98% (USP).

(o) 

**Vitamin D3 [2H3] (isotopically labeled).**—Cat. No. S3077-1.0 (1 mg/mL in ethanol), purity ≥97% (Isosciences, King of Prussia, PA).

(p) 

**Vitamin D3 [2H3] (isotopically labeled).**—Cat. No. S5014-0.1 (1 mg/mL in ethanol), purity ≥98% (Isosciences).

**D. Reagent Preparation**

(a) 

**Ethanol (40%, v/v).**—Dilute 800 mL 200-proof ethanol to 1 L.

(b) 

**KOH (50%, w/v).**—(1) Dissolve 500 g KOH pellets in 500 mL water KOH (1 M).

(2) Dissolve 56 g KOH pellets in water and dilute to 1 L.

(c) 

**Mobile phase A1: 2 mM NH4COOH.**—Dissolve 0.126 g ammonium formate in water and dilute to 1 L.

(d) 

**Mobile phase B1: 2 mM NH4COOH–MeOH.**—Dissolve 0.126 g ammonium formate in methanol and dilute to 1 L.

(e) 

**Strong wash solution: 80% MeOH:20% purified H2O.**—Dilute 800 mL methanol to 1 L with water.

(f) 

**Weak wash solution: 90% purified H2O:10% MeOH.**—Dilute 100 mL methanol to 1 L with water.

(g) 

**Internal standard stock solution vitamin D2 [2H3] (0.1 mg/mL).**—Dilute 1 mg/mL vitamin D2 [2H3] in 200-proof ethanol and dilute to 10 mL.

(h) 

**Internal standard stock solution vitamin D3 [2H3] (0.1 mg/mL).**—Dilute 1 mg/mL vitamin D3 [2H3] in 200-proof ethanol and dilute to 10 mL.

(i) 

**80:20 Methylene chloride/isopropanol solution.**—Dilute 800 mL methylene chloride to 1 L with isopropanol.

(2) 

**99.8:0.2 Methylene chloride/isopropanol solution.**—Dilute 2 mL isopropanol to 1 L with methylene chloride.

(3) 

**Vitamin D2 [2H3]/vitamin D3 [2H3] internal standard working stock solution (1000 ng/mL).**—Dilute 250 µL internal standard stock solution vitamin D2 [2H3] (0.1 mg/mL) and 250 µL internal standard stock solution vitamin D3 [2H3] (0.1 mg/mL) to 25 mL with 200-proof ethanol.

(4) 

**Vitamin D2 stock solution (0.1 mg/mL).**—Dilute 10 mg vitamin D2 in 200-proof ethanol to 100 mL.

(5) 

**Vitamin D3 stock solution (0.1 mg/mL).**—Dilute 10 mg vitamin D3 in 200-proof ethanol to 100 mL.

(6) 

**Vitamin D2/vitamin D3 standard working stock solution (1000 ng/mL).**—Dilute 250 µL vitamin D2 stock solution (0.1 mg/mL) and 250 µL vitamin D3 stock solution (0.1 mg/mL) in 200-proof ethanol to 25 mL.

(7) 

**Vitamin D mixed reference standard (200 ng/mL).**—Dilute 1 mL vitamin D2 [2H3]/vitamin D3 [2H3] internal standard working stock solution (1000 ng/mL) and 1 mL vitamin D2/vitamin D3 standard working stock solution (1000 ng/mL) to 5 mL.

**E. Preparation of Test Samples**

(a) 

**General preparations and instructions.**—

(1) Homogenize samples in air-tight, dark containers stored at or below 8°C.

(2) 

**Handling and storage of samples should be in accordance with good laboratory practices.** Allow refrigerated or frozen...
samples to come to room temperature before weighing.

(3) Perform all laboratory work under yellow or golden fluorescent lighting conditions for vitamin analysis using either low actinic or covered clear glassware.

(4) Perform all laboratory work in a hood with the exception of the Genevac Rocket evaporation systems, sample reconstitution with methanol, and sample analysis on the LC-MS/MS.

(5) Consult the laboratory equipment or analytical instrument operating manual for preventative and routine maintenance of the equipment utilized in this method.

(b) Sample preparation.—(1) Turn on the 6 × 500 mL heating mantle 10 min prior to intended use to reach temperature equilibrium.

(2) Dispense an appropriate quantity of sample into a 500 mL round-bottom flask based on Table 2011.12A as a guide. Record the weight to 0.001 g for food and 0.0001 g for vitamin D premix samples.

(3) Add a stir bar and a few boiling chips to each flask, and add 0.5 g ascorbic acid and 0.5 g pyrogallic acid to each boiling flask.

(4) Dispense 200 μL vitamin D₂ [²H₃]/vitamin D₃ [²H₃] internal standard working stock solution (1000 ng/mL) to each boiling flask for food samples and method blank. For vitamin premix samples, add 2 mL vitamin D₂ [²H₃]/vitamin D₃ [²H₃] internal standard working stock solution (1000 ng/mL) to each boiling flask.

(5) Dispense 80 mL of 200-proof ethanol and 20 mL of 50% KOH to each boiling flask using a bottle dispenser. Attach a reflux condenser to each flask. Turn the magnetic stirrers, cooling water, and nitrogen gas overflow on.

(6) Reflux samples for 15 min at ~95°C.

(7) After saponification, remove from heating mantle and add 50 mL Nanopure water. Allow the sample to equilibrate to room temperature.

(8) Quantitatively transfer the contents of the boiling flask to a 500 mL separatory funnel with 50 mL 40% (v/v) ethanol solution. Repeat this rinse step. Add 75 mL heptane, and shake the separatory funnel for 1 min. Let the phases separate, and transfer the upper heptane phase to a 250 mL separatory funnel. Repeat the extraction procedure once, and combine the extracts. Wash the combined heptanes phases once with 50 mL 1 M KOH solution, then twice with 50 mL 40% ethanol, and finally with a 100 mL portion of water. Shake separatory funnel vigorously for 30 s at every washing step.

(9) Transfer the heptane phase to an evaporator flask with a few granules of BHT and evaporate in the Genevac Rocket evaporator using a control temperature of 40°C and a chiller temperature of ~10°C at 20 mbar of vacuum. Dissolve the residue in 1 mL 99.8:0.2 methylene chloride/isopropanol.

(10) Wash the SPE column by filling the cartridge reservoir (~5.5 mL) with 80:20 methylene chloride/isopropyl alcohol solution, followed by a full cartridge reservoir (~5.5 mL) of 99.8:0.2 methylene chloride/isopropanol solution.

(11) Transfer the solution from the evaporation flask to the SPE cartridge.

(12) Rinse the evaporator flask with 1 mL of 99.8:0.2 methylene chloride/isopropyl alcohol solution, and transfer the rinse to the SPE column. Elute the SPE column with 30 mL 99.8:0.2 methylene chloride/isopropyl alcohol solution into a receiving vessel. Quantitatively transfer the eluant collected into evaporator flask containing a few grains of BHT.

(13) Evaporate the samples on the Genevac Rocket evaporator to dryness.

(14) Reconstitute the samples with 1 mL methanol, and vortex mix well. Transfer the sample into a 3 mL disposable syringe equipped with a 13 mm syringe filter. Filter the sample into an HPLC vial containing an insert and assay by UPLC-MS/MS.

(15) For premix samples, reconstitute with 1 mL methanol, and vortex mix well. Quantitatively transfer the contents with a 9 in. disposable transfer pipet into a 10 mL volumetric flask. Repeat rinsing the flask until the volumetric is full. Stopper, mix, and transfer an aliquot using a disposable transfer pipet into a 3 mL disposable syringe equipped with a 13 mm syringe filter. Filter the sample into an HPLC vial containing an insert and assay by UPLC-MS/MS.

F. Operating Conditions

(a) UPLC operating conditions.—See Tables 2011.12B and C.

(b) MS/MS operating conditions.—See Tables 2011.12D and E.

G. Calculations

(a) Calculate the response factor for the vitamins in the vitamin D reference standard solution with respect to the corresponding isotopically labeled internal standards.

\[ RF = \frac{P A_a \times C_a}{P A_i \times C_i} \]  \hspace{1cm} (1)

where \( RF \) = response factor; \( P A_a \) = peak area of analyte; \( C_a \) = concentration of respective internal standard; \( P A_i \) = peak area of respective internal standard; and \( C_i \) = concentration of analyte.

(b) Calculate the concentration (μg/100 g) of the vitamins found in the samples.

\[ C = \frac{P A_a \times C_a}{P A_i \times RF_a} \times \frac{V}{W} \times \frac{1}{1000} \]  \hspace{1cm} (2)

where \( C \) = concentration of analyte in sample μg/100 g; \( P A_a \) = peak area of analyte in sample; \( C_a \) = concentration of respective internal standard added to sample (ng); \( P A_i \) = peak area of respective internal standard in sample; \( RF_a \) = response factor of analyte calculated from Equation 1; \( V \) = volume of sample diluted to mL; \( W \) = weight of sample in g; 1/1000 = conversion factor from ng to μg, e.g., 1000 ng = 1 μg; and 100 = conversion factor to 100 g.

(e) Report the concentration of vitamin D found as the total of vitamins D₂ and D₃.

Reference: J. AOAC Int. xxx(2012)

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**Figure 1**. Vitamins D₂ and D₃ linear regression.
Results and Discussion

System Linearity Study

The linearity of vitamins D2 and D3 was performed in the range of 0.005–50 μg/mL. The data demonstrate a linear response for the instrument over the concentration range studied (Figure 1).

LOD and LOQ

The calculation of LOD and LOQ of the assay were based on the SD of the slope response and the slope (S) of the calibration curve. For infant formula, the LOD and LOQ were calculated from the injection solution LOD and LOQ using a typical 1 g sample and 1 mL final solution. The LOD was found to be 0.20 and 0.47 for vitamins D2 and D3, respectively. The LOQ was found to be 0.61 and 1.44 for vitamins D2 and D3, respectively.

Adjusting sampling weight and final solution volume will give a greater sensitivity. See Table 1 for details.

\[
\text{LOD} = 3.3 \frac{\text{SD}}{S} \quad \text{LOQ} = 10 \frac{\text{SD}}{S}
\]

Precision

The method precision was evaluated by analyzing different food matrixes in triplicate over 5 days. Most of the foods selected for testing contained vitamin D3, with the exception of irradiated mushrooms. Also notable is that whole eggs contained both vitamins D2 and D3. The results from this testing are shown in Table 2.

Accuracy

Four matrixes were analyzed at four levels according to the method to show accuracy. The % recovery was calculated from the slope of the linear regression. See Table 3 for the results.

The accuracy was examined by a comparison of the mean LC-MS/MS precision results to the HPCL-UV analysis of the samples and the label claim of the products used in the validation study. Table 4 shows a comparison of the label claim with the results obtained by UPLC-UV and LC-MS/MS.

Robustness

To test the robustness of the method, two different lots and lengths of columns (Waters UPLC HSS C18, 100 mm, Table 1. LOD and LOQ results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD, ng/mL inj. soln</th>
<th>LOQ, ng/mL inj. soln</th>
<th>LOD, μg/100 g infant formula</th>
<th>LOQ, μg/100 g infant formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D2</td>
<td>2.0</td>
<td>6.1</td>
<td>0.20</td>
<td>0.61</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>4.7</td>
<td>14.4</td>
<td>0.47</td>
<td>1.44</td>
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</table>

The LOQ was verified in nonfortified ready-to-eat (RTE) cereal, mushrooms, and yogurt spiked with 0.5 μg/100 g at the recommended sample preparation weights for the assay.

Table 2. Precision results (μg/100 g)

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<th>AOAC sector</th>
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<td>1–1</td>
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<td>1.99</td>
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</table>
S/N 010738038154 12; and 50 mm, S/N 011730124156 71) were used with no significant impact on the results. The evaluation of an alternative stationary phase, Waters UPLC HSS T3 column (100 mm, S/N 0116392741) gave poor peak symmetry and, therefore, was not recommended. Various manufacturers and lots of materials used in the mobile phase preparation and SPE cleanup were performed with no aberrations observed. Also used intermittently throughout the study were two Waters Quattro Premier XE Micromass LC-MS/MS units (S/N VAB 1100 and S/N 800).

The sample solution stability was inherent to the validation study due to accessibility of the extraction equipment or the LC-MS/MS; finding samples stored post-SPE in methanol at 5°C in enclosed containers were stable for 5 days. No significant difference in vitamin D content was observed in samples stored in the dark in an enclosed container of heptane containing butylated hydroxytoluene or dried down in enclosed rotary evaporator flask after 3 days at room temperature, e.g., stoppered rocket flask in cabinet or left in the unit over the weekend.

Also tested were various grain matrixes. These samples included Wheat Chex, Rice Chex, and Corn Chex. There was no significant matrix interference present.

**Measurement Uncertainty**

The measurement uncertainty was calculated for the internal reference material (IRM) by the combined use of the precision data ($n = 15$) from the validation and the analytical competence data ($n = 18$) obtained during technical transfer of the method according to the formula below:

$$
\mu = k\sigma
$$

where $\mu = $ measurement uncertainty, vitamin D µg/100 g; $k =$ coverage factor of 2 obtained from Student's $t$ tables with respect to degrees of freedom; $\sigma =$ SD of data points. See Table 5 for the results.

**References**


(3) Centers for Disease Control and Prevention (July 2008) http://www.cdc.gov/nutritionreport/part_2b.html

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### Table 3. Results of accuracy testing

<table>
<thead>
<tr>
<th>Identity</th>
<th>Food matrix</th>
<th>AOAC food triangle sector</th>
<th>Vitamin D$_2$ recovery, %</th>
<th>Vitamin D$_3$ recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRM 25F</td>
<td>RTE cereal</td>
<td>5</td>
<td>100.8</td>
<td>102.4</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>Vegetable</td>
<td>7</td>
<td>93.4</td>
<td>104.2</td>
</tr>
<tr>
<td>NIST SRM 1849</td>
<td>Infant/adult nutritional</td>
<td>6</td>
<td>100.9</td>
<td>105.5</td>
</tr>
<tr>
<td>Yogurt</td>
<td>Dairy</td>
<td>5</td>
<td>95.5</td>
<td>106.2</td>
</tr>
</tbody>
</table>

### Table 4. Comparison of label claims to testing with UPLC-UV and LC-MS/MS

<table>
<thead>
<tr>
<th>Matrix</th>
<th>HPLC-UV</th>
<th>Label claim</th>
<th>LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td>14.68</td>
<td>10.73</td>
<td>13.08</td>
</tr>
<tr>
<td>Egg</td>
<td>6.93</td>
<td>4.48</td>
<td>8.30</td>
</tr>
<tr>
<td>Tuna</td>
<td>3.03</td>
<td>2.68</td>
<td>2.38</td>
</tr>
<tr>
<td>NIST SRM 1849</td>
<td>—</td>
<td>25.10</td>
<td>24.75</td>
</tr>
<tr>
<td>Cheese</td>
<td>5.58</td>
<td>4.75</td>
<td>4.50</td>
</tr>
<tr>
<td>Bread</td>
<td>2.18</td>
<td>2.13</td>
<td>1.95</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>19.10</td>
<td>11.78</td>
<td>13.35</td>
</tr>
<tr>
<td>RTE cereal</td>
<td>5.00</td>
<td>—</td>
<td>6.10</td>
</tr>
<tr>
<td>Yogurt</td>
<td>1.25</td>
<td>1.20</td>
<td>1.85</td>
</tr>
</tbody>
</table>

### Table 5. RTE cereal (IRM 25F) measurement uncertainty

<table>
<thead>
<tr>
<th>Mean</th>
<th>$\sigma$</th>
<th>$k$</th>
<th>$\mu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.89</td>
<td>0.653</td>
<td>2.04</td>
<td>1.33</td>
</tr>
</tbody>
</table>

S/N 010738038154 12; and 50 mm, S/N 011730124156 71) were used with no significant impact on the results. The evaluation of an alternative stationary phase, Waters UPLC HSS T3 column (100 mm, S/N 0116392741) gave poor peak symmetry and, therefore, was not recommended. Various manufacturers and lots of materials used in the mobile phase preparation and SPE cleanup were performed with no aberrations observed. Also used intermittently throughout the study were two Waters Quattro Premier XE Micromass LC-MS/MS units (S/N VAB 1100 and S/N 800).

The sample solution stability was inherent to the validation study due to accessibility of the extraction equipment or the LC-MS/MS; finding samples stored post-SPE in methanol at 5°C in enclosed containers were stable for 5 days. No significant difference in vitamin D content was observed in samples stored in the dark in an enclosed container of heptane containing butylated hydroxytoluene or dried down in enclosed rotary evaporator flask after 3 days at room temperature, e.g., stoppered rocket flask in cabinet or left in the unit over the weekend.

Also tested were various grain matrixes. These samples included Wheat Chex, Rice Chex, and Corn Chex. There was no significant matrix interference present.

**Measurement Uncertainty**

The measurement uncertainty was calculated for the internal reference material (IRM) by the combined use of the precision data ($n = 15$) from the validation and the analytical competence data ($n = 18$) obtained during technical transfer of the method according to the formula below:

$$
\mu = k\sigma
$$

where $\mu = $ measurement uncertainty, vitamin D µg/100 g; $k =$ coverage factor of 2 obtained from Student’s $t$ tables with respect to degrees of freedom; $\sigma =$ SD of data points. See Table 5 for the results.

**References**


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