Simultaneous Determination of Vitamins D2 and D3 by LC-MS/MS in Infant Formula and Adult Nutritionals: First Action 2011.13

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During the “Standards Development and International Harmonization: AOAC INTERNATIONAL Mid-Year Meeting” held on June 29, 2011, an Expert Review Panel (ERP) on behalf of AOAC INTERNATIONAL adopted the method “Simultaneous Determination of Vitamins D2 and D3 by LC-MS/MS in Infant Formula and Adult Nutritionals” as an AOAC Official First Action method. Vitamins D2 and D3 are extracted from the sample using pentane–ether; the extract is collected and dried under nitrogen. Vitamin D is separated from interfering compounds using UPLC, and quantitated using tandem mass spectrometry (MS/MS). Preliminary data showed the intermediate precision ranged from 3.34–8.05% and an accuracy range of 98.5–111% over the samples tested for vitamin D2. For vitamin D3, the intermediate precision ranged from 2.37–5.45% and accuracy ranged from 96.4–104% over the four matrices evaluated. The analytical range for the method is bounded by the concentrations of the working standards, 21–270 ng/mL, and is equivalent to 0.168–2.16 mcg/100 g in ready-to-feed product. The practical method quantitation limit is 0.168 mcg/100 g product with method detection limit of 60 ng/100 g product. The ERP reviewed the data and determined that the performance characteristics of the method met the standard method performance requirements, and therefore the method was granted First Action status.

Vitamins D2 and D3

Vitamins D2 and D3 are an essential part of bone health. The majority of dietary vitamins D2 and D3 comes from fortified foods such as milk products (2). Because of the need to assure the levels of supplements in food products are accurate, testing methods are needed that are user-friendly, less time-consuming, and accurate. Because of this need, the method “Simultaneous Determination of Vitamins D2 and D3 in Infant Formula and Adult Nutritionals by LC-MS/MS” was submitted for consideration for approval under AOAC’s new path to Official MethodsSM. The method was reviewed by an ERP at the Mid-Year Meeting and approved as Official First Action MethodSM 2011.13.

AOAC Official Method 2011.13 Vitamins D2 and D3 in Infant Formula and Adult Nutritionals LC-MS/MS First Action 2011

(Applicable to the determination of vitamins D2 and D3 in infant formula and adult/pediatric nutritional formula by LC-MS/MS.)

Caution: Refer to the Material Safety Data Sheets (MSDS) of chemicals prior to use and follow the suggested personal protective equipment.

A. Principle

The test sample is weighed directly into a 50 mL glass centrifuge tube and inoculated with a stable isotope labeled internal standard. The sample is saponified at 75°C for 30 min with potassium hydroxide to break up the product matrix and facilitate analyte extraction. Vitamin D is extracted using a pentane–ether solvent, which is collected and dried under nitrogen. The dried extract is reconstituted and filtered prior to...
analysis. Vitamin D is separated from interfering compounds using UPLC and quantitated using tandem mass spectrometry. Elevated temperature saponification allows sample preparation to be completed in less than 3 h. Instrument analysis time is 12 min/sample.

**B. Apparatus**

(a) **Column.**—Waters HSST 3 1.7 μm, 2.1 × 150 mm (Waters Corp., Milford, MA) or equivalent.  
(b) **Liquid chromatograph.**—Waters Acquity UPLC Binary Solvent Manager capable of 15 000 psi or equivalent.  
(c) **Detector.**—Waters Quattro Premier XE, Xevo triple quadrupole mass spectrometer, or equivalent.  
(d) **Injector.**—Waters Acquity sample manager with integrated column oven or equivalent.  
(e) **Nitrogen generator.**—Peak Scientific (Billerica, MA) Model NM30LA or equivalent.  
(f) **Data system.**—Waters MassLynx, latest revision or equivalent.  
(g) **Centrifuge tubes.**—Glass, 50 mL with Teflon-lined screw cap.  
(h) **Centrifuge.**  
(i) **Water bath.**—Capable of 75 ± 2°C.  
(j) **Nitrogen source.**—Purity >99.7% for solvent extract evaporation.  
(k) **Vortex mixer.**  
(l) **Evapo-Rac Evaporator.**—Cole-Parmer (Vernon Hills, IL), Model N-01610-35 or equivalent.  
(m) **Balance.**—Readable to at least 0.001 g. (Mettler-Toledo XS-204, Columbus, OH, or equivalent).  
(n) **Syringe filters.**—PTFE 0.45 μm.  
(o) **Pipets.**—100, 250, and 1000 μL adjustable.  
(p) **Pipet tips.**

**C. Reagents**

(a) **Solvents.**  
(1) **Pentane.**—HPLC grade.  
(2) **Ethyl ether.**—Anhydrous. Refer to the appropriate MSDS and follow the precautions as substance is hazardous.  
(3) **Acetonitrile.**—HPLC grade.  
(4) **Acetone.**—HPLC grade.  
(5) **Methanol.**—HPLC grade (for sample preparation).  
(6) **Methanol.**—LC-MS grade (for LC-MS/MS mobile phase preparation).  
(7) **Sodium ascorbate.**—Sodium L-ascorbate, crystalline, ≥98%.  
(8) **Distilled or deionized laboratory water.**—Purified water, USP; ≥18MΩcm/cm.  
(b) **Solutions.**  
(1) **Potassium hydroxide.**—45%.  
(2) **Phosphoric acid.**—85%.  
(3) **Ammonium formate.**—≥99.0%.  
(4) **Iso-octane.**—≥99.0%.

**D. Standards**

(a) **Vitamin D₃.**—USP (Rockville, MD) reference standard No. 1310 (cholecalciferol = vitamin D₃). Purity = 40 000 IU/mg. (Consult current USP literature for current Lot No.)  
(b) **Trideuterated vitamin D₃, ²H₃-D₃.**—Iso-Sciences (King of Prussia, PA), Cat. No. 3077, ≥97% purity and 97% incorporation of stable-isotope incorporation.  
(c) **Vitamin D₂.**—USP reference standard No. 1239005 (ergocalciferol = vitamin D₂). Purity = 40 000 IU/mg. (Consult current USP literature for current Lot No.)  
(d) **Trideuterated vitamin D₂, ²H₃-D₂.**—Iso-Sciences, Cat. No. 5014, ≥98.0% purity and 97% stable-isotope incorporation.

**E. Solutions Preparation**

(a) **Mobile phase.**—(1) **Mobile phase A.**—2 mM ammonium formate (aqueous). Quantitatively transfer 126 (±5) mg ammonium formate into a 1 L volumetric flask and add approximately 5 mL laboratory water to dissolve and fill to volume with laboratory water.  
(2) **Mobile phase B.**—2 mM ammonium formate in methanol. Quantitatively transfer 126 (±5) mg ammonium formate into a 1 L volumetric flask and add approximately 5 mL laboratory water to dissolve and fill to volume with laboratory HPLC grade methanol.

(3) **Extraction solution.**—Ether–pentane (20 : 80).  
(b) **Vitamin D₃ stock standard (approximately 48 000 ng/mL).**—Aliquot a portion of the solution into glass vials with Teflon lined screw caps. Store at −10°C.Expiration: 8 weeks.  
(1) **Weigh 24 (±1) mg vitamin D₃.**  
(2) **Transfer into a 500 mL volumetric flask.**  
(3) **Dissolve and bring to volume using iso-octane.**  
(c) **Vitamin D₂ stock standard (approximately 48 000 ng/mL).**—Aliquot a portion of the solution into glass vials with Teflon lined screw caps. Store at −10°C.Expiration: 8 weeks.  
(1) **Weigh 24 (±1) mg vitamin D₂.**  
(2) **Transfer into a 500 mL volumetric flask.**  
(3) **Dissolve and bring to volume using iso-octane.**

(d) **Vitamin D₂⁻D₃ mixed intermediate standard (ISTD; approximately 540 ng/mL).**—Prepare fresh each day. Expiration: 48 h.  
(1) **Fill a 50 mL volumetric flask with approximately 25 mL LC-MS grade methanol.**

**Table 2011.13A. Volumes required for working standard solutions**

<table>
<thead>
<tr>
<th>Std</th>
<th>ISTD volume, μL</th>
<th>SIIS volume, μL</th>
<th>Volume MeOH, μL</th>
<th>Volume water, μL</th>
<th>Final volume, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS1</td>
<td>45</td>
<td>75</td>
<td>1780</td>
<td>100</td>
<td>2</td>
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<tr>
<td>WS2</td>
<td>90</td>
<td>75</td>
<td>1740</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>WS3</td>
<td>150</td>
<td>75</td>
<td>1680</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>WS4</td>
<td>500</td>
<td>75</td>
<td>1326</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>WS5</td>
<td>1000</td>
<td>75</td>
<td>825</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2011.13B. Approximate sample weights**

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Sample size, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ready to feed</td>
<td>12</td>
</tr>
<tr>
<td>Concentrated liquids</td>
<td>6</td>
</tr>
<tr>
<td>Powder products</td>
<td>12</td>
</tr>
</tbody>
</table>

* Reconstitute powder samples by weighing 25 g into 200 g laboratory water and mix thoroughly to generate a homogeneous mixture prior to sampling for analysis.

* Concentrated liquids are diluted 1:1 with laboratory water after weighing into sample tube.
(2) Transfer 560 μL of each stock standard solution into the 50 mL volumetric flask.

(3) Dilute to 50 mL with LC-MS grade methanol.

(e) Trideuterated vitamin D2/D3, 2H3-D2 / 2H3-D3, stable-isotope labeled internal standard solution (SIIS; approximately 5000 ng/mL).—The prepared solution is transferred into single-use vials and tightly sealed. Store at −70°C. Solution can be used indefinitely until noticeable degradation of the solution concentration is observed.

(1) Quantitatively transfer the entire contents each of an ampule of trideuterated D2 and D3 (1 mg) to a 200 mL volumetric flask using LC-MS grade methanol to aid in dissolution of the standards as well as to wet the flask. Dilute to 200 mL with HPLC grade methanol once the contents of both ampules have been completely transferred to the flask.

(f) Working standards.—Prepare fresh on day of analysis. Expiration: 48 h. (1) Add 100 μL water, 75 μL SIIS mixture, the specified volume of native standard mix (ISTD), and methanol per Table 2011.13A.

(2) Cap and mix well.

(3) Transfer to an amber autosampler vial and cap.

F. Procedure—Sample Preparation

(a) Accurately weigh designated sample size into a 50 mL centrifuge tube (see Table 2011.13B).

(b) Add 75 μL SIIS and vortex for 10 s to mix.

(c) Saponification.—(1) Add about 0.4 ± 0.1 g sodium ascorbate and vortex for a minimum of 10 s.

(2) Add 6 ± 0.3 mL HPLC grade methanol and vortex for 15 s.

(3) Add 4 ± 0.3 mL of 45% potassium hydroxide solution and immediately vortex for a minimum of 20 s to thoroughly mix contents.

(4) Place tubes in a preheated water bath at 75 ± 2°C for a minimum of 30 min making sure to vortex for approximately 5 s at approximately 10 and 20 min intervals.

(5) After 30 min, remove the tubes from the water bath and promptly place into an ice bath for a minimum of 30 min to bring them rapidly to room temperature.

(d) Liquid–liquid extraction.—(1) Add 5 ± 0.3 mL acetonitrile to each tube, cap, and vortex at a moderate speed for a minimum of 5 s.

(2) Add 22 ± 1 mL extraction solvent (20 + 80, ether–pentane) and shake in a wide, semicircular arc 20 times. Invert the tubes with each stroke.

(3) Briefly centrifuge at moderate speed (approximately 300 × g for 1.5 min) to complete layer separation.

(4) Draw off the clear ether–pentane and transfer the top layer to a 50 mL conical centrifuge tube, leaving behind a few milliliters of the ether–pentane mixture.

(5) Evaporate to dryness the transferred ether–pentane layer in a 40 ± 4°C water bath, using a flow of nitrogen over the sample.

(6) Remove the centrifuge tubes from the water bath as soon as evaporation is complete. Observe the extract as it should
Table 2011.13E. Mass analysis parameters

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Retention time, typical, min</th>
<th>Molecular ion (precursor)</th>
<th>Product ion</th>
<th>Dwell, s</th>
<th>Cone voltage</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D₃</td>
<td>5.3</td>
<td>385.5</td>
<td>259.2</td>
<td>0.080</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>²H₃-Vitamin D₃</td>
<td>5.3</td>
<td>388.5</td>
<td>259.2</td>
<td>0.080</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Vitamin D₂</td>
<td>5.1</td>
<td>397.5</td>
<td>69.1</td>
<td>0.080</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>²H₃-Vitamin D₂</td>
<td>5.1</td>
<td>400.5</td>
<td>69.1</td>
<td>0.080</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

appear as a white or yellow film. Make sure the extract is completely dried before reconstitution.

(7) Immediately reconstitute with 1.9 mL methanol and vortex for 10 s or until the solids have dissolved.

(8) Add 100 μL laboratory water and vortex for an additional 5 s.

(9) Filter reconstituted sample into HPLC autosampler vial using a PTFE syringe filter.

(10) Inject onto the equilibrated chromatographic system.

G. Instrument Operating Conditions

(a) UPLC conditions.—See Table 2011.13C.

(b) Mass spectrometer conditions.—See Tables 2011.13D and 2011.13E.

(c) UPLC analysis.—After verifying equilibration of the UPLC system, inject the working standards (WS1 to WS5) followed by a reagent blank, control sample, and sample extracts.

Inject working standards approximately every 5 h of sample extraction analysis (e.g., 25 samples with analysis cycle time of 12 min) injected after the analysis of the last sample extract.

Notes: Calibration curves must have a correlation coefficient, r², of >0.990. Calibration curve residuals must be <15% for WS1 and <10% for WS2–WS5.

H. Calculations and Results

(a) Calculation of working standard concentrations.—Calculate the concentration of vitamin D in the working standards from the following equation:

\[ C = \frac{V \times W}{500 \times \left( \frac{1000\mu g}{mg} \times \frac{1000\mu g}{mg} \times \frac{5600\mu L}{50\mu L} \times \frac{V_{\text{ISTD}}(\mu L)}{2\mu L} \right)} \]

where W = weight of vitamin D used to make the stock standard solution, in mg; V_{\text{ISTD}} = volume of ISTD used, in mL.

(b) Standard curve calculation using linear regression and quantitation of vitamin D in samples.—(1) Use peak heights for both vitamin D and ²H₃-D for each working standard level to generate a linear regression line.

(2) Calculate the regression by plotting peak height response ratios for each working standard vs vitamin D concentration. The response ratio is defined as the ratio of vitamin D peak height divided by ²H₃-D peak height.

(c) Product calculation:

\[ \text{Vitamin D (μg/kg)} = \frac{C \times V}{S} \]

where C = vitamin concentration (ng/mL) from standard curve; V = volume (mL) of methanol to reconstitute extracts; S = sample size in grams.

Reference: J. AOAC Int. 95, XXX(2012)

Results and Discussion

The method performance for vitamin D₃ was determined in several matrices. Accuracy was determined by comparing the results to the target values established by LC-UV measurements. Samples were analyzed in duplicate over an 8 day interval using two instruments. Data show the intermediate precision ranged from 3.34 to 8.05% and an accuracy range of 98.5–111% over all the sample types with vitamin D₃ concentrations in the final analytical sample of 12–270 ng/mL. The method chromatography facilitates the coelution of previtamin D₃ with vitamin D₃. As such, product-to-product variability in the thermal production of previtamin D₃ is effectively addressed without the need to separately detect and quantify the previtamin D₃ isomer that can be present in amounts as low as a few percent relative to vitamin D₃. This shows the repeatability of the method to determine vitamin D₃ to be acceptable. See Table 1 for results of all the sample types.

The method quantitation limit (MQL) is a “practical” limit due to the fact that the method is designed to measure a fortified nutrient in these product matrices. As such, the practical MQL for this method is bounded by the concentration of the lowest working standard concentration, or 12 ng/mL or 190 ng/100 g in an RTF product.

Table 1. Method performance summary for D₃

<table>
<thead>
<tr>
<th>Product type</th>
<th>D₃, μg/kg</th>
<th>Intermediate precision, %</th>
<th>Accuracy, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk-based IF RTF</td>
<td>12.7</td>
<td>3.51</td>
<td>101</td>
</tr>
<tr>
<td>Milk-based IF RTF</td>
<td>13.9</td>
<td>6.03</td>
<td>98.5</td>
</tr>
<tr>
<td>Milk-based IF RTF</td>
<td>36.8</td>
<td>6.31</td>
<td>104</td>
</tr>
<tr>
<td>Milk-based IF Pwd</td>
<td>93.5</td>
<td>8.05</td>
<td>106</td>
</tr>
<tr>
<td>Milk-based IF Pwd</td>
<td>1190</td>
<td>5.04</td>
<td>111</td>
</tr>
<tr>
<td>Soy-based IF RTF</td>
<td>11.9</td>
<td>3.79</td>
<td>103</td>
</tr>
<tr>
<td>Hydrolysate IF RTF</td>
<td>9.75</td>
<td>7.00</td>
<td>105</td>
</tr>
<tr>
<td>Hydrolysate IF Pwd</td>
<td>103</td>
<td>5.76</td>
<td>110</td>
</tr>
<tr>
<td>Elemental IF Pwd</td>
<td>118</td>
<td>6.77</td>
<td>107</td>
</tr>
<tr>
<td>Adult nutritional RTD</td>
<td>14.8</td>
<td>5.85</td>
<td>110</td>
</tr>
<tr>
<td>Adult nutritional RTD</td>
<td>14.0</td>
<td>5.17</td>
<td>104</td>
</tr>
<tr>
<td>Adult nutritional RTD</td>
<td>11.4</td>
<td>3.34</td>
<td>100</td>
</tr>
<tr>
<td>Adult nutritional RTD</td>
<td>43.0</td>
<td>7.57</td>
<td>109</td>
</tr>
<tr>
<td>Adult nutritional Pwd</td>
<td>407</td>
<td>3.84</td>
<td>104</td>
</tr>
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</table>

²H₃ = Infant formula, RTF = ready-to-feed, Pwd = powder, RTD = ready-to-drink.
Method performance for vitamin D3 was determined in several matrices. Accuracy was determined by using overspike recovery evaluation. Samples were analyzed in triplicate over a 3 day interval using two instruments. Data show the intermediate precision ranged from 2.37 to 8.45% and accuracy ranging from 96.4–104% over the four matrixes with vitamin D2 concentrations in the final analytical sample of 12–270 ng/mL. This shows the repeatability of the method to determine vitamin D2 to be acceptable. The method chromatography facilitates the coelution of previtamin D2 with vitamin D2. As such, product-to-product variability in the thermal production of previtamin D2 is effectively addressed without the need to separately detect and quantify pre-vitamin D2 isomers that can be present in amounts as low as a few percent relative to vitamin D2. See Table 2 for results of all sample types.

See Figures 1 and 2 for representative chromatograms.

As a result of the AOAC new pathway approval of this method, the method will remain First Action for approximately 2 years before it is determined if the method meets requirements for Final Action. This allows time to generate additional information and allows the method to be used in a practical setting.

References

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

Table 2. Method performance summary for D2

<table>
<thead>
<tr>
<th>Product type</th>
<th>Accuracy, % recovery</th>
<th>Intermediate precision, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk-based IF RTF</td>
<td>96.4</td>
<td>4.11</td>
</tr>
<tr>
<td>Milk-based IF Pwd</td>
<td>100</td>
<td>2.89</td>
</tr>
<tr>
<td>Soy-based IF</td>
<td>100</td>
<td>2.37</td>
</tr>
<tr>
<td>Hydrolysate IF RTF</td>
<td>104</td>
<td>8.45</td>
</tr>
</tbody>
</table>

*IF = Infant formula, RTF = ready-to-feed, Pwd = powder, RTD = ready-to-drink.

Figure 1. Typical data vitamin D3 chromatogram: (a) working standard solution; (b) calibration curve; (c) infant formula; and (d) adult nutritional.

Figure 2. Typical data vitamin D2 chromatogram: (a) working standard solution; (b) calibration curve; and (c) infant formula.