INFANT FORMULA AND ADULT NUTRITIONALS

# Determination of Vitamins $D_2$ and $D_3$ in Infant Formula and Adult Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS): First Action 2011.12

**JACK STEVENS**
General Mills, Inc., 9000 Plymouth Ave N., Minneapolis, MN 55427
**DAWN DOWELL**
AOAC INTERNATIONAL, 481 N. Frederick Ave, Suite 500, Gaithersburg, MD 20877

**The method for the "Determination of Vitamins $D_2$ and $D_3$ 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 $D_2$ and $D_3$). 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 $D_2$ and 102.4–106.2% for vitamin $D_3$. The LOD and LOQ for vitamin $D_2$ were reported as 0.20 and 0.61 µg/100 g, respectively; for vitamin $D_3$, 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.**

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The method was approved by the Expert Review Panel on Infant Formula and Adult Nutritionals as First Action. *See* "Standards News," (2011) *Inside Laboratory Management*, July/August issue.
The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.
Corresponding author's e-mail: Jack.Stevens@genmills.com
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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 $D_2$ and $D_3$

Vitamin $D_2$, ergocalciferol, and vitamin $D_3$, 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 $D_2$ and $D_3$ 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.

## AOAC Official Method 2011.12
## Vitamins $D_2$ and $D_3$ in Infant Formula and Adult Nutritionals

### Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS) First Action 2011

(Applicable to the determination of vitamin D in infant formula and adult nutritionals by UPLC-MS/MS.)

*Caution:* Before working with chemicals, consult Material Safety Data Sheets for instructions on safety precautions. Use all personal protective equipment when required.

**Table 2011.12A. Sample dispensation guidelines for vitamin D analysis**

| Vitamin D samples | Quantity, g |
|---|---|
| Ready-to-eat cereal | >5 |
| NIST 1849 Infant/Adult Nutritional Formula | 1 |
| Bread | >5 |
| Mushrooms | >10 |
| Eggs | 2–3 |
| Yogurt | 20–25 |
| Cheese | 2–3 |
| Fish | 2–3 |
| Butter | 3–5 |
| Milk, RTF infant formula | 20–25 |
| Premix | <0.005 |

### *A. Principle*

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, followed by liquid–liquid extraction into *n*-heptane. Extraneous materials present in the *n*-heptane fraction are removed by performing a sequential liquid–liquid extraction with dilute potassium hydroxide in water, 40% ethanol in water, and water, respectively. The *n*-heptane fraction is filtered through sodium sulfate to remove residual water and evaporated to dryness 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. The eluant from the SPE columns are evaporated to dryness and reconstituted in methanol, and the samples assayed using a methanol and ammonium formate gradient on a UPLC-MS/MS system equipped with a C18 column. Vitamin D is reported as the quantity of vitamins $D_2$ and $D_3$ present.

### *B. Apparatus*

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

**Table 2011.12B. Inlet method vitamin D**

| Parameter | Value |
|---|---|
| Column | HSS C18 UPLC 2.1 × 100 mm, 1.8 µm |
| Column temperature | 40°C |
| Sample temperature | 8°C |
| Sample loop | Full loop; offline = 1.0 min |
| Flow rate | 0.4 mL/min |
| Injection volume | 20 µL |
| Seal wash | 1.0 min |
| Run time | 6.0 min |
| Wash solvents | Weak (600 µL); strong (600 µL) |

(**a**) *Analytical balance.*—Capable of weighing to 4 decimal places.

(**b**) *Pipets.*—100–1000 µL.

(**c**) *Graduated cylinders.*—100 and 1000 mL.

(**d**) *UPLC HSS C18 column.*—2.1 × 100 mm, 1.8 µm particle size (Waters Corp., Milford, MA.)

(**e**) *UPLC system.*—Acquity or equivalent (Waters Corp.).

(**f**) *Quattro Premier mass spectrometer.*—Waters Corp.

(**g**) *Nalgene separatory funnels.*—250 and 500 mL.

(**h**) *Disposable funnel.*

(**i**) *Glass wool.*

(**j**) *Connecting adaptors.*—14/20 Top (outer); 24/40 bottom (inner).

(**k**) *Genevac Rocket™ evaporation system.*—Genevac, Inc. (Gardiner, NY; www.genevac.com).

(**l**) *Water and nitrogen manifold.*

(**m**) *Heating mantle.*—6 × 500 mL.

(**n**) *Bull nose evaporation flasks.*

(**o**) *Amber autosampler vials.*—300 µL.

(**p**) *Autosampler vial caps.*—11 mm crimp seals.

(**q**) *Refrigerator.*—Explosion proof.

(**r**) *Flow meter.*—Thermo Scientific, Inc. (Pittsburgh, PA; www.thermofisher.com).

(**s**) *Bottle top dispensers.*—20–100 mL.

(**t**) *Boiling chips.*—Hengar micro granules (VWR International LLC, Battavia, IL).

(**u**) *Magnetic Teflon coated stir bars.*

(**v**) *Flat-bottom boiling flasks.*—500 mL.

(**w**) *Disposable weigh boats.*

(**x**) *Disposable weigh paper.*

(**y**) *Disposable borosilicate tubes.*—16 × 125 mm.

(**z**) *Bakerbond silica gel SPE cartridges.*—1000 mg silica in a 6 mL column.

(**aa**) *Pall Acrodisc syringe filters.*—0.45 µm, 13 mm (VWR International LLC).

(**bb**) *Transfer disposable pipets.*—9 in. (length) × 6 mL (volume; VWR International LLC).

(**cc**) *Pipettor tips.*—200 and 1000 µL.

(**dd**) *Analog vortex mixer.*

(**ee**) *Timer.*

(**ff**) *Visiprep™ vacuum manifold.*—SPE 12 port (Sigma-Aldrich, St. Louis, MO).

(**gg**) *Bench-top shaker.*—Glas-Col, LLC (Terre Haute, IN).

(**hh**) *Separatory funnel holder.*—Glas-Col, LLC.

(**ii**) *PTFE magnetic stir bar retriever.*—VWR International LLC.

(**jj**) *B-D disposable syringes.*—3 mL.

### *C. Reagents*

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

(**a**) *n-Heptane.*—HPLC grade; No. JT9177-03 (J.T. Baker,

**Table 2011.12C. UPLC gradient**

| Time, min | % A1, 2 mM $NH_4COOH$ | % B1, 2 mM $NH_4COOH$:MeOH | Gradient curve |
|---|---|---|---|
| 0 | 5 | 95 | — |
| 1 | 0 | 100 | 6 |
| 2 | 0 | 100 | 6 |
| 3 | 5 | 95 | 6 |

**Table 2011.12D. MS method: Vitamin D[a]**

| Analyte | Parent, *m/z* | Daughter, *m/z* | Cone voltage | Collision voltage |
|---|---|---|---|---|
| Vitamin $D_2$ [$^2H_3$] (internal standard) | 399.89 | 69.10 | 20 | 30 |
| | 399.89 | 109.31 | 20 | 30 |
| Vitamin $D_2$ | 397.6 | 69.10 | 20 | 30 |
| | 397.6 | 106.98 | 20 | 30 |
| | 397.6 | 271.30 | 20 | 30 |
| Vitamin $D_3$ [$^2H_3$] (internal standard) | 388.7 | 370.6 | 20 | 20 |
| | 388.7 | 259.6 | 20 | 20 |
| Vitamin $D_3$ | 385.5 | 107.15 | 20 | 20 |
| | 385.5 | 259.10 | 20 | 20 |

[a] Ionization mode: APCI+, span 0, interchannel delay 0.005, interscan time 0.005 s, dwell 0.1 s, APCI Probe 550°C.

**Table 2011.12E. UPLC-MS/MS operating parameters**

| Source parameter | Value | Analyzer parameter | Value |
|---|---|---|---|
| Corona | 5 | LM resolution 1 and 2 | 5 |
| Cone, V | 20 | HM resolution 1 and 2 | 5 |
| Extractor, V | 10 | Ion energy 1 | 0.1 |
| RF lens, V | 0 | Ion energy 2 | 10 |
| Desolvation temp., ±°C | 350 | Entrance, V | 0 |
| Source temp., °C | 150 | Collision, V | 30 |
| Desolvation gas, L/h | 350 | Exit, V | 0 |
| Cone gas flow, L/h | 50 | Multiplier | 850 |
| Probe temp., °C | 550 | Collision gas flow | 0.5 |

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. MK882903 (Phillipsburg, NJ).

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

(**g**) *Isopropanol.*—HPLC grade; EMD Omnisolv No. PX1834-6.

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

(**i**) *Methanol.*—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.*—Granular 12-60 mesh; J.T. Baker No. JT3375-5.

(**l**) *Purified water.*—(>18 MΩ) Nanopure.

(**m**) *Vitamin $D_3$ (cholecalciferol).*—Cat. No. 1131009, purity ≥98% (USP, Rockville, MD).

(**n**) *Vitamin $D_2$ (ergocalciferol).*—Cat. No. 1239005, purity ≥98% (USP).

(**o**) *Vitamin $D_3$ [$^2H_3$] (isotopically labeled).*—Cat. No. S3077-1.0 (1 mg/mL in ethanol), purity ≥97% (Isosciences, King of Prussia, PA).

(**p**) *Vitamin $D_2$ [$^2H_3$] (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 $NH_4COOH$.*—Dissolve 0.126 g ammonium formate in water and dilute to 1 L.

(**d**) *Mobile phase B1: 2 mM $NH_4COOH$–MeOH.*—Dissolve 0.126 g ammonium formate in methanol and dilute to 1 L.

(**e**) *Strong wash solution: 80% MeOH:20% purified $H_2O$.*—Dilute 800 mL methanol to 1 L with water.

(**f**) *Weak wash solution: 90% purified $H_2O$:10% MeOH.*—Dilute 100 mL methanol to 1 L with water.

(**g**) *Internal standard stock solution vitamin $D_2$ [$^2H_3$] (0.1 mg/mL).*—Dissolve 1 mg/mL vitamin $D_2$ [$^2H_3$] in 200-proof ethanol and dilute to 10 mL.

(**h**) *Internal standard stock solution vitamin $D_3$ [$^2H_3$] (0.1 mg/mL).*—Dissolve 1 mg/mL vitamin $D_3$ [$^2H_3$] in 200-proof ethanol and dilute to 10 mL.

(*1*) *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 $D_2$ [$^2H_3$]/vitamin $D_3$ [$^2H_3$] internal standard working stock solution (1000 ng/mL).*—Dilute 250 µL internal standard stock solution vitamin $D_2$ [$^2H_3$] (0.1 mg/mL) and 250 µL internal standard stock solution vitamin $D_3$ [$^2H_3$] (0.1 mg/mL) to 25 mL with 200-proof ethanol.

(*4*) *Vitamin $D_2$ stock solution (0.1 mg/mL).*—Dissolve 10 mg vitamin $D_2$ in 200-proof ethanol to 100 mL.

(*5*) *Vitamin $D_3$ stock solution (0.1 mg/mL).*—Dissolve 10 mg vitamin $D_3$ in 200-proof ethanol to 100 mL.

(*6*) *Vitamin $D_2$/vitamin $D_3$ standard working stock solution (1000 ng/mL).*—Dilute 250 µL vitamin $D_2$ stock solution (0.1 mg/mL) and 250 µL vitamin $D_3$ 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 $D_2$ [$^2H_3$]/vitamin $D_3$ [$^2H_3$] internal standard working stock solution (1000 ng/mL) and 1 mL vitamin $D_2$/vitamin $D_3$ standard working stock solution (1000 ng/mL) to 5 mL.

*Note*: Stock and working stock solutions are stable in the dark at –20°C for 1 year. Stock solutions should be brought to room temperature before use. Prepare the vitamin D mixed reference standard daily.

### *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 $D_2$ [$^2H_3$]/vitamin $D_3$ [$^2H_3$] 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 $D_2$ [$^2H_3$]/vitamin $D_3$ [$^2H_3$] 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.

$$RF = \frac{PA_a \times C_{is}}{PA_{is} \times C_a} \qquad (1)$$

where $RF$ = response factor; $PA_a$ = peak area of analyte; $C_{is}$ = concentration of respective internal standard; $PA_{is}$ = peak area of respective internal standard; and $C_a$ = concentration of analyte.

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

$$C = \frac{PA_a \times C_{is}}{PA_{is} \times RF_a} \times \frac{V}{W} \times \frac{1}{1000} \times 100 \qquad (2)$$

where $C$ = concentration of analyte in sample µg/100 g; $PA_a$ = peak area of analyte in sample; $C_{is}$ = concentration of respective internal standard added to sample (ng); $PA_{is}$ = 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.

(**c**) Report the concentration of vitamin D found as the total of vitamins $D_2$ and $D_3$.

Reference: *J. AOAC Int.* xxx(2012)

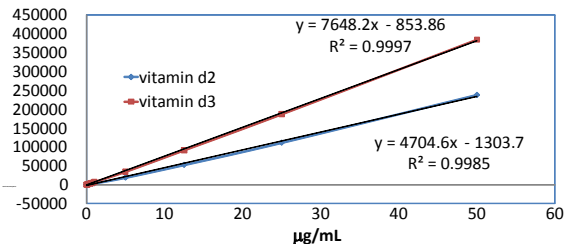


**Figure 1. Vitamins $D_2$ and $D_3$ linear regression.**

**Table 1. LOD and LOQ results[a]**

| Analyte | LOD ng/mL, inj. soln | LOQ ng/mL, inj. soln | LOD, µg/100 g infant formula | LOQ, µg/100 g infant formula |
|---|---|---|---|---|
| Vitamin $D_2$ | 2.0 | 6.1 | 0.20 | 0.61 |
| Vitamin $D_3$ | 4.7 | 14.4 | 0.47 | 1.44 |

[a] 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.

## Results and Discussion

### *System Linearity Study*

The linearity of vitamins $D_2$ and $D_3$ 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 l mL final solution. The LOD was found to be 0.20 and 0.47 for vitamins $D_2$ and $D_3$, respectively. The LOQ was found to be 0.61 and 1.44 for vitamins $D_2$ and $D_3$, respectively. Adjusting sampling weight and final solution volume will give a greater sensitivity. *See* Table 1 for details.

$$LOD = 3.3\frac{SD}{S} \qquad LOQ = 10\frac{SD}{S}$$

### *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 $D_3$, with the exception of irradiated mushrooms. Also notable is that whole eggs contained both vitamins $D_2$ and $D_3$. 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 2. Precision results (µg/100 g)**

| AOAC sector | 1 | 4 | 4 | 5 | 5 | 5 | 6 | 7 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Day No. | Butter | Egg | Cheese | Yogurt | Ready-to-eat cereal | Bread | NIST 1849 | Mushroom | Tuna |
| 1–1 | 13.22 | 8.81 | 5.57 | 1.99 | 6.73 | 2.10 | 25.95 | 11.47 | 2.15 |
| 1–2 | 12.10 | 9.46 | 5.51 | 2.19 | 6.05 | 2.34 | 26.64 | 11.90 | 2.57 |
| 1–3 | 12.16 | 8.94 | 4.91 | 1.94 | 6.38 | 1.99 | 26.54 | 11.86 | 2.46 |
| 2–1 | 11.57 | 8.49 | 4.27 | 1.87 | 6.22 | 1.66 | 22.96 | 13.59 | 1.99 |
| 2–2 | 12.02 | 8.64 | 4.08 | 1.96 | 6.47 | 2.07 | 23.19 | 14.78 | 2.23 |
| 2–3 | 12.67 | 9.15 | 4.10 | 1.85 | 5.21 | 1.96 | 24.75 | 14.78 | 2.05 |
| 3–1 | 13.66 | 7.34 | 4.45 | 1.57 | 7.20 | 1.87 | 23.41 | 12.87 | 2.07 |
| 3–2 | 14.05 | 8.88 | 4.15 | 2.09 | 5.34 | 1.72 | 26.99 | 12.09 | 1.98 |
| 3–3 | 13.15 | 7.43 | 3.89 | 1.64 | 5.48 | 1.56 | 24.30 | 12.05 | 3.03 |
| 4–1 | 13.14 | 7.39 | 4.16 | 1.56 | 5.44 | 1.82 | 24.99 | 11.93 | 2.25 |
| 4–2 | 15.71 | 7.93 | 5.31 | 1.82 | 5.89 | 1.62 | 24.53 | 10.99 | 2.72 |
| 4–3 | 13.34 | 7.80 | 4.28 | 1.62 | 7.16 | 2.40 | 21.10 | 14.49 | 2.69 |
| 5–1 | 13.15 | 7.53 | 4.45 | 2.32 | 5.88 | 2.17 | 25.40 | 15.30 | 2.24 |
| 5–2 | 12.70 | 7.69 | 4.35 | 1.58 | 5.87 | 1.91 | 24.99 | 16.36 | 2.51 |
| 5–3 | 13.48 | 9.05 | 4.06 | 1.66 | 6.26 | 2.21 | 25.57 | 15.94 | 2.73 |
| Mean | 13.07 | 8.30 | 4.50 | 1.84 | 6.10 | 1.96 | 24.75 | 13.36 | 2.38 |
| σ | 1.00 | 0.74 | 0.55 | 0.24 | 0.62 | 0.26 | 1.59 | 1.77 | 0.32 |
| RSD, % | 7.63 | 8.90 | 12.25 | 12.94 | 10.10 | 13.10 | 6.43 | 13.26 | 13.52 |

**Table 3. Results of accuracy testing**

| Identity | Food matrix | AOAC food triangle sector | Vitamin $D_2$ recovery, % | Vitamin $D_3$ recovery, % |
|---|---|---|---|---|
| IRM 25F | RTE cereal | 5 | 100.8 | 102.4 |
| Mushrooms | Vegetable | 7 | 93.4 | 104.2 |
| NIST SRM 1849 | Infant/adult nutritional | 6 | 100.9 | 105.5 |
| Yogurt | Dairy | 5 | 95.5 | 106.2 |

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

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

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

| Mean | σ | *k* | μ |
|---|---|---|---|
| 5.89 | 0.653 | 2.04 | 1.33 |

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

## References

(1) Sullivan, D. (2012) *J. AOAC Int.* **95**, 287–290. http://dx.doi.org/10.5740/jaoacint.Sullivan_Intro

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