Analysis of Vitamin D in Milk and Infant Formula using UHPLC/MS/MS per AOAC Method 2011.11

Olga Shimelis, Principal R&D Scientist, Supelco
olga.shimelis@sial.com

Fortification of milk, dairy products, infant and adult nutritionals with vitamin D is required in many countries to provide additional dietary vitamin D. The fortification of milk in the USA, for example, is done at 400 International Units (IU) per eight ounces of milk. The recently adopted AOAC method 2011.11 describes the procedure for the analysis of vitamin D in fortified infant formula. This method was used in the current work to measure vitamin D in the fortified whole milk and in an infant formula sample. Two forms of vitamin D are recognized: vitamin D_2 or ergocalciferol and vitamin D_3 or cholecalciferol. Although both forms may be present in the fortified food, vitamin D_3 is more commonly used for fortification of dairy products. Vitamin D hydroxy-metabolites can also be present in food products at low levels. While quantitative analysis was performed for vitamins D_2 and D_3, the samples were scanned for the presence of 25-hydroxy metabolites.

Method

AOAC method 2011.11 was followed for analysis of vitamin D in both milk and infant formula. Vitamin D_2 and D_3 standards and their deuterated analogues were used. The UHPLC column was Titan C18, 10 cm x 2.1 mm packed with 1.9 µm particles. The HPLC method was adopted from the standard AOAC method, including mobile phases, gradient, and flow rates. Baseline separation between vitamin D_3 and D_2 peaks, and between all vitamins and matrix component peaks was obtained on the Titan C18 UHPLC column. Analysis of vitamin D was done using MS/MS with APCI detection. MS transitions used in this study are shown in **Table 1**.

Table 1. MS Parameters Used for the Measurement of Vitamin D Forms

Compound	Q1	Q3
Vitamin D_3	385.5	259.2
Vitamin D_3	385.5	159.2
Vitamin D_2	397.5	125.2
Vitamin D_2	397.5	107.2
Vitamin D_3-d_3	388.5	259.2
Vitamin D_2-d_3	400.5	125.2
25-OH-Vitamin D_3	383.4	257.2
25-OH-Vitamin D_2	395.2	269.2

The sample preparation for both milk and infant formula samples was done through saponification, extraction, evaporation, and reconstitution of extracts into LC-compatible solvents. The sample preparation steps following AOAC method 2011.11 are shown in **Figure 1**.

Figure 1. Preparation Method for Milk and Nutritional Supplements

Saponification	• To 30 mL of milk or infant formula add 40 mL of reagent-grade alcohol containing 2% pyrogallol, isotope D_3 I.S., and 20 mL of 50% w/v KOH • Mix overnight at room temperature
Extraction	• Add to separatory funnel along with 30 mL of hexane containing 12.5 mg/mL BHT and shake to extract • Wash hexane fraction with 20 mL of water:50% w/v KOH (85:15)
Sample Preparation	• Remove 10 mL of hexane extract and dry under nitrogen • Reconsitute in 1 mL of acetonitrile:water (70:30) with 5-minute sonication • Filter through 0.45 µm PTFE membrane

Table 2. Found Concentrations (IU/mL) for Vitamin D with % RSD (n=3)

Sample	Vitamin D_2	Vitamin D_3
Whole milk	0.16 (41%)	4.76 (4%)
Infant formula	0	5.29 (0.2%)

Results

Chromatography of the vitamin D and hydroxyvitamin D compounds is presented in **Figures 2** and **3**, respectively. Analyses of vitamin D_2 and D_3 in extracted milk and infant formula are shown in **Figures 4** and **5**, respectively. No Vitamin D_2 was found in infant formula while a low amount of D_2 was found in milk. Concentrations of Vitamin D_3 in milk and infant formula were found to be close to the expected fortification levels of at least 4.2 IU/mL (**Table 2**). No hydroxyvitamin D forms were found in samples of either milk or infant formula. The lowest level for calibration curve was 0.2 IU/mL which would correspond to 0.5 ng of vitamins per mL of milk.

Conclusions

Presented here is a method for the determination of nutritionally important vitamin D forms as standards and extracted from milk and infant formula after saponification following AOAC method 2011.11.

Figure 2. UHPLC/MS Analysis Vitamin D Standards at 1 IU/mL on Titan C18

Vitamin D_3 is shown as 385/259 parent/daughter transition and vitamin D_2 is shown as 397/125 transition using the same Y-scale.

column: Titan C18, 10 cm × 2.1 mm I.D., 1.9 µm (577124-U)
mobile phase: [A] 0.1% (v/v) formic acid in methanol: water (20:80); [B] 0.1% (v/v) formic acid in methanol
gradient: 60 to 90% B in 0.4 min, to 100% B in 0.3 min, held for 7.8 min, to 60% B, held for 1.5 min
flow rate: 0.25 mL/min for 5.55 min, to 0.50 mL/min for 3.84 min, to 0.25 mL/min for 0.7 min
column temp.: 25 °C
detector: MS/MS, APCI(+), MRM as indicated in Table 1
injection: 5 µL
sample: vitamin D_2 and D_3, 1 IU/mL

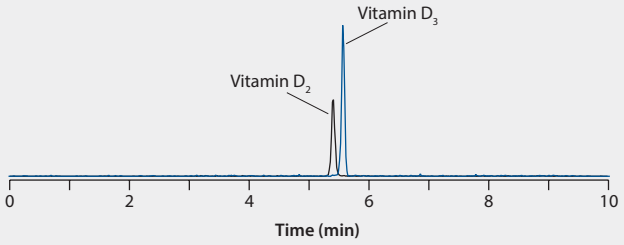


Figure 3. UHPLC/MS Analysis of 25-OH-Vitamin D Standards at 250 ng/mL on Titan C18

25-OH-Vitamin D_3 (red trace) is shown as 383/257 parent/daughter transition and 25-OH-vitamin D_2 (blue trace) is shown as 395/269 transition using the same Y-scale. Conditions same as in Figure 2.

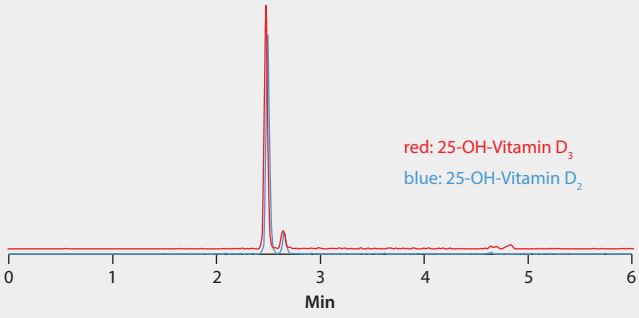


Figure 4. UHPLC/MS Analysis Vitamin D in Milk on Titan C18

Vitamin D_3 is shown as 385/259 parent/daughter transition and vitamin D_2 is shown as 397/125 transition using the same Y-scale. Conditions as in Figure 2.

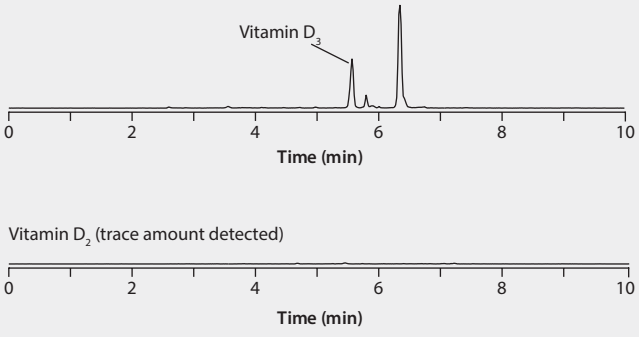
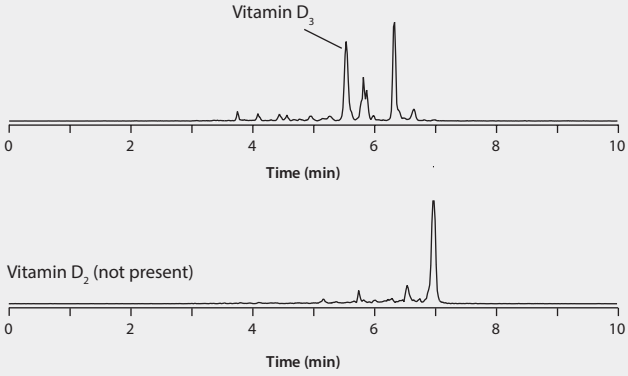


Figure 5. UHPLC/MS Analysis Vitamin D in Infant Formula on Titan C18

Vitamin D_3 is shown as 385/259 parent/daughter transition and vitamin D_2 is shown as 397/125 transition using the same Y-scale. Conditions as in Figure 2.



Featured Products

Description	Cat. No.
UHPLC Column	
Titan C18, 10 cm × 2.1 mm I.D., 1.9 µm particle size	577124-U
Mobile Phase Components	
Methanol, LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS, 1 L, 2 L	14262
Water, LC-MS Ultra CHROMASOLV, tested for UHPLC-MS, 1 L, 2 L	14263
Formic acid, LC-MS Ultra eluent additive, 1 mL, 2 mL	14265
Standards	
Ergocalciferol (vitamin D_2)	47768
Cholecalciferol (vitamin D_3)	47763
Vitamin D_3 (6,19,19-d_3) solution	740284
Vitamin D_2 (6,19,19-d_3) solution	739847
25-Hydroxyvitamin D_2	17937
25-Hydroxyvitamin D_3	17938
Saponification and Extraction Solvents and Reagents	
Hexane, CHROMASOLV, for HPLC, ≥97%	34859
Water, CHROMASOLV Plus, for HPLC, ≥99.9%	34877
Acetonitrile, CHROMASOLV Plus, for HPLC, ≥99.9%	34998
Reagent alcohol (ethanol)	362808
Pyrogallol, ACS reagent	254002
Potassium hydroxide, ACS reagent, ≥85%, pellets	221473
2,6-Di-*tert*-butyl-4-methylphenol (BHT), ≥99.0% (GC), powder	B1378
Glassware and Accessories	
Aldrich® separatory funnel with glass stopcock. capacity 250 mL	Z548030
Acrodisc® syringe filters; PTFE membrane, diam. 13 mm, pore size 0.45 µm	Z259888