

Measurement of Vitamin D in Milk and Infant Formulas Using Automated Online Sample Preparation with Liquid Chromatography/Tandem Mass Spectrometry

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Key Words

Transcend TLX-1, TurboFlow technology, TSQ Access MAX, Vitamin D, Infant formula, Milk

Goal

To develop a rapid and sensitive automated online sample preparation LC-MS/MS method to measure vitamin D in infant formula.

Introduction

Vitamin D, including vitamin D₂ and D₃ (Figure 1), is essential to calcium absorption and bone health. Current studies suggest vitamin D could help prevent a wide range of disorders.¹ Although vitamin D can be synthesized by the human body from exposure to sunlight, this process can be impaired by many factors.² Therefore, fortification of vitamin D in some dietary sources becomes necessary. The Institute of Medicine (IOM) of the National Academies recommends an average vitamin D intake of 400 IU/day (10 µg/day) vitamin D for infants 12 months of age and younger. This recommendation increases to 600 IU/day for older children and adults. The intake of excessive amounts of vitamin D can, however, lead to a potentially serious condition, vitamin D toxicity, also called hypervitaminosis D. It is thus critical to measure the amount of vitamin D in foods, occurring both naturally and from fortification.

The level of vitamin D in food has been determined using gas chromatography (GC)³, high-performance liquid chromatography (HPLC)⁴, and other physicochemical methods. The use of liquid chromatography/tandem mass spectrometry (LC-MS/MS) has gained popularity in vitamin D analysis due to its selectivity, specificity, and reliability. Traditional sample preparation for LC-MS/MS analysis of vitamin D can be time and labor intensive, often involving saponification, liquid extraction, solvent evaporation, manual solid phase extraction, and pre-concentration. In addition, thermal isomerization of vitamin D to previtamin D can occur during saponification, which usually complicates LC/MS analysis.

In this application note, we describe an easy and comprehensive LC-MS/MS method using a Thermo Scientific Transcend TLX-1 system powered by Thermo Scientific TurboFlow technology to measure vitamin D levels in infant formula. TurboFlow™ technology eliminates most traditional sample preparation steps.

Experimental

Stock Solutions

Vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol) were purchased from Sigma (St Louis, MO). The internal standards (IS), vitamin D₂-[³H] and vitamin D₃-[³H] were obtained from IsoSciences (King of Prussia, PA). A stock mixture was prepared in methanol at 10 µg/mL for both analytes.

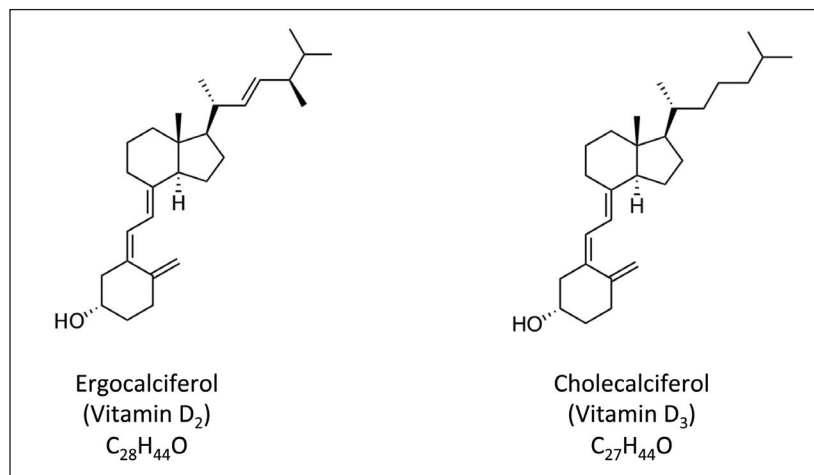


Figure 1. Chemical structures of vitamin D

Matrix Standard Curve

Due to the difficulty obtaining infant formula without vitamin D fortification, low-fat half and half (HH), a blend of milk and cream purchased from a local food store, was used as a blank matrix in which to prepare a standard curve. Five grams of HH spiked with internal standards (IS) were extracted using 5 mL of ethanol followed by 5 minutes of ultrasonication. Methanol was added to bring the total volume to 15 mL. The resultant solution was then transferred to multiple 2 mL polypropylene tubes and centrifuged at 13000 RPM (at 25 °C) for 10 minutes. The supernatant was used to prepare the matrix calibrators and QC samples. The matrix calibrant concentrations ranged from 0.5 ng/mL to 100 ng/mL. The final IS concentrations were 25 ng/mL and 10 ng/mL for vitamin D₂ and D₃, respectively. Each milliliter of supernatant corresponds to 0.33 g HH as the unit conversion.

Infant Formula Sample Preparation

Solid Powder

Canned infant formula powders from various manufacturers were obtained from a local food store. The infant formula solution was prepared by adding 5 mL of water to 5 g of infant formula powder, vortexed, and then brought to a total final volume of 10 mL by adding more water. Two milliliters of this solution was transferred into a new 50 mL centrifuge tube. The extraction was performed using 5 mL each of ethanol and methanol and followed by 10 minutes of ultrasonication. Centrifugation was performed at 7500 RPM (at 25 °C) for 10 minutes to separate the precipitated solids from the supernatant. The supernatant was filtered through a 0.45 µm membrane filter.

Liquid Formula

Liquid infant formula was also obtained from a local food store. Fifteen milliliters of liquid formula were extracted using 5 mL each of ethanol and methanol followed by vortexing for 1 minute. This mixture was then centrifuged at 7500 RPM (at 25 °C) for 10 minutes to separate the precipitated solids from the supernatant. One-half milliliter of supernatant was transferred into a 2 mL polypropylene tube and 0.5 mL of methanol was added, followed by centrifugation at 13000 RPM (at 25 °C) for 10 minutes. The supernatant was then filtered through a 0.45 µm membrane filter.

LC/MS Method

TurboFlow Method Parameters

Column:	TurboFlow C18-P XL 0.5 x 50 mm
Injection Volume:	50 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol
Solvent D:	1:1:1 acetonitrile isopropyl alcohol : acetone (v:v:v)

HPLC Method Parameters

Analytical Column:	Thermo Scientific Hypersil GOLD 2.1 x 50 mm, 3 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters

MS:	Thermo Scientific TSQ Access MAX triple stage quadrupole
Polarity:	Positive mode
MS Ionization Source:	Atmospheric-pressure chemical ionization (APCI)
Discharge Current:	3.5 KV
Sheath Gas Pressure (N ₂):	40 arbitrary units
Auxiliary Gas Pressure (N ₂):	15 arbitrary units
Vaporizer Temperature:	375 °C
Capillary Temperature:	310 °C
Collision Gas Pressure:	1.5 mTorr
Q1 Peak Width:	0.7 Da
Q3 Peak Width:	0.7 Da

The LC method schematic view in Thermo Scientific Aria operating software (OS) is shown in Figure 2. The fragment ions and corresponding mass spectrometer parameters are listed in Table 1. For vitamin D₂, the product ion *m/z* 107 was selected as quantifier due to less interference and relatively high intensity. The entire experiment was controlled by Aria™ OS version 1.6.3. The data were processed using Thermo Scientific TraceFinder software version 1.1.

Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	30	2.00	Step	20.0	80.0	-	-	=====	out	1.00	Step	98.0	2.0
2	0.50	120	0.15	Step	100.0	-	-	-	T	in	0.90	Step	98.0	2.0
3	2.50	20	2.00	Step	-	-	-	100.0	=====	out	1.00	Ramp	40.0	60.0
4	2.83	20	2.00	Step	-	100.0	-	-	=====	out	1.00	Ramp	20.0	80.0
5	3.17	60	2.00	Step	-	-	-	100.0	=====	in	1.00	Ramp	10.0	90.0
6	4.17	90	2.00	Step	-	100.0	-	-	=====	out	1.00	Ramp	-	100.0
7	5.67	30	2.00	Step	-	-	-	100.0	=====	in	1.00	Step	-	100.0
8	6.17	30	2.00	Step	-	100.0	-	-	=====	in	1.00	Step	-	100.0
9	6.67	240	2.00	Step	20.0	80.0	-	-	=====	out	1.00	Step	98.0	2.0

Figure 2. TurboFlow method schematic diagram as viewed in Aria OS software

Vitamin	Parent ion (m/z)	Quantifier (m/z)	Tube Lens (V)	Collision Energy (V)	Qualifier (m/z)	Tube Lens (V)	Collision Energy (V)
Vitamin D ₂	397.3	107.1	87	29	159.2	87	22
Vitamin D ₂ -[² H ₃]	400.3	107.1	90	32	-	-	-
Vitamin D ₃	385.3	259.2	106	15	91.1	106	53
Vitamin D ₃ -[² H ₃]	388.3	259.2	76	15	-	-	-

Results and Discussion

Prior to determining the calibration curve, the matrix blank was screened to ensure there was no detectable vitamin D. Figure 3 shows the representative chromatograms of both analytes at 10 ng/mL (3.0 µg/100 g), indicating good signal intensity and chromatographic peak shape. Matrix-matched calibration standards showed linear response of two orders of magnitude ($r^2 > 0.99$) for both analytes as shown in Figure 4. The limits of quantitation (LOQ) were determined at 1.0 ng/mL (0.3 µg/100 g) and 0.5 ng/mL (0.15 µg/100 g) for vitamin D₂ and D₃, respectively. All coefficients of variation (CV) (n=3) were less than 20% for the LLOQ and less than 15% for all other points of the curve.

The results of an analysis of a series of actual infant formula samples are presented in Table 2. Since none of the product labels mentioned the presence of vitamin D₂, only vitamin D₃ was measured. Vitamin D₃-[²H₃] was used to minimize the impact of matrix interference. Comparing the claimed and experimentally obtained values of vitamin D, less than 15% difference was observed for all samples. These results are satisfactory considering the vitamin D content tends to decrease with time and changes in storage conditions⁵. Excellent analytical reproducibility was demonstrated with CVs (n=6) of no more than 11% for 5 tested samples. It should be noted that since the results obtained from real samples were calculated against the standard calibration curve from HH matrix, some deviation may occur. Overall, the current LC-MS/MS strategy was able to detect vitamin D present in the actual samples, proving to be a fast and efficient screening tool.

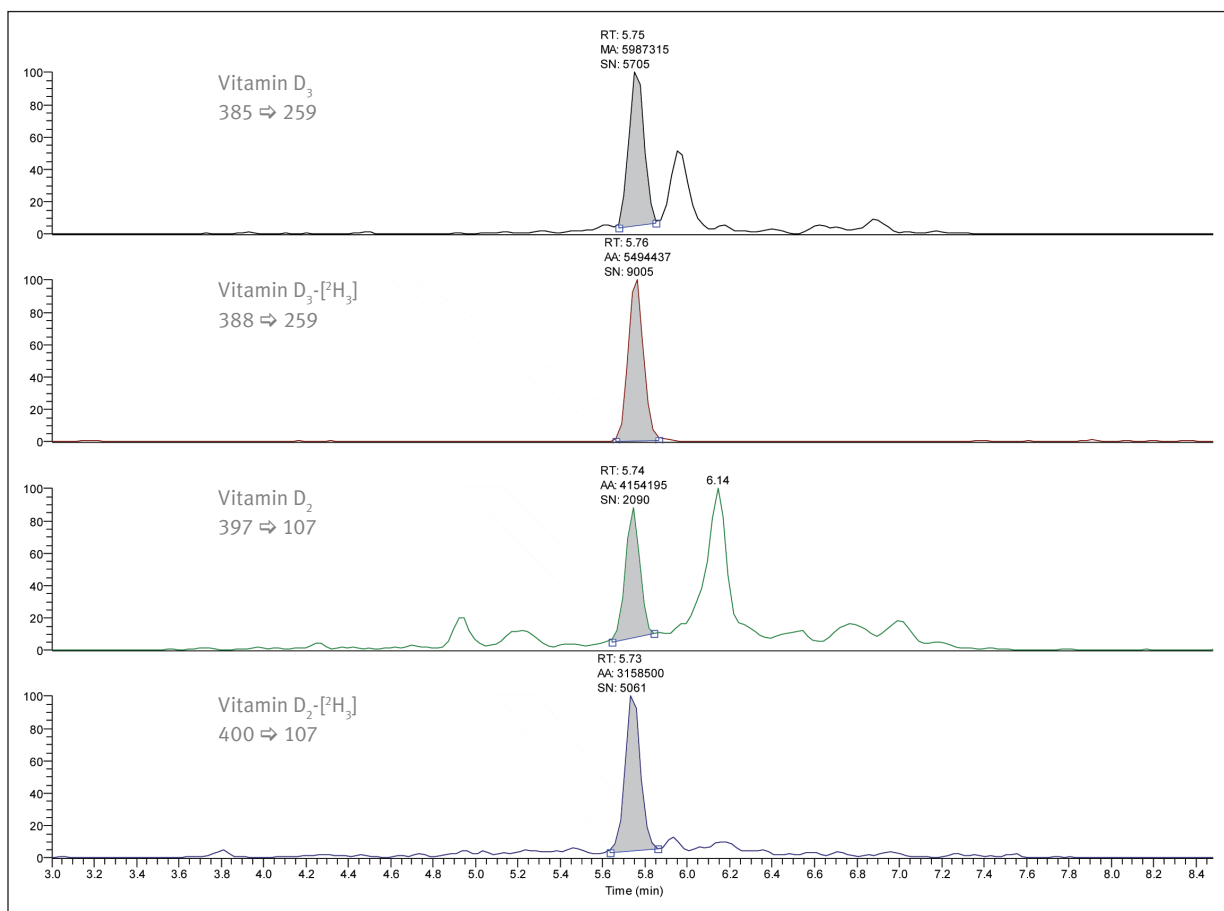


Figure 3. Example chromatogram of 10 ng/mL calibration standard

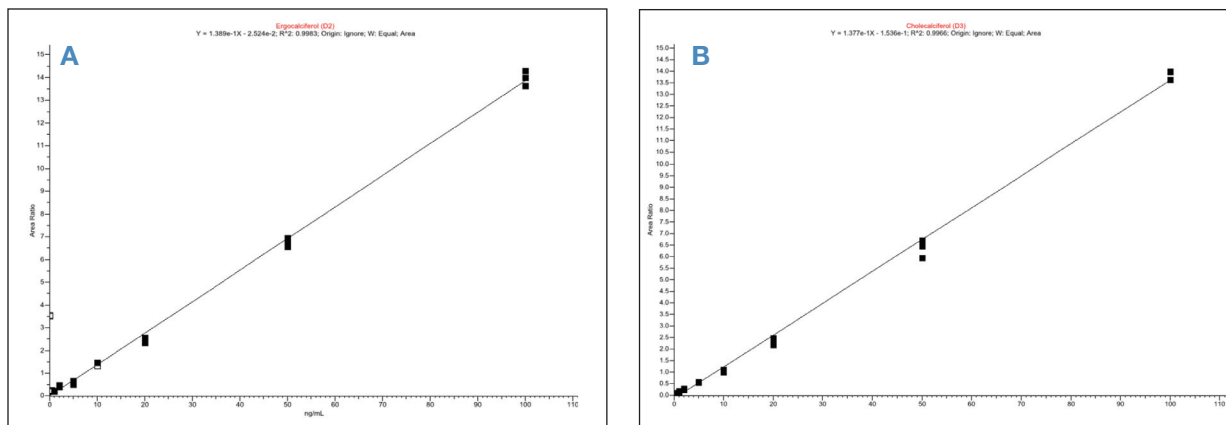


Figure 4. Linear regression curve of vitamin D₂ (A) and D₃ (B) standards based on area ratio with individual internal standards

Table 2. Results obtained for vitamin D in real infant formula samples

Sample Number	Powder or liquid	Form of vitamin D on label	Claimed (IU/5 fl oz)	LC-MS/MS (ng/mL)	Calculated (IU/5 fl oz)	Coefficient of variation (n=6) (%)
1	Powder	D ₃	75	10.4	67.8	7.8
2	Powder	D ₃	60	8.2	54.1	7.9
3	Powder	D ₃	50	6.6	44.6	8.7
4	Powder (Soy-based)	D ₃	60	8.3	55.4	6.9
5	Liquid	D ₃	45	3.3	40.7	11.0

Conclusion

A quick, automated online sample preparation LC-MS/MS method has been developed that is sensitive enough to measure vitamin D content in infant formula. Because this method eliminates the saponification step, no toxic or environmentally unfriendly solvents were used in sample preparation. Good recoveries were achieved for both powdered and liquid infant formula. The sample throughput can be improved by multiplexing the two methods on different LC channels using a Transcend™ TLX-2 (or TLX-4) system. Future work will focus on the application of this methodology to other food matrices and references.

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