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Application Note

Determination of Vitamin D and Previtamin D in Food Products

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Abstract

This application note demonstrates an improvement in the current LC-MS method for vitamin D analysis. It demonstrates the determination of total vitamin D by individually measuring the vitamin D and previtamin D in food products. Previtamin D was directly measured in the total vitamin D analysis, eliminating the error that could arise from not measuring previtamin D. This method will be less affected by the heating history of food products.

Benefits

- Determination of both previtamin D and vitamin D.
- More accurate and precise determination of total vitamin D analysis.

Introduction

Vitamin D is a fat-soluble vitamin that promotes calcium absorption and maintains adequate serum calcium and phosphate concentrations to enable normal mineralization of bone and to prevent hypocalcemic tetany.[1] The most common vitamin D compounds are vitamin D_3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol). Their structures are shown in Figure 1. Vitamin D can be produced endogenously when ultraviolet (UV) light strikes the skin and triggers vitamin D synthesis. Recent studies revealed that humans might not produce adequate supplies of vitamin D from exposure to sunlight alone, so it is important to supplement vitamin D intake through diet.[2] The U.S. Food and Drug Administration (FDA) revised the food labeling regulations in 2016 to make the vitamin D content a required item on the nutrition or supplement facts labels for conventional food and dietary supplements.[3] The change in labeling regulation is aimed to promote vitamin D awareness among consumers.

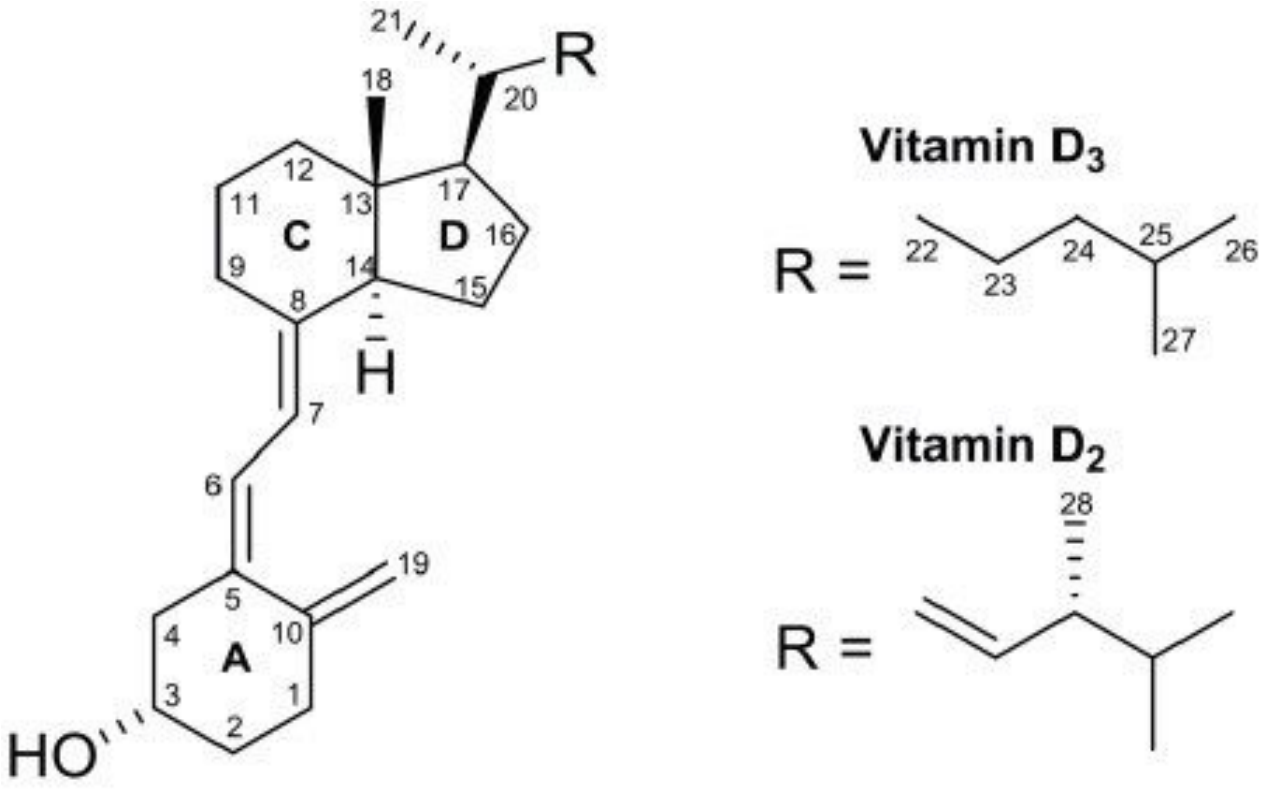

Figure 1. Structures of vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol).

Existing standard methods for vitamin D analysis involve saponification, liquid-liquid extraction (LLE), sample clean-up, and liquid chromatography (LC)-UV determination. The most challenging aspect in vitamin D analysis is the diverse interferences from sample matrix. A large number of lipid-like compounds are co-extracted with the vitamin D, and even after extensive sample clean-up, there are still numerous interferences that co-elute and interfere with the vitamin D quantitation. Recently, to simplify the sample preparation and to improve the analysis, a derivatization reaction with 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) and mass spectrometry (MS) were adopted in a new AOAC standard method.[4] This new method has provided much better analytical performance for vitamin D analysis. However, previtamin D is not measured in this new standard.

It is known that vitamin D can thermally isomerize to previtamin D. This transformation is reversible (Figure 2), and both forms are biologically active. It has been reported that the relative content of previtamin D could be up to 22% of the total vitamin D at 80 °C.[5] Therefore, it is prudent to individually determine previtamin D and vitamin D contents in the analysis of vitamin D in foods. This application note demonstrates the determination of total vitamin D by individually measuring the vitamin D and previtamin D in food products.

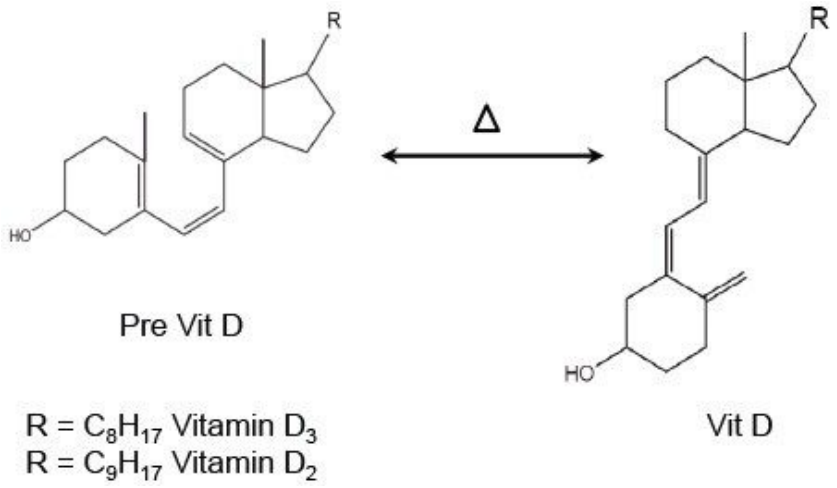

Figure 2. Reversible thermal isomerization of previtamin D to vitamin D. The equilibration constant and equilibration time depends on temperature.[5]

Experimental

Samples

Vitamin D_3 and vitamin D_2 were purchased from Sigma-Aldrich. Stable isotope labeled cholecalciferol (6,19,19-d_3) (SIL-D_3) was purchased from Cambridge Isotope Labs (Tewksbury, MA) and used as the internal standard (IS). Infant formula reference material NIST 1849a was purchased from National Institute of Science and Technology (NIST, Gaithersburg, MD). PTAD, potassium hydroxide (KOH), pyrogallol (or 1,2,3-trihydroxybenzene), butylated hydroxytoluene (BHT), formic acid, and absolute ethanol were purchased from Sigma-Aldrich. Food products, such as non-fat dry milk powder (fortified with A and D), infant formulas (milk based and soy based), oatmeal, and fish oil were purchased from local market. All sample preparation was carried out in subdued light and in amber glass vials.

Standard calibration solutions

The Vitamin D standards (vitamin D_3 and vitamin D_2) were dissolved in absolute ethanol to form 1 mg/mL stock solutions. Portions of these vitamin D stock solutions were used for the purity check using a UV/Vis spectrophotometer. Aliquots of the vitamin D stock solutions were mixed and diluted with acetonitrile (ACN) to form vitamin D mix stock solutions. The vitamin D mix stock solutions were spiked with IS (SIL-D_3), and

diluted with ACN to obtain a series of standard calibration solutions ranging from 1 ppb to 500 ppb (or ng/mL). The IS level was kept at constant concentration in these standard solutions. These calibration solutions underwent the derivatization step as described below.

Sample solutions

About 0.5 g (weighted to 0.001 g) of samples were separately spiked with IS (20 ng SIL-D_3), mixed with 4 mL of water and 16 mL of pyrogallol ethanolic solution (2 g/100 mL). 8 mL of KOH (50%) solution was then added and mixed. The solutions turned black after mixing. The solutions were put in a hot water bath (75 °C) for 1 hour with periodical mixing every 30 min. After the solutions were cooled to room temperature in an ice bath, 12 mL of hexanes (with 12.5 mg/L BHT) was added, mixed, and centrifuged. The hexane layer portion was taken and washed with 8 mL water 4 times. A centrifuge (1500 rpm for 2 min) was used to aid the phase separation. These extracts underwent derivatization as described next.

Derivatization

100 µL of each standard calibration solution or 6 mL of the hexanes extract from each sample was dried with a gentle nitrogen stream at 30 °C, then mixed with 0.6 mL PTAD solution (1 mg/mL in ACN). The mixtures were kept at room temperature in the dark for 40 minutes. The derivatization reaction was quenched with 0.4 mL water and the sample was filtered with a 0.2 µm PTEF syringe filter before injection.

LC conditions

System:	ACQUITY UPLC H-Class with Xevo TQ-S micro MS System
Software:	MassLynx v4.1
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm
Column temp.:	40 °C
Mobile phases:	A) Water (0.1% formic acid); B) ACN (0.1% formic acid)
Inj Vol.:	10 µL

Flow rate:	0.60 mL/min
Run time:	8.5 min

MS conditions

Polarity:	ESI+
Capillary (kV):	1.2
Source temp.:	150 °C
Desolvation temp.:	500 °C
Cone gas flow (L/Hr):	0
Desolvation gas flow (L/Hr):	1000

Gradient

	Time (min)	%A	%B	Curve
1	Initial	80	20	initial
2	0.25	80	20	6
3	2.75	0	100	6
4	6.5	0	100	6
5	6.6	80	20	6

Table 1. Elution gradient.

	MRM	Dwell (secs)	Cone volt	Col. energy	Delay (secs)	Compound	Note
1	560.3>161.0	0.032	43	36	Auto	D_3:PTAD	Qualifier
2	560.3>298.1	0.032	43	19	Auto	D_3:PTAD	Quantifier
3	560.3>365.3	0.032	43	21	Auto	preD_3:PTAD	Qualifier
4	560.3>383.3	0.032	43	13	Auto	preD_3:PTAD	Quantifier
5	563.2>301.2	0.032	43	16	Auto	SIL-D_3:PTAD	Quantifier
6	563.2>386.3	0.032	43	11	Auto	preSIL-D_3:PTAD	Quantifier
7	572.3>311.8	0.032	43	15	Auto	D_2:PTAD	Qualifier
8	572.3>377.3	0.032	43	19	Auto	preD_2:PTAD	Qualifier
9	572.3>395.3	0.032	43	9	Auto	preD_2:PTAD	Quantifier
10	572.3>448.2	0.032	43	9	Auto	D_2:PTAD	Quantifier

Table 2. MRM parameters.

Results and Discussion

Determination of vitamin D and previtamin D

Figure 3 shows the typical MRM chromatograms of vitamin D and previtamin D in standard mixtures and in infant formula samples. The Retention times of the vitamin D derivatives (D_3:PTAD, D_2:PTAD, and SIL-D_3 :PTAD) and the previtamin D derivatives (preD_3:PTAD, preD_2:PTAD, and SIL-preD_3:PTAD) are 3.50 min and 3.67 min, respectively.

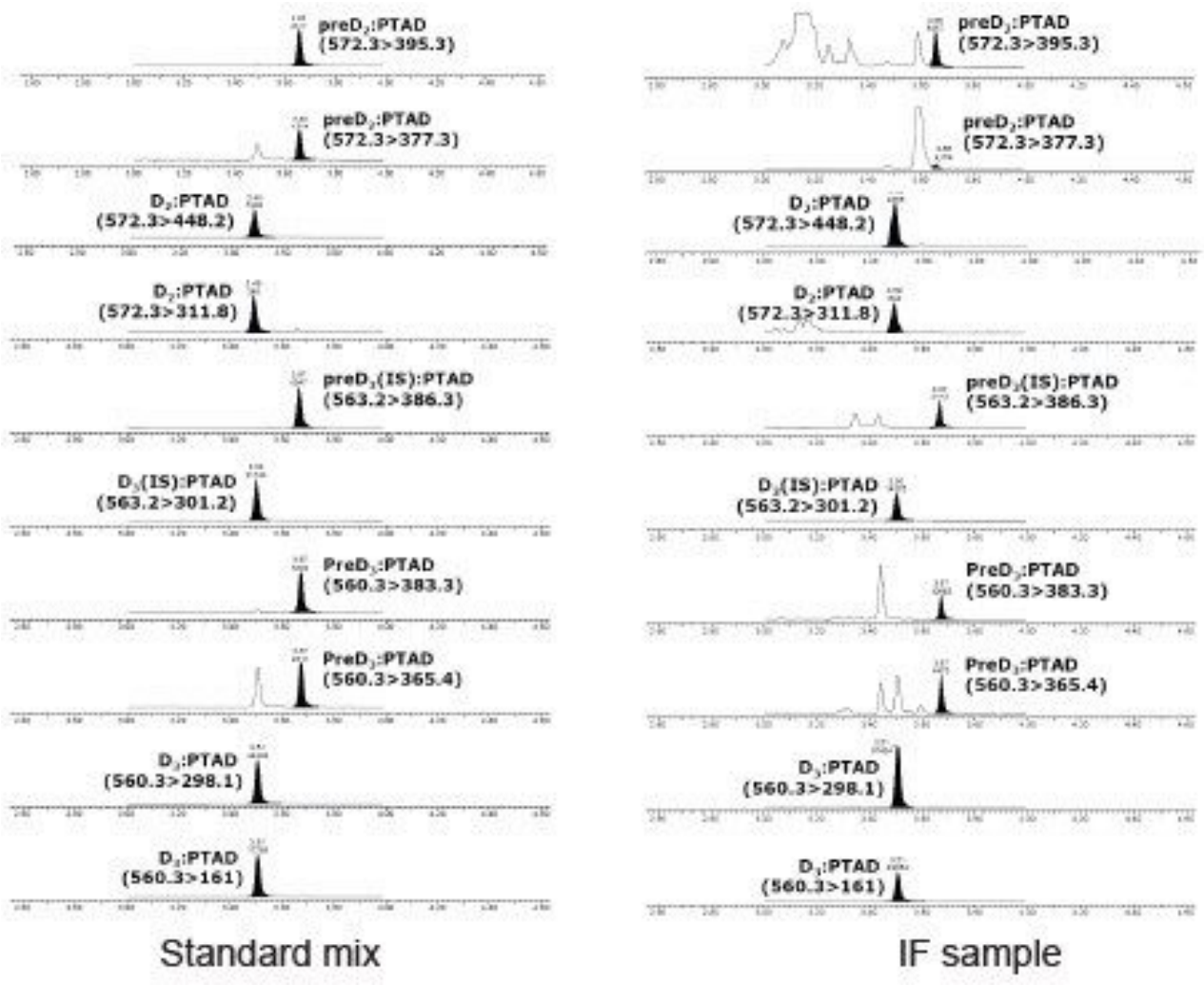

Figure 3. Typical MRM chromatograms of vitamin D and previtamin D in standard mix solutions and infant formula samples. Vitamin Ds (3.50 min) are separated from previtamin Ds (3.67 min).

There is no pure standard for previtamin D. In order to quantify the previtamin D, the relative response factors of the vitamin D over the previtamin D were determined in a simple experiment as follows. A solution with certain concentrations of the vitamin D_3, vitamin D_2, and SIL-D_3 was split into two portions. One portion was kept at room temperature, while the other portion was heated and maintained at 75 °C for 1 hour. Because of the isomerization equilibration, the heated portion would have increased previtamin D and decreased vitamin D contents than those in the unheated portion. Since the total vitamin D content in the two portions was the same, the relative response factor of the vitamin D over the previtamin D was calculated by the following equation:

Rel. Response Factor = (VitD Peak Area Unheated–VitD Peak Area heated)/(PreD Peak Area Heated–PreD Peak Area Unheated) - Eq. (1)

The Rel. Response Factors for vitamin D_3, vitamin D_2, and SIL-D_3 were determined each time the samples were analyzed.

Method of calibration and quantitation in vitamin D analysis

Total vitamin D is calculated as the sum of the previtamin D and the vitamin D contents. The total vitamin D peak area was calculated according to the following equation:

Total VitD Peak Area = VitD Peak Area + Rel. Response Factor x PreD Peak Area Eq. (2)

In the calibration process, the Total VitD Peak Area ratios of the analyte over the IS were plotted against their total vitamin D concentration ratios (analyte over IS). A linear regression through zero fitted the data points very well. Figure 4 shows a typical calibration plot. The R^2 values of 0.999 and 0.997 were obtained (Fig. 4) for vitamin D_3 and vitamin D_2, respectively. The calibration ranges were 0.0004 mg/kg to 0.2 mg/kg for vitamin D_3, and 0.002 to 0.2 mg/kg for vitamin D_2. These ranges are comparable to the AOAC standard method.[4]

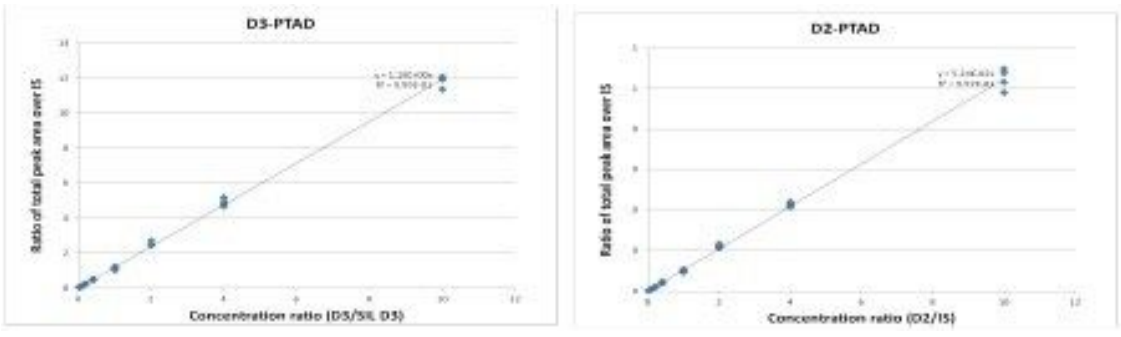

Figure 4. Calibration plots for vitamin D_3 and vitamin D_2.

The total vitamin D (D_3 or D_2) content in samples was calculated using the following equation:

Total Vitamin D = (Total VitD Peak Area x mass of spiked IS in sample)/Total VitD Peak Area (IS) x Slope in Calibration Curve x mass of sample - Eq. (3)

where the Total VitD Peak Area is calculated according to equation 2. The IS concentration in calibration solutions were kept at 50 ppb (or 50 ng/mL), and the spiked mass of IS in samples were kept at 0.020 µg. The total vitamin D results are in mg/kg unit.

Method performance and analysis results

The limit of detection (LOD) and limit of quantitation (LOQ) were estimated based on the peak area standard deviation (SD) in oatmeal and in solvent at low concentrations near the LOQ (Table 3). The LOD was estimated at 3 times the SD in peak area and the LOQ was estimated at 10 times the SD in peak areas. The LOQ values for vitamin D_3 and vitamin D_2 were estimated at 0.01 mg/kg and 0.02 mg/kg in oatmeal, and 0.0003 mg/kg and 0.002 mg/kg in solvent, respectively. The LOQ values are comparable to the existing standard.[4]

	Oatmeal		Solvent	
	D_3	D_2	D_3	D_2
LOD (mg/kg)	0.003	0.006	0.0001	0.0007
LOQ (mg/kg)	0.01	0.02	0.0003	0.002

Table 3. The estimated LOD and LOQ in the vitamin D analysis in oatmeal and in solvent.

The NIST reference material 1849a was measured and the average value and relative standard deviation (RSD) were compared with the reference values (Table 4). Excellent accuracy (102.6%) and repeatability (RSD 2.4%) was obtained. A spiking experiment was performed on the infant formula (at 0.09 mg/kg) and the oatmeal (at 0.02 and 0.09 mg/kg) and results are shown in Table 5. The recovery of the two spiking levels ranged from 98% to 117%. Besides the infant formula, other types of foodstuff, such as non-fat dry milk powder fortified with vitamin D, soy based infant formula, chocolate fortified with vitamin D, oatmeal, and fish oil were tested. Table 6 shows the results of three replicate measurements of the total vitamin D contents of these food products. The mean and the RSD results for the vitamin D_3 and D_2 are listed in the table. The vitamin D values on nutrition or supplement facts sheet of these foods were converted to numbers in mg/kg

and listed in Table 6 for comparison. The determined vitamin D concentrations for milk and oatmeal were in agreement with their label claim for vitamin D values (less than 9% in difference). The result for soy based infant formula was 52% higher than the label value, which is not uncommon for food product testing. The result for the fortified chocolate was high (70% higher than the label value), and the cause is unknown and needs further investigation. The nutrition fact information for the fish oil product was not available for comparison.

	Measurements			Average			Ref. values	Accuracy
		Mean	SD	Mean	SD	RSD		
Vitamin D_3 (mg/kg)	1	0.116	0.003	0.114	0.003	2.4%	0.111 ± 0.017	102.6%
	2	0.107	0.002					
	3	0.118	0.003					

Table 4. Vitamin D analysis results for NIST 1849a reference material and comparison to its reference values.

	Infantformula		Oatmeal	
	D_3	D_2	D_3	D_2
Original (mg/kg)	0.116	0.030	0.000	0.000
spike level 1 (0.02 mg/kg)	N/A	N/A	100%	102%
spike level 2 (0.09 mg/kg)	116%	98%	110%	117%
Average	116%	98%	105%	110%

Table 5. Recovery data on infant formula and oatmeal samples.

Sample (mg/kg)	Non-fat dry milk fortified with Vitamin A and D		Infant formula (soy based)		Chocolate fortified with Vitamin D		Oatmeal		Fish oil	
	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
Vitamin D_3	0.118	1.6%	0.089	1.1%	3.030	1.3%	0.000	N/A	0.190	7.1%
Vitamin D_2	0.000	N/A	0.015	4.3%	0.025	28.5%	0.000	N/A	0.011	31.9%
Total vitamin D	0.118		0.103		3.055		0		0.200	
Label vitamin D	0.109		0.068		1.786		0		N/A	

Table 6. Vitamin D analysis results for different food products. The total vitamin D values on the nutrition and supplement fact sheets on some food products are also listed.

Benefits of measuring previtamin D in the vitamin D analysis

To emphasis the need to consider previtamin D in total vitamin D measurements, the same two sets of sample data were processed using two different methods of quantitation. A comparison of the methods is summarized in Table 7. In method A, total vitamin D was quantified without using the previtamin D peak area. This is the same data processing method that the standard method used.[4] In method B, total vitamin D was quantified using both the previtamin D and the vitamin D peak areas in the calibration and the quantitation, which is the new method that we propose to use in this study. One can see that in Table 7, method A allowed 11–12% difference for the standards prepared at different conditions (high temperature, HT, vs. room temperature, RT) while method B only had 1–2% difference. For samples with different saponification conditions (HT saponification vs. RT saponification), method A showed a larger difference (3–6%) than method B did (1–3%). Table 7 data proves that method B is less affected by the previtamin D concentration variation. The bottom line is that without measuring the previtamin D concentration, the total vitamin D analysis result could carry a large error that could be contributed to previtamin D formation during the manufacturing, transportation, or storage of food products.

	Method A[3]		Method B[3]	
	D_3	D_2	D_3	D_2
Standard (RT)[1]	0.0092	0.0092	0.0095	0.0096
Standard (HT)[1]	0.0103	0.0102	0.0097	0.0096
Difference between RT and HT treatment	12%	11%	2%	1%
Sample[2] (HT saponification)	0.303	0.191	0.299	0.189
Sample[2] (RT saponification)	0.285	0.185	0.303	0.194
Difference between RT and HT saponification	-6%	-3%	1%	3%

Note: 1) Standard was split into two parts. One is kept in RT. The other was heated at 75 °C for 1 hour (HT).
2) Samples from the same food product was split into two parts. One was saponified at 75 °C for 1 hour (HT saponification), the other was saponified at RT overnight (RT saponification).
3) Method A does not include the previtamin Ds. Method B includes the previtamin Ds in the total vitamin Ds. The results are in mg/kg unit.

Table 7. Comparison of two vitamin D methods in the event of different heating history.

Conclusion

This application note demonstrates an improvement in the current LC-MS method for vitamin D analysis. Previtamin D was directly measured in the total vitamin D analysis, eliminating the error that could arise from not measuring previtamin D. This method will be less affected by the heating history of food products. Therefore, potential errors due to conversion of vitamin D to previtamin D are eliminated when accidental situations occur during the manufacturing, transportation, or storage of food products.

The results of vitamin D analysis for the NIST reference sample showed excellent accuracy (102.6%), and repeatability (2.4%). The recovery data from oatmeal and infant formula ranged from 98% to 117%. The LOQs in oatmeal were 0.01 mg/kg and 0.02 mg/kg for vitamin D_3 and vitamin D_2, and 0.0003 mg/kg (D_3) and 0.002 mg/kg (D_2) in solvent. A variety of food products, such as non-fat dry milk power, infant formula (soy based), chocolate, oatmeal, and fish oil samples have been successfully tested with this method. Good agreement with the label values have been observed for the infant formula, dry milk powder, and oatmeal.

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