ANALYSIS OF VITAMIN D AND PREVITAMIN D IN FOODS

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INTRODUCTION

The U.S. Food and Drug Administration (FDA) revised the food labeling regulations in 2016, which requires the vitamin D content to be listed on the nutrition or supplement facts labels for conventional food and dietary supplements.(1) The change in labeling regulation is aimed to promote vitamin D awareness among consumers.

Recently, a new AOAC standard method which uses a derivatization reaction in the sample prep and a mass spectrometry in quantitation is available. (2) This new method has provided much better analytical performance for vitamin D analysis than previous methods. However, previtamin D is not measured in this new standard.

Previtamin D is bioactive, and it is known that Vitamin D can thermally isomerize to Previtamin D (Figure 1). It has been reported that the relative content of previtamin D could be up to 22% of the total vitamin D at 80°C.(3) Therefore, it is prudent to count previtamin D content in the analysis of vitamin D in foods.

This work demonstrates the determination of total vitamin D by individually measuring the vitamin D and previtamin D in food products.

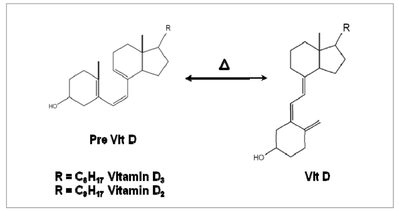

Figure 1. Reversible thermal isomerization of previtamin D to vitamin D. The equilibration constant and equilibration time depends on temperature.

METHODS

Sample Preparation:

0.5 g sample mixed with 20 ng I.S. (Cholecalciferol-d3), 4 mL water, 16 mL pyrogallol ethanolic solution (2 g/100 m), and 8 mL of KOH (50%) solution.

Saponification:
75°C for 1 hour

L-L extraction:
12 mL hexanes, extracts are washed with 8 mL water 4 times

Derivatization:
6 mL of hexanes extracts (or 100 µL standard calibration solution) blow dried with N^2 at 30oC, react with 0.6 mL PTAD solution (1 mg/mL in ACN) in dark for 40 min, and quenched with 0.4 mL water.

UPLC 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 50mm 1.7 um
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:	6.5 min

Elution 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

MS Conditions:

Polarity:	ESI+
Capillary (kV):	1.2
Source Temperature (°C):	150
Desolvation Temperature (°C):	500
Cone Gas Flow (L/Hr):	0
Desolvation Gas Flow (L/Hr):	1000

MRM parameters:

	MRM	Dwell (secs)	Cone Volt	Col. Energy	Delay (secs)	Compound	Note
1	560.3>161.0	0.032	43	36	Auto	D3:PTAD	Qualifier
2	560.3>298.1	0.032	43	19	Auto	D3:PTAD	Quantifier
3	560.3>365.3	0.032	43	21	Auto	preD3:PTAD	Qualifier
4	560.3>383.3	0.032	43	13	Auto	preD3:PTAD	Quantifier
5	563.2>301.2	0.032	43	16	Auto	SIL-D3:PTAD	Quantifier
6	563.2>386.3	0.032	43	11	Auto	preSIL-D3:PTAD	Quantifier
7	572.3>311.8	0.032	43	15	Auto	D2:PTAD	Qualifier
8	572.3>377.3	0.032	43	19	Auto	preD2:PTAD	Qualifier
9	572.3>395.3	0.032	43	9	Auto	preD2:PTAD	Quantifier
10	572.3>448.2	0.032	43	9	Auto	D2:PTAD	Quantifier

RESULTS

Determination of vitamin D and previtamin D

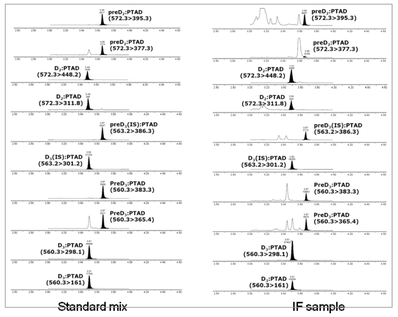

Figure 2. Typical MRM chromatograms of vitamin D and previtamin D in standard mix solutions and infant formula samples. Vitamin Ds (3.50 min) are separated from previtamin Ds (3.67 min).

Relative response factors
The relative response factor of the vitamin D over the previtamin D was calculated by the following equation:

$$Rel.Response\ Factor = \frac{VitD\ Peak\ Area\ Unheated \times PreD\ Peak\ Area\ Heated}{PreD\ Peak\ Area\ Heated \times PreD\ Peak\ Area\ Unheated} \quad \text{Eq. (1)}$$

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

Including the previtamin D in the calibration and quantitation for vitamin D

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:

$$Total\ VitD\ Peak\ Area = VitD\ Peak\ Area + Rel.\ Response\ Factor \times PreD\ Peak\ Area \quad \text{Eq. (2)}$$

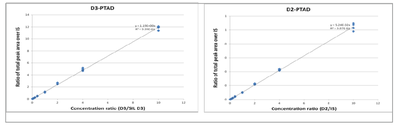

Figure 3. Calibration plots for vitamin D_3 and vitamin D_2.

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 3 shows a typical calibration plot. The R^2 values of 0.999 and 0.997 were obtained 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)

RESULTS

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

$$\text{Vitamin D} = \frac{Total\ VitD\ Peak\ Area \times mass\ of\ spiked\ IS\ in\ sample}{Total\ VitD\ Peak\ Area\ (IS) \times Slope\ in\ Calibration\ Curve \times mass\ of\ sample} \quad \text{Eq. (3)}$$

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

Table 1. *The estimated LOD and LOQ in the vitamin D analysis in oatmeal and in solvent.*

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

		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 3. *Recovery on vitamin D_3 and D_2 in different food products.*

Sample Type		Original (mg/kg)	Spiked level (mg/kg)	Measured level (mg/kg)	Recovery (%)
Infant Formula	D_3	0.098	0.101	0.191	92.1%
	D_2	0.000	0.097	0.099	102.1%
Oatmeal	D_3	0.000	0.108	0.114	105.6%
	D_2	0.000	0.104	0.125	120.2%
Milk Powder	D_3	0.104	0.109	0.211	98.2%
	D_2	0.000	0.105	0.097	92.4%
Sesame Cereal	D_3	0.000	0.108	0.107	99.1%
	D_3	0.001	0.104	0.105	100.0%
Soy protein powder	D_3	0.000	0.107	0.101	94.4%
	D_3	0.000	0.103	0.097	94.2%
Energy Bar	D_3	0.000	0.021	0.021	100.0%
	D_2	0.011	0.021	0.032	100.0%
Canned Tuna	D_3	0.002	0.022	0.024	100.0%
	D_2	0.000	0.021	0.022	104.8%
Coca powder	D_3	0.000	0.111	0.113	101.8%
	D_2	0.008	0.107	0.121	105.6%

Table 4. *Vitamin D analysis results for different food products. The vitamin D values on the nutrition and supplement fact sheets on food products are also listed.*

Samples	Non-fat dry milk fortified with Vitamin A and D		Infant Formula (Soy based)		Infant Formula (milk based)		Energy Bar		Canned Tuna	
	Average (ug/kg)	RSD (%)	Average (ug/kg)	RSD (%)	Average (ug/kg)	RSD (%)	Average (ug/kg)	RSD (%)	Average (ug/kg)	RSD (%)
Vitamin D_3	104	1.4%	83	6.2%	70	6.8%	0		2	7.5%
Vitamin D_2	0		0		0		11	5.6%	0	
Total Vitamin D	104		83		70		11		2	
Vitamin D content on label	109		68		68		31		10	
Accuracy	95%		122%		102%		36%		19%	

DISCUSSION

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 1). 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.(2)

The NIST reference material 1849a was measured and the average value and relative standard deviation (RSD) were compared with the reference values (Table 2). Excellent accuracy (102.6%) and repeatability (RSD 2.4%) was obtained. A spiking experiment was performed on several food products and results are shown in Table 3. The recovery of the spiking experiments ranged from 92% to 106%, except for oatmeal, the recovery for vitamin D_2 was 120%.

Table 4 shows the results of measurements of the total Vitamin D contents in food products. The vitamin D values on nutrition or supplement facts sheet of these foods were converted to numbers in µg/kg and listed in Table 4 for comparison. The determined vitamin D concentrations for milk and infant formulas were in agreement with their label claim for vitamin D values (less than 22% in difference). The result for energy bar and canned tuna fish were significant lower than the label claims. The cause is unknown and needs further investigation.

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 5. 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 5, 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 5 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.

Table 5. *Comparison of two vitamin D methods in the event of different heating history.*

	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
Diff. between RT and HT treatment	12%	11%	2%	1%
Sample (HT saponification)[2]	0.303	0.291	0.299	0.189
Sample (RT saponification)[2]	0.285	0.285	0.303	0.194
Diff. between HT and RT 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.

CONCLUSION

This study 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 potential errors due to conversion of vitamin D to previtamin D. This method is less affected by the heating history of food products. Any accidental situation that causes the shifting of the equilibrium between the vitamin D and previtamin D in foods will not cause error in the vitamin D analysis.

The results of vitamin D analysis for the NIST reference sample showed excellent accuracy (102.6%), and repeatability (2.4%). The recovery data from selected foods ranged from 92% to 105%, except for the oatmeal. 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 formulas (milk and soy based), energy bar, and canned fish have been successfully tested with this method. Good agreement with the label values have been observed for the infant formulas and dry milk powder.

References

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