Estimation of Fat Soluble Vitamins, Ergocalciferol, and Cholecalciferol in Edible Oil

Using the Agilent 6470 Triple Quadrupole LC/MS System Coupled to an Agilent 1290 Infinity II LC System

Application Note

Food Testing and Agriculture

Authors

Dr. Priti Amritkar
Envirocare Labs Pvt. Ltd.
Thane, India

Prasanth Joseph and Dr. Samir Vyas
Agilent Technologies, Inc.
Bangalore, India

Abstract

Ergocalciferol and cholecalciferol are two different forms of vitamin D. Vitamin D is one of the essential fat soluble vitamins that play a major role in many biological activities in the human body. A major source of vitamin D is sunlight exposure to the skin. Most people get their vitamin D requirements through a combination of solar conversion and by maintaining a various and balanced diet. Liver, fish, eggs, and milk, as well as other dairy products are rich in vitamin D. In dairy products, vitamin D exists as cholecalciferol. To maintain the vitamin D requirements of the population, ergocalciferol and cholecalciferol are used to fortify food ingredients such as cooking oil. Edible oil is one of the best food commodities to serve as a vehicle to easily reach consumers and reduce issues related to vitamin deficiency. Analysis of ergocalciferol and cholecalciferol in edible oil is challenging because of the complexity of the matrix. For the accurate and simultaneous quantitation of these vitamins, an analytical method was developed using the Agilent 6470 triple quadrupole LC/MS system with APCI ionization installed. Sample preparation is based on saponification, followed by extraction with hexane. The evaporated hexane residue was reconstituted in methanol before instrumental analysis.

Agilent Technologies

Introduction

Vitamin D deficiency is one of the most common nutrient deficiencies. It is a well known cause of rickets, a bone disease seen in children. Taking vitamin D supplements has been shown to have numerous benefits related to cancer, bone health, mental health, and autoimmune diseases. Vitamin D from the diet, or dermal synthesis from sunlight, is biologically inactive. Activation requires enzymatic conversion (hydroxylation) in the liver and kidney. Minor differences in the chemistry of the side chains between the two forms of vitamin D result in differences in the site of hydroxylation, and leads to the production of unique biologically active metabolites.

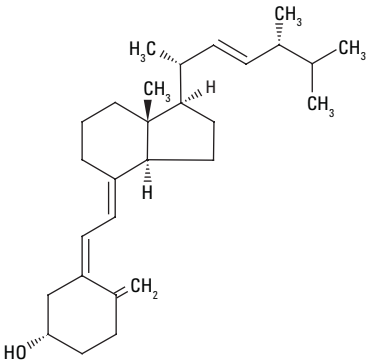

Figure 1. Chemical structure of ergocalciferol, one of the major forms of vitamin D.

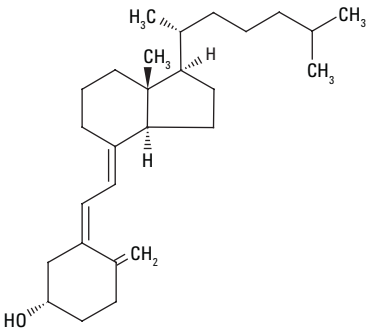

Figure 2. Chemical structure of cholecalciferol, one of the major forms of vitamin D.

Chemicals and reagents

- Ergocalciferol certified reference material
- Cholecalciferol certified reference material
- Hydrochloric acid, concentrated
- Ethanol: 95 %
- *n*-Hexane
- Pyrogallol
- Glass beads
- Aluminum foil
- 39 % KOH: 39 g of potassium hydroxide pellets dissolved in 100 mL water (freshly prepared on the day of analysis)

Apparatus and glassware

- **Heating mantle:** With sufficient heating surface area to handle multiple reflux apparatus setups preferred
- **Reflux condensers:** With adapters (if necessary) to attach 250-mL round-bottom boiling flasks
- **Volumetric flasks:** Amber-colored, 100 mL
- **Nitrogen blanket apparatus:** A supply of nitrogen gas with appropriate tubing and connectors to provide a constant atmosphere blanket in the reflux apparatus during saponification

Mobile phase

- **Mobile phase A:** 5 mM ammonium acetate with 0.02 % acetic acid (AcOH) in water
- **Mobile phase B:** 5 mM ammonium acetate with 0.02 % acetic acid in methanol

Operating conditions

Parameter	Value	
Column	Agilent InfinityLab Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 µm)	
Column temperature	40 °C	
Flow rate	0.5 mL/min	
Injection volume	20 µL	
Gradient program	Time (min)	% B
	0	50
	2	50
	4	100
	9	100
	9.5	50
	10	50

Sample preparation

The sample preparation involved a saponification process, which was the addition of concentrated potassium hydroxide to an ethanol solution of sample, followed by reflux heating at 70 °C. This process breaks down the fat to release the matrix-bound vitamins. These released vitamins were extracted with hexane. The extracted hexane layer was evaporated completely, and reconstituted in methanol. Figure 3 shows the entire sample preparation.

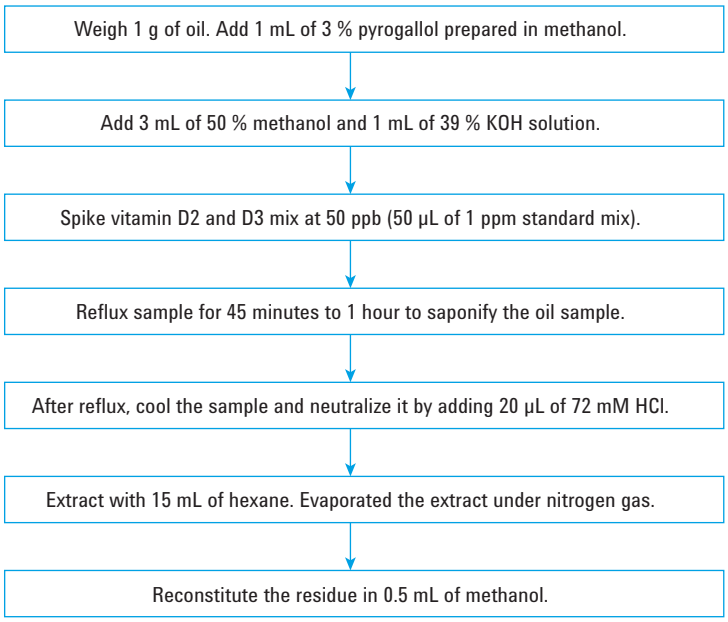

Figure 3. Sample preparation flow chart.

Analytical technique

Instrumentation

- Agilent 1290 Infinity II quaternary pump with built-in degassing unit (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II LC multisampler (G7167B)
- Agilent InfinityLab Poroshell 120 EC-C18 (p/n 699975-302)
- Agilent 6470 triple quadrupole LC/MS system (G6470A)

MRM Parameters

Sl. no.	Analyte ID	Parent	Product	Fragmentor	Collision energy
1	Ergocalciferol	385.35	107.1	90	40
	Ergocalciferol	385.35	91	90	76
2	Cholecalciferol	397.01	367.3	90	8
	Cholecalciferol	397.01	259	90	16

Q1MS (APCI POS) scan data of ergocalciferol and cholecalciferol standards mixture

A LC/MS/MS method was developed on an Agilent 6470 LC/MS triple quadrupole system installed with an APCI source operated in positive ionization mode. Figure 4 shows the MS spectrum of the standards mixture. Matrix-matched calibration curves were made for both ergocalciferol and cholecalciferol from 10 to 500 ng/g (Figures 7 and 8, respectively). Calibration curves were found to be linear within the previously mentioned concentration ranges, with a minimum correlation coefficient of 0.9940. Figures 5 and 6 show representative chromatograms of ergocalciferol and cholecalciferol in oil matrix.

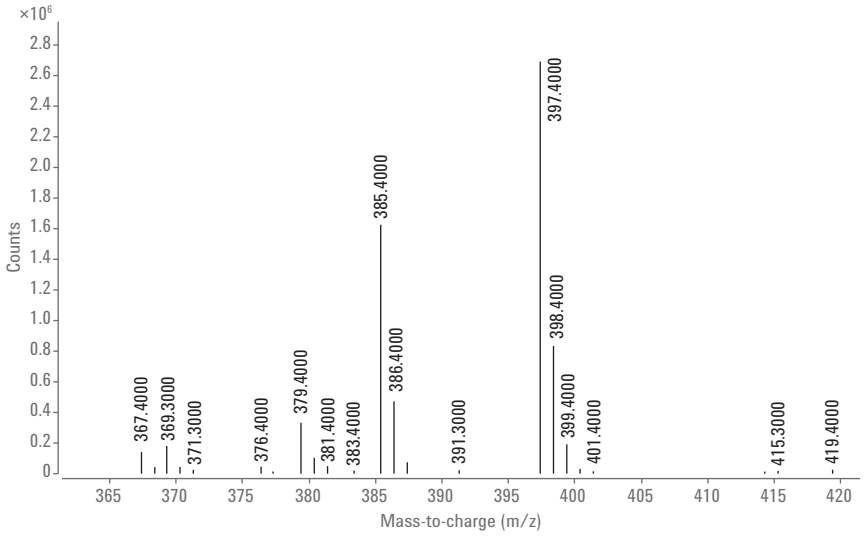

Figure 4. Ergocalciferol (*m/z* 397.4), cholecalciferol (*m/z* 385.4), and its isotopic pattern is seen in APCI.

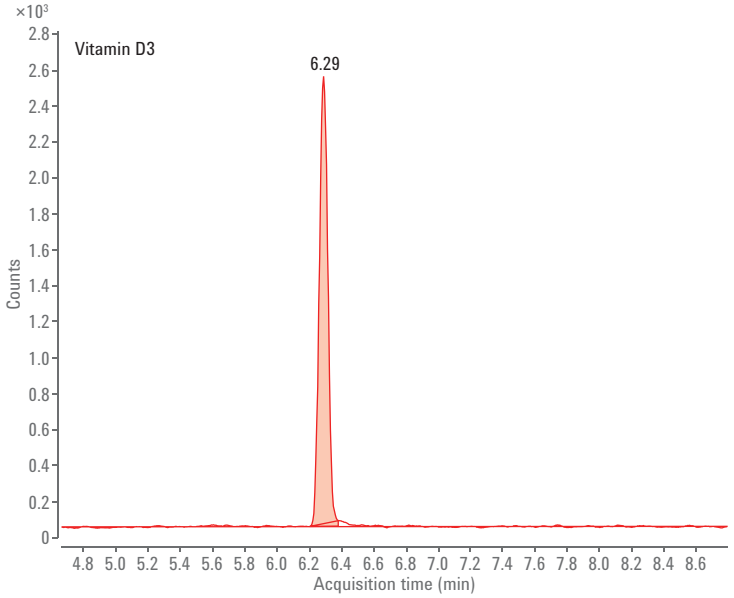

Figure 5. Representative chromatogram of vitamin D3 in oil matrix.

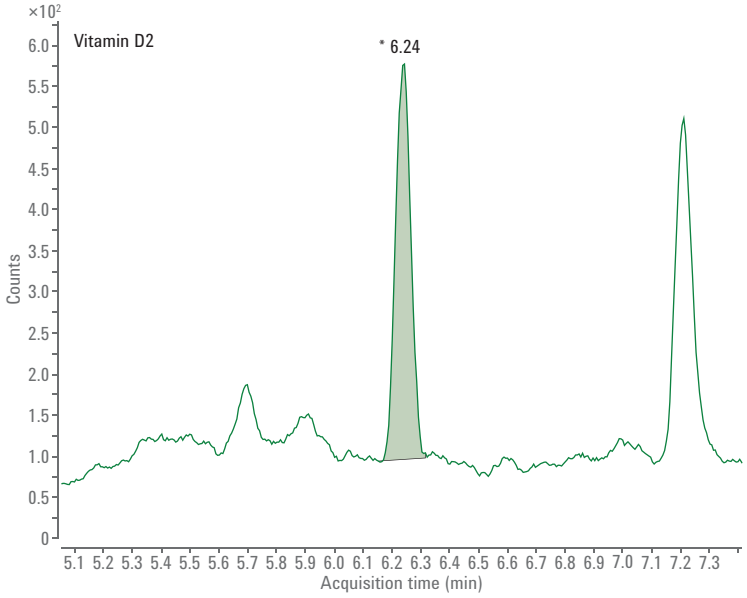

Figure 6. Representative chromatogram of vitamin D2 in oil matrix.

Different concentration levels are prepared for both vitamin D2 and vitamin D3, and plotted to generate the calibration curves.

Chromatograms of the different dilutions used for the calibration curves are merged. Figure 9 shows the combined chromatogram which demonstrates a linear increase of response, resulting in an excellent correlation coefficient (R^2) of 0.99.

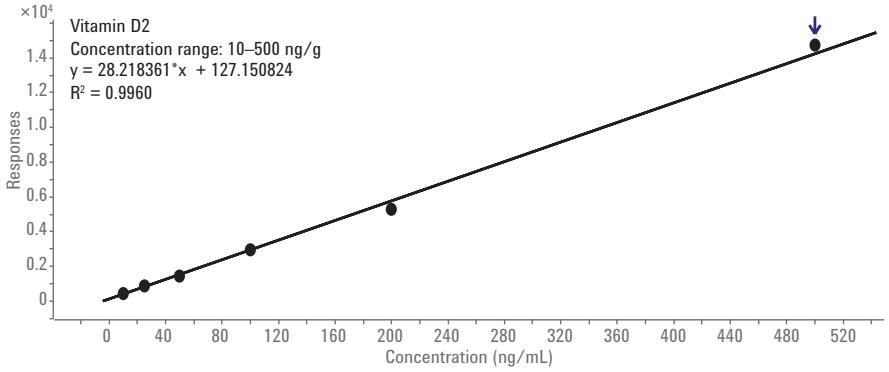

Figure 7. Matrix-matched calibration curve for ergocalciferol (10 to 500 ng/mL).

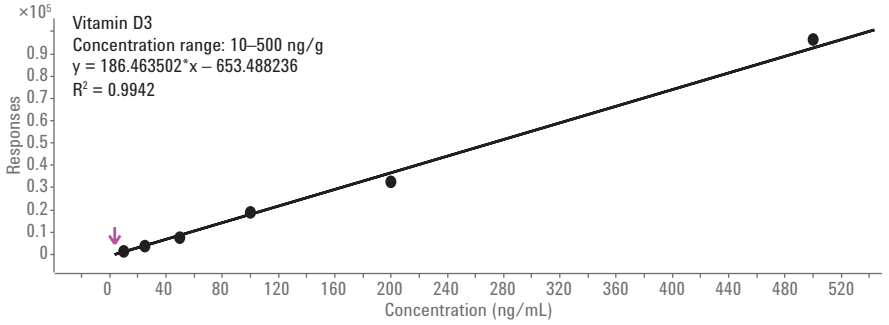

Figure 8. Matrix-matched calibration curve for cholecalciferol (10 to 500 ng/mL).

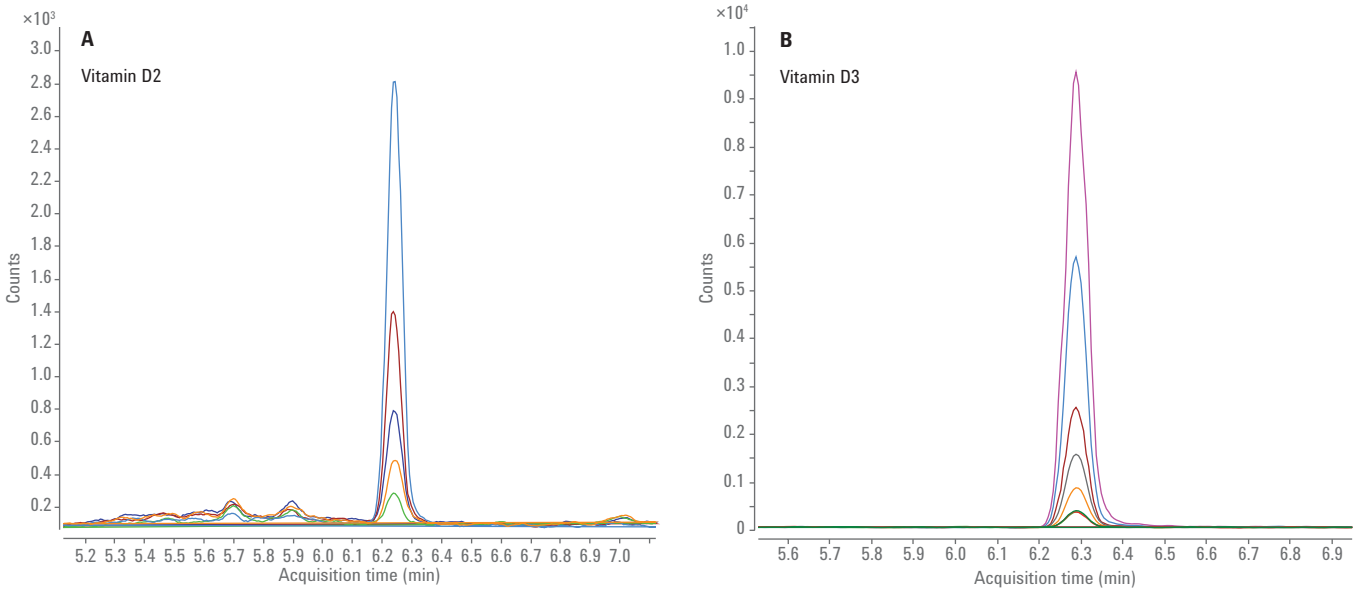

Figure 9. Overlay of chromatograms of vitamin D2 and vitamin D3 at different concentration levels, used to generate the calibration curves.

Reproducibility

System reproducibility at the limit of quantification (LOQ)

The LOQ was determined as 50 ng/g for both vitamin D2 and vitamin D3 (Tables 1 and 2). System reproducibility was checked using six repeated injections of extracted oil sample spiked at 50 ng/g level. The response %RSDs of six injections of vitamin D2 and vitamin D3 were found to be 4.7 % and 3.9 % respectively.

Table 1. System reproducibility data for vitamin D2 at 50 ng/g spike level.

	Vitamin_D2 results					
Sample	Type	Acquisition date, time	RT	Response	Calculated concentration (ng/g)	Final concentration (ng/g)
SPIKE_50PPB	Sample	8/7/2016, 11:10	6.237	1226	38.9511	38.9511
SPIKE_50PPB	Sample	8/7/2016, 11:21	6.237	1352	43.4028	43.4028
SPIKE_50PPB	Sample	8/7/2016, 11:32	6.243	1243	39.5556	39.5556
SPIKE_50PPB	Sample	8/7/2016, 11:43	6.243	1281	40.8935	40.8935
SPIKE_50PPB	Sample	8/7/2016, 11:54	6.237	1206	38.2179	38.2179
SPIKE_50PPB	Sample	8/7/2016, 12:05	6.243	1335	42.7987	42.7987
					Avg.	40.6366
					Std.	1.925794961
					%CV	4.739065181

Table 2. System reproducibility data for vitamin D3 at 50 ng/g spike level.

Sample	Vitamin_D3 results					
Name	Type	Acquisition date, time	RT	Response	Calculated concentration	Final concentration
SPIKE_50PPB	Sample	8/7/2016, 11:10	6.288	5846	45.4195	45.4195
SPIKE_50PPB	Sample	8/7/2016, 11:21	6.288	5609	43.5802	43.5802
SPIKE_50PPB	Sample	8/7/2016, 11:32	6.288	5484	42.6091	42.6091
SPIKE_50PPB	Sample	8/7/2016, 11:43	6.288	5364	41.6749	41.6749
SPIKE_50PPB	Sample	8/7/2016, 11:54	6.288	5366	41.6948	41.6948
SPIKE_50PPB	Sample	8/7/2016, 12:05	6.288	5166	40.137	40.137
					Avg.	42.51925
					Std.	1.663976
					%CV	3.913465

Spike recovery

To evaluate the efficiency of the sample extraction, spike recovery studies were conducted for both vitamin D2 and vitamin D3 in oil samples at two different spike levels: 50 and 100 ng/g (Tables 4 and 5). Average recoveries of 91.5 % and 87 % for vitamin D2 and vitamin D3 were calculated.

Chromatograms of vitamin D2 and vitamin D3 of the recovery samples spiked at 50 ng/g are given in Figure 10. The matrix interferences are higher with vitamin D2 compared to vitamin D3. Table 5 clearly shows that the method adopted for sample preparation demonstrated good recovery, more than 80 % for both ergocalciferol and cholecalciferol at a spike level of 50 ng/g.

Table 3. Quantification table for vitamin D2, showing the spike recovery concentration at 50 and 100 ng/g.

Sample				Vitamin_...	Vitamin_D2_1 Results				
Name	Type	Level	Acq. Date-Time	Exp. Conc.	RT	Resp.	Calc. Conc.	Final Conc.	Accuracy
BLANK_MM	Samp...		8/7/2016 9:54 AM		5.953	76	0.0000	0.0000	
MM_STD_10 PPB	Cal	1	8/7/2016 10:05 AM	10.0000	6.243	419	10.3383	10.3383	103.4
MM_STD_25 PPB	Cal	2	8/7/2016 10:16 AM	25.0000	6.243	901	27.4095	27.4095	109.6
MM_STD_50 PPB	Cal	3	8/7/2016 10:27 AM	50.0000	6.243	1421	45.8379	45.8379	91.7
MM_STD_100 PPB	Cal	4	8/7/2016 10:38 AM	100.0000	6.243	2956	100.2360	100.2360	100.2
MM_STD_200 PPB	Cal	5	8/7/2016 10:49 AM	200.0000	6.243	5285	182.7709	182.7709	91.4
MM_STD_500 PPB	Cal	6	8/7/2016 10:59 AM	500.0000	6.243	14756	518.4074	518.4074	103.7
SPIKE_50PPB	Samp...		8/7/2016 11:10 AM		6.237	1226	38.9511	38.9511	
SPIKE_50PPB	Samp...		8/7/2016 11:21 AM		6.237	1352	43.4028	43.4028	
SPIKE_50PPB	Samp...		8/7/2016 11:32 AM		6.243	1243	39.5556	39.5556	
SPIKE_50PPB	Samp...		8/7/2016 11:43 AM		6.243	1281	40.8935	40.8935	
SPIKE_50PPB	Samp...		8/7/2016 11:54 AM		6.237	1206	38.2179	38.2179	
SPIKE_50PPB	Samp...		8/7/2016 12:05 PM		6.243	1335	42.7987	42.7987	
SPIKE_100PPB	Samp...		8/7/2016 12:16 PM		6.243	2671	90.1479	90.1479	
SPIKE_100PPB	Samp...		8/7/2016 12:27 PM		6.243	2583	87.0427	87.0427	
SPIKE_100PPB	Samp...		8/7/2016 12:38 PM		6.243	2734	92.3752	92.3752	
SPIKE_100PPB	Samp...		8/7/2016 12:49 PM		6.243	2513	84.5543	84.5543	
SPIKE_100PPB	Samp...		8/7/2016 1:00 PM		6.237	2551	85.8897	85.8897	
SPIKE_100PPB	Samp...		8/7/2016 1:11 PM		6.237	2954	100.1924	100.1924	

Table 4. Quantification table for vitamin D3, showing the spike recovery concentration at 50 and 100 ng/g.

Sample				Vitamin_...	Vitamin_D3_1 Results				
Name	Type	Level	Acq. Date-Time	Exp. Conc.	RT	Resp.	Calc. Conc.	Final Conc.	Accuracy
BLANK_MM	Blank		8/7/2016 9:54 AM		6.246	0	0.0000	0.0000	
MM_STD_10 PPB	Cal	1	8/7/2016 10:05 AM	10.0000	6.288	1532	10.8663	10.8663	108.7
MM_STD_25 PPB	Cal	2	8/7/2016 10:16 AM	25.0000	6.288	3595	24.0435	24.0435	96.2
MM_STD_50 PPB	Cal	3	8/7/2016 10:27 AM	50.0000	6.288	7842	51.1725	51.1725	102.3
MM_STD_100 PPB	Cal	4	8/7/2016 10:38 AM	100.0000	6.288	14626	94.5068	94.5068	94.5
MM_STD_200 PPB	Cal	5	8/7/2016 10:49 AM	200.0000	6.288	29800	191.4286	191.4286	95.7
MM_STD_500 PPB	Cal	6	8/7/2016 10:59 AM	500.0000	6.288	80143	512.9823	512.9823	102.6
SPIKE_50PPB	Sample		8/7/2016 11:10 AM		6.288	6734	44.0981	44.0981	
SPIKE_50PPB	Sample		8/7/2016 11:21 AM		6.288	6834	44.7325	44.7325	
SPIKE_50PPB	Sample		8/7/2016 11:32 AM		6.288	6474	42.4379	42.4379	
SPIKE_50PPB	Sample		8/7/2016 11:43 AM		6.288	6383	41.8531	41.8531	
SPIKE_50PPB	Sample		8/7/2016 11:54 AM		6.288	6129	40.2333	40.2333	
SPIKE_50PPB	Sample		8/7/2016 12:05 PM		6.288	6660	43.6212	43.6212	
SPIKE_100PPB	Sample		8/7/2016 12:16 PM		6.288	12896	83.4555	83.4555	
SPIKE_100PPB	Sample		8/7/2016 12:27 PM		6.288	14699	94.9719	94.9719	
SPIKE_100PPB	Sample		8/7/2016 12:38 PM		6.288	14430	93.2492	93.2492	
SPIKE_100PPB	Sample		8/7/2016 12:49 PM		6.288	13909	89.9223	89.9223	
SPIKE_100PPB	Sample		8/7/2016 1:00 PM		6.288	13776	89.0766	89.0766	
SPIKE_100PPB	Sample		8/7/2016 1:11 PM		6.288	14269	92.2241	92.2241	

Conclusion

A sensitive LC/MS/MS method was developed for the quantification of two forms of vitamin D: ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3), fat soluble vitamins. This multiple reaction monitoring (MRM) based method was found to be specific in a complex matrix such as edible oil. Adopted APCI ionization technology in positive ionization mode reduced the matrix effect. Sample preparation was based on saponification of the oil sample, followed by liquid-liquid extraction with hexane. The method demonstrated good reproducibility and decent recovery. This method can be adopted by commercial testing labs involved in the analysis of vitamin D, required for nutritional labeling of edible oils.

References

1. Stevens; Dowell. Determination of Vitamins D2 and D3 in Infant Formula and Adult Nutritionals by Ultra Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC MS/MS). *J. AOAC Int.* **2012**, *95(3)*, First Action 2011.12.

2. Staffas; Nyman. Determination of Cholecalciferol (Vitamin D3) in Selected Foods by Liquid Chromatography, NMKL1 Collaborative Study. *J. AOAC Int.* **2003**, *86(2)*.

3. LC/MS/MS Determination of Vitamin D in Food, *Agilent Technologies Application Note*, publication number 5990-8627EN, **2011**.

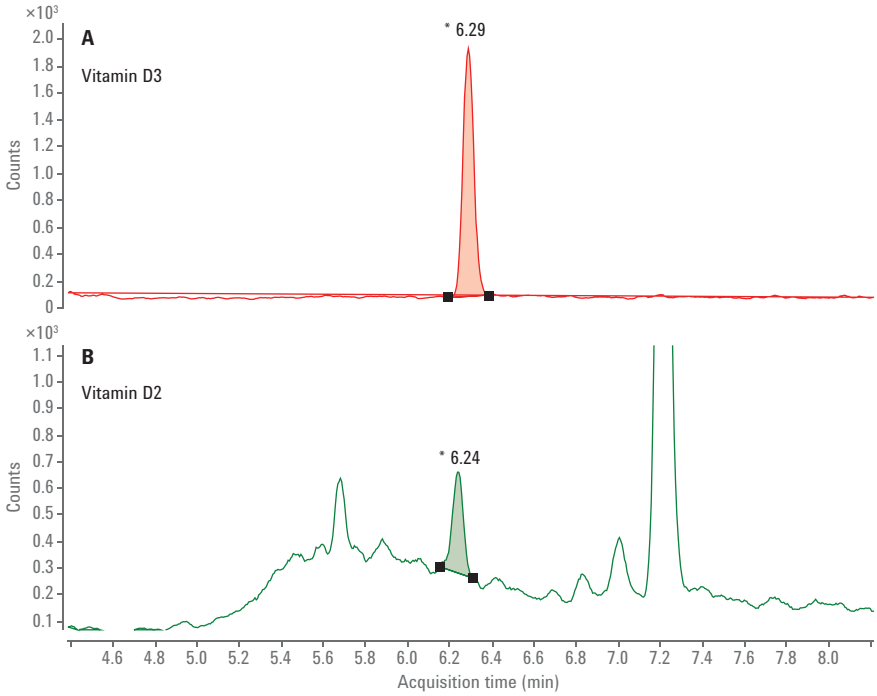

Figure 10. Chromatograms of vitamin D2 and vitamin D3 in an oil sample, spiked at 50 ng/g.

Table 5. Recoveries of vitamin D2 and vitamin D3 in edible oil.

	Average % recovery	
Analyte	50 ng/g spike level	100 ng/g spike level
Vitamin D2	81.26	90.03
Vitamin D3	85.65	90.47

www.agilent.com/chem

Published in the USA, September 1, 2017
5991-8328EN

Agilent Technologies