Application Note

Food

Simultaneous Determination of Vitamin D2 and D3 in Milk

Agilent 1290 Infinity II LC with Agilent 6470 LC/TQ system in food fortification

Authors

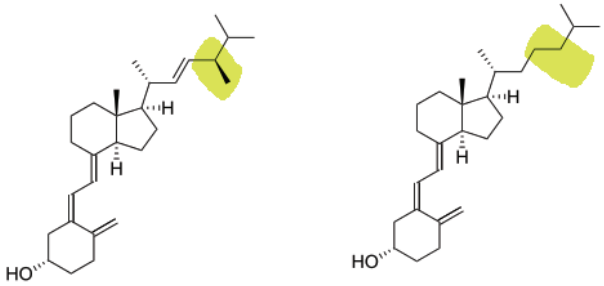
Amod Prakash Yadav, Vikrant Goel, Nitin Vig, and Saikat Banerjee
Agilent Technologies, Inc.

Rita Singh and Dr. Meenakshi Tripathi,
FARE Lab,
Gurgaon, India

Abstract

Vitamin D plays an important role in metabolic pathways, including calcium and phosphorus homeostasis and is a precursor to steroidal hormones. Liver, fish, eggs, and dairy products are rich in vitamin D. Among some developing nations, milk and cooking oils are fortified with vitamin D as a cost-effective solution to preventing malnutrition. It is available in plants as ergocalciferol (vitamin D2) and in animals as cholecalciferol (vitamin D3).

LC/MS/MS-based quantitative analysis is more popular than traditional analytical methods for quantification, due to better specificity and higher sensitivity. However, analysis of D2 and D3 in animal food, plant foods and fortified foods face challenges such as fast degradation and poor reproducibility. Analysis complexity has further increased due to the diversity of milk and severe matrix effects. The developed methodology has demonstrated sensitivity at 0.4 ng/g levels, has high reproducibility, and is useful for routine testing laboratories involved in such analysis.



Ergocalciferol (Vitamin D2) **Cholecalciferol (Vitamin D3)**

Figure 1. Vitamins D2 and D3.

Introduction

There are various published methods for analysis of vitamins D2 and D3. The high matrix effects in food-based samples are analyzed through the use of an APCI source in LC/MS/MS methods. Despite all efforts, the diverse nature of milk with variable fat content has posed a threat to analytical data. Saponification with ethanolic potassium hydroxide is important in handling the variable ratio of fats, while the addition of pyrogallol is critical to prevent oxidation of analytes during repeated washing steps. Derivatization by 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is useful to increase sensitivity of both the analytes, while use of deuterated internal standards (IS) is vital for recovery analysis.

Reagents and chemicals

- Acetonitrile (Honeywell, LC/MS, 34967)
- Methanol (Honeywell, LC/MS, 34966)
- Water (Millipore, Milli-Q)
- Vitamin D2 (ergocalciferol, CAS No. 50146)
- Vitamin D3 (cholecalciferol, CAS No. 67970)
- D2-d3-IS (Susex Research, 1311259-89-8)
- D3-d6-IS (Susex Research, 118584-54-6,67-97-0)
- PTAD (CAS No. 4233-33-4)
- Formic acid (HPLC Grade)
- KOH (AR Grade)
- Pyrogallol (AR Grade)
- Acetone (HPLC Grade)
- Ethanol (HPLC Grade)
- Isooctane (AR Grade)

Standards and reagents preparation

1. To prepare the PTAD solution (10 mg/mL), transfer 50 mg of PTAD into a 5 mL volumetric flask and add 4 mL of acetone. Following dissolution, add acetone to reach the final volume. This solution is stable for 24 hours.
2. To prepare the KOH solution (50%, w:v), dissolve 100 g of potassium hydroxide in 200 mL of water. This solution is stable for one month.
3. To prepare the ethanolic pyrogallol solution (1%, w:v), dissolve 5 g of pyrogallol in 400 mL of ethanol. Following dissolution, add ethanol to reach the final volume. This solution is stable for 24 hours.
4. To prepare internal standards, weigh 1 mg of IS (D2 and D3) in a 100 mL volumetric flask. Dilute with ethanol and vortex. Following dissolution, add ethanol to reach the final volume.
5. Measure the absorbance of an aliquot of SIL-D2 or SIL-D3 at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.
6. Pipette 1 mL of each of the stocks into the same volumetric flask (10 mL) and dilute with acetonitrile to obtain working internal standards (1 ppm). Store all dilutions at 4 °C.
7. To prepare standards, weigh 10 mg of D2 or D3 in different 10 mL volumetric flasks. Dilute with ethanol. Following dissolution, add ethanol to obtain a 1 mg/mL stock solution. Mix 1 mL of individual stock in a volumetric flask (100 mL). Dilute with ethanol. Following dissolution, add ethanol to reach a 10 ppm standard. The working standard is obtained by diluting 1 mL of 10 ppm mix to 10 mL in acetonitrile. Store all dilutions at 4 °C.
8. For derivatization, add respective volumes of vitamin D2 or D3 standard, 250 µL of internal standard, 5 mL of acetonitrile, and 750 µL of PTAD into a 25 mL amber-colored volumetric flask. Vortex for one minute and leave for 5 minutes in darkness.
9. For dilution, add 6.25 mL of Milli-Q water in each derivatized flask and dilute with acetonitrile to reach 25 mL. The dilution protocol is described in Table 1.

Table 1. Dilution protocol for standard preparation.

Level	Standard Concentration	Standard Volume	IS Volume	ACN	PTAD	Water (Dilution)	ACN (Dilution)	Final Concentration
1	1 ppm	10 µL	250 µL	5 mL	75 µL	6.25 mL	13.415 mL	0.4 ppb
2	1 ppm	50 µL	250 µL	5 mL	75 µL	6.25 mL	13.375 mL	2.0 ppb
3	1 ppm	250 µL	250 µL	5 mL	75 µL	6.25 mL	13.175 mL	10 ppb
4	1 ppm	500 µL	250 µL	5 mL	75 µL	6.25 mL	12.925 mL	20 ppb
5	1 ppm	1000 µL	250 µL	5 mL	75 µL	6.25 mL	12.425 mL	40 ppb

Instrumentation

- Agilent 1290 Infinity II High Speed Pump (G7104A)
- Agilent 1290 Infinity II vial sampler (G7129B)
- Agilent 1290 Infinity II MCT (G7130A)
- 6470 LC/TQ with AJS ion source (G6470A).

The HPLC parameters with gradient and column are shown in Table 2. The source and MRM parameters for the Agilent 6470 LC/TQ are shown in Table 3.

Table 2. HPLC gradient method.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 EC-C18 (p/n 699975-302)
Column Temperature	40 °C
Injection Volume	10 µL
Autosampler Temperature	10 °C
Flushing Solution	Methanol/water (80/20)
Mobile Phases	A) 0.1% formic acid in water B) 100% methanol
Flow Rate	300 µL/min
Gradient	Time (min) %A %B 0.0 50 50 2.0 50 50 4.0 0 100 9.0 0 100 9.5 50 50 11.0 50 50

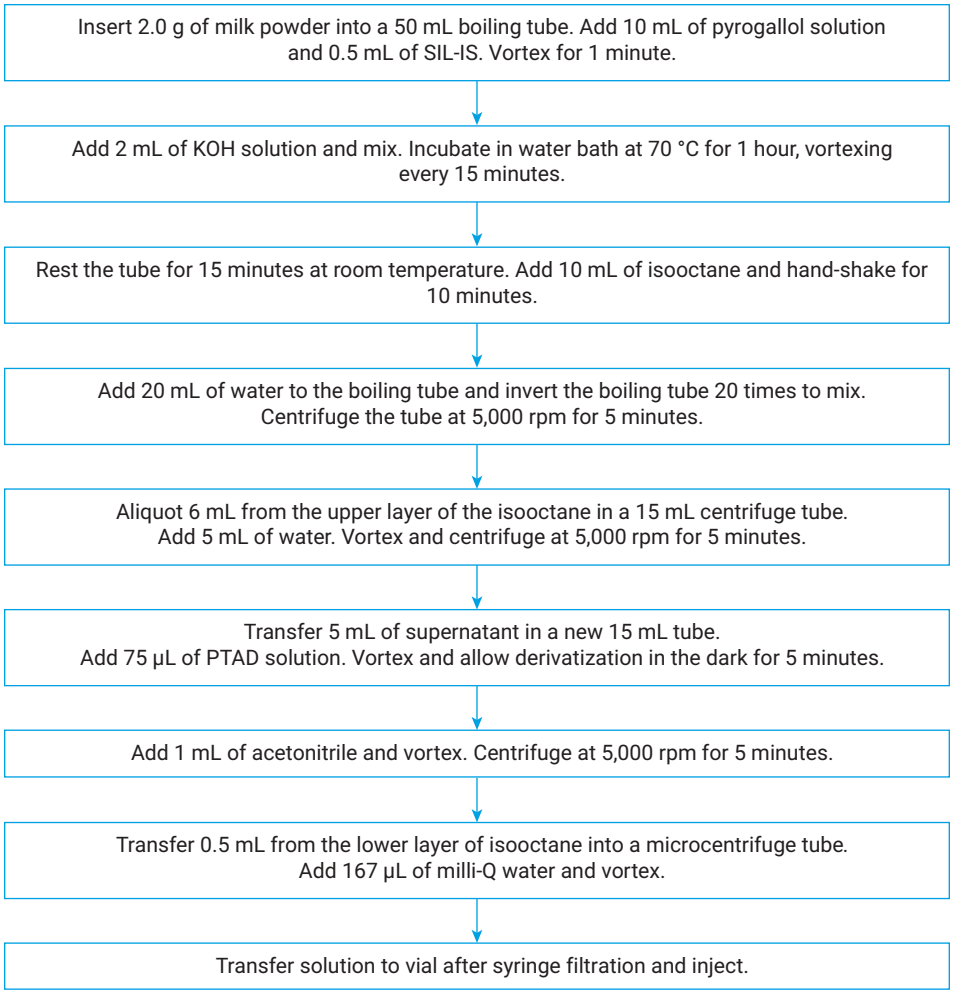


Figure 2. Sample preparation workflow.

Table 3. LC/TQ conditions.

Parameter	Value
Ionization Mode	AJS (+ ve)
Nebulizer Gas	45 psi
Drying Gas	5 L/min at 300 °C
Heath Gas	11 L/min at 250 °C
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
CAV	5 V
Dwell Time	50 ms
Resolution	Unit/Unit

Analyte	Precursor *m/z*	Frag (V)	Quantifier *m/z* (CE)	Qualifier *m/z* (CE)
Vit. D2	572.4	120	298.1 (15)	280.0 (32)
Vit. D2-IS	575.4	110	301.1 (18)	283.1 (35)
Vit. D3	560.5	110	298.1 (18)	280.0 (32)
Vit. D3-IS	566.5	110	298.2 (17)	280.1 (30)

Results and discussion

As shown in Figure 3, the standards and samples had good response for both analytes, with vitamin D2 eluting out of column earlier than vitamin D3. The matrix blank did not show any interference at the target RTs.

Reproducible chromatography was obtained from 0.4 to 40 ppb, as shown in the overlay of both analytes in Figure 4. To measure repeatability, samples were prepared at the 10 ppb spike level in five different milk samples, and their %RSD for final concentration was <2.5.

A calibration linearity plot was generated for relative response versus relative concentration across 0.4 to 40 ppb concentration levels (Figure 5). Figure 5 shows the calibration table with one quantifier and qualifier each. The multiple reaction monitoring (MRM) ratios for the analyte and internal standard are shown in Figures 6 and 7, in accordance with SANTE/12682/2019 regulations.

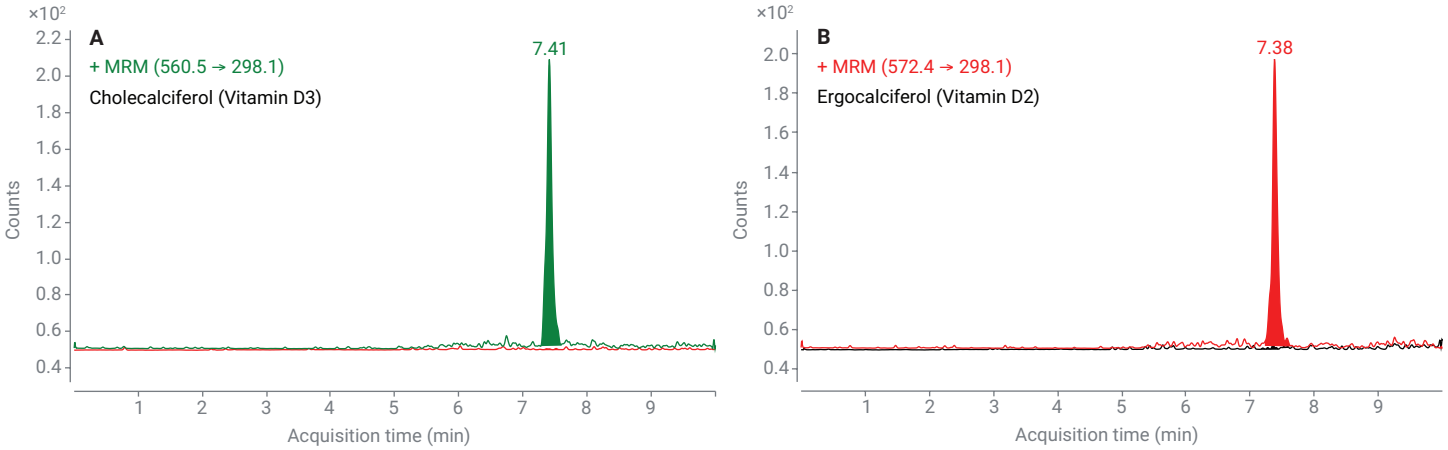


Figure 3. Sensitivity of vitamins D2 and D3 (0.4 ppb in matrix versus blank).

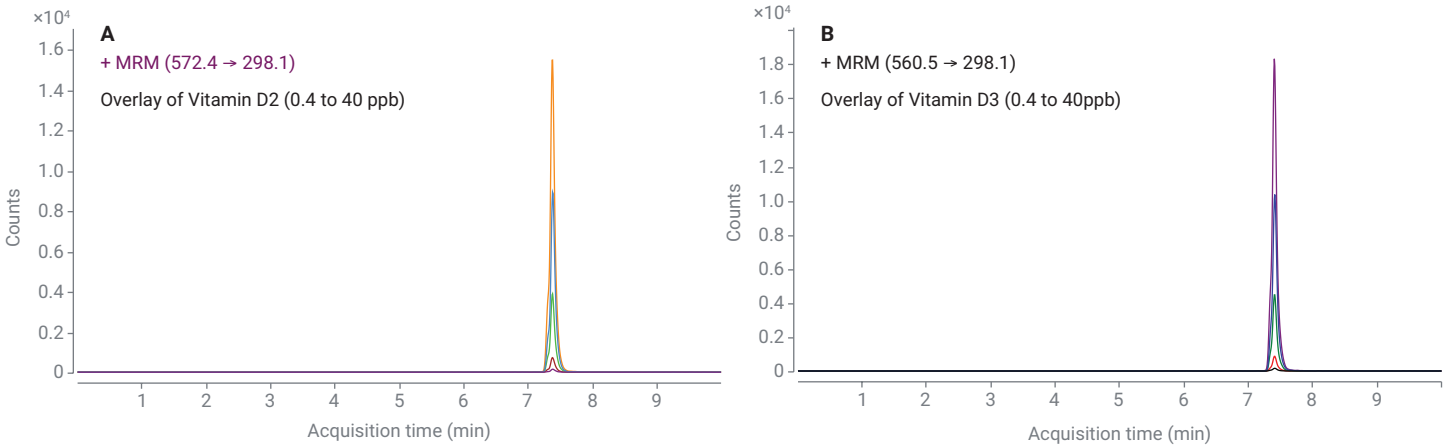


Figure 4. Overlay of various concentrations of vitamins D2 and D3.

Recovery study and quantitation in milk samples

Powdered milk samples, with no vitamin D added, were spiked at the 10 ppb level and extracted as per the protocol in Figure 2. The values of response for analytes and respective internal standards were used to calculate individual analyte concentrations as per the formula described in Equation 1.

Equation 1.

$$D = \frac{D\ Area}{IS\ Area} \times \frac{IS\ Conc}{Slope} \times \frac{IS}{S}$$

D = vitamin D2 or D3 concentration in the sample in ng/g

D Area = area response for D2 or D3 in sample

IS Area = area response for SIL-D2 or SIL-D3 in sample

IS Conc = concentration of D2 or D3 in ng/g

Slope = slope of D2 or D3 from calibration curve

IS = volume of the SIL-IS spiked into the sample in mL

S = weight of milk sample in grams

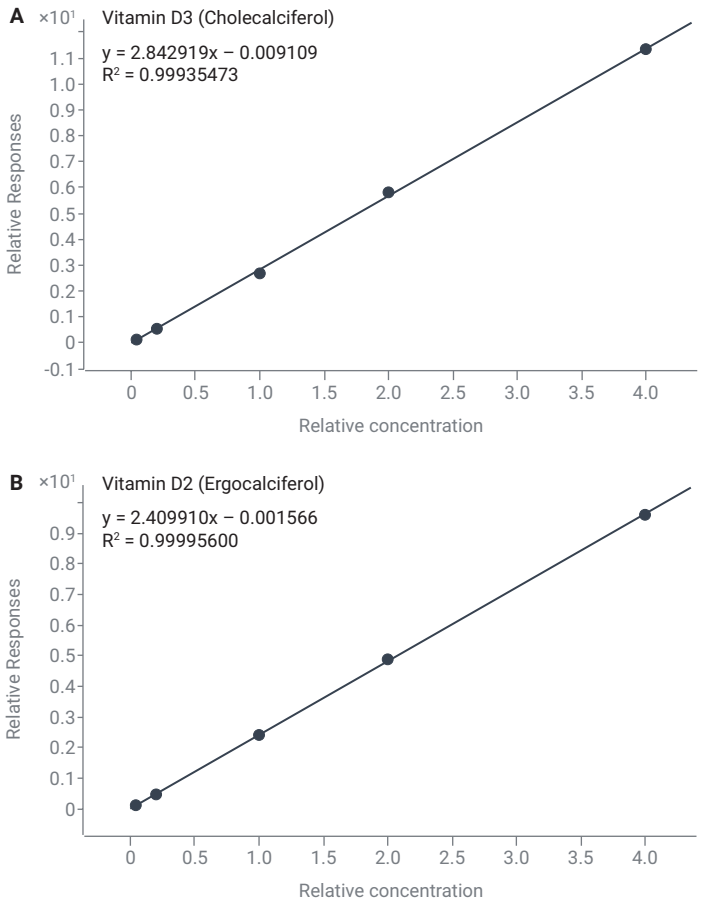


Figure 5. Linearity plot from 0.4 to 40 ppb (R^2 >0.999, across five levels).

Sample				VIT D2-E...	VIT D2-Ergo Results						Qualifie...	VIT D2-Ergo I...		Qualifie...	
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy	RR	Ratio	MI	RT	Resp.	Ratio	MI
	Blank.d	Blank			7.37	5.3		0.48		0.1162	56.9		7.38	45.8		
	Spiked_Sample_01.d	Sample			7.40	7334.7		0.39		0.0930	31.5		7.39	78872.5	31.1	
	Spiked_Sample_02.d	Sample			7.39	7908.0		0.39		0.0936	31.0		7.39	84443.9	31.1	
	Spiked_Sample_03.d	Sample			7.39	8277.1		0.39		0.0923	30.6		7.38	89635.9	31.4	
	Spiked_Sample_04.d	Sample			7.39	7801.4		0.40		0.0969	31.0		7.38	80492.2	31.9	
	Spiked_Sample_05.d	Sample			7.40	8971.9		0.41		0.0984	30.0		7.39	91215.7	30.2	
	Vit D mix Std Cal L1.d	Cal	1	0.4	7.39	826.3		0.40	98.8	0.0946	32.8		7.38	8733.2	31.2	
	Vit D mix Std Cal L2.d	Cal	2	2.0	7.38	4107.1		2.03	101.7	0.4902	32.1		7.38	8377.9	32.7	
	Vit D mix Std Cal L3.d	Cal	3	10.0	7.38	21681.7		9.89	98.9	2.3852	30.0		7.38	9090.2	31.7	
	Vit D mix Std Cal L4.d	Cal	4	20.0	7.39	49560.7		20.15	100.8	4.8628	29.8		7.38	10191.8	32.0	
	Vit D mix Std Cal L5.d	Cal	5	40.0	7.38	88800.0		39.93	99.8	9.6355	29.7		7.37	9215.9	31.3	

Figure 6. Calibration table of vitamin D2 from 0.4 to 40 ppb.

Sample				VIT D3-C...	VIT D3-Chole Results						Qualifie...		VIT D3-Chole I...		Qualifie...	
	Data File	Type	Level	Exp. Conc.	RT	Resp.	MI	Calc. Conc.	Accuracy	RR	Ratio	MI	RT	Resp.	Ratio	MI
	Blank.d	Blank			7.39	0.9		0.13		0.0266	26...		7.41	32.8	63.8	
	Spiked_Sample_01.d	Sample			7.42	8654.9		0.37		0.0955	32.8		7.42	90605.5	34.6	
	Spiked_Sample_02.d	Sample			7.42	8912.0		0.37		0.0943	32.8		7.41	94550.2	34.6	
	Spiked_Sample_03.d	Sample			7.42	9392.6		0.37		0.0946	32.8		7.41	99259.7	34.8	
	Spiked_Sample_04.d	Sample			7.42	8986.5		0.39		0.1004	31.4		7.41	89486.1	34.4	
	Spiked_Sample_05.d	Sample			7.42	10258.5		0.38		0.0982	31.8		7.42	104482.5	34.9	
	Vit D mix Std Cal L1.d	Cal	1	0.4	7.41	928.7		0.41	101.7	0.1055	32.5		7.40	8805.8	36.5	
	Vit D mix Std Cal L2.d	Cal	2	2.0	7.41	4897.5		2.03	101.3	0.5662	32.9		7.40	8650.5	35.2	
	Vit D mix Std Cal L3.d	Cal	3	10.0	7.41	25132.4		9.44	94.4	2.6745	31.6		7.40	9396.9	32.8	
	Vit D mix Std Cal L4.d	Cal	4	20.0	7.41	57034.1		20.50	102.5	5.8215	30.8		7.40	9797.1	36.6	
	Vit D mix Std Cal L5.d	Cal	5	40.0	7.40	101967.2		40.02	100.1	11.37...	30.7		7.40	8965.7	35.6	

Figure 7. Calibration table of vitamin D3 from 0.4 to 40 ppb.

As shown in Table 4, the calculated concentration for vitamin D2 among five samples is within 9.57 to 10.19 ppb with %RSD of 2.49 and average recovery of 98.26%. Similarly, Table 5 for vitamin D3 shows that the values are within 8.29 to 8.83 ppb with %RSD of 2.44 and an average recovery of 84.91%.

Table 4. Vitamin D2 in samples and their recoveries.

Sample	Area of Sample	Area of IS	Calculated concentration (ppb)	Percent Recovery
Spiked Sample 01	7334.70	78,872.50	9.63	96.34
Spiked Sample 02	7908.00	84,443.90	9.70	97.01
Spiked Sample 03	8277.10	89,635.90	9.57	95.66
Spiked Sample 04	7801.40	80,492.20	10.04	100.40
Spiked Sample 05	8971.90	91,215.70	10.19	101.89

Table 5. Vitamin D3 in samples and their recoveries.

Sample	Area of Sample	Area of IS	Calculated Concentration (ppb)	Percent Recovery
Spiked Sample 01	8654.90	90605.50	8.40	83.96
Spiked Sample 02	8912.00	94550.20	8.29	82.85
Spiked Sample 03	9392.60	99259.70	8.32	83.18
Spiked Sample 04	8986.50	89486.10	8.83	88.27
Spiked Sample 05	10258.50	104482.50	8.63	86.30

Conclusion

The developed method is useful for the quantification of two forms of vitamin D: ergocalciferol and cholecalciferol. An MRM-based ESI-LC/MS/MS method was found to be specific for the two analytes in milk matrix. In addition to the use of internal standards, sample preparation based on saponification of milk matrix followed by derivatization proved to be useful in observing low limits of detection, high sensitivity, and high recoveries.

Performing sample preparation across market samples showed decent recovery for both analytes. This method can be used by routine commercial testing laboratories or milk and dairy industries for quantitative estimation of vitamins D2 and D3 at fortification levels.

References

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