Analysis of vitamin D in cheese by APCI LC-MS/MS using the Bruker EVOQ™ LC-TQ Elite mass spectrometry system

Abstract

A rapid, simple and robust method has been developed to determine vitamin D levels in cheese using the Bruker EVOQ™ LC-TQ Elite MS/MS system configured with an APCI source. Excellent sensitivity has been achieved for vitamin D3 with a limit of detection (LOD) below 0.1 µg/kg (ppb) and a method reporting limit of 0.5 µg/kg (ppb). Calibration was performed for levels from 0.5–1000 ppb, giving linearity of $R^2 > 0.999$ and RSD < 25%. For validation of the method, a statistical analysis of precision and accuracy was conducted based on the analytical quality criteria of ISO 17025, with a precision of < 3.5% and recovery of 90–95% obtained. The method is ready for implementation in food quality control process laboratories.

Keywords:
Vitamin D3 (Cholecalciferol)
Vitamin D2 (Ergocalciferol)
Cheese
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Workstation ver. 8.2.1
TASQ.1.4

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Introduction

Vitamin D is an essential liposoluble vitamin that exists in very different forms, of which vitamin D2 (ergocalciferol) and D3 (cholecalciferol) (Figure 1) have greatest physiological importance. Vitamin D2 is found in plants and fungi, while vitamin D3 is naturally produced in animals, including humans. Most vitamin D (as vitamin D3) is produced in the skin through exposure to sunlight. Only a limited number of foods naturally contain vitamin D (mainly salmon, herring, liver, eggs and dairy products), though at low levels.

For this reason, health authorities around the world [1], primarily in regions with long winters and little sunlight, recommend dietary vitamin D supplements, particularly for more vulnerable population groups such as young children, pregnant women and the elderly. The food industry is therefore permitted to develop certain vitamin D-enriched foods, with vitamin D3 being the most widely used because it binds more tightly to its receptors in the body and is therefore more effective at raising and maintaining levels of vitamin D.

One food that is frequently vitamin D-enriched is cheese. In addition to its naturally occurring vitamin D content, cheese offers the advantage that it is a globally accepted foodstuff and that, from an industrial perspective, the process of enriching it with liposoluble vitamins (such as A, D, E and K) is a simple one.

Vitamin D plays a fundamental role in regulating calcium homeostasis and in bone mineralization, as well as in promoting intestinal absorption of calcium. Vitamin D deficiencies can result in a higher risk of illnesses as varied as insulin resistance and diabetes, cancer, autoimmune diseases, cardiovascular disease and mortality due to various causes [2].

However, excessive vitamin D intake can have a negative impact on health, resulting in hypercalcaemia, hypercalciuria, or kidney problems. Accurately determining vitamin D levels in enriched foods is therefore very important [3]. The European Food Safety Authority (EFSA) has established that adequate intake (AI) should be 15 µg per day for adults and 10 µg per day for babies between 7 and 11 months of age [4].

The EFSA has established upper intake levels (ULs) for vitamin D for different population groups [6], as summarized in Table 1.

For these reasons, interest in analysis of this nutrient has increased dramatically in recent years, and relevant analysis criteria have tightened to avoid exceeding permitted enrichment levels. A methodology for analyzing vitamin D 25-OH, a metabolite commonly used for monitoring vitamin D concentration in human serum and plasma, has previously been developed by Bruker [5]. In this application note, a robust and simple method has

Table 1: Tolerable upper intake levels for vitamin D. Source: EFSA

Summary of Tolerable Upper Intake Levels (UL) for Vitamin D

Age (years)	UL for vitamin D (µg/day)
0–1	25
1–10	50
11–17	100
Adults (>18)	100

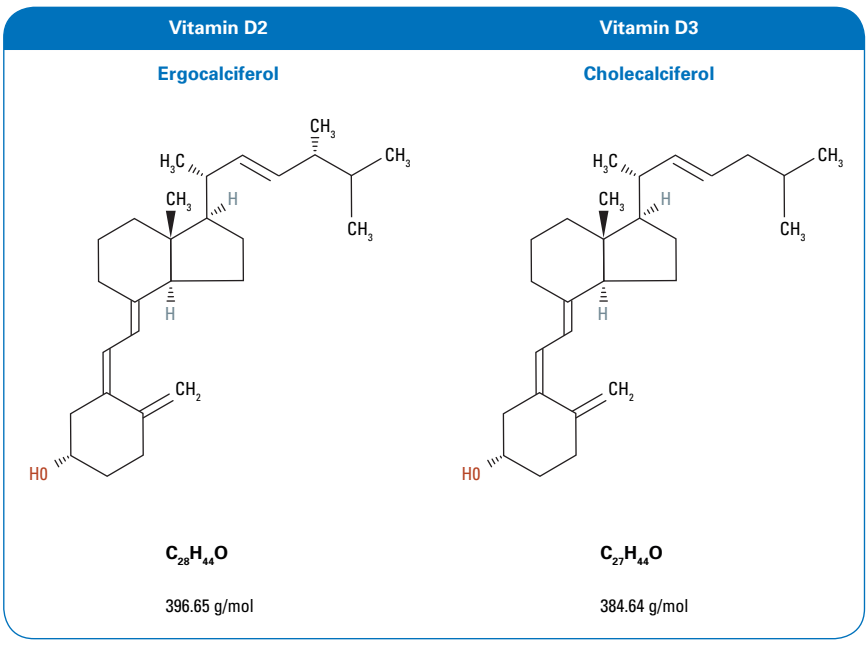

Figure 1: Chemical structure for vitamins D2 and D3

Table 2: Mass Spectrometry Method Conditions

Instrument Conditions	Bruker EVOQ™ LC-TQ Elite MS/MS system	
APCI	Spray current: (+/-) 100 µA	
Cone Temperature	250 °C	
Cone Gas Flow	15 psi	
Nebulizer Gas Flow	30 psi	
Heated Probe Temperature	250 °C	
Probe Gas Flow	25 psi	
CID Gas	Ar, 2.0 mtorr	
Detector Mode	EDR	
Liquid Chromatography	**Bruker Elute™ UHPLC system**	
LC Column	Bruker Intensity Solo C18 100 x 2.1 mm (P/N:BRKHSC18022100)	
Mobile Phase A	Water + 0.1% formic acid	
Mobile Phase B	Methanol + 0.1% formic acid	
Flow Rate	400 µL/min	
Injection Volume	10 µL	
Column Oven Temperature	35 °C	
Gradient	Time (min) 0.0 0.5 1.0 5.2 5.3 8.5	Mobile phase A (%) 90 90 0 0 90 90
Total Run Time	9 min	
Software	Hystar 4.1/Bruker MSWS 8.2.1/TASQ 1.4 processing software	

been developed to determine vitamin D3 and D2 levels in enriched cheese samples using the Bruker EVOQ™ LC-TQ Elite MS/MS system.

Experimental

Sample Preparation

A 10 g sample of commercially available cheese is homogenized and weighed in an Erlenmeyer flask. A 2% pyrogallol solution is added (40 mL), along with 20 mL of a 50% KOH solution, and a vitamin D2 solution (IS, at a final concentration of 10 ppb). An N_2 stream is introduced and the sample is agitated overnight for saponification. The resulting solution is extracted with 30 mL of hexane to which 12.5 mg/L of butylhydroxytoluene [BHT] has previously been added. A 10 mL portion of the extract is evaporated and reconstituted with 1 mL of an acetonitrile/water mixture (70/30). The final extract is filtered through a 0.45 µm PTFE filter. Vitamin D2 and D3 standards were supplied by Sigma-Aldrich (Merck, Darmstad, Germany)

Methodology

MS conditions are summarized in Table 2. The measured MRM transitions for vitamins D2 and D3 are shown in Table 3, and an example MRM chromatogram is shown in Figure 2.

Results and discussion

Linearity

The linearity of the method was demonstrated with an extracted matrix-matched (quark cheese) calibration curve, meaning the standards had undergone the extraction process described above. Seven solutions were prepared with the following concentrations: 0.5 ppb, 1 ppb, 5 ppb, 10 ppb, 50 ppb, 100 ppb and 1000 ppb. Various blank matrices had been injected at an earlier stage to verify the absence of vitamin D.

Figure 3 shows the excellent linearity offered by this method. The signal for

Table 3: MRM transitions for vitamins D2 and D3

Compound Name	RT (min.)	Precur-sor Ion	Quan Ion	CE (V)	Confirm Ion 1	CE (V)	Confirm Ion 2	CE (V)
Vitamin D2 (IS)	5.02	397.60	69.10	-15	107.00	-15	271.30	-10
Vitamin D3	5.17	385.50	259.10	-10	159.10	-20	-	-

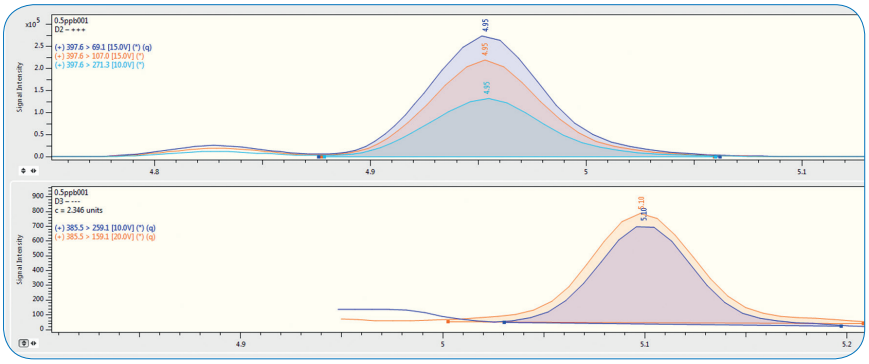

Figure 2: MRM chromatogram of vitamin D2 (top) and D3 (bottom) at 50 ppb level

vitamin D3 was linear across the range under study, with a coefficient of determination of $R^2 > 0.999$ and RSD < 25%. Taking into account that the difference between lower and higher calibration levels exceeds three orders of magnitude, the linearity criteria that are generally required are confidently exceeded.

Sensitivity and Detection Limits

The Limit of Quantification (LOQ) for vitamin D3 was verified at 0.5 µg/kg (ppb), the limit of calibration established the method. Figure 4 shows the comparison of the 0.5 ppb peak signal against the blank quark cheese matrix, demonstrating unambiguous signal at the first calibration level.

To establish the experimental LOD, decreasing concentrations of vitamin D3 were injected into the matrix, down to 0.1 µg/kg (ppb). Three replicates were injected at this level. As shown in Figure 5, a S/N ratio of > 100 was achieved for all replicates at 0.1 ppb, giving a clear definition of both the quantification and the confirmation ions. An experimental LOD < 0.1 µg/kg (ppb) can therefore be established.

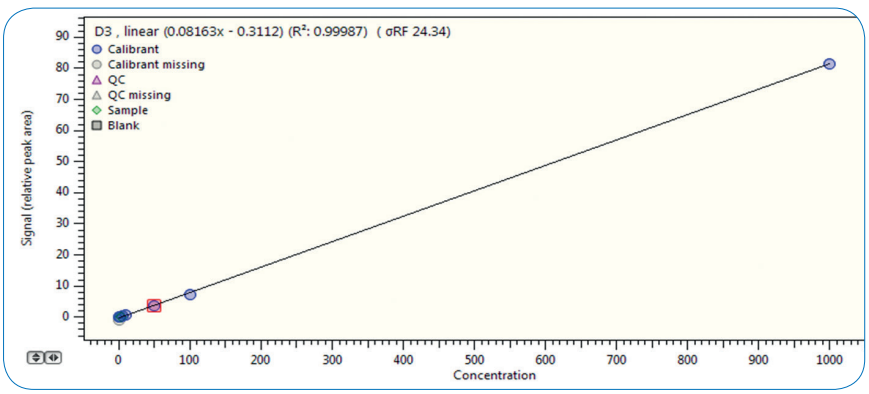

Figure 3: Calibration curve for vitamin D3 in the concentration range of 0.5-1000 µg/kg (ppb)

Precision and Repeatability

Precision, expressed as repeatability, was assessed by analyzing five replicates (n=5) from each of the five different vitamin D3 concentrations at 0.5 ppb, 1 ppb, 10 ppb, 50 ppb and 100 ppb.

Figure 6 provides a graph (calculated automatically by the Bruker TASQ software) of vitamin D3 deviation values

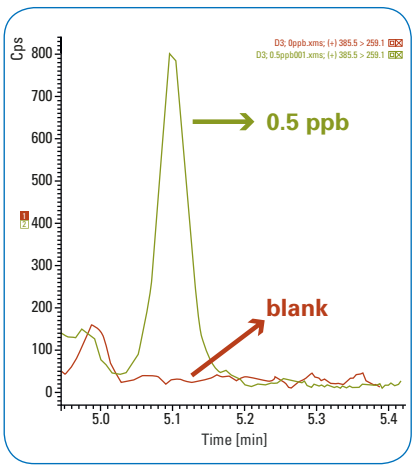

Figure 4: Comparison between LOQ 0.5 ppb peak signal and blank matrix

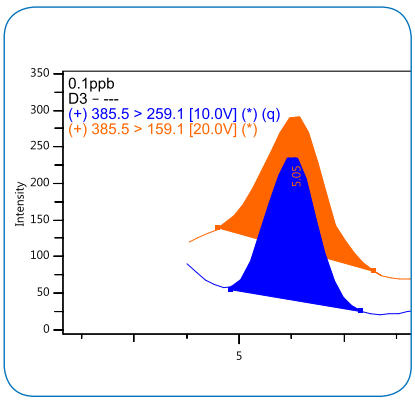

Figure 5: MRM chromatogram of vitamin D3 at 0.1 µg/kg (ppb) level

Table 4: Recovery for cheese samples spiked with vitamin D3

Vitamin D3 recovery from spiked cheese samples							
Samples/Replicates		**Replicate 1**	**Replicate 2**	**Replicate 3**	**Replicate 4**	**Replicate 5**	**RSD (%)**
Quark cheese (spiked with 250 ppb of vitamin D3)	Vitamin D3 results (ppb)	236.2	236.5	232.2	233.5	223.9	2.2
	Recovery (%)	94.5%	94.6%	92.9%	93.4%	89.6%	
Cottage cheese (spiked with 100 ppb of vitamin D3)	Vitamin D3 results (ppb)	92.9	91.9	94.3	96.3	95.8	2.0
	Recovery (%)	92.9%	91.9%	94.3%	96.3%	95.8%	

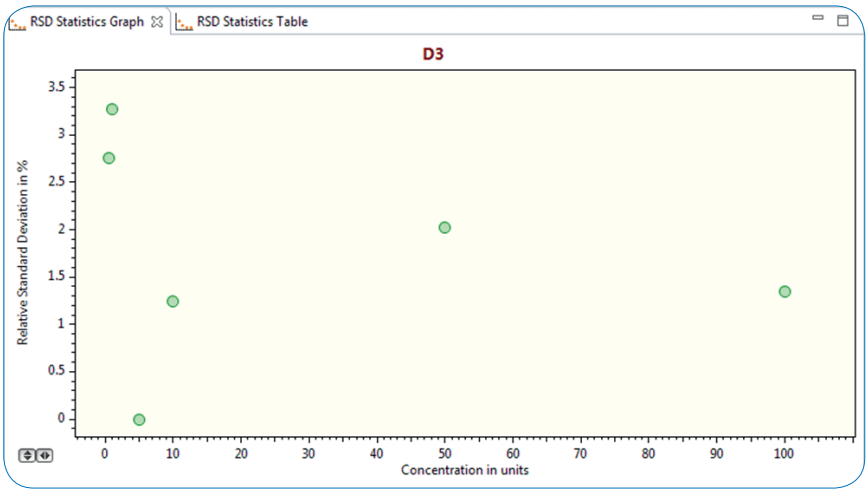

Figure 6: RSD statistics graph (n=5) for vitamin D3 concentrations at 0.5 ppb, 1ppb, 10 ppb, 50 ppb and 100 ppb levels

(RSD) measured across all replicates (n=5) for all of the above indicated concentrations. As shown, the RSDs are below 3.5% across the concentration range under study, substantially exceeding the generally accepted quality criteria.

Accuracy

In order to assess the accuracy of the method, five replicates were analyzed (n=5) from two commercial cheese samples spiked with vitamin D3. Sample 1 was a fresh cheese (quark) spiked with 250 µg/kg (ppb) of vitamin D3, while Sample 2 was a semi-soft cheese (cottage cheese) spiked with 100 µg/kg (ppb). None of the cheese samples contained detectable levels of natural vitamin D. Table 4 shows the results for the five replicates of the two analyzed samples. Recoveries were between 90% and 95%, indicating a high level of accuracy.

Conclusion

A rapid and robust method has been developed to determine vitamin D levels in cheeses using triple quadrupole LC-MS with an APCI interface. The versatility of the Bruker EVOQ™ LC-TQ Elite MS/MS system, with the option of two ionization modes (HESI and APCI) in a single chamber, offers additional flexibility in routinely combining vitamin D analysis with other analyses performed in quality control laboratories.

The proposed method enables rapid and selective analyses for vitamin D. The excellent sensitivity, with a limit of detection < 0.1 µg/kg (ppb), in combination with extensive linearity indicates that vitamin D can be reliably determined in enriched foods (including baby foods) without compromising the analytical quality of the results, in accordance with current regulations.

Learn More

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www.bruker.com/evoq-lc

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