LC/MS/MS Determination of Vitamin D in Food

The Measure of Confidence

Agilent Technologies

Introduction

Vitamin D is an essential fat soluble vitamin. Vitamin D itself is biologically inactive and must be converted by the liver and kidneys to 1α, 25 – dihydroxyvitamin D. It helps regulate levels of calcium and phosphate in the body and promotes healthy bone development. Vitamin D deficiency in children causes rickets in which the bones soften, becoming weakened and deformed. In adults the deficiency is called osteomalacia which causes the bones to become brittle.

In Europe the main source of vitamin D is obtained from sunlight exposure to the skin where ultraviolet B converts 7-dehydrocholesterol to vitamin D_3. In some countries it is possible to obtain the full vitamin D requirement from sunlight, however the majority of people obtain their vitamin D requirements through a combination of solar conversion and by eating a varied and balanced diet.

Foods naturally rich in vitamin D include fatty fish such as herrings, salmon and pilchards. Liver, eggs and milk and other dairy products also contain Vitamin D. In all cases above it is a version of Vitamin D called cholecalciferol (Vitamin D_3) which is present. Solar irradiation of ergosterol in plants, yeast and fungi can also produce Vitamin D in smaller amounts which in this case is present as a compound called ergocalciferol (Vitamin D_2).

Manufacturers may fortify certain foods with either D_2 or D_3 though D_3 is most common. Fortification is done because many governments across Europe recommend that diet be supplemented for certain groups including children aged between six months and five years old, pregnant and breastfeeding women, and people aged over sixty five. However Vitamin D toxicity can occur due to excessive intake of supplements. This may produce a dangerous increase in the level of calcium in the blood resulting in potential kidney failure. Hence manufacturers must carefully control the final concentration of Vitamin D in products or relevance

Methodology

Campden BRI have developed the capability to very sensitively monitor Vitamin D_2 and D_3 simultaneously in food extracts using Agilent LC QQQ (6460). As stated it is usually only Vitamin D_3 that is of interest in many food products. Whether natural or fortified this is certainly the case with milk, other dairy products and infant formula and supplements. Hence Campden BRI have evolved it's capability to show how the Vitamin D_2 channel can be used as an internal standard through both extraction and analysis of these particular food products, to deliver quantitation of Vitamin D_3 with new levels of accuracy and reliability and at the same time afford a relatively simple extraction.

The scope of this method has been demonstrated to also be suitable for the determination of Vitamin D_3 in a wide range of other foodstuffs including fish, spreads and supplements (data not shown here). This is achieved simply by modifying the weight of sample taken and the volume of ethanolic KOH solution.

Vitamin D_2 can also be monitored using the same approach (data not shown here) either by switching to an external calibration approach or by using Vitamin D_3 as the internal standard.

HPLC Conditions

		Gradient:	Time	% B
Mobile Phases:	A – Distilled Water			
	0.0 2 % acetic acid,		0	50
	5 mM Ammonium Acetate		2	50
	B – Methanol		4	100
	0.02 % acetic acid,		9	100
	5 mM Ammonium Acetate		9.5	50
			10	50
Column:	Agilent Eclipse PAH (PN 959741-918), 2.1 x 50 mm, 1.8 µm			
Flow rate:	0.4 mL/min			

Gradient setting.

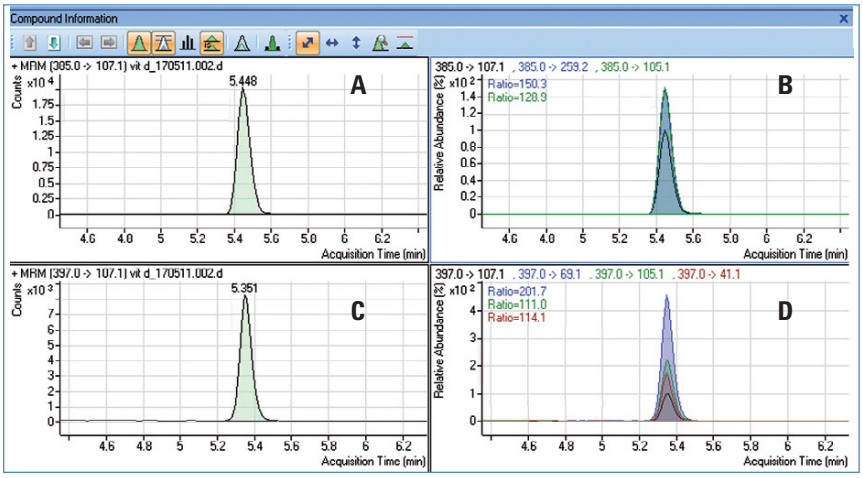
Mass Spectrometer Settings and Jet Stream Parameters

Gas Temperature: 200 ˚C **Sheath Gas Temperature: 400 ˚C**
Gas Flow: 8 L/min **Sheath Gas Flow: 12 L/min**
Nebulizer: 45 psi

Compound Name	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy	Polarity
Vitamin D_2	397	107	70	74	20	Positive
Vitamin D_2	397	105	70	74	50	Positive
Vitamin D_2	397	69	70	74	24	Positive
Vitamin D_2	397	41	70	74	50	Positive
Vitamin D_3	385	259	70	83	8	Positive
Vitamin D_3	385	107	70	83	28	Positive
Vitamin D_3	385	105	70	83	40	Positive

Quantifier and Qualifier MRM Transitions of Vitamin D_2 and D_3.

Results: Extracted Ions from Vitamin Standard



Vitamin D molecules produce a number of useful diagnostic ions. MassHunter software allows their ratios to be automatically monitored and flagged if out of specification contributing to the confidence in results.
A) Vitamin D_3 Quantifier Ion, B) Vitamin D_3 Qualifier Ion Ratios,
C) Vitamin D_2 Quantifier Ion, D) Vitamin D_2 Qualifier Ion Ratios.

Sample Preparation Steps – Milk

15 g of milk+ internal standard (vit D_2)+15 mL 39 % ethanolic KOH

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Mix, heat at 60˚C for 40 minutes

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Transfer contents to separating funnel. Wash flask with 60 mL hexane, add to separating funnel.

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Shake for 90 seconds. Remove aqueous layer. Wash with a further 15 mL two aliquots of water.

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Pass the hexane through sodium sulphate, evaporate to dryness and reconstitute in 10 mL methanol.

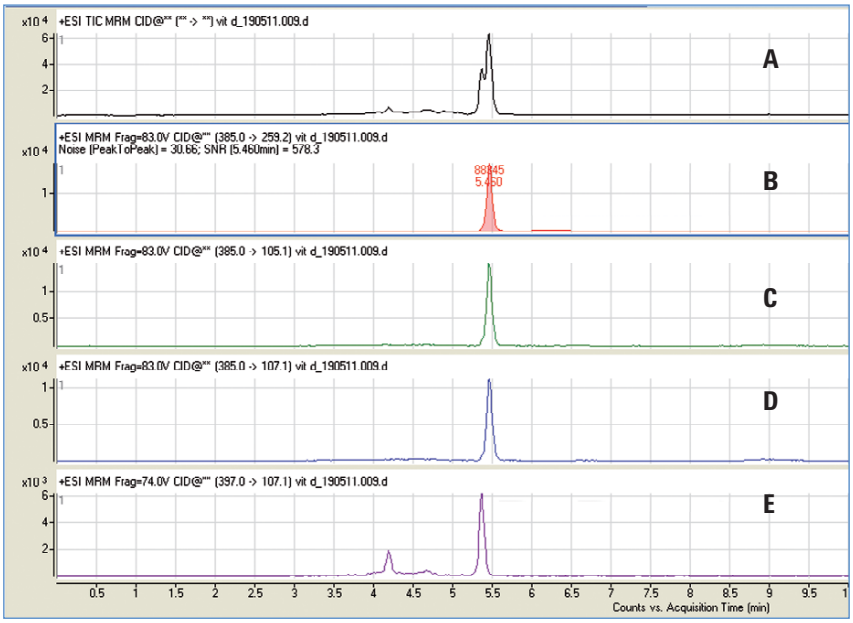
↓

Filter through a 0.02 µm PTFE membrane filter.

↓

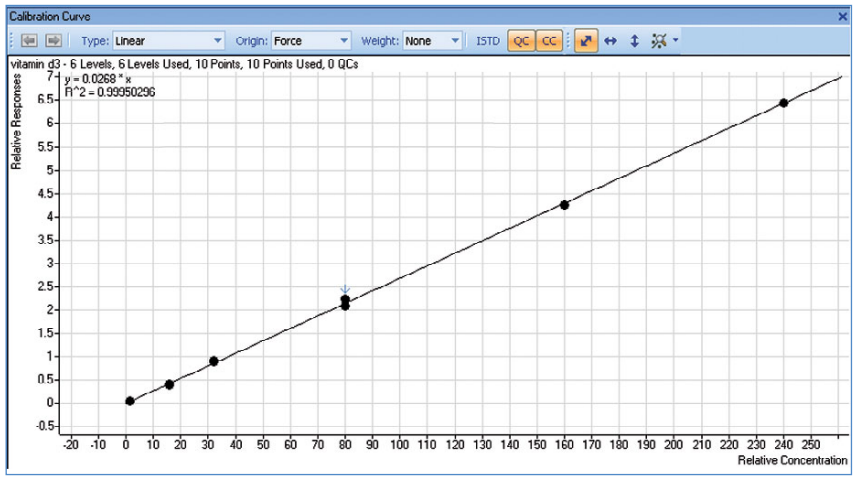
LC/MS/MS Determination

Results:
Extracted Ions from Fortified Milk Sample



Selectivity of the method is illustrated with the required ion traces. A) TIC, B) Vitamin D_3 Quantifier Ion, C+D) Vitamin D_3 Qualifier Ions E) Vitamin D_2 Internal Standard Quantifier Ion.

Calibration Curve



Representative Vitamin D_3 Calibration Curve within 1.0 – 250 ppb.

Vitamin D Method Performance Criteria

Mean Recovery	94.7
% RSD	3.4
LOD	0.1 ppb
LOQ	0.3 ppb
Linearity Range	0.1 – 250 ppb
R^2	>0.999
LOD S/N =3	
LOQ S/N=10	

Performance data of fortified milk samples at 100 µg/kg (n=9).

Conclusion

Vitamin D determination can be problematic and challenging because of the very low levels naturally present in food, and the presence of interfering lipid components within the food matrix.

Many techniques have been used to determine vitamin D in foods including colormetric, spectrometric and gas chromatographic procedures. HPLC with UV detection is a widely used technique but lacks specificity and sensitivity.

This method using the 6460 QQQ provides a sensitive, selective and reproducible procedure to quantify vitamin D in milk, milk powder and dairy products. The Agilent Eclipse PAH (PN 959741-918) column produced excellent peak shape for D_2 and D_3 and enabled the two isomers to be well resolved from potential interfering compounds. The estimated LOQ of 0.3 ppb means that the analysis is working well within the required sensitivity.

The scope of this method has been demonstrated to be suitable for the determination of D_2 or D_3 in a wide range of foodstuffs including fish, spreads and supplements by modifying the weight of sample taken and the volume of ethanolic KOH solution.

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Published in Germany, August 1, 2011
5990-8627EN

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