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

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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.
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.

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

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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.
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.

Analytical technique
Instrumentation
- Agilent 1290 Infinity II quaternary pump with built-in degassing unit (G7120A)
- Agilent 1290 Infinity II multicolonmn 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)

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.

MRM Parameters

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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.

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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.

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

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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


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