

# Measuring Phytosterols in Health Supplements by LC/MS

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## Overview

**Purpose:** Develop a method for the extraction of phytosterols from health supplements. A liquid chromatography-mass spectrometry (LC/MS) method for analysis is used to demonstrate linear response and quantitate extraction results.

**Methods:** Saponification of the supplements followed by liquid-liquid extraction (LLE) separated the analytes from the matrix. Liquid chromatography along with mass selective detection was used to quantitate individual components.

**Results:** Good extraction efficiency combined with linear calibration gave results that were in good agreement with dose levels claimed by supplement manufacturers.

## Introduction

Plant sterols, phytosterols, are claimed to help lower cholesterol levels in humans. In the year 2000, the United States Food & Drug Administration (US FDA) approved a health claim relating phytosterol ester or phytostanol ester consumption to reduced risk for coronary heart disease.<sup>1</sup> Thus, there is increased interest in qualitative and quantitative analysis of phytosterols in food products. Phytosterols are typically measured by gas chromatography after derivatization.<sup>2</sup> In this study, phytosterols were injected onto an LC-MS for direct analysis.

This application demonstrates the use of MS detection with LC for determination of several phytosterols and their extraction from some commercial health supplements. To separate the analytes from the matrix, health supplement pills were saponified in ethanolic potassium hydroxide. After the pill was completely dissolved, the solution was neutralized with acetic acid. Hexane was then used to extract the target compounds. The extract solvent was evaporated. Then samples were reconstituted and diluted to an appropriate concentration range for analysis. Chromatographic separation was optimized, but mass selectivity was required to accurately quantitate the composition of the supplements. Linear calibration and measurements for the supplements are presented.

## Methods

**Supplement extraction:** Health supplement pills were obtained locally from a vitamin store. Each pill was placed in a 50 mL centrifuge tube and 10 mL of 2M ethanolic potassium hydroxide was added. Samples were then sonicated for 30 minutes. After each supplement was completely dissolved, an equal volume of 2M acetic acid was added to neutralize the solution. Subsequently 5 mL of Hexane was added to each centrifuge tube and the samples spun at 3,000 G for 10 minutes. The supernatant was decanted and saved while another 5 mL of hexane was added for a second extraction. The extraction was done 3 times. Each time, the supernatant was separated and all three combined and then evaporated to dryness. Samples were reconstituted with 5 mL of hexane and sonicated briefly to ensure complete dissolving. Further dilution was required to bring the concentrations into range of the calibration. Thus, a 20-fold dilution was used to prepare a 100x sample and a subsequent 10-fold dilution to produce a 1000x dilution. Extracts were stored at 5 °C.

**Reagent and Chemicals:** Stock solutions at 1000 ppm for the six sterols were made up in hexane. Cholesterol (p/n C8667, CAS 57-88-5), Campesterol (p/n C5157, 474-62-4), Brassicasterol (B4936, 474-67-9), Lupeol (p/n L5632, 545-47-1) and B-Sitosterol (p/n S1270, 83-46-5) were obtained from Aldrich.

A working stock solution of the combined six phytosterols was made by combining the above stock solutions to achieve a 100 ppm primary dilution stock. Sequential dilutions of this stock were used to prepare calibration standards at concentrations of 0.5, 1, 2, 5, 10, 20, 50 and 100 part-per-million (ppm or ng/mL).

Acetonitrile (HPLC grade, AH015-4) and methanol (230-4) were obtained from Burdick & Jackson. Hexane was purchased from Aldrich (p/n 320315)

Ethanolic potassium hydroxide was made by mixing 15 mL of 45% w/w KOH concentrate (Fisher, SP236) with Ethanol (VWR, BDH 1160) to make a volume of 100 mL. An amber bottle was used to minimize light exposure and the solution discarded if not used in two weeks.

## Chromatography

A Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) system was used. This system included a LPG-3600 Ternary Pump, WPS-3000T Autosampler, and a FLM-3100 Column Oven.

A Thermo Scientific Acclaim C30 column was used to separate the compounds under isocratic conditions. The column was thermostated at 30 °C. The solvent mixture was held constant at 50:50 MeOH/ACN. The autosampler delivered a 5 µL injection while the flow rate was 0.35 mL/min. A retention time was established for each compound. The divert valve directed flow from the column to waste or to the MS for analysis as described below. Total run time was 12 minutes, with all peaks of interest eluting between 6 and 11 minutes.

| Divert Valve (to MS) | Time (min) | Position |
|----------------------|------------|----------|
|                      | 0.0        | to waste |
|                      | 4.5        | to MS    |
|                      | 12.0       | to waste |

The divert valve was used for the first 4.5 minutes, shunting the LC flow to waste to allow impurities from the extraction that were not retained on the column to bypass the MS. The valve was then switched to direct flow to the MS for analysis. A Thermo Scientific Dionex AXP Auxiliary Pump provided a continuous stream of liquid flowing through the MS source so that it would remain wet at all times.

## Mass Spectrometry

A Thermo Scientific MSQ Plus single quadrupole mass spectrometer was used for analysis. The atmospheric-pressure chemical ionization (APCI) interface proved to be most effective at ionizing the relatively neutral compounds. Selectivity is inherently present due to the difference in *m/z* ratio of the analytes serving to reduce potential interferences that might be present in the extracted samples. Sensitivity is greatly enhanced through the use of the selective ion monitoring (SIM) scan function. Water loss from the protonated molecular ion was the most strongly observed species for all the analytes. Thus, the SIM scans represent  $[M+H-H_2O]^+$ , or a target *m/z* of the pseudo-molecular ion minus water. Details of the SIM scan parameters are detailed in Table 1.

## Data Analysis

Integrated control of the LC and MS hardware was accomplished through the Thermo Scientific Dionex Chromeleon Chromatography software (version 6.8 SR10). This software also provided tools for data acquisition, processing and report generation.

TABLE 1. Table of SIM scan parameters\*

| Analyte        | t <sub>R</sub> (min) | m/z   | Dwell time (s) | Cone Voltage (V) |
|----------------|----------------------|-------|----------------|------------------|
| Cholesterol    | 8.0                  | 369.2 | 0.2            | 50               |
| Brassicasterol | 7.5                  | 381.2 | 0.2            | 45               |
| Campesterol    | 9.0                  | 383.2 | 0.2            | 50               |
| Stigmasterol   | 9.0                  | 395.3 | 0.2            | 50               |
| B-Sitosterol   | 10.2                 | 397.3 | 0.2            | 40               |
| Lupeol         | 6.8                  | 409.4 | 0.2            | 40               |

\* The optimum settings and responses may vary on different instruments, and thus optimization of the MSQ Plus™ mass spectrometer source conditions and acquisition parameters is highly recommended for best results.

## Results

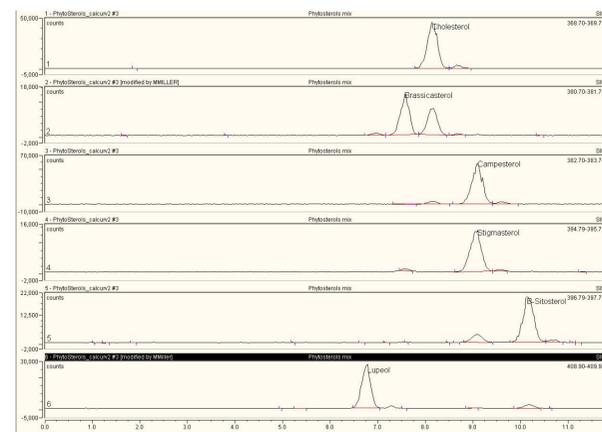
This method was used to quantify the target analytes in clean water as well as the recovery of extractions from health supplement pills

Calibration curves were generated from standards with concentrations from 0.5 to 50 ppm. All compounds showed a correlation coefficient (*r*<sup>2</sup>) greater than 0.9997. Calibration ranges were from 0.5 to 100 ppm for a 5 µL injection, giving a load range of 2.5 to 500 µg on column. Results are summarized in Table 2. A typical separation is shown in the chromatogram in Figure 1.

Table 2. Calibration and Coefficient of Determination

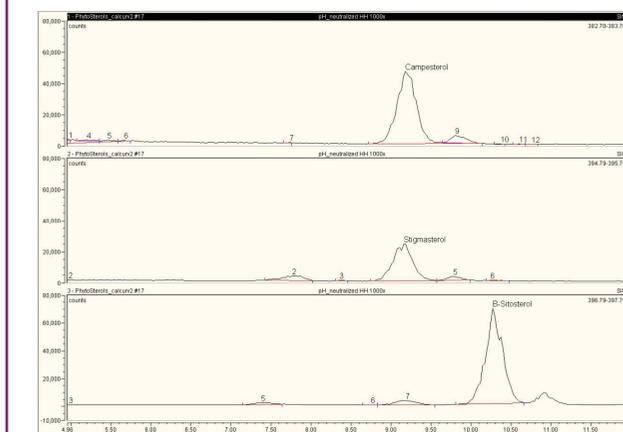
| Analyte        | Calibration Range (ppm) | Fitting | r <sup>2</sup> |
|----------------|-------------------------|---------|----------------|
| Cholesterol    | 0.5-100                 | Linear  | 0.9997         |
| Brassicasterol | 0.5-100                 | Linear  | 0.9997         |
| Campesterol    | 0.5-100                 | Linear  | 0.9999         |
| Stigmasterol   | 0.5-100                 | Linear  | 0.9999         |
| B-Sitosterol   | 0.5-100                 | Linear  | 0.9999         |
| Lupeol         | 0.5-100                 | Linear  | 0.9999         |

FIGURE 1. Separation of analytes in clean standard



Two health supplements were analyzed for recovery and compared with labeled amount. Measured recoveries for total amount of phytosterols were 80% for one supplement and 88% for the other. Figure 2 shows one supplement and the three detected phytosterols present after extraction.

FIGURE 2. Chromatogram of extracted health supplement after 1000x dilution



## Conclusion

This application demonstrates the use of the Acclaim™ C30 column coupled with the UltiMate™ 3000 HPLC and MSQ Plus™ detector to separate and quantitate the phytosterols in this method. Use of MS detection allows confirmation of analyte identity. Quantitation showed linear response for the analytes. This shows the capability of using the LC/MS combination to analyze nutraceutical health supplements for phytosterol content.

## References

- Code of Federal Regulations, *Title 21 Part 101 Subpart E, 101.83 Health claims: plant sterol/stanol esters and risk of coronary heart disease* (2000).
- Winkler, J.K., Rennick, K.A., Eller, F.J. and Vaughn, S.F. Phytosterol and tocopherol components in extracts of corn distillers dried grain. *J. Agric. Food Chem.*, **55**, 6482-6486 (2007).