Rapid and Direct Analysis of Free Phytosterols by Reversed Phase HPLC with Electrochemical Detection

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Overview

Purpose: In order to measure cholesterol and free phytosterols from biological samples scientists require a sensitive approach. A simple, accurate and rapid UHPLC method was developed for the analysis of these compounds using liquid chromatography and an amperometric electrochemical cell with a boron-doped diamond (BDD) working electrode. This allowed the accurate quantification of these analytes to sub-nanogram levels.

Methods: This method describes an approach for direct analysis of phytosterols and cholesterol from whole blood using UHPLC chromatographic techniques with a robust electrochemical cell using a BDD electrode.

Results: The method enables the rapid separation of various phytosterols and cholesterol at low levels from whole blood without significant matrix interferences.

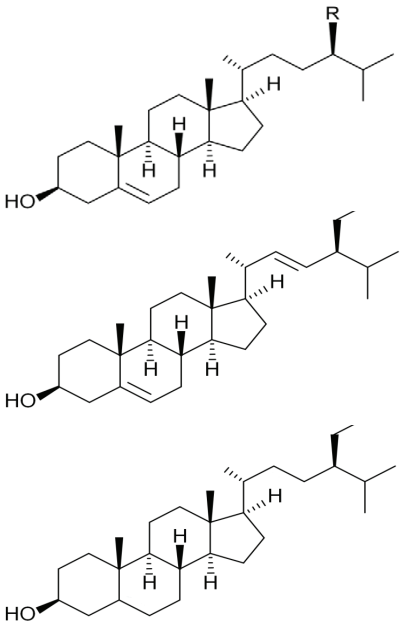
Introduction

Phytosterols are a group of naturally occurring steroid alcohols found in plants which can occur in foods as the free form or as esters with fatty acids/cinammic acid or glycosides. They are key structural components of plant cell membranes, assuming the role that cholesterol plays in mammalian cells. There is considerable interest in phytosterols as dietary supplements as they are reported to lower cholesterol levels and also have a positive impact on cardiovascular diseases. However, recent research suggests that phytosterol supplementation may aggravate atherosclerosis and lead to aortic valve stenosis.

The chemical structures of five standards, cholesterol, campesterol, stigmasterol, β-sitosterol, and stigmastanol, are shown in Figure 1. These compounds lack a good chromophore, requiring UV detection below 210 nm, or they need to be derivatized with TMS for GC analysis. Phytosterols measured by gas chromatography is time-consuming since it requires saponification of the sample, several extractions steps, and then derivatization. Presented here is a simplified method using reversed phase high-performance liquid chromatography (HPLC) and electrochemical detection using a boron doped diamond electrode The latter technique requires careful selection of internal standards, complete extractions, and good reaction yields for quantitative results. These processes can significantly add to the time required and analysis costs for sample analysis.

Five standards, campesterol, cholesterol, stigmasterol, ß-sitosterol, and stigmastano were resolved in < 5 min. For quantitation of phytosterols, the ECD offers high precision, typically < 3% RSD, and a simplified sample preparation, not requiring any extraction, derivatization, or the need for internal standards. Considering the growing amount of research that is ongoing with the biological effects of phytosterols in the

FIGURE 1. Structures of five phytosterols.



Cholesterol, R = H
Campesterol, R = CH_3
b-Sitosterol, R = CH_2CH_3

Stigmasterol

Stigmastanol

diet, a simple but reliable analytical method for the determination of content and purity of samples and standards is a significant factor. The HPLC method with electrochemical detection is simple to implement, has good linearity and sensitivity, and is capable of measuring numerous free phytosterols in biological extracts. This approach can be used to examine product purity, supplement content, and adulteration. The use of the BDD electrode extends the range of analytes to those whose structure are normally considered to be electrochemically inert when using conventional carbon- or metal-based working electrodes. The electrochemical detector with the boron doped diamond electrode provides sensitive detection with a Limit of Detection (LOD) ≤ 1 ng on column for all analytes.

Methods

Analytical Conditions for Phytosterol Analysis

Column:	Thermo Scientific™ Accucore™ C8 column, 2.6 µm, 2.1 × 100 mm
Pump Flow Rate:	1.00 mL/min
Column Temperature:	50.0 °C
Mobile Phase A:	0.1% trifluoroacetic acid, 50 mM lithium perchlorate in water
Mobile Phase B:	0.1% trifluoroacetic acid, 50 mM lithium perchlorate in acetonitrile
Injection Volume:	2 µL standards and samples
Gradient	

Time	%A	%B
-5.00	22.0	78.0
0.00	22.0	78.0
5.00	10.0	90.0
5.50	22.0	78.0
6.50	22.0	78.0

Cell Potential:	Thermo Scientific™ Dionex™ 6041RS ultra Amperometric Analytical Cell with BDD electrode at +1900 mV
Clean Cell Potential	+1950 mV
Cell Clean Duration:	20.0 s
Filter Constant:	2.0 s
Sample Prep:	5–20 µL whole blood + 500 µL Mobile Phase B, mix and spin for 10 minutes at 13,000 RPM. The supernatant was transferred into an autosampler vial and placed onto the autosampler. tray

FIGURE 2A. Thermo Scientific™ Dionex™ UltiMate™ 3000 Electrochemical detector
FIGURE 2B. 6041RS ultra Amperometric Analytical Cell with BDD electrode
FIGURE 2C. Thermo Scientific ™ Dionex ™ nanoViper ™ fingertight capillary fittings

A

B

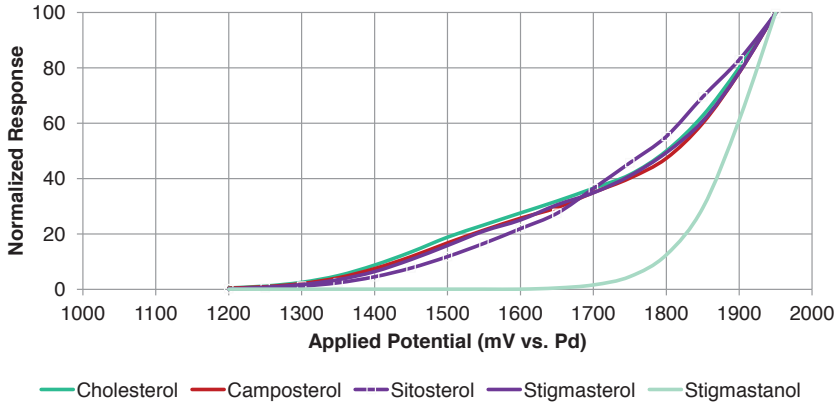
C

Results and Discussion

An instrumental prerequisite for trace electrochemical analysis is that the HPLC system must be inert (free from leachable transition metals) in order to achieve optimal sensitivity using an electrochemical detector. The system uses biocompatible materials in the flow path to reduce the influence of metal that can contribute to elevated background currents at the electrochemical cell.

The 6041RS amperometric cell (Figure 2B) provides the unique electrochemical capabilities of the boron-doped diamond which enables the oxidation of organic compounds using higher electrode potentials than other working electrode materials. NanoViper fingertight fittings (Figure 2C) were employed to cope with the higher pressures due to smaller column particles. These fingertight, virtually zero-dead-volume (ZDV) capillaries can operate at pressures up to 14,500 psi and are much safer to use than PEEK tubing which can slip when using elevated pressures. They are made of PeekSil™ tubing and are available in small internal dimensions to minimize chromatographic band spreading. Capillaries used on this system were 150 micron ID for all connections made prior to the autosampler valve and 100 micron ID for those made after the injector valve.

FIGURE 3. Current-Voltage Curve for phytosterols using the BDD electrode.



The direct electrochemical detection of phytosterol compounds using a boron-doped diamond electrochemical cell has been previously described.[1] The applied potential used for this study (+1900 mV) was sufficient to oxidize all phytosterol analytes evaluated as shown in Figure 3. Advantages of this approach include a stable electrode surface and method simplicity since no sample derivatization is required. After each analysis the electrode surface was regenerated by a 20 second clean cell pulse at +1950 mV. After a 2 minute re-equilibration at +1900 mV the electrode was once again stable and could be used for the analysis of the next sample. In the current method, the whole blood sample was added to mobile phase B (high acetonitrile), mixed and then centrifuged. The clear supernatant was then transferred into an autosampler vial and placed on the autosampler. Rapid UHPLC analysis as shown in Figure 4 enables processing of samples within five minutes. Some additional time to re-equilibrate the column is then required.

The calibration curves for phytosterol standards are shown in Figure 5. Good linearity of response to different concentrations was obtained with correlation coefficients ranging from R^2 = 0.992 – 0.997 for the five compounds evaluated (Table 1) over the range of 0.5 – 20 μg/mL. The percent relative standard deviation (%RSD) for the calibration curves (six concentration points) are also shown in Table 1. The RSD values ranged from 7.2% to 9.5%, indicating that the BDD electrode provided suitable stability.

Since phytosterols can exist as fatty acid esters, hydrolysis with base can liberate these phytosterols for analysis. Other sample types containing phytosterols can be prepared for HPLC analysis by saponification with potassium hydroxide to reduce acylglycerols to fatty acids prior to analysis in order to remove potential buildup of lipophilic material being retained on the analytical column (data not shown).

FIGURE 4. Overlay of analytical standards (1 – 40 ng on column)

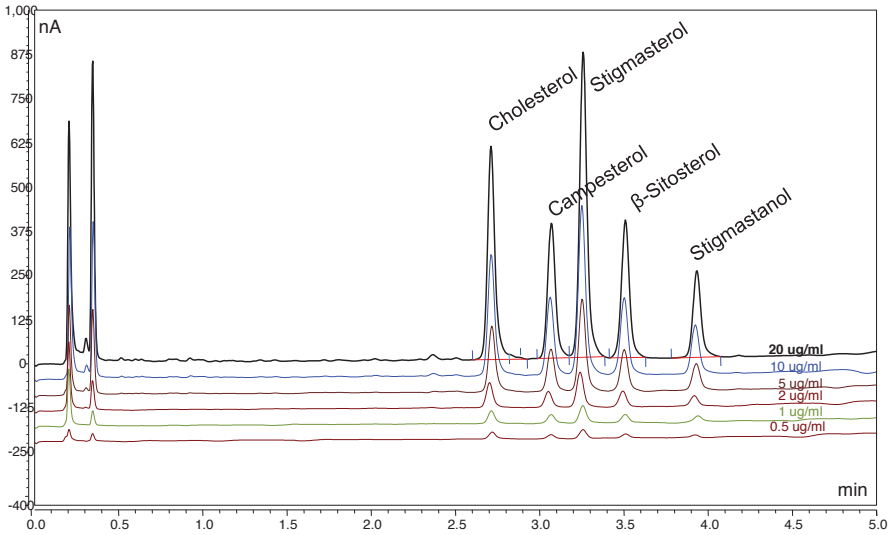


FIGURE 5. Calibration curves for standards (0.5 – 20 *µ*g/mL)

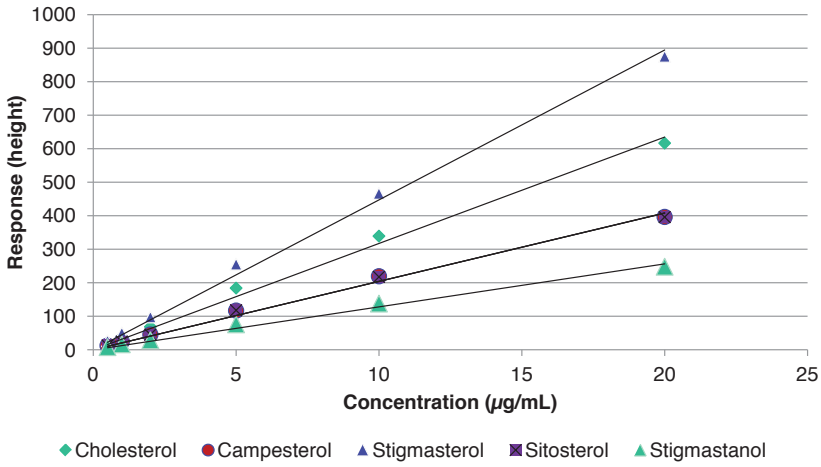
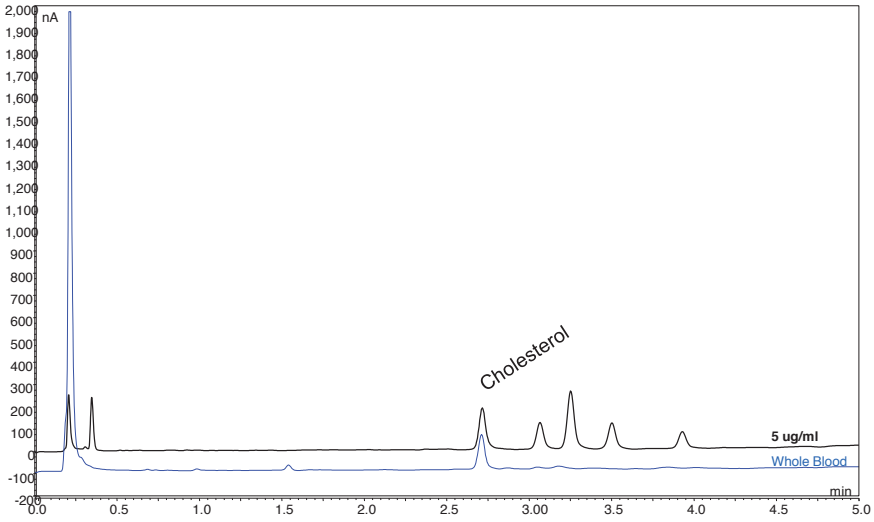


Table 1. Regression data for standard calibration curves

Peak Name	Ret.Time (min)	Number of Points	Rel.Std.Dev. %	Coeff.of Determination	Slope
Cholesterol	2.72	12	8.73	0.9943	31.74
Campesterol	3.068	12	9.4045	0.9942	20.43
Stigmasterol	3.258	12	7.1536	0.9965	44.72
Sitosterol	3.51	12	8.4131	0.9943	20.38
Stigmastanol	3.923	12	9.5449	0.9923	12.82

Enhanced peak shape (narrow peak width) in addition to improved design features of the 6041RS cell with a BDD electrode provides good sensitivity. The small cell volume of only 50 nL contributes to low background currents which helps minimize the noise of the electrochemical cell. The analysis of cholesterol in whole blood was performed and is shown in Figure 6.

FIGURE 6. Overlay of standard (red trace) vs. whole blood (blue trace).



An overlay of chromatograms for a standard mixture of phytosterols and a sample of deproteinized whole blood is shown in Figure 6. The levels of cholesterol was easily measured in small samples of whole blood (<20 μL) using the UHPLC method described. The mean level of cholesterol in one subject was calculated at 182.3 mg/dL with a %RSD of 1.91% (n=6) which is within the range of reported levels using other techniques (Table 1).[3]

Table 3. Whole Blood Cholesterol.

	Concentration ($\bar{X}$; n= 6)	%RSD
Cholesterol	182.3 mg/dL	1.91

Conclusions

- The Boron Doped Diamond electrode provides a suitable tool to measure compounds that require elevated electrode potentials to oxidize. In this case phytosterols are determined with high sensitivity (LOD ≤ 1 ng on column for all analytes).
- The method for the analysis for the measurement of phytosterols proved to be simple and reliable with sufficient sensitivity for their measurement in deproteinized whole blood. Compared to GC techniques, the present method is more robust, and does not need a time consuming derivatization step.
- The levels of cholesterol detected in whole blood are within the range of reported levels using other techniques.

References

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