Rapid quantification of free and esterified phytosterols in human serum using APPI-LC-MS/MS

Jan Lembcke, Uta Ceglarek, Georg Martin Fiedler, Sven Baumann, Alexander Leichtle, Joachim Thiery

Rapid quantification of free and esterified phytosterols in human serum using APPI-LC-MS/MS

Jan Lembcke,[1] Uta Ceglarek,[1] Georg Martin Fiedler, Sven Baumann, Alexander Leichtle, and Joachim Thiery[2]

Institute of Laboratory Medicine, Clinical Chemistry, and Molecular Diagnostics, University Hospital, Leipzig, Germany

Abstract **A novel analytical platform based on liquid chromatography and tandem mass spectrometry using atmospheric pressure photoionization was applied for the simultaneous quantification of free and esterified β-sitosterol, campesterol, brassicasterol, and stigmasterol. The total time for sample pretreatment and analysis could be reduced from ~3 h [gas chromatography-mass spectrometry (GC-MS)] to 15 min. The detection limits of the different phytosterols ranged between 0.25 and 0.68 μg/l. Linear ranges were between 1 and 1,000 μg/l. The within-run and between-run variabilities ranged between 1.4% and 9.9%. The analytical sensitivity was at least 150-fold higher compared with GC-MS. Our new method allows a rapid and simultaneous determination of free and esterified phytosterols in serum.**—Lembcke, J., U. Ceglarek, G. M. Fiedler, S. Baumann, A. Leichtle, and J. Thiery. **Rapid quantification of free and esterified phytosterols in human serum using atmospheric pressure photoionization liquid chromatography-MS/MS.** *J. Lipid Res.* 2005. 46: 21–26.

Phytosterols are common components of plant foods, especially vegetable oils, seeds, nuts, and cereals (1). They are structurally similar to cholesterol, differing only in the number of carbons or double bonds in the side chain. An average Western diet contains ~200–400 mg of phytosterols per day (e.g., β-sitosterol, campesterol, stigmasterol, and brassicasterol). All serum and tissue phytosterols are derived exclusively by intestinal absorption. Therefore, serum levels of phytosterols reflect dietary plant sterol intake and intestinal absorption (2). The individual differences in plasma phytosterol concentrations are highly heritable (3). Compared with dietary cholesterol, the intestinal absorption rate of dietary phytosterols is markedly lower, because the bulk of absorbed phytosterols is immediately secreted into the intestine by the ATP-binding cassette half-transporters ABCG5 and ABCG8 of the enterocytes (4). Mutations in either of the transporter genes have been identified as the cause of sitosterolemia, a rare autosomal recessive lipid disorder that is characterized by markedly increased serum phytosterol concentrations (e.g., β-sitosterol > 50 mg/l) as a consequence of hyperabsorption and impaired biliary secretion of neutral sterols (5–7). These patients develop premature coronary heart disease (8, 9). A recent study in patients admitted for elective coronary artery bypass graft surgery supports the hypothesis that even slightly increased campesterol (3.8 ± 1.6 mg/l) and β-sitosterol (3.1 ± 1.1 mg/l) concentrations in serum may contribute to the risk of coronary heart disease (10).

Sensitive analytical methods are necessary to detect physiological phytosterol concentrations and even slightly increased concentrations in human serum. Currently available methods for the measurement of phytosterols in serum are based on GC-MS. However, this analytical platform is time-consuming and requires a laborious pretreatment procedure (hydrolysis, liquid-liquid extraction, and derivatization) and a large sample volume (11, 12). Recently described liquid chromatography-mass spectrometry (LC-MS) methods for the determination of cholesterol and oxidized metabolites in human plasma also include time-consuming extraction and hydrolysis steps (13, 14).

The aim of our study was to develop a rapid tandem mass spectrometric method for the simultaneous quantification of free and esterified phytosterols combined with a simple one-step sample pretreatment for small sample volumes. For this purpose, we used a novel atmospheric pres-

sure photoionization (APPI) technique combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (15).

MATERIALS AND METHODS

Chemicals and reagents

Stigmasterol, β-sitosterol, campesterol, and brassicasterol were purchased from Steraloids (Newport, RI). Labeled phytosterol standards could not be used for the method development because of the lack of a commercial supplier. Thus, [25,26,26,26,27,27,27-$^2H_7$]-cholesterol (Euriso-top, Saarbrücken, Germany) was used as an internal standard. [3,4-$^{13}C_2$]cholesterol was obtained from Cambridge Isotope Laboratory (Andover, MA). Cholesterol, cholesteryl stearate, toluene, and all other chemicals were obtained from Sigma-Aldrich (München, Germany).

Sample collection

Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation of pooled serum (16). Blood samples were collected from 49 healthy volunteers with a mean age of 22.5 years (20–25 years). After coagulation and centrifugation at 1,400 g, serum was stored in polypropylene tubes at −80°C until analysis.

Standard and sample preparation

Stock solutions of all phytosterols and cholesterol were prepared in isopropanol (1 g/l).

Four calibrators were prepared by mixing and diluting the phytosterol stock solutions with methanol containing 750 mg/l [$^{13}C_2$]cholesterol to final concentrations of 0.1, 0.5, 2, and 10 mg/l for β-sitosterol, campesterol, brassicasterol, and stigmasterol. [$^{13}C_2$]cholesterol was added to all methanolic standard solutions in the same concentration range as in serum samples. In-house-made LPDS controls containing 0.5, 2, and 10 mg/l of each phytosterol and 750 mg/l [$^{13}C_2$]cholesterol were used to calculate accuracy. Daily precision was determined using an in-house control preparation of pooled serum (final mean concentration, 2.14, 5.80, 0.62, and 1.41 mg/l for β-sitosterol, campesterol, brassicasterol, and stigmasterol, respectively). Internal standard solution (100 μg/l [$^2H_7$]cholesterol in methanol) was prepared from a stock solution of 1 g/l in isopropanol.

For the quantification of free and esterified phytosterols, 20 μl of calibrators, controls, and serum samples were mixed with 980 μl of internal standard solution. After centrifugation for 10 min at 11,400 g, the supernatant was transferred into a glass vial.

For the quantification of total sterol concentration, 10 μl of internal standard (100 mg/l) and 20 μl of butylated hydroxyl toluene (1 g/l) were added to 20 μl of a cholesterol-cholesteryl stearate standard solution (51.7 μmol/l each) and to the serum control. After mixing with 2 ml of freshly prepared 1 M ethanolic sodium hydroxide, hydrolysis was performed for 1 h at 68°C. Thereafter, 1 ml of deionized water was added. The sample was extracted twice with 3 ml of cyclohexane. The extract was dried at 65°C under a stream of nitrogen and reconstituted with 200 μl of isopropanol.

LC-MS/MS

The chromatographic system consisted of a series 200 autosampler, a column oven, and a binary micro pump system from Perkin-Elmer (Rotgau-Jügesheim, Germany). Chromatographic separation was performed using a Chromolith SpeedRod RP-18e (50 × 4.6 mm) monolithic column (Merck KGaA, Darmstadt, Germany) at 40°C. Supernatants (25 μl) were injected onto the analytical column, which was equilibrated with methanol-water

(75:25, v/v) at a flow rate of 600 μl/min. After 1 min under these isocratic conditions, a linear gradient step to 100% isopropanol was performed in 1 min and kept for 3.5 min. After chromatographic separation, the system was reequilibrated with the starting solvent mixture for 4.5 min.

A MDS SCIEX API 3000 triple quadrupole mass spectrometer with a Photospray™ from Applied Biosystems (Darmstadt, Germany) was used. For APPI measurements, the eluent was introduced into the ion source without solvent splitting at 600 μl/min and 400°C. Ions were formed in positive ion mode with a lamp voltage of 1,700 V and an orifice voltage of 45 V. Toluene was used as ionization dopant at a flow rate of 90 μl/min delivered by an additional Perkin-Elmer series 200 binary pump.

Recovery

The investigation of potential interfering effects of serum matrix components and the determination of the analytical recovery was done using spiked LPDS containing 2 mg/l β-sitosterol, campesterol, brassicasterol, stigmasterol, or [$^2H_7$]cholesterol. After sample pretreatment and HPLC separation, the mass spectrometer response of the analytes was compared with the signal response of a methanolic standard solution.

Because of the lack of phytosterol ester standards, a methanolic standard mixture of cholesterol (1 mg/l) and cholesteryl stearate (1.65 mg/l) was used to evaluate the LC-MS/MS measurements. The total sterol concentration was calculated by adding the free and esterified sterol content and compared with the result obtained after hydrolysis. Therefore, the standard solution containing cholesterol and cholesteryl stearate was analyzed before and after alkaline saponification.

Detection limit and linearity

Standard curves were determined by plotting the peak-area ratio of the external calibrators and the internal standards against the calibrator concentration. The limit of detection was calculated from methanolic standard solutions using a signal-to-noise ratio of 3. The linear range was investigated up to a phytosterol concentration of 1,000 μg/l.

Variability and accuracy data

Variability was assessed using an in-house-made control from pooled serum. Within-run variability was determined by measuring controls 10 times in one run. Between-run variability was calculated by measuring the control levels on 10 consecutive working days.

Accuracy was determined using LPDS standards containing 0.5, 2, and 10 mg/l β-sitosterol, campesterol, brassicasterol, and stigmasterol. Carry-over effects were routinely controlled by injecting double blanks and blanks before and after each analytical series.

RESULTS

Mass spectrometry

Phytosterols formed [M+H-H_2O]$^+$ ions during the APPI process. After fragmentation, characteristic product ions were detected (multiple reaction monitoring). The respective transitions were as follows: m/z 397.4/257.3 for β-sitosterol, m/z 383.4/161.3 for campesterol, m/z 381.4/297.3 for brassicasterol, m/z 395.4/297.3 for stigmasterol, and m/z 376.4/161.3 for [$^2H_7$]cholesterol. For the corresponding sterol esters, the same mass transitions could be used because of ester bond cleavage during the photoion-
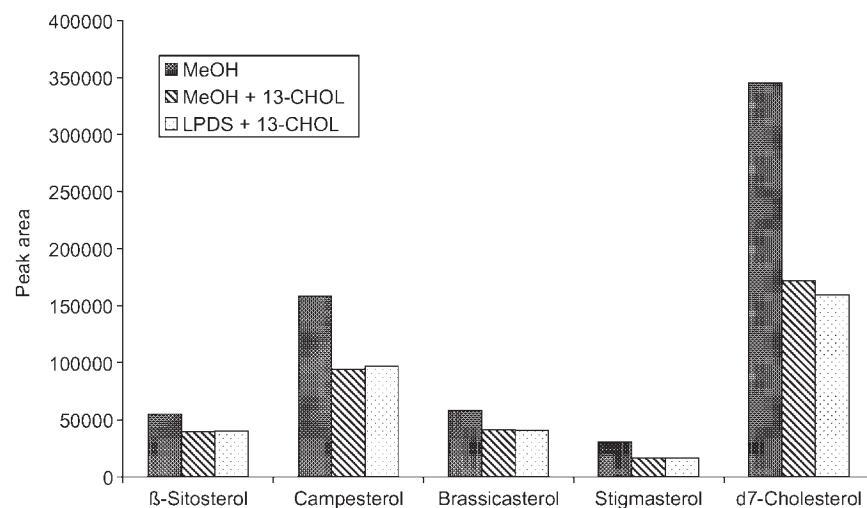
Fig. 1. Comparison of signal suppression effects by excess cholesterol ($[^{13}C_2]$cholesterol; c = 0.75 g/l) on the signals of phytosterols (c = 2 mg/l for each analyte) in methanol (MeOH) and lipoprotein-deficient serum (LPDS).

ization process, as observed for the ionization of cholesteryl stearate.

The influence of different solvents (methanol, isopropanol, acetonitrile, and water) and additives such as ammonium acetate and formic acid on the signal intensity was investigated. The highest signal intensities could be obtained using pure methanol or isopropanol. The use of ionization dopants such as anisole and acetone showed no better ionization characteristics than toluene.

To investigate the potential ion-suppressing effects of the physiological excess cholesterol in serum, signal intensities of a methanolic standard solution and LPDS containing β-sitosterol, campesterol, brassicasterol, stigmasterol, and $[^2H_7]$cholesterol (each 2 mg/l in methanol) in the presence of 0.75 g/l $[^{13}C_2]$cholesterol were compared with the standard without excess cholesterol. The ratios for the mean signal intensities with and without excess cholesterol were 0.72, 0.60, 0.71, 0.54, and 0.49 for β-sitosterol, campesterol, brassicasterol, stigmasterol, and $[^2H_7]$-cholesterol, respectively. Therefore, $[^{13}C_2]$cholesterol was added to all methanolic calibrators, standards, and LPDS, simulating serum sample conditions and adapting the ionization behavior shown in **Fig. 1**. The phytosterol/$[^2H_7]$-cholesterol ratios were not influenced by excess cholesterol in a range from 0.25 to 1 g/l.

LC-MS/MS

Characteristic chromatograms (total ion current) of our serum control without and after hydrolysis are presented in **Fig. 2A, B**. Because of the high sensitivity and specificity of the LC-MS/MS method, the coeluting phytosterols could be quantified without any additional chromatographic separation step within an analysis time of 6 min. Mass transitions for β-sitosterol, campesterol, brassicasterol, stigmasterol, and their corresponding esters are shown in Fig. 2C, E, G, and I, respectively. In contrast, the corresponding mass transitions of phytosterols after hydrolysis and LC-MS/MS are presented in Fig. 2D (β-sitosterol),

2F (campesterol), 2H (brassicasterol), and 2J (stigmasterol). In Fig. 2K, L, the internal standard ($[^2H_7]$-cholesterol) without and after hydrolysis is presented.

Recovery

To evaluate the LC-MS/MS method, a standard solution containing 20 mg of cholesterol and 33.8 mg of cholesteryl stearate (equivalent to 20 mg of cholesterol) was analyzed with and without hydrolysis. The calculated analytical recovery was 97.5% for free cholesterol, 97.7% for cholesterol originated from cholesteryl stearate, and 90.1% for total cholesterol after hydrolysis.

The extraction efficiency of our method was determined by comparing the signal intensities of phytosterol-spiked LPDS standard solutions and methanolic standards. The analytical recovery was 100.3% for β-sitosterol, 102.1% for campesterol, 98.9% for brassicasterol, 98.3% for stigmasterol, and 97.9% for $[^2H_7]$cholesterol.

Detection limits and linearity

The limits of detection were calculated using a signal-to-noise ratio of 3. Detection limits were 0.24, 0.28, 0.42, and 0.68 μg/l for β-sitosterol, campesterol, brassicasterol, and stigmasterol, respectively. The standard curves were linear throughout the calibration range for all phytosterols, with Pearson correlation coefficients of $r \geqslant 0.999$.

Variability and accuracy

The variability data of the LC-MS/MS method for free, esterified, and total sterols of our in-house serum control are presented in **Table 1**. Within-run coefficients of variation ranged between 2.4% to 9.1%, and between-run coefficients of variation ranged between 3.9% and 9.9%.

The accuracy data were calculated by measuring spiked LPDS controls (0.5, 2, and 10 mg/l of each phytosterol). The mean accuracy was 100.3% (96.0–107.0%) for β-sitosterol, 99.7% (95.0–107.0%) for campesterol, 97.8% (96.4–
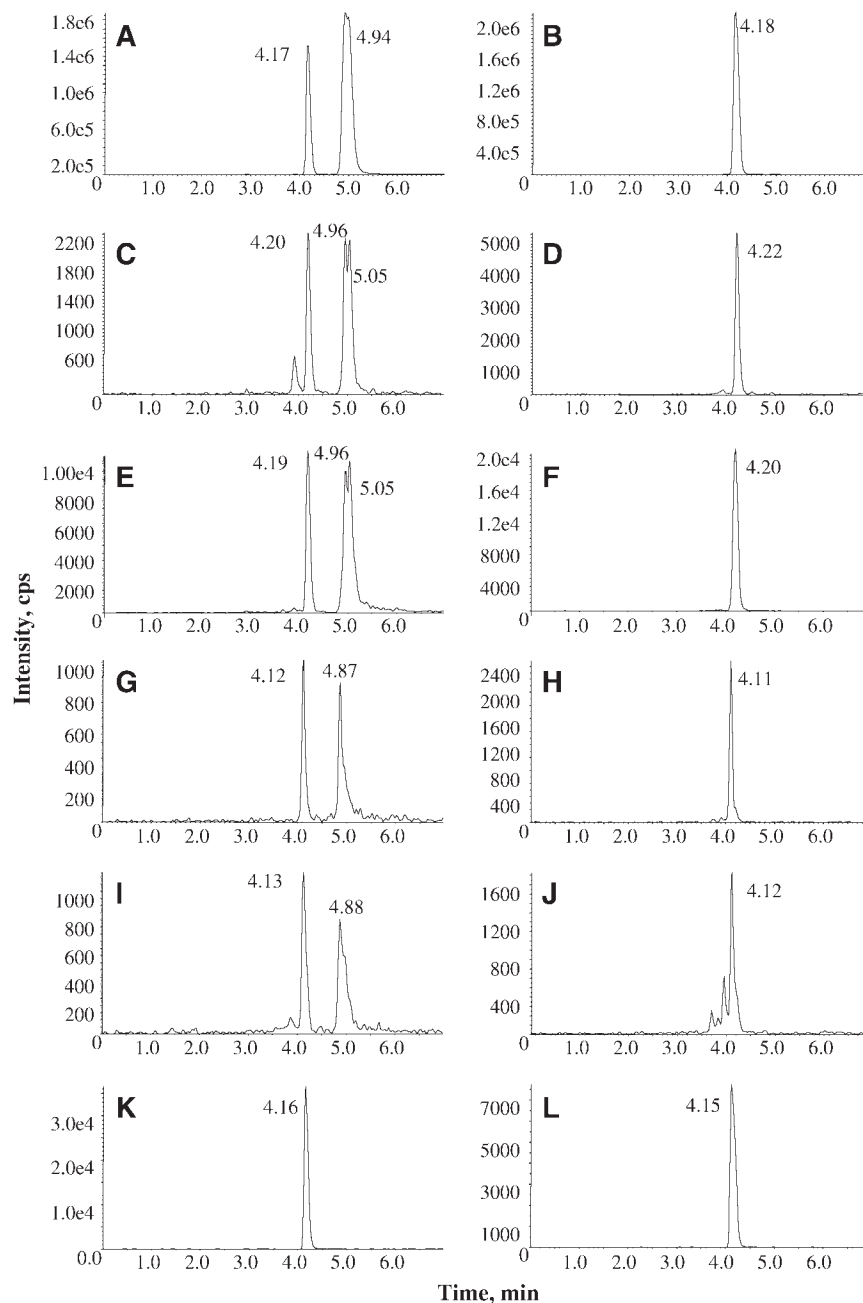
Fig. 2. Chromatographic run of the serum control without and after hydrolysis. A: Free sterols [retention time(t_R) = 4.17 min] and sterol esters (t_R = 4.94 min). B: Total sterols after hydrolysis (t_R = 4.18 min). C: Selected transitions of β-sitosterol (t_R = 4.20 min) and β-sitosterol esters (t_R = 4.96 and 5.05 min). D: Total β-sitosterol (t_R = 4.22 min). E: Selected transitions of campesterol (t_R = 4.19 min) and campesterol esters (t_R = 4.96 and 5.05 min). F: Total campesterol (t_R = 4.20 min). G: Selected transitions of brassicasterol (t_R = 4.12 min) and brassicasterol esters (t_R = 4.87 min). H: Total brassicasterol (t_R = 4.12 min). I: Selected transitions of free stigmasterol (t_R = 4.13 min) and stigmasterol ester (t_R = 4.87 min). J: Total stigmasterol (t_R = 4.12 min). K and L: Selected transitions of [$^2H_7$]cholesterol (t_R = 4.16 min) without hydrolysis (K) and after hydrolysis (L).

104.0) for brassicasterol, and 100.0% (96.4–108.0%) for stigmasterol.

Determination of normal levels of phytosterols

Phytosterol concentrations in serum samples from 49 young (<25 years of age) volunteers were measured for the determination of normal levels. Results are summa-

rized in **Table 2**. The means of the total concentrations were 2.43 for β-sitosterol, 4.53 for campesterol, 0.54 for brassicasterol, and 1.37 mg/l for stigmasterol. The percentages of the esterified phytosterol fractions were 65, 66, 58, and 71% for β-sitosterol, campesterol, brassicasterol, and stigmasterol, respectively. The mean of the total cholesterol concentration was 2.13 g/l, and the percentage of esterified cholesterol was 74%.

TABLE 1. Variation data of the phytosterol determination in the pooled serum control after sample preparation and atmospheric pressure photoionization-LC-MS/MS

Analyte	Within Run		Between Run	
	Mean	Coefficient of Variation	Mean	Coefficient of Variation
	mg/l	%	mg/l	%
β-Sitosterol				
Free	0.69	6.7	0.70	9.2
Esterified	1.43	9.1	1.44	9.9
Total	2.12	6.0	2.14	8.6
Campesterol				
Free	1.57	3.8	1.50	7.0
Esterified	3.53	2.9	3.39	5.8
Total	5.10	2.4	5.80	6.7
Brassicasterol				
Free	0.24	7.3	0.25	6.7
Esterified	0.41	5.5	0.38	5.1
Total	0.64	4.1	0.62	3.9
Stigmasterol				
Free	0.38	4.6	0.36	6.7
Esterified	1.08	5.7	1.02	7.8
Total	1.46	4.3	1.41	7.4

LC-MS/MS, liquid chromatography-tandem mass spectrometry.

DISCUSSION

We describe a new rapid APPI-LC-MS/MS method for the simultaneous quantification of free β-sitosterol, campesterol, brassicasterol, stigmasterol, and their corresponding esters in serum samples. Because of the high sensitivity of the MS/MS detection, the elaborating sample extraction procedure and hydrolysis for GC-MS can be omitted. The workup procedure for a single sample could be reduced from 3 h to 15 min. Detection of the different phytosterols was performed using specific multiple reaction monitoring transitions. Free and esterified phytosterols showed the same mass transitions. The fragmentation of sterols is very complicated because of their polycyclic structure. Thus, the identification of direct fragments is difficult because of the specific mechanisms of fragmentation (i.e.,

TABLE 2. Free and esterified phytosterol concentrations in serum samples from 49 volunteers determined with LC-MS/MS

Analyte	Mean	SD	Range
	mg/l		mg/l
β-Sitosterol			
Free	0.85	0.36	0.32–2.29
Esterified	1.57	0.62	0.68–3.78
Total	2.43	0.96	1.01–6.07
Campesterol			
Free	1.57	0.63	0.55–4.73
Esterified	2.96	0.96	1.41–7.54
Total	4.53	1.56	1.96–12.27
Brassicasterol			
Free	0.23	0.10	0.10–0.73
Esterified	0.31	0.11	0.14–0.76
Total	0.54	0.20	0.27–1.49
Stigmasterol			
Free	0.40	0.19	0.16–1.23
Esterified	0.98	0.21	0.47–1.48
Total	1.37	0.31	0.64–2.28

retro-Diels-Alder reaction, neutral molecule elimination) (17).

The chromatography was optimized to separate free sterols and sterol esters within 6 min. At present, we cannot exclude the possibility that oxidized phytosterols are not sufficiently separated. However, in a recent study by Grandgirard et al. (18), the concentration of oxidized phytosterols in the plasma of healthy human subjects ranged only between 4.8 and 57.2 μg/l. In patients with phytosterolemia, the percentage of oxidized β-sitosterol was ~1.4% (19). The method is limited for the specific measurements of phytosterol isomers because of the same chromatographic and mass spectrometric properties. However, there is no need for the detection of serum phytosterol isomers because there are no known natural phytosterol isomers.

The use of labeled phytosterols as internal standards is an important requirement of the LC-MS/MS analysis. Unfortunately, the commercially available labeled phytosterols are only a sterol mixture of low purity. Therefore, we started the development of the method using $[^2H_7]$-labeled cholesterol as an internal standard for quantification. Because of the similar structure and polarity, it was adequate as an internal standard, as we showed for the quantification of all phytosterols (Fig. 1). The phytosterol/$[^2H_7]$-cholesterol ratios were not influenced by excess cholesterol in a range from 0.25 to 1 g/l.

Our new LC-MS/MS setup for the detection of phytosterols showed a high sensitivity, which was at least 150-fold higher compared with the GC-MS platform (11). The method showed linearity over a wide range (from 1 to 1,000 μg/l). Therefore, low phytosterol and high cholesterol concentrations might be analyzed in serum samples simultaneously.

Validation data showed good within-day and between-day precision for both free and esterified sterols. Accuracy data for free phytosterols ranged between 97.8% and 100.3%. Accuracy experiments for phytosterol esters could not be performed because no commercial standards were available.

Serum phytosterol levels in 49 healthy volunteers, measured with our novel LC-MS/MS platform, were in the same range as those reported by other authors using GC-MS. Campesterol serum mean concentrations of 3.2 mg/l (20) and 5.2 mg/l (1.5–9.7 mg/l) (21) have been reported. For β-sitosterol, mean serum concentrations of 2.7 mg/l (20) and 3.6 mg/l (0.8–6.6 mg/l) (21) were found. In comparison, we found a mean total campesterol serum concentration of 4.53 mg/l (1.96–12.27 mg/l) and a mean total β-sitosterol concentration of 2.43 mg/l (1.01–6.07 mg/l). No data for brassicasterol and stigmasterol serum concentrations have been reported to the present.

In conclusion, our new analytical platform allows a rapid determination of phytosterols and their corresponding esters in small serum volumes without extensive sample pretreatment. Therefore, our new method is especially suited for large-scale clinical and animal studies to evaluate the role of phytosterols as an additional coronary heart disease risk factor.

REFERENCES

1. Weihrauch, J. L., and J. M. Gardner. 1978. Sterol content of foods of plant origin. *J. Am. Diet. Assoc.* **73:** 39–47.
2. Miettinen, T. A., R. S. Tilvis, and Y. A. Kesaniemi. 1990. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am. J. Epidemiol.* **131:** 20–31.
3. Boomsma, D. I., H. M. Princen, R. R. Frants, J. A. Gevers Leuven, and H. J. Kempen. 2003. Genetic analysis of indicators of cholesterol synthesis and absorption: lathosterol and phytosterols in Dutch twins and their parents. *Twin Res.* **6:** 307–314.
4. Lee, M. H., K. Lu, and S. B. Patel. 2001. Genetic basis of sitosterolemia. *Curr. Opin. Lipidol.* **12:** 141–149.
5. Berge, K. E., H. Tian, G. A. Graf, L. Yu, N. V. Grishin, J. Schultz, P. Kwiterovich, B. Shan, R. Barnes, and H. H. Hobbs. 2000. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science.* **290:** 1771–1775.
6. Heimer, S., T. Langmann, C. Moehle, R. Mauerer, M. Dean, F. U. Beil, K. von Bergmann, and G. Schmitz. 2002. Mutations in the human ATP-binding cassette transporters ABCG5 and ABCG8 in sitosterolemia. *Hum. Mutat.* **20:** 151.
7. Salen, G., K. von Bergmann, D. Lutjohann, P. Kwiterovich, J. Kane, S. B. Patel, T. Musliner, P. Stein, and B. Musser. 2004. Ezetimibe effectively reduces plasma plant sterols in patients with sitosterolemia. *Circulation.* **109:** 966–971.
8. Salen, G., I. Horak, M. Rothkopf, J. L. Cohen, J. Speck, G. S. Tint, V. Shore, B. Dayal, T. Chen, and S. Shefer. 1985. Lethal atherosclerosis associated with abnormal plasma and tissue sterol composition in sitosterolemia with xanthomatosis. *J. Lipid Res.* **26:** 1126–1133.
9. Katayama, S., T. Satoh, T. Yagi, N. Hirose, Y. Kurita, T. Anzai, Y. Asakura, T. Yoshikawa, H. Mitamura, and S. Ogawa. 2003. A 19-year-old man with myocardial infarction and sitosterolemia. *Intern. Med.* **42:** 591–594.
10. Sudhop, T., B. M. Gottwald, and K. Von Bergmann. 2002. Serum plant sterols as a potential risk factor for coronary heart disease. *Metabolism.* **51:** 1519–1521.
11. Phillips, K. M., D. M. Ruggio, and J. A. Bailey. 1999. Precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum by capillary gas-liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **732:** 17–29.
12. Kuksis, A. 2001. Plasma non-cholesterol sterols. *J. Chromatogr. A.* **935:** 203–236.
13. Burkard, I., K. M. Rentsch, and A. von Eckardstein. 2004. Determination of 24S- and 27-hydroxycholesterol in plasma by high-performance liquid chromatography-mass spectrometry. *J. Lipid Res.* **45:** 776–781.
14. Plat, J., H. Brzezinka, D. Lutjohann, R. P. Mensink, and K. von Bergmann. 2001. Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. *J. Lipid Res.* **42:** 2030–2038.
15. Robb, D. B., T. R. Covey, and A. P. Bruins. 2000. Atmospheric pressure photoionization: an ionization method for liquid chromatography-mass spectrometry. *Anal. Chem.* **72:** 3653–3659.
16. Teupser, D., J. Thiery, A. K. Walli, and D. Seidel. 1996. Determination of LDL- and scavenger-receptor activity in adherent and nonadherent cultured cells with a new single-step fluorometric assay. *Biochim. Biophys. Acta.* **1303:** 193–198.
17. Pelillo, M., G. Galletti, and G. Lercker. 2000. Mass spectral fragmentations of cholesterol acetate oxidation products. *Rapid Commun. Mass Spectrom.* **14:** 1275–1279.
18. Grandgirard, A., L. Martine, L. Demaison, C. Cordelet, C. Joffre, O. Berdeaux, and E. Semon. 2004. Oxyphytosterols are present in plasma of healthy human subjects. *Br. J. Nutr.* **91:** 101–106.
19. Plat, J., H. Brzezinka, D. Lutjohann, R. P. Mensink, and K. von Bergmann. 2001. Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. *J. Lipid Res.* **42:** 2030–2038.
20. Teunissen, C. E., J. De Vente, K. von Bergmann, H. Bosma, M. P. van Boxtel, C. De Bruijn, J. Jolles, H. W. Steinbusch, and D. Lutjohann. 2003. Serum cholesterol, precursors and metabolites and cognitive performance in an aging population. *Neurobiol. Aging.* **24:** 147–155.
21. Stalenhoef, A. F., M. Hectors, and P. N. Demacker. 2001. Effect of plant sterol-enriched margarine on plasma lipids and sterols in subjects heterozygous for phytosterolaemia. *J. Intern. Med.* **249:** 163–166.