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Analysis of Plant Sterol and Stanol Esters in Cholesterol-Lowering Spreads and Beverages Using High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization–Mass Spectroscopy

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Plant sterol and stanol esters were separated on a Luna hexyl-phenyl column using a gradient of acetonitrile (90−100%) in water. The eluted compounds were detected by atmospheric pressure chemical ionization (APCI)−mass spectroscopy (MS) in the positive mode. Sterol and stanol esters produced $[M + H - HOOCR]^+$ ions. Application of the hyphenated technique—LC-MS—allowed differentiation between a number of esters of sitosterol, campesterol, stigmasterol, and (tentatively) avenasterol, as well as sitostanol and campestanol esters. With cholesteryl decanoate used as the internal standard, the method showed good linearity, precision, and reproducibility. The method required minimal sample pretreatment and can be applied to samples with high water content (juices) as well as samples with high oil content (margarine spreads). The method could be useful for the analysis of sterol and stanol esters in fortified food products.

KEYWORDS: Sterol esters; stanol esters; cholesterol-lowering food; orange juice; Benecol; Take Control; liquid chromatography−atmospheric pressure chemical ionization−mass spectroscopy

INTRODUCTION

An elevated blood cholesterol level is a well-known risk factor for coronary heart disease, so this area is receiving a lot of attention from health and medical authorities. Cholesterol levels are typically managed by dietary changes (e.g., diets low in saturated fats) and/or by use of the statin drugs. A number of recent studies have shown the beneficial effect of a diet enriched in natural plant sterol esters (SE) or stanol esters on blood cholesterol levels in humans, mainly through the inhibition of intestinal cholesterol absorption (*1*−*5*). Recently, the U.S. Food and Drug Administration (FDA) allowed the marketing of margarine products that contain sterol esters (Take Control) and stanol esters (Benecol) due to the positive clinical and safety information available on these products. Our interest was focused on the development of a line of beverages fortified with the plant sterols.

As a part of this work, an analytical method applicable for the analysis of the SE was required.

The typical analytical methods used to determine the SE content of products are based on the application of gas chromatography (GC) or high-performance liquid chromatography (HPLC). For GC analysis, the material is typically subjected to alkaline hydrolysis, followed by isolation of the sterol fraction by solvent extraction, solid-phase extraction, or preparative thin layer chromatography (TLC) (*6*−*12*). The isolated sterols can be analyzed in free or silylated forms. These methods are time and labor intensive and do not provide information regarding the fatty acid composition of the SE. Although GC methods for the analysis of intact SE have been described (*13*, *14*), they can suffer from thermal instability of the SE during analysis (*13*).

Another approach for the analysis of SE is based on the application of reversed-phase HPLC. There are a number of published articles describing HPLC of CE (*14*, *16*), and SE (*15*, *17*, *18*). All of them involve chromatography on HPLC columns packed with C18 modified silica gels. Elution of the highly hydrophobic SE requires application of nonaqueous mobile phases, usually consisting of acetonitrile and stronger organic eluents such as propionitrile or 2-propanol (*15*, *17*), although a three-component nonaqueous mobile phase is also reported (*18*).

The eluted compounds can be detected using an ultraviolet (UV) (*16*) or evaporative light scattering detector (*18*), if the samples are first subjected to preliminary fractionation to remove triglycerides.

Coupling HPLC to a mass spectroscopy (MS) detector provides a tool for the identification of incompletely separated SE, as well as solves the problems associated with coelution of SE and the neutral lipids. Early papers (*13*, *15*) describe HPLC-MS of a total sterol esters fraction, isolated from plasma lipoproteins by preparative TLC. Application of this approach to the plasma of phytosterolemia patients led to the detection of several campesterol, sitosterol, and avenasterol esters (*13*).

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The authors also noted the necessity of background subtraction to compensate for solvent impurities associated with propionitrile.

To the best of our knowledge, there have been no publications describing an HPLC-MS method suitable for the analysis of intact plant SE in food products. To develop a simple and reliable method, we have investigated the chromatography of SE on an HPLC column packed with hexyl-phenyl-modified silica gel. Unlike previously described C18 sorbents, hexyl-phenyl medium is less hydrophobic, which could therefore allow elution of SE by commonly used water−organic mixtures. Moreover, the hexyl-phenyl phase, besides providing pure hydrophobic interactions, is capable of $\pi-\pi$ interactions with the double bonds present in the sterol esters, thereby implying higher selectivity in comparison to C18 phases. Here we describe the combination of the suggested HPLC method with atmospheric pressure chemical ionization−mass spectroscopy (APCI-MS) in positive mode and its application for the analysis of plant sterol and stanol esters in fortified spreads and beverages.

MATERIALS AND METHODS

Chemicals. All organic solvents were of HPLC grade and were purchased from G. J. Chemical (Newark, NJ). HPLC grade water was purchased from Burdick and Jackson (Muskegon, MI). Cholesteryl decanoate was purchased from Sigma (St. Louis, MO). Cholesteryl stearate, cholesteryl palmitate, cholesteryl linoleate, cholesteryl oleate, and a mixture of plant sterols were purchased from Matreya, Inc. (State College, PA).

Materials. A mixture of plant sterol esters (SE) were obtained from Cargill Co. (Minneapolis, MN) and were kept at 4 °C. The pulped orange juice samples (SunPure, Lakeland, FL) were fortified with an emulsion of plant sterol esters (prepared in house) to achieve a concentration of 0.23−0.25% (w/w).

Cholesterol-lowering margarine spread 1 (containing sterol esters) and margarine spread 2 (containing stanol esters) were purchased in a local supermarket.

Instrumentation. HPLC-APCI-MS was performed on an HP 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a quadrupole mass selective detector (MSD). The sterol esters were separated on a Luna hexyl-phenyl HPLC column, 100 mm × 2.0 mm i.d., 3 μm particle size (Phenomenex, Torrance, CA). The following chromatographic conditions were used: flow rate, 0.6 mL/min; linear gradient of acetonitrile in water from 90 to 100% in 15 min; postrun equilibration time, 5 min. The temperature of the column compartment was 35 °C. The sterol esters were detected by APCI-MS in positive mode. Optimized settings of the MSD were as follows: fragmentator voltage, 80 V; vaporizer temperature, 400 °C; drying gas temperature, 300 °C; nebulizer pressure, 40 psi; corona current, 8 μA; capillary voltage, 3000 V. Unless indicated otherwise, the MSD was set to operate in scan mode over the m/z range of 350−410.

GC of the saponified sterol fraction was performed on a DB-5 30 m × 0.25 mm (df = 0.25 μm i.d.) fused silica capillary column (J&W Scientific, Folsom, CA) housed inside an Agilent 6890 gas chromatograph. Samples were injected in split mode [split ratio (SR) 50] at 300 °C. Sterols were separated in isothermal mode (300 °C) in 10 min. Identification of the eluted peaks was achieved by interfacing the gas chromatograph to an Agilent MSD 5973. The mass spectrometer was operated at 280 °C in scan mode between m/z 40 and 500, and the energy of electron impact was 70 eV. For peak quantitation, the capillary column was connected to a flame ionization detector (FID), operated at 300 °C. FID percentage peak area determined the fraction of individual sterol in the total sterol mixture.

Calibration Curve for the Determination of SE by HPLC-APCI-MS. The mixture of SE was dissolved in ethyl acetate containing 0.05 mg/mL of cholesteryl decanoate as an internal standard (IS). The SE concentrations in the calibration solutions were 0.05, 0.1, 0.5, and 1 mg/mL.

The four-point calibration curve was generated in the range 250 ng−5 μg of total SE per injection (5 μL injection volume). Each calibration point was done in triplicate. The ratio of integrated total ion current of the peaks of SE to the area of the peak of the internal standard was used in construction of the calibration curve. Linear regression analysis was performed in coordinates ratio of areas (SE/IS)−ratio of concentrations (SE/IS). To establish precision and accuracy, working solutions of SE were analyzed independently from generation of the calibration curve, and back-calculation of the concentrations of the standards was performed.

Orange Juice Sample Preparation. The samples of orange juice were accurately weighed in glass vials (∼5 g), followed by the addition of ethyl acetate (20.0 mL) containing 0.05 mg/mL of cholesterol decanoate as IS. The samples were vigorously agitated and sonicated for 20 min and then left for 30 min to allow phase separation. Portions of the upper phase (∼3 mL) were taken out and centrifuged at 1000g for 5 min. Clear supernatants were filtrated (0.45 μm PTFE filters) and analyzed by HPLC-APCI-MS. The injection volume was 5 μL.

The blank orange juice samples were analyzed in a similar way, using ethyl acetate for extraction.

Margarine Spread Preparation. Samples of spreads in triplicate (200 mg) were accurately weighed in a volumetric flask (25 mL), and the volume was adjusted by ethyl acetate containing 0.05 mg/mL of cholesteryl decanoate as IS. Samples were sonicated (5 min), and hydrophilic material was filtered (0.45 μm). The prepared samples were analyzed by HPLC-APCI-MS.

RESULTS AND DISCUSSION

Mass Selective Detector Setting Optimization. There are very few studies describing the application of electrospray ionization (ESI) for the analysis of sterol esters. Direct injection of cholesterol esters in a chloroform/methanol mixture containing ammonium acetate (10 mM) led to formation adducts with ammonium ions (*9*). There were no data regarding the possibility of performing chromatographic separation of SE using this type of eluent. Recently the formation of ions of protonated cholesteryl acetate was observed in an HPLC-ESI-MS experiment (*20*) if the mobile phase (acetonitrile/water = 80:20) contained a low concentration of formic acid (0.02%). As mentioned before, the elution of long-chain esters of sterols requires application of either nonaqueous mobile phases or, as we report here, mobile phases with low water content (<10%). In general, a very high organic content is not favorable for ESI (for a review see ref *21*). It has to be noted that the response factors for the cholesterol esters in the described ESI conditions depend on the structure of the fatty acid moieties. In the case of the plant SE, the variability both in the fatty acids and in sterol moieties requires the application of numerous commercially unavailable standards for quantification purposes.

Similar to the previously reported data (*13*, *14*) we found that in APCI conditions SE produced ions ascribed as $[M + H - HOOCR]^+$. We also found that individual cholesterol esters in HPLC-APCI-MS have the following relative response factors: 1.00 (16:0 ester), 1.03 (18:0 ester), and 1.05 (18:1 and 18:2). The relative independence of the response factors of SE from the structures of the fatty acid moieties simplifies quantification. The aim of our work was to develop a simple method for the quality control of plant SE (with known fatty acid composition) in fortified food products, and thus the absence of molecular ions of SE in APCI conditions was deemed not to be crucial.

The MSD settings for the detection of SE were optimized using flow injection analysis. The SE solution (5 mg/mL) was injected (2 μL) in the mobile phase (5% of water in CH_3CN) pumped at a flow rate of 0.6 mL/min. The MSD was set to scan in the m/z range from 200 to 900. The effect of the

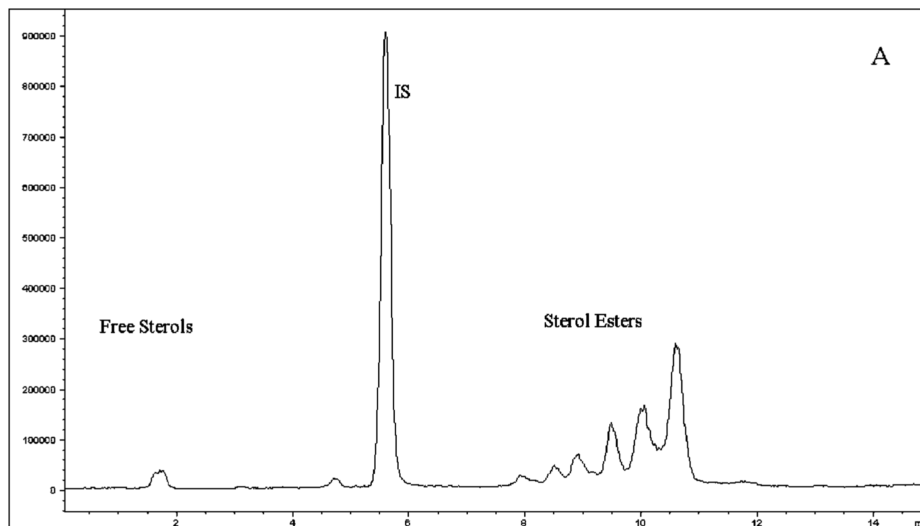

Figure 1. Chromatogram of sterol esters with internal standard (IS). Conditions: column, Luna hexyl-phenyl, 100 mm × 2.0 mm i.d., 3 μm; flow rate, 0.6 mL/min; linear gradient of acetonitrile in water from 90 to 100% in 15 min.

following parameters was investigated: corona current, fragmentator voltage, capillary voltage, nebulizer pressure, and vaporizer temperature. Optimized values are referred to under Materials and Methods.

High-Performance Liquid Chromatography of Sterol Esters. It was found that the separation of SE on a hexyl-phenyl phase could be achieved using isocratic elution by 90% of acetonitrile in water; however, there was a noticeable broadening of the late-eluted peaks. Therefore, gradient elution was used. A representative chromatogram of the mixture of SE (500 ng per injection) with internal standard (250 ng) is shown in **Figure 1**. The position of the free sterols on the chromatogram was established by injection of a plant sterol mixture and confirmed by characteristic ions corresponding to sterol nuclei. Starting the gradient elution from a mobile phase with lower acetonitrile content allowed separation of free major sterols (data not shown) but greatly increased the total run time and therefore was not pursued further in this work. The SE mixture was injected as a solution in ethyl acetate, a stronger than mobile phase solvent. The narrow-bore column used (2.0 mm) allowed injection of up to 10 μL of the ethyl acetate solution without distortion of the SE peaks, indicating satisfactory robustness of the chromatographic separation.

The elution profile of SE reflects differences both in the sterol moieties and in the fatty acid composition. To establish the dependence of the elution profile of SE from the structure of the fatty acid moiety, we investigated the separation of the corresponding fatty acid esters of cholesterol. The obtained chromatogram is shown in **Figure 2**. The elution order of CE on the phenyl-hexyl phase is similar to the order obtained in nonaqueous reversed phase chromatography on C18 phases (for a review see ref *7*).

The pair cholesteryl palmitate (16:0) and cholesteryl oleate (18:1) can be partly resolved in isocratic mode (90% acetonitrile). The fatty acid part of the SE used to fortify orange juice was derived from canola oil. Considering the low content of 16:0 acid in canola oil (~4%) (*19*), achieving the partial resolution of this pair at the cost of greatly increased run time was deemed to be unwarranted. Assignment of the molecular species of SE was done on the basis of the elution profile of cholesterol esters and the known composition of fatty acids derived from canola oil.

Ion extraction corresponding to m/z windows of $[M + H - HOOCR]^+$ ions of total ion current (TIC) of the SE chromato-

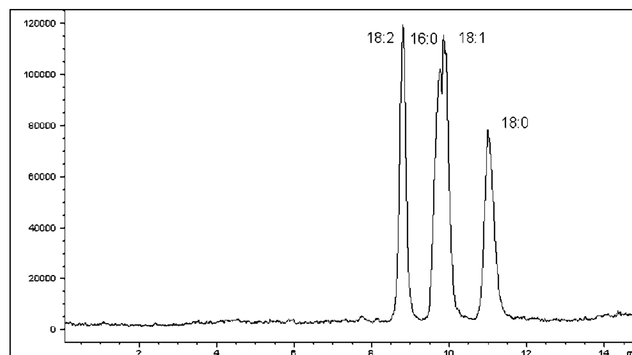

Figure 2. Chromatogram of mixture of CE of 18:2, 16:0, 18:1, and 18:0 fatty acids.

gram revealed the presence of four major peaks of esters of sitosterol (m/z 397−398; **Figure 3A**) and campesterol (m/z 383−384; **Figure 3B**). Ion extraction in the m/z 395−396 window revealed a more complex picture (**Figure 3C**), presumably due to the separation of stigmasterol and avenasterol esters. Due to the lack of the commercially available avenasterol esters, work on further identification was not performed.

To calculate the SE composition, the response factor for the sterols was established. First, the percentage of corresponding ion current in TIC was calculated using the equation

$$S_i = I_i/I_{TIC} \times 100$$

where S_i −is the percent of the ion current of the sterol, I_i is the integrated area of the extracted ion current in the m/z window corresponding to $[M + H - HOOCR]^+$, and I_{TIC} is the integrated area of total ion current (m/z range 350−410).

The response factor was established by comparing the calculated S_i values with data obtained by GC-FID (**Table 1**).

Table 2 represents the calculated composition of molecular species of SE in the analyzed preparation. The following equation was used

$$SE_i = I_i/I_{TIC} \times K_i \times 100$$

where K_i is a response factor.

The calculated fatty acid composition of SE was very close to literature data (*19*) for canola oil, confirming that at the

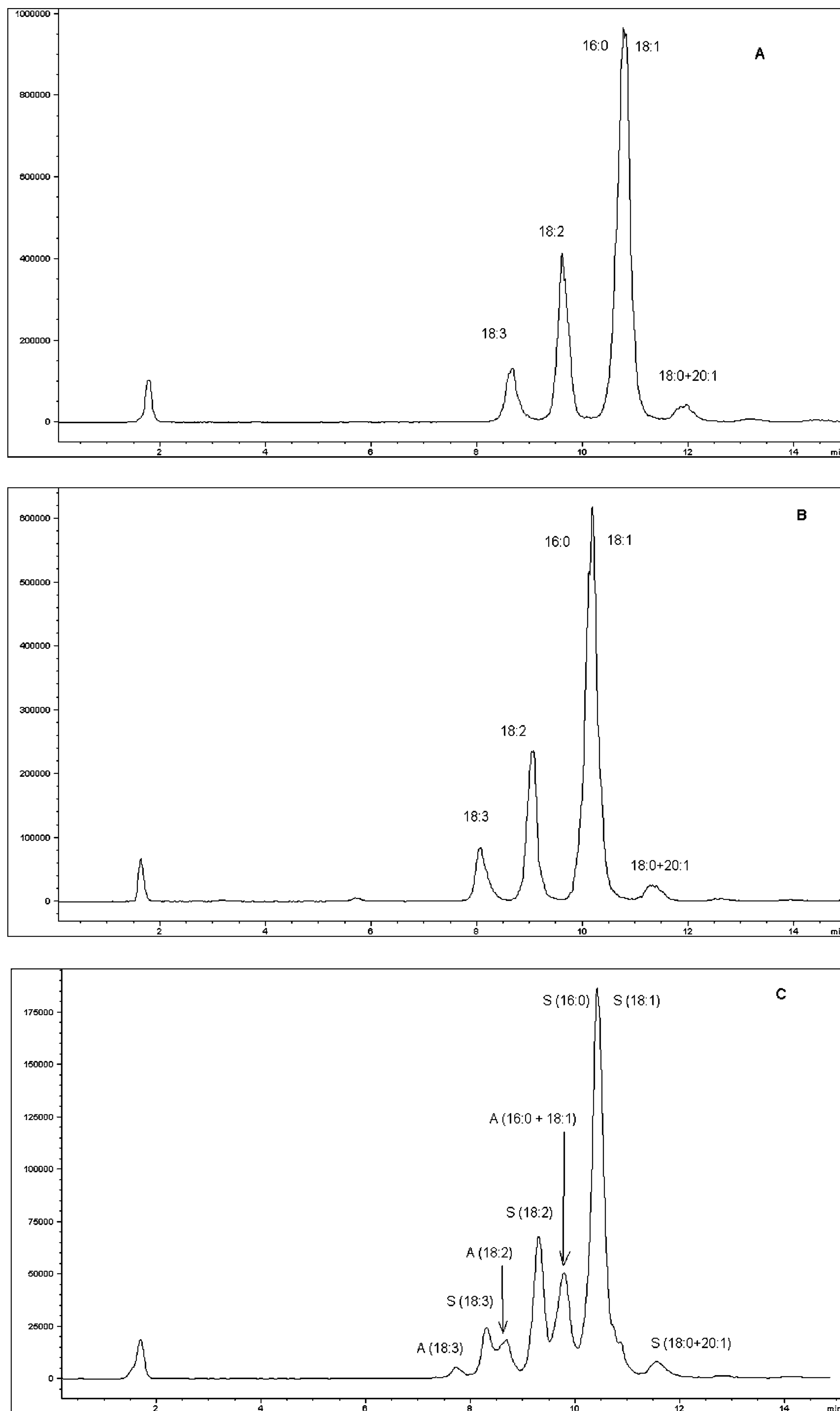

Figure 3. Ion extraction from total ion current chromatogram (see **Figure 1**) of mixture of SE and IS: (A) in *m*/*z* window 397–398 (sitosterol); (B) in *m*/*z* window 383–384 (campesterol); (C) in *m*/*z* window 395–396, where "S" and "A" correspond to esters of stigmasterol and tentatively avenasterol.

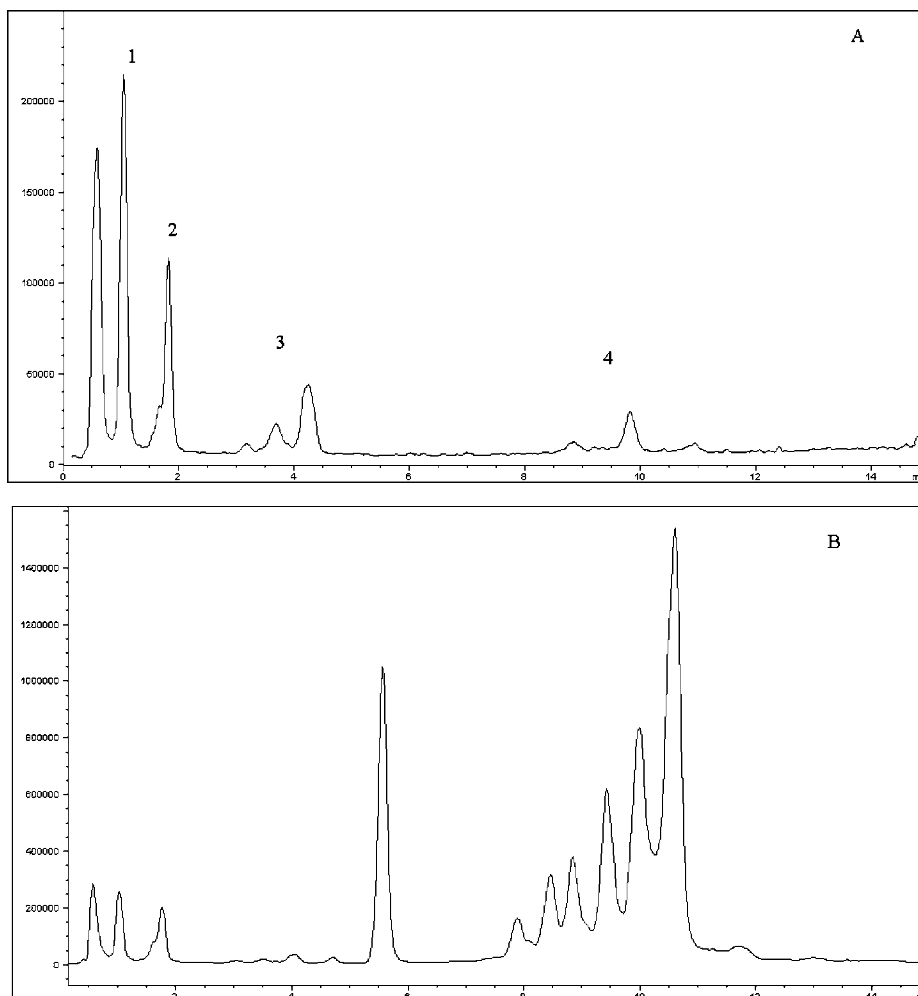

Figure 4. (A) TIC chromatogram of extract of blank orange juice: 1, 3, unknown; 2, free sterols; 4, SE. (B) Total ion current chromatogram of extract of fortified orange juice.

Table 1. Response Factors of Sterols

	ion current (%) in TIC (S_i)	sterol content (SC_i,%) by GC-FID	response factor[a] K_i
sitosterol	38.0	50.0	1.32
avenasterol[b]	1.7	ND[c]	3.05[d]
stigmasterol	7.4	22.6	3.05
campesterol	22.0	27.4	1.25

[a] Response factor was calculated as $K_i = SC_i/S_i$. [b] Tentative assignment. [c] Not determined. [d] Assumed to be equal to stigmasterol.

Table 2. Fatty Acids and Sterol Composition of the Sterol Ester Mixture (Cargill) and Fatty Acid Composition of Canola Oil (*19*)

sterol base	free	18:3	18:2	16:0 + 18:1	18:0 + 20:1	total of sterol content, %
sitosterol	1.86	3.56	10.06	33.11	1.41	50.00
avenasterol	0.00	0.22	1.32	3.64	0.00	5.18
stigmasterol	0.94	1.36	4.50	15.29	0.51	22.60
campesterol	0.80	1.98	6.07	17.92	0.63	27.40
total from above	3.60	7.13	21.96	69.95	2.55	
canola oil (%)		8.6	20	67 (4 + 63)	3.7 (1.8 + 1.9)	

described APCI conditions response factors of SE do not depend on the structure of fatty acid moieties.

Linearity, Precision, and Accuracy of the Method. The linear calibration was performed in the range 250−5000 ng of total SE mixture per injection. The limit of detection (S/N > 3) was found to be 25 ng of total SE mixture, and the limit of quantification (S/N > 10) was 100 ng of SE per injection. The following parameters were obtained: intercept, −0.2492; slope, 0.6287; and correlation coefficient, 0.9985, which indicates good linearity of the method. The method also has good precision—day to day reproducibility (RSD) was 6.6% (n = 16).

HPLC-APCI-MS Analysis of Sterol Esters Content in Fortified Orange Juice Samples. The developed method was applied to the determination of the SE content in orange juice samples fortified with an emulsion of sterol esters (see Materials and Methods). HPLC-APCI-MS analysis of the blank orange juice (**Figure 4A**) revealed that it contained free sterols and sterol esters, as well as some other unidentified compounds producing mass spectra with ion patterns similar to those of sterol nuclei. The complete separation and identification of all orange juice sterol species was beyond the scope of this work. The combined content of free sterols and sterol ester was estimated to be <2% compared to added SE, and thus for the simplicity of experimental design their contribution was neglected. It was found that extraction with ethyl acetate is highly efficient, with recovery ranging from 97 to 103% and an average recovery of 99% (n = 6, SD = 2%). Representative chromatograms of fortified samples are shown in **Figure 4B**. As can be seen, there was no interference of matrix components with the IS peak.

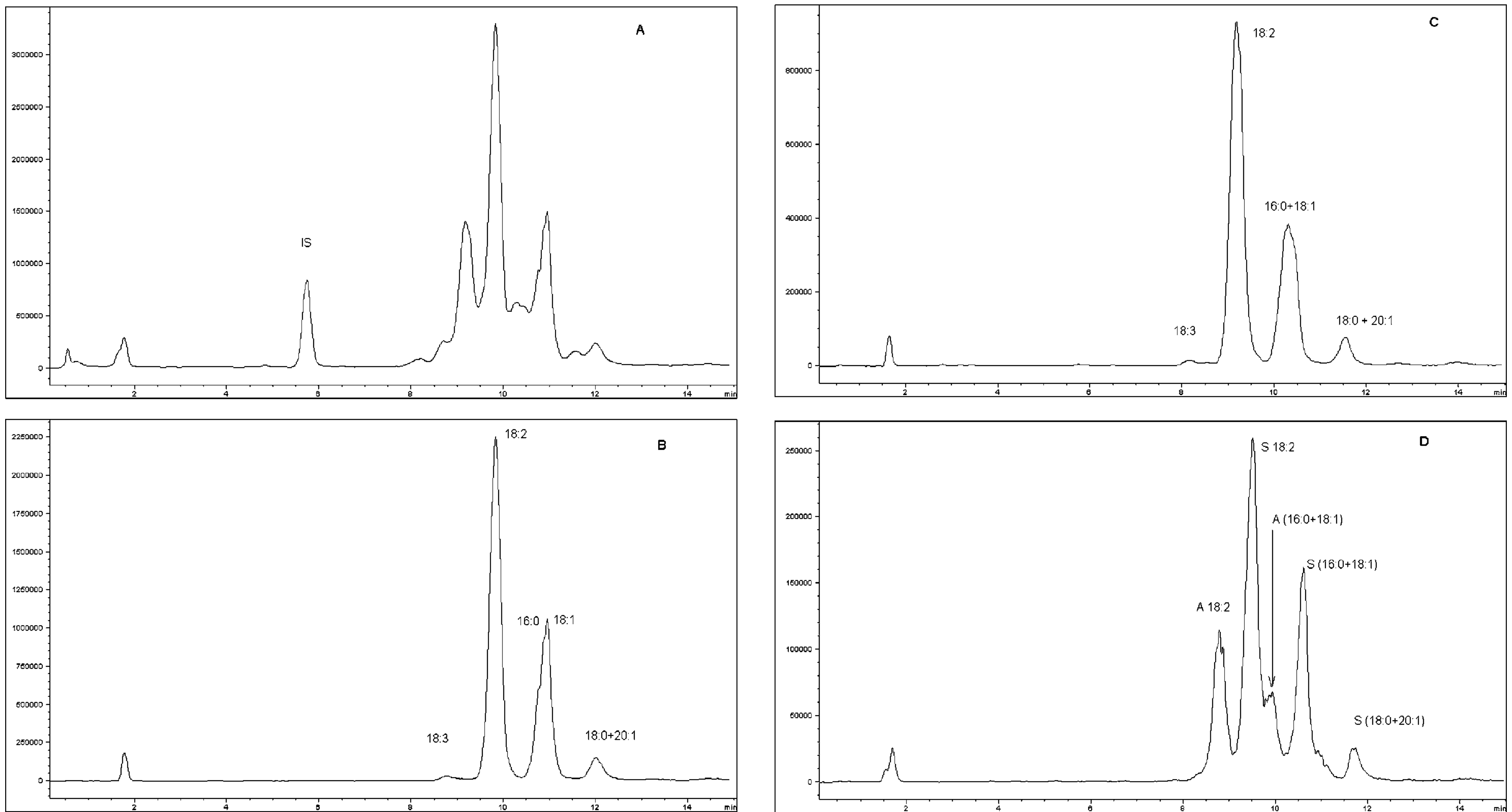

Figure 5. (A) TIC chromatogram of spread 1 with IS; (B, C, D) ion extractions corresponding to sitosterol, campesterol, and stigmasterol/avenosterol esters, respectively.

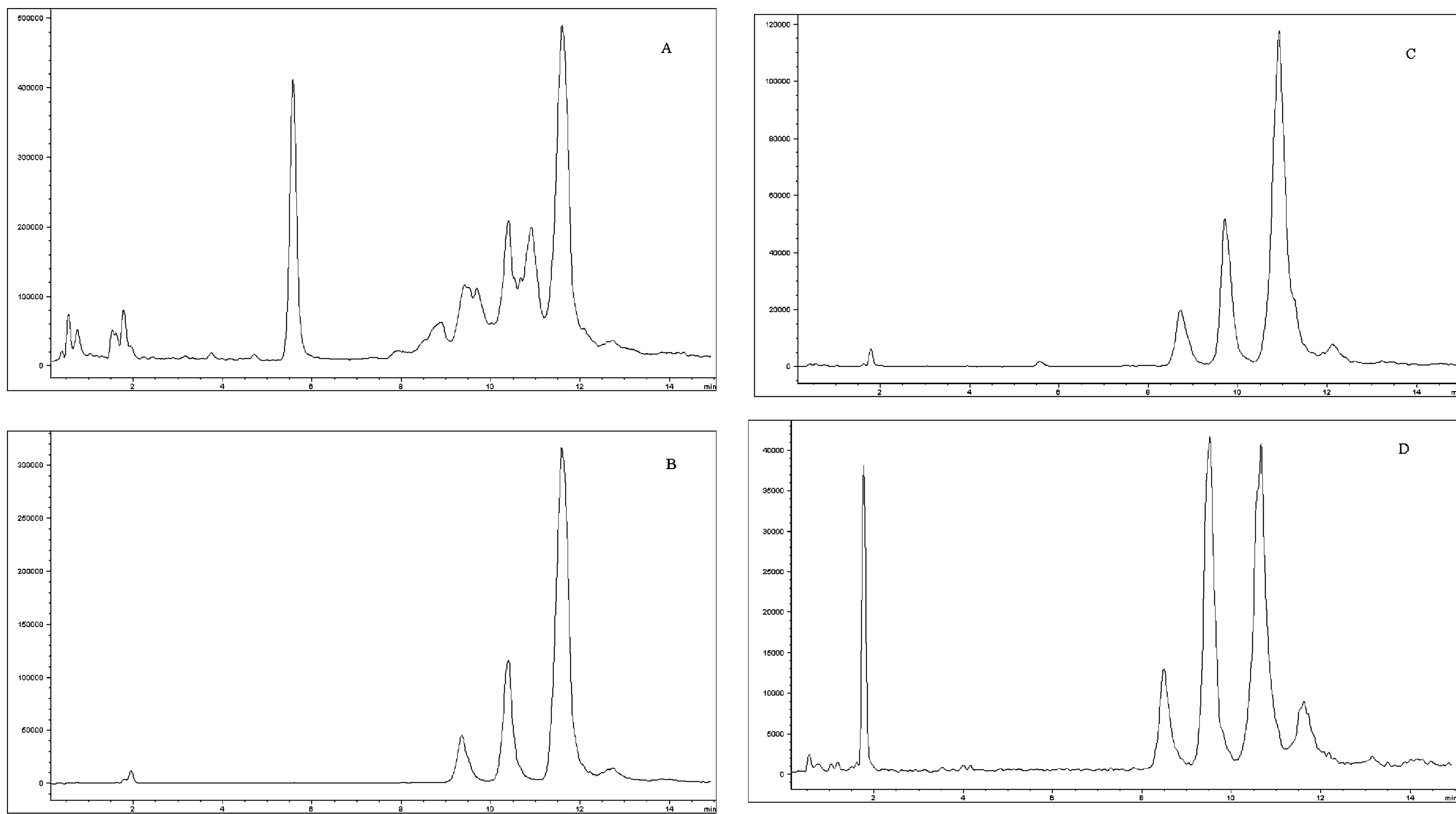

Figure 6. (A) TIC chromatogram of spread 2 sample with IS; (B, C, D) ion extractions corresponding sitostanol, campestanol, and sitosterol nucleus, respectively.

APCI-MS Analysis of Sterol Esters Content in Cholesterol-Lowering Margarine Spreads. We also investigated the applicability of the method to the analysis of SE content in commercial margarine spreads 1 and 2. The spreads were almost completely soluble in ethyl acetate, and after a small amount of hydrophilic constituents had been filtered out, the obtained samples were analyzed without any further workup. The major components of the spreads are triglycerides from vegetable oils. The initial scan of the eluate in the *m*/*z* range 350−900 revealed that there was overlapping of peaks of SE with, tentatively, peaks of triglycerides (data are not shown). Setting the mass spectrometry detector to scan in the *m*/*z* range 350−410 allowed specific detection of SE peaks. The representative TIC chromatograms, obtained in scan mode over the *m*/*z* range 350−410, as well as the ion extraction chromatograms, are shown in **Figures 5** (spread 1) and **6** (spread 2). The content of sterol esters in spread 1 was found to be 12.7% (±0.6%), which is close to the manufacturer's data (12%). The following sterol composition was found: sitosterol, 47.2% (±2.8); stigmasterol + avenasterol, 23.8% (±1.2); and campesterol, 25.5% (±1.9).

In the case of spread 2 we found ions [M + H − HOOCR] corresponding to two types of stanol esters: sitostanol (*m*/*z* 399, 400) and campestanol (*m*/*z* 385, 386), as well as sitosterol esters. It should be noted that sitosterol esters are different in fatty acid composition from stanol esters.

Conclusion. The described method consisits of sample preparation and HPLC separation of the sterol esters coupled with an APCI-MS detector. Depending on the type of matrix, sample preparation requires only simple extraction of the total neutral lipids or, in case of spreads, the dissolved samples after filtering can be directly analyzed by HPLC-MS. Ethyle acetate was a very efficient solvent for the extraction of SE from fortified orange juice samples, as indicated by high recovery values. HPLC of sterol esters was performed on a hexyl-phenyl bound phase, which, unlike C18 sorbents, can separate SE using a gradient of acetonitrile in water. The peaks of sterol esters were detected by APCI-MS in the positive mode. Using cholesteryl decanoate as an internal standard, the method showed good linearity, precision, and accuracy. The method can be used for the analysis of sterol and stanol esters content in fortified food products with high water or high oil content.

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