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Identification of Acyl Chain Oxidation Products upon Thermal Treatment of a Mixture of Phytosteryl/-stanyl Linoleates

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ABSTRACT: A mixture of phytosterols/-stanols, consisting of 75% β-sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol, and 1% others, was esterified with linoleic acid. The resulting mixture of phytosteryl/-stanyl linoleates was subjected to thermal oxidation at 180 °C for 40 min. A silica solid-phase extraction was applied to separate a fraction containing the nonoxidized linoleates and nonpolar degradation products (heptanoates, octanoates) from polar oxidation products (oxo- and hydroxyalkanoates). In total, 15 sitosteryl, sitostanyl, and campesteryl esters, resulting from oxidation of the acyl chain, could be identified by GC-FID/MS. Synthetic routes were described for authentic reference compounds of phytosteryl/-stanyl 7-hydroxyheptanoates, 8-hydroxyoctanoates, 7-oxoheptanoates, 8-oxooctanoates, and 9-oxononanoates, which were characterized by GC-MS and two-dimensional NMR spectroscopy. The study provides data on the formation and identities of previously unreported classes of acyl chain oxidation products upon thermal treatment of phytosteryl/-stanyl fatty acid esters.

KEYWORDS: *phytosteryl ester oxidation, solid-phase extraction, NMR spectroscopy, core aldehydes, fatty acid cleavage*

INTRODUCTION

Since the authorization of a yellow fat spread enriched with phytosteryl fatty acid esters as a novel food in the European Union in 2000,[1] these functional ingredients have been added to a broad spectrum of foods due to their cholesterol-lowering properties.[2,3] Fat-based foods are enriched with fatty acid esters of phytosterols/-stanols due to their enhanced solubility in lipid media.[4] The esters are obtained by esterification of phytosterols/-stanols with saturated as well as unsaturated fatty acids of plant oil origin.[5] Similarly to the structurally related cholesterol and its fatty acid esters, phytosterols as well as their fatty acid esters are susceptible to oxidation in the steroid ring due to their unsaturation between C-5 and C-6. Oxidation reactions of phytosterols are catalyzed by light, transition metals, water, heat, and oxygen.[6] Previous studies almost exclusively focused on the analysis of secondary oxidation products, mainly 7-keto, 7-hydroxy, and 5,6-epoxy derivatives of the phytosterol moieties, which are commonly referred to as "phytosterol oxidation products" (POPs).[6,7] The occurrence of POPs in enriched foods, estimations of their daily intake, and their biological effects have recently been reviewed.[8] Comprehensive data sets on the formation of POPs in foods during heating conditions representing typical ways of preparing foods in the home are available.[9] However, the commonly used fatty acid ester mixtures of phytosterols/-stanols are prone to oxidation not only in the steroid moieties but also in the fatty acid moieties. In a recent study the decreases of a spectrum of individual phytosteryl/-stanyl esters upon heating of phytosteryl/-stanyl fatty acid ester-enriched margarines and the formation of POPs were simultaneously followed.[10] In the phytosteryl ester-enriched margarine only approximately 20% of the ester losses could be explained by the formation of POPs; in the phytostanyl ester-enriched margarine the POPs accounted for even <1% of the decreased esters. This indicated that oxidative modifications in the fatty acid moieties may account for part of the degraded esters, particularly for those of unsaturated fatty acids.

The oxidation behavior of cholesteryl esters has been thoroughly investigated.[11,12] In vitro studies on the oxidation of low- and high-density lipoprotein and isolated cholesteryl esters with copper or *tert*-butyl hydroperoxide resulted in cholesteryl ester hydroperoxides as well as so-called cholesteryl ester core aldehydes that consist of sterols and oxysterols esterified to scission products of oxidized fatty acids with terminal carbonyl groups.[13−15] The most abundant cholesteryl ester core aldehydes resulting from cholesteryl linoleate oxidation were cholesteryl 9-oxononanoate and cholesteryl 8-oxooctanoate. These types of oxidized fatty acid moieties, typically analyzed as fatty acid methyl esters after transesterification, are also well-known products formed upon thermo-oxidation and frying of fats, oils, and unsaturated fatty acid methyl esters.[16−18]

On the other hand, knowledge of the compounds resulting from oxidative modifications in the fatty acid parts of phytosteryl/-stanyl esters is very scarce. There are investigations on the formation and decomposition of phytosteryl ester hydroperoxides.[19] Another study following the formation of sitosteryl oleate oxidation products upon heating of phytosteryl ester-enriched margarine reported only the presence of sitosteryl 9,10-dihydroxystearate.[20] The fact that no other acyl chain oxidation products of phytosteryl/-stanyl fatty acid esters have been described is probably also due to the unavailability of the respective reference compounds.

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Therefore, the aim of the present study was to identify acyl chain oxidation products upon heat treatment of a mixture of phytosteryl/-stanyl linoleates, taking the existing body of knowledge on oxidations of cholesteryl fatty acid esters and of fats and oils into account. Crucial steps of the investigations were (i) the development of a method suitable to separate the nonoxidized esters from the polar oxidation products, (ii) the synthesis of authentic reference compounds of steryl/-stanyl esters with oxidized fatty acid moieties, and (iii) the identification of the oxidation products in the heat-treated mixture by means of capillary gas chromatography−mass spectrometry.

MATERIALS AND METHODS

Chemicals and Reagents. A mixture of phytosterols/-stanols, named "β-sitosterol" and consisting of 75% sitosterol, 12% sitostanol, 10% campesterol, 2% campestanol, and 1% others, was purchased from Acros Organics (Morris Plains, NJ, USA). Linoleic acid (≥99%), octanoic acid (≥98%), heptanoic acid (≥96%), pyridine (anhydrous, ≥99.8%), trimethylsilyldiazomethane (2 M in hexane), 3-chloroperbenzoic acid (*m*-CPBA, ≥77%), cyclooctanone (≥98%), cycloheptanone (≥98%), 2-iodoxybenzoic acid (45 wt %), sodium borohydride (≥96%), 4-toluenesulfonic acid monohydrate (4-TsOH, ≥98%) and pyridinium chlorochromate (≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). The silylation reagent BSTFA/TMCS (99:1) was purchased from Supelco (Bellefonte, PA, USA). Dicyclohexylcarbodiimide (DCC, 1 M in dichloromethane, > 99%) and 4-(dimethylamino)pyridine (DMAP, puriss.) were ordered from Fluka Analytical (Steinheim, Germany). Aleuritic acid (9,10,16-trihydroxypalmitic acid, ≥94%) was bought from Santa Cruz Biotechnology (Heidelberg, Germany). Methyl *tert*-butyl ether (MTBE) was supplied by Evonik Industries AG (Essen, Germany) and hexane (HiPerSolv Chromanorm) was purchased from VWR International (Darmstadt, Germany).

Synthesis of Phytosteryl/-stanyl Linoleates. The synthesis of the mixture of phytosteryl/-stanyl linoleates was carried out according to a previously described protocol.[21] Briefly, 100 mg of the mixture of phytosterols/-stanols ("β-sitosterol") and 170 mg of linoleic acid were heated in a sealed, nitrogen-flushed 2 mL glass vial for 25 h at 180 °C. The excess of acid was removed with 2.5 mL of potassium hydroxide (1 M), and the esters were extracted with 3 × 2.5 mL hexane/MTBE (3:2, v/v). Final purification on a 500 mg silica solid-phase extraction (SPE) column (Supelco) with cyclohexane as an eluent resulted in 47 mg of a mixture of phytosteryl/-stanyl linoleates (yield = 29%) as a colorless oil. The phytosterol/phytostanol distribution of the mixture corresponded to that of the "β-sitosterol" sample used for esterification. The purity of the linoleates determined by ^{1}H NMR and GC-FID was ≥98%.

Synthesis of Phytosteryl/-stanyl Octanoates and Heptanoates. Phytosteryl/-stanyl octanoates and heptanoates were synthesized according to the same procedure as described for the linoleates. The reaction of the phytosterol/-stanyl mixture "β-sitosterol" (100 mg) and octanoic acid/heptanoic acid (90 mg) resulted in 50 mg of phytosteryl/-stanyl octanoates (yield = 38%) and 61 mg of phytosteryl/-stanyl heptanoates (yield = 48%), respectively, as white solids. The sterol/stanol distributions corresponded to that of the "β-sitosterol" sample used for esterification. No impurities were observed in ^{1}H NMR and GC-FID analyses. The mass spectra of synthesized octanoates were in agreement with previously published spectra.[21] Full NMR signal assignments could be achieved for the quantitatively dominating sitosteryl esters (for atom numbering, see Figure 1). Both esters exhibited the same NMR signals for the steryl moiety (^{13}C/^{1}H): δ 139.70 (St-5), 122.59/5.39 (St-6, 1H, d), 73.67/4.63 (St-3, 1H, m), 56.68/1.00 (St-14, 1H, m), 56.02/1.10 (St-17, 1H, m), 50.01/0.98 (St-9, 1H, m), 45.81/0.93 (St-24, 1H, m), 42.31 (St-13), 39.72/1.17;2.03 (St-12, 2H, m), 38.16/2.33 (St-4, 2H, d), 37.01/1.13;1.86 (St-1, 2H, m), 36.60 (St-10), 36.17/1.37 (St-20, 1H, m), 33.92/1.32 (St-22, 2H, m), 31.91/1.96;2.03 (St-7, 2H, m), 31.86/1.31 (St-8, 1H, m), 29.12/1.32 (St-25, 1H, m), 28.27/1.59;1.87 (St-2, 2H, m), 27.82/1.57;1.88 (St-16, 2H, m), 26.03/1.19 (St-23, 2H, m), 24.31/1.04;1.60 (St-15, 2H, m), 23.05/0.90 (St-28, 2H, m), 21.03/1.01;1.51 (St-11, 2H, m), 19.85/0.89 (St-26/27, 3H, d), 19.34/1.04 (St-19, 3H, s), 19.04/0.89 (St-26/27, 3H, d), 18.79/0.94 (St-21, 3H, d), 12.00/0.89 (St-29, 3H, t), 11.87/0.70 (St-18, 3H, s). The NMR signals of the fatty acid parts were as follows (^{13}C/^{1}H): sitosteryl heptanoate δ 173.37 (FA-1), 34.74/2.29 (FA-2, 2H, t), 31.49/1.32 (FA-5, 2H, m), 28.81/1.32 (FA-4, 2H, m), 25.05/1.64 (FA-3, 2H, m), 22.51/1.31 (FA-6, 2H, m), 14.07/0.88 (FA-7, 3H,t); sitosteryl octanoate δ 173.35 (FA-1), 34.76/2.29 (FA-2, 2H, t), 31.69/1.3 (FA-6, 2H, m), 29.1/1.32 (FA-4/5, 2H, m), 28.96/1.32 (FA-4/5, 2H, m), 25.08/1.63 (FA-3, 2H, m), 22.62/1.3 (FA-7, 2H, m), 14.1/0.9 (FA-8, 3H, t).

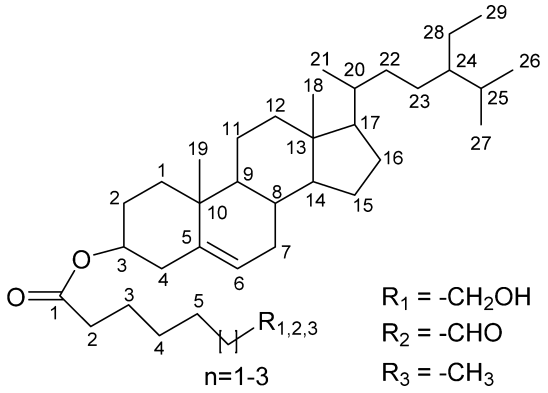

Figure 1. General structures and atom numbering of acyl chain oxidation products of sitosteryl linoleate.

Synthesis of 9-Oxononanoic Acid. 9-Oxononanoic acid was synthesized according to a previously described method via diol cleavage of aleuritic acid (9,10,16-trihydroxypalmitic acid).[22] Briefly, 6 g of potassium periodate in 300 mL of sulfuric acid (1 M) was quickly added to 8 g of aleuritic acid in methanol/water (200 + 200 mL) at 40 °C. After 10 min of stirring, the mixture was cooled to 15 °C in a methanol/ice bath and extracted immediately with 2 × 400 mL of diethyl ether. The combined organic layers were extracted with 2 × 100 mL saturated sodium hydrogen carbonate solution, and the combined aqueous layers were acidified with concentrated hydrochloric acid. The aqueous solution was extracted with 2 × 100 mL of diethyl ether, and the combined ether layers were washed with 2 × 100 mL of an aqueous sodium chloride solution (10%) and dried over magnesium sulfate. After removal of the solvent under a nitrogen stream, 3.2 g of a colorless oil was obtained, which was used for the subsequent esterification step.

Synthesis of Phytosteryl/-stanyl 9-Oxononanoates. The esterification was carried out as previously described for the synthesis of cholesteryl 9-oxononanoate.[23] 9-Oxononanoic acid (200 mg), DMAP (5.6 mg), and the phytosterol/-stanyl mixture "β-sitosterol" (195 mg) were dissolved in 4 mL of dichloromethane, 1 mL of DCC solution was added, and the mixture was stirred for 24 h at room temperature. The mixture was filtered in the dark and dissolved in 10 mL of dichloromethane, and the solution was subsequently washed with 25 mL of hydrochloric acid (0.5 M) and 25 mL of saturated sodium hydrogen carbonate solution. The organic layer was dried over magnesium sulfate and filtered. For final purification, the esterified product was dissolved in 5 mL of cyclohexane. The sample was loaded onto a 500 mg silica SPE column, preconditioned with 6 mL of cyclohexane. The esters were eluted with 15 mL of heptane/MTBE (98:2, v/v). After evaporation of the solvent, 203 mg of a mixture of phytosteryl/-stanyl 9-oxononanoates was obtained as a white solid. The phytosterol/-stanol distribution of the mixture corresponded to that of the "β-sitosterol" sample used for esterification. The purity of the oxoesters was determined to be ≥92% by ^{1}H NMR and GC-FID. NMR signals of the fatty acid moiety (^{13}C/^{1}H): δ 202.87/9.79 (FA-9, 1H, t), 173.22 (FA-1), 43.89/2.44 (FA-8, 2H, d of t), 34.63/2.29 (FA-2, 2H, t), 29.02/1.35 (FA-4/5/6, 2H, m), 28.97/1.35 (FA-4/5/6, 2H, m),

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28.89/1.35 (FA-4/5/6, 2H, m), 27.82/1.61 (FA-7, 2H, m), 24.95/1.63 (FA-3, 2H, m).

Synthesis of 8-Oxooctanoic Acid and 7-Oxoheptanoic Acid. 8-Oxooctanoic acid and 7-oxoheptanoic acid were synthesized on the basis of a previously published method[24] via Baeyer−Villiger reaction of the corresponding cycloalkanones, lactone opening, and subsequent oxidation of the hydroxyacids. Cyclooctanone and cycloheptanone (2.079 and 1.392 g, respectively) were dissolved in 10 mL of dichloromethane, and *m*-CPBA (2.152 and 1.392 g, respectively) was added. After 6 days of stirring at room temperature, the reaction was quenched by the addition of 500 μL of saturated sodium thiosulfate solution. The organic layer was washed with 3 × 20 mL saturated sodium hydrogen carbonate solution and 2 × 20 mL of an aqueous sodium chloride solution (10%), dried over magnesium sulfate, and filtered. After evaporation of the solvent under nitrogen, the following colorless liquids (purities determined by GC) were obtained: 2-oxononanone (723 mg; 20% lactone and 80% educt) and 2-oxocanone (563 mg; 21% lactone and 79% educt). For lactone opening, the reaction products were dissolved in 2 mL of dioxane, treated with 15 mL of sodium hydroxide (3 M), and stirred overnight at room temperature. The mixtures were washed with 15 mL of ethyl acetate, and the pH was adjusted to 3.5 with hydrochloric acid (25%). The mixtures were extracted with 2 × 20 mL of ethyl acetate, washed with 2 × 20 mL of saturated sodium hydrogen carbonate solution, dried over magnesium sulfate, filtered, and evaporated under nitrogen stream. 8-Hydroxyoctanoic acid (168 mg) and 7-hydroxyheptanoic acid (130 mg) were obtained as white solids. After silylation, GC purities of ≥90% were determined for both acids. 8-Hydroxyoctanoic acid and 7-hydroxyheptanoic acid were dissolved in dimethyl sulfoxide (3.7 and 3.2 mL, respectively), before 2-iodoxybenzoic acid (463 and 440 mg, respectively) was added. The mixtures were stirred at room temperature for 4 h, the reactions were quenched with distilled water, and the mixtures were filtered. After extraction with 2 × 20 mL of ethyl acetate, drying over magnesium sulfate, filtering, and evaporation under nitrogen stream, 165 mg of 8-oxooctanoic acid and 125 mg of 7-oxoheptanoic acid were obtained as white solids.

Synthesis of Phytosteryl/-stanyl 8-Oxooctanoates and 7-Oxoheptanoates. Esterifications of the phytosterol/-stanyl mixture "β-sitosterol" with 8-oxooctanoic acid and 7-oxoheptanoic acid were performed as described for the 9-oxononanoates. After esterification, the esters were purified by silica SPE as described for the 9-oxononanoates, but the esters were eluted with MTBE. After purification, 43.5 mg of a mixture of phytosteryl/-stanyl 8-oxooctanoates and 26 mg of a mixture of phytosteryl/-stanyl 7-oxoheptanoates were obtained as colorless oils. The phytosterol/-stanol distribution of the mixture corresponded to that of the "β-sitosterol" sample used for esterification. The purities of the oxoesters determined by ^{1}H NMR and GC-FID were ≥80% for 8-oxooctanoates and ≥70% for 7-oxoheptanoates. NMR signals of the fatty acid moieties (^{13}C/^{1}H): sitosteryl 7-oxoheptanoate, δ 202.59/9.79 (FA-7, 1H, t), 173 (FA-1), 43.68/2.46 (FA-6, 2H, d of t), 34.57/2.31 (FA-2, 2H, t), 28.57/1.28 (FA-4/5, 2H, m), 27.81/1.59 (FA-4/5, 2H, m), 24.73/1.62 (FA-3, 2H, m); sitosteryl 8-oxooctanoate, δ 203/9.78 (FA-8, 1H, t), 173 (FA-1), 43.82/2.45 (FA-7, 2H, d of t), 34.56/2.29 (FA-2, 2H, t), 28.82/1.36 (FA-4/5, 2H, m), 28.8/1.36 (FA-4/5, 2H, m), 27.82/1.66 (FA-6, 2H, m), 24.8/1.65 (FA-3, 2H, m).

Reduction of Oxoesters to the Corresponding Hydroxyesters. The oxoesters were reduced to the hydroxyesters according to a procedure described for the reduction of 9-oxononanoic acid.[25] The oxoesters (5−10 mg) were dissolved in 200 μL of dioxane and cooled in an ice bath. Sodium borohydride (5 mg) was added to the solution and stirred for 10 min at room temperature. Crushed ice was added to quench the reaction, and the hydroxides were extracted with 2 × 3 mL hexane/MTBE (3:2, v/v). After drying over magnesium sulfate and solvent evaporation under a nitrogen stream, 8.4 mg of a mixture of phytosteryl/-stanyl 8-hydroxyoctanoates was obtained as a white solid and 4.5 mg of a mixture of 7-hydroxyheptanoates as a colorless oil. The phytosterol/-stanol distributions of the mixtures corresponded to those of the oxoesters used for reduction. The purities of the hydroxyesters determined by ^{1}H NMR and GC-FID (after silylation) were ≥82% for 8-hydroxyoctanoates and ≥80% for 7-hydroxyheptanoate. NMR signals of the fatty acid moieties (^{13}C/^{1}H): sitosteryl 7-hydroxyheptanoate, δ 173.25 (FA-1), 62.94/3.67 (FA-7, 2H, t), 34.6/2.32 (FA-2, 2H, t), 32.55/1.6 (FA-6, 2H, m), 28.9/1.39 (FA-4/5, 2H, m), 28.86/1.31 (FA-4/5, 2H, m), 25.4/1.66 (FA-3, 2H, m); sitosteryl 8-hydroxyoctanoate, δ 173.29 (FA-1), 63.06/3.66 (FA-8, 2H, t), 34.64/2.29 (FA-2, 2H, t), 32.71/1.58 (FA-7, 2H, m), 29.21/1.37 (FA-4/5, 2H, m), 29.02/1.29 (FA-4/5, 2H, m), 25.66/1.37 (FA-6, 2H, m), 24.95/1.66 (FA-3, 2H, m).

Synthesis of Stigmasta-3,5-dien-7-one. "β-Sitosterol" (740 mg) was acetylated with 3 mL of acetic acid anhydride in 30 mL of pyridine at room temperature for 12 h. The solvent was removed under reduced pressure, and sitosteryl acetate was used for the synthesis of 7-ketositosteryl acetate as described by Geoffroy et al.[26] Briefly, 7.4 g of pyridinium chlorochromate was added to a suspension of 18 g of Celite in 90 mL of benzene, followed by the addition of sitosteryl acetate. The reaction mixture was refluxed for 24 h. After cooling to room temperature, the reaction mixture was filtered on a fritted glass funnel, and the filtrate was carefully washed with 3 × 15 mL of ethyl acetate. After removal of the solvents under reduced pressure, sitosteryl acetate (623 mg) was removed from the reaction mixture on a silica gel column (30 g) with ethyl acetate/hexane (5:95, v/v), followed by the elution of 7-ketositosteryl acetate (90 mg) with ethyl acetate/hexane (10:90, v/v). Then, an aliquot of 23 mg of 7-ketositosteryl acetate and 52 mg of *p*-toluenesulfonic acid, dissolved in 5 mL of toluene, was refluxed for 2 h according to the method of Abramovitch and Micetich[27] The mixture was dissolved in diethyl ether and washed with an aqueous sodium carbonate solution and distilled water. After evaporation to dryness, 20 mg of stigmasta-3,5-dien-7-one was obtained as a yellow oil (purity determined by GC = 75%).

Thermal Treatment of the Mixture of Phytosteryl/-stanyl Linoleates. The mixture of phytosteryl/-stanyl linoleates was weighed into a 2 mL brown glass vial without a lid (12 ± 0.2 mg) and oxidized in a heating block (VLM Metal, Bielefeld, Germany) at 180 °C for 40 min. After thermal treatment, the samples were cooled to room temperature before undergoing further sample preparation.

Solid-Phase Extraction. The heated linoleates were dissolved in 3 mL of cyclohexane, and the solution was loaded onto a Supelclean 500 mg silica SPE column (Supelco), which had been preconditioned with 6 mL of hexane. The nonoxidized linoleates and nonpolar oxidation products were eluted from the SPE column, attached to a vacuum chamber, with 21 mL of cyclohexane at a flow rate of approximately 1 mL/min (fraction 1). Subsequently, the polar oxidation products were eluted with 6 mL of MTBE (fraction 2). The solvents of both fractions were evaporated under a gentle nitrogen stream. The residue of fraction 1 was dissolved in 1 mL of hexane/MTBE (3:2, v/v) and subjected to GC-FID/GC-MS analysis. The residue of fraction 2 was silylated with 100 μL of pyridine and 100 μL of BSTFA/TMCS for 20 min at 80 °C. The silylation reagent was evaporated under a gentle nitrogen stream, and the residue was dissolved in 500 μL of hexane/MTBE (3:2, v/v) and subjected to GC-MS/FID analysis.

GC-FID Analysis. The analysis of the SPE fractions (1 μL injection volume) was performed using a 6890N GC equipped with an FID (Agilent Technologies, Böblingen, Germany). The separations were carried out on a 30 m × 0.25 mm i.d., 0.1 μm film, Rtx-200MS fused silica capillary column (Restek, Bad Homburg, Germany). The temperature of the injector was set to 280 °C, and hydrogen was used as carrier gas with a constant flow rate of 1.5 mL/min. The split flow was set to 11 mL/min, resulting in a split ratio of 1:7.5. The oven temperature was programmed as follows: initial temperature, 100 °C; programmed at 15 °C/min to 310 °C (2 min) and then at 1.5 °C/min to 340 °C (3 min). The FID temperature was set to 340 °C, and nitrogen was used as makeup gas with a flow rate of 25 mL/min. Data acquisition was performed by ChemStation B.04.03.

GC-MS Analysis. Mass spectra were recorded with a Finnigan Trace GC ultra coupled with a Finnigan Trace DSQ mass spectrometer (Thermo Electro Corp., Austin, TX, USA). Mass spectra were obtained by positive electron-impact ionization at 70 eV in the scan

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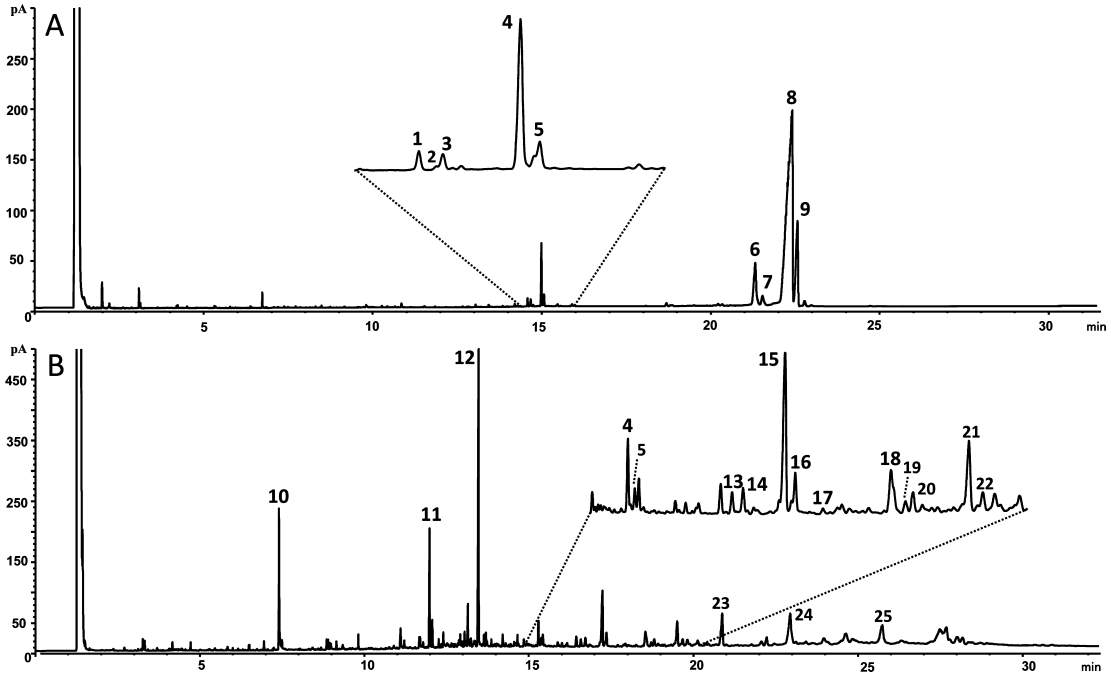

Figure 2. Capillary gas chromatographic separations of (A) the unpolar and (B) the polar fractions obtained via SPE from the heated phytosteryl/-stanyl linoleate mixture. **1**, sitosteryl heptanoate; **2**, sitostanyl heptanoate; **3**, campesteryl octanoate; **4**, sitosteryl octanoate; **5**, sitostanyl octanoate; **6**, campesteryl linoleate; **7**, campestanyl linoleate; **8**, sitosteryl linoleate; **9**, sitostanyl linoleate; **10**, linoleic acid; **11**, sitosterol; **12**, stigmasta-3,5-dien-7-one; **13**, sitosteryl 7-hydroxyheptanoate; **14**, campesteryl 8-hydroxyoctanoate; **15**, sitosteryl 8-hydroxyoctanoate; **16**, sitostanyl 8-hydroxyoctanoate; **17**, sitosteryl 7-oxoheptanoate; **18**, sitosteryl 8-oxooctanoate; **19**, sitostanyl 8-oxooctanoate; **20**, campesteryl 9-oxononanoate; **21**, sitosteryl 9-oxononanoate; **22**, sitostanyl 9-oxononanoate; **23**/**24**, α/β-7-hydroxysitosteryl linoleate (tentative); **25**, α/β-5,6-epoxysitosteryl linoleate (tentative).

Table 1. Characteristic GC-MS Fragment Ions of Steryl/Stanyl Ester Oxidation Products

acyl chain oxidation product[a]	RT[b] (min)	molecular ion $[M]^+$	characteristic fragments $[m/z]$
1, sitosteryl heptanoate	14.565	526 (−)	397 (33), 396 (100), 381 (27), 275 (15), 255 (22), 213 (16)
2, sitostanyl heptanoate	14.636	528 (6)	513 (0.3), 399 (10), 398 (41), 383 (35), 344 (10), 257 (18), 215 (100)
3, campesteryl octanoate	14.662	526 (−)	383 (36), 382 (100), 367 (33), 261 (22), 255 (26), 213 (20)
4, sitosteryl octanoate	14.972	540 (−)	397 (49), 396 (100), 381 (46), 275 (34), 255 (40), 213 (34)
5, sitostanyl octanoate	15.049	542 (4)	527 (1), 399 (21), 398 (53), 383 (39), 344 (11), 257 (18), 215 (100)
13, sitosteryl 7-hydroxyheptanoate[c]	16.606	614 (−)	397 (47), 396 (100), 381 (24), 275 (14), 255 (17), 213 (14), 524 (0.3)
14, campesteryl 8-hydroxyoctanoate[c]	16.740	614 (−)	383 (39), 382 (100), 367 (23), 261 (13), 255 (15), 213 (26)
15, sitosteryl 8-hydroxyoctanoate[c]	17.277	628 (−)	397 (43), 396 (100), 381 (18), 275 (12), 255 (17), 213 (14), 538 (0.2)
16, sitostanyl 8-hydroxyoctanoate[c]	17.398	630 (0.2)	399 (17), 398 (42), 383 (32), 344 (5), 257 (17), 215 (100), 540 (0.3)
17, sitosteryl 7-oxoheptanoate	17.736	540 (−)	525 (0.1), 397 (35), 396 (100), 381 (28), 275 (14), 255 (20), 213(19)
18, sitosteryl 8-oxooctanoate	18.590	554 (−)	397 (50), 396 (100), 381 (41) 275 (20), 255 (24), 213 (18)
19, sitostanyl 8-oxooctanoate	18.761	556 (0.9)	541 (0.7), 399 (14), 398 (50), 383 (36), 344 (4), 257 (14), 215 (100)
20, campesteryl 9-oxononanoate	18.856	554 (−)	383 (30), 382 (100), 367 (27), 261 (12), 255 (14), 213 (13),
21, sitosteryl 9-oxononanoate	19.559	568 (−)	397 (32), 396 (100), 381 (25), 275 (13), 255 (15), 213 (12),
22, sitostanyl 9-oxononanoate	19.722	570 (1.3)	555 (0.3), 399 (21), 398 (58), 383 (36), 344 (7), 257 (16), 215 (100)

[a]Numbers correspond to those in the chromatogram shown in Figure 2. [b]Retention times on Rtx-200MS. [c]As trimethylsilyl ether.

mode at unit resolution from 40 to 750 Da. The interface was heated to 330 °C and the ion source to 250 °C. Helium was used as carrier gas with a constant flow rate of 1.0 mL/min. The other GC conditions were the same as described for GC-FID analysis. Data acquisition was perfomed by Xcalibur 3.063 software.

NMR Spectroscopy. The compounds were dissolved in 0.5 mL of deuterated chloroform. ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 and 126 MHz, respectively, with Avance-HD 500 spectrometers (Bruker, Billerica, MA, USA) operating at 27 °C. ^{1}H-detected experiments including two-dimensional COSY, NOESY, HSQC, and HMBC were measured with an inverse ^{1}H/^{13}C probe head; direct ^{13}C measurements were performed with a QNP ^{13}C/^{31}P/^{29}Si/^{19}F/^{1}H cryoprobe. All experiments were done in full automation using standard parameter sets of the TOPSPIN 3.2 software package (Bruker). ^{13}C NMR spectra were recorded in proton-decoupled mode. Data processing was typically done with the MestreNova software.

RESULTS AND DISCUSSION

Thermal Treatment and Sample Preparation. Phytosteryl/-stanyl linoleates prepared via esterification of a commercially available mixture of sterols (75% β-sitosterol, 10% campesterol) and stanols (12% sitostanol, 2% campestanol) were used as model substrates for the thermal treatment. Such esters account for a large proportion of the phytosteryl/-stanyl fatty acid ester mixtures being added to foods.[10]

Nonoxidized phytosteryl/-stanyl linoleates were anticipated to be still quantitatively dominating in the thermally treated sample.

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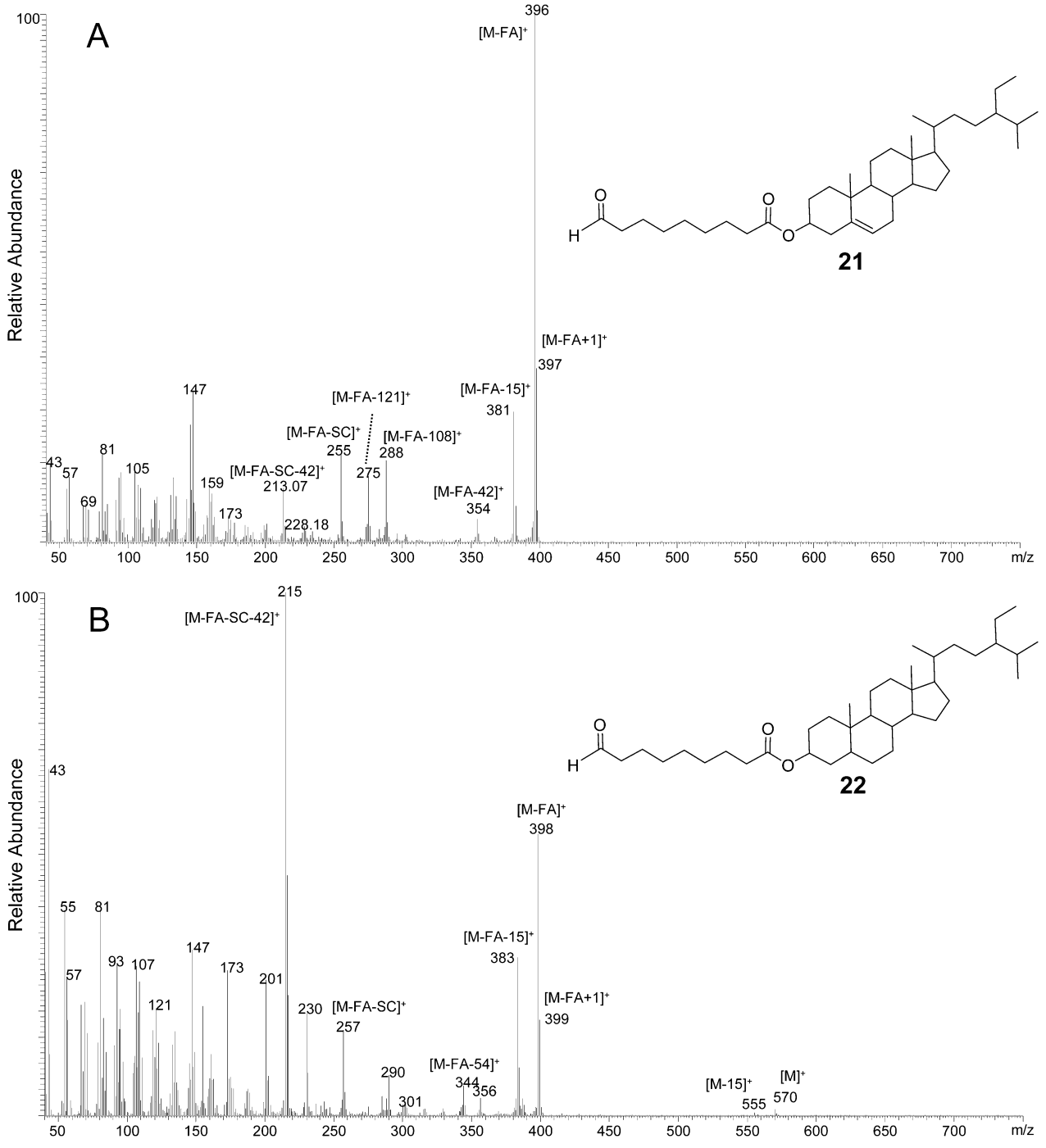

Figure 3. Electron-impact ionization spectra of (A) sitosteryl 9-oxononanoate and (B) sitostanyl 9-oxononanoate. $[M]^+$, molecular ion; [FA], fatty acid; [SC], sterol/stanol side chain.

Therefore, as a first step, an SPE method was developed to preconcentrate the acyl chain oxidation products by removal of the nonoxidized esters. The oxidized esters were expected to be more polar than the starting esters. Accordingly, the strategy was based on an elution of the nonoxidized esters from a silica SPE column with a nonpolar eluent in a first fraction, followed by a second eluting step for the polar oxidation products. In previous studies, SPE methods have also been described for the preconcentration of cholesteryl ester hydroperoxides formed upon heating of cholesteryl esters and for the separation of oxidized fatty acid methyl esters into nonpolar and polar fractions.[17,19,28] In the course of the method development several nonpolar solvents with increasing elution powers were tested to find an eluent just capable of eluting the nonoxidized linoleates from the silica column without causing the elution of polar oxidation products. The compounds retained on the column were subsequently eluted with MTBE, and both fractions were checked for nonoxidized linoleates by GC-FID/MS. When using heptane or hexane as elution solvents for the first fraction, remainders of nonoxidized esters were still detectable in the MTBE fraction. Using cyclohexane, nonoxidized linoleates could be effectively removed from the silica column in the first elution step (Figure 2A), and no nonoxidized linoleates were detected in the second fraction after elution with MTBE (Figure 2B).

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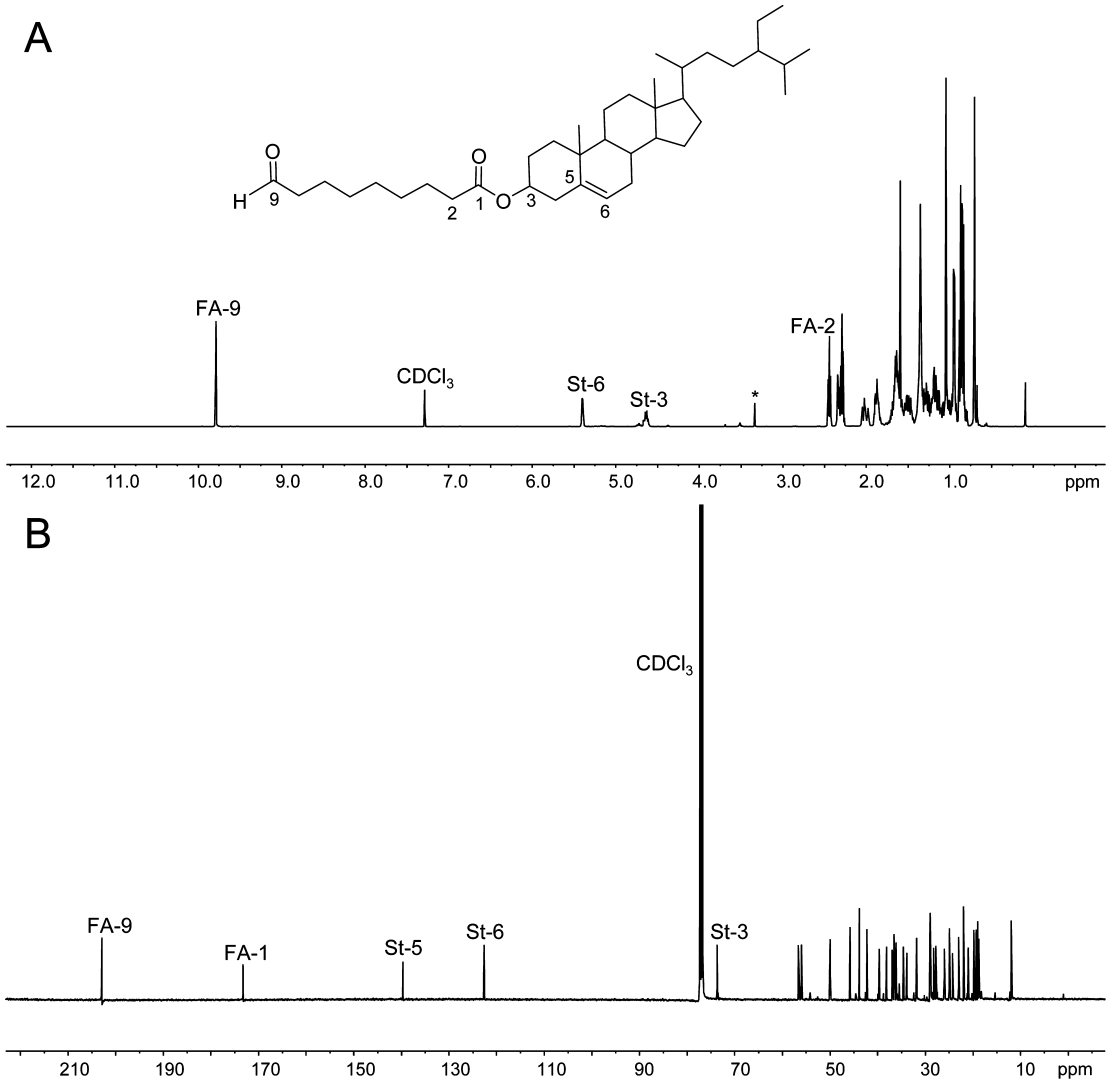

Figure 4. NMR spectra of synthesized sitosteryl 9-oxononanoate. (A) ^{1}H NMR; characteristic proton signals of the sterol moiety and the fatty acid moiety: δ 9.79 (FA-9, 1H, t), 5.39 (St-6, 1H, m), 4.63 (St-3, 1H, m), 2.29 (FA-2, 2H, t). The asterisks represents an impurity. (B) ^{13}C NMR; characteristic carbon signals of the sterol moiety and the fatty acid moiety: δ 202.87 (FA-9), 173.22 (FA-1), 139.7 (St-5), 122.59 (St-6), 73.67 (St-3).

Identification of Oxidation Products. The capillary gas chromatographic analysis of fraction 1 revealed that in addition to the remaining nonoxidized linoleates several minor peaks were detectable (Figure 2A). Peaks 1−5 were identified via GC-MS as heptanoates and octanoates of sitosterol, campesterol, and sitostanol (Table 1).

In fraction 2, three major peaks were detected (Figure 2B). Peaks 10 and 11 were identified as trimethylsilyl derivatives of linoleic acid and β-sitosterol, respectively. According to GC and ^{1}H NMR analysis, both were not contained as impurities in the synthesized mixture of phytosteryl/-stanyl linoleates used for the heating experiment. Their occurrence might be explained by a potential formation via hydrolysis during the heating procedure. On the other hand, the presence of linoleic acid may also be related to the formation of compound **12**. On the basis of comparison of the retention time and the mass spectrum to those of a synthesized reference compound, peak 12 was assigned as stigmasta-3,5-dien-7-one. This compound has previously been identified in heartwood and in the smoke of incense.[27,29] In previous investigations involving the cleavage of the ester bond as part of the analytical procedure, 7-ketositosterol has been reported as a major oxidation product resulting from the thermal treatment of sitosteryl esters.[30,31] The respective 7-ketositosteryl linoleate was not detected in the present study; stigmasta-3,5-dien-7-one might be explained, for example, as degradation product formed from 7-ketositosteryl linoleate via liberation of linoleic acid. On the other hand, the analogous oxidation product stigmasta-3,5,22-trien-7-one has also been identified upon thermo-oxidation of free stigmasterol,[32] indicating that compound **12** might also be the degradation product of intermediately formed β-sitosterol.

In addition to these three quantitatively dominating compounds, GC-MS analysis of fraction 2 revealed the presence of a series of minor peaks exhibiting fragmentation patterns similar to those described for nonoxidized phytosteryl/-stanyl esters.[21,33,34] The respective part of the chromatogram is enlarged in Figure 2B, and the MS data are listed in Table 1. The base peaks m/z 396 observed for compounds **13**, **15**, **17**, **18**, and **21** and m/z 382 for compounds **14** and **20** correspond to those described to result from the cleavage of the fatty acid from sitosteryl and campesteryl esters, respectively.[33,34] The base peak m/z 215 observed for compounds **16**, **19**, and **22** has been described as being generated by cleavage of the fatty acid

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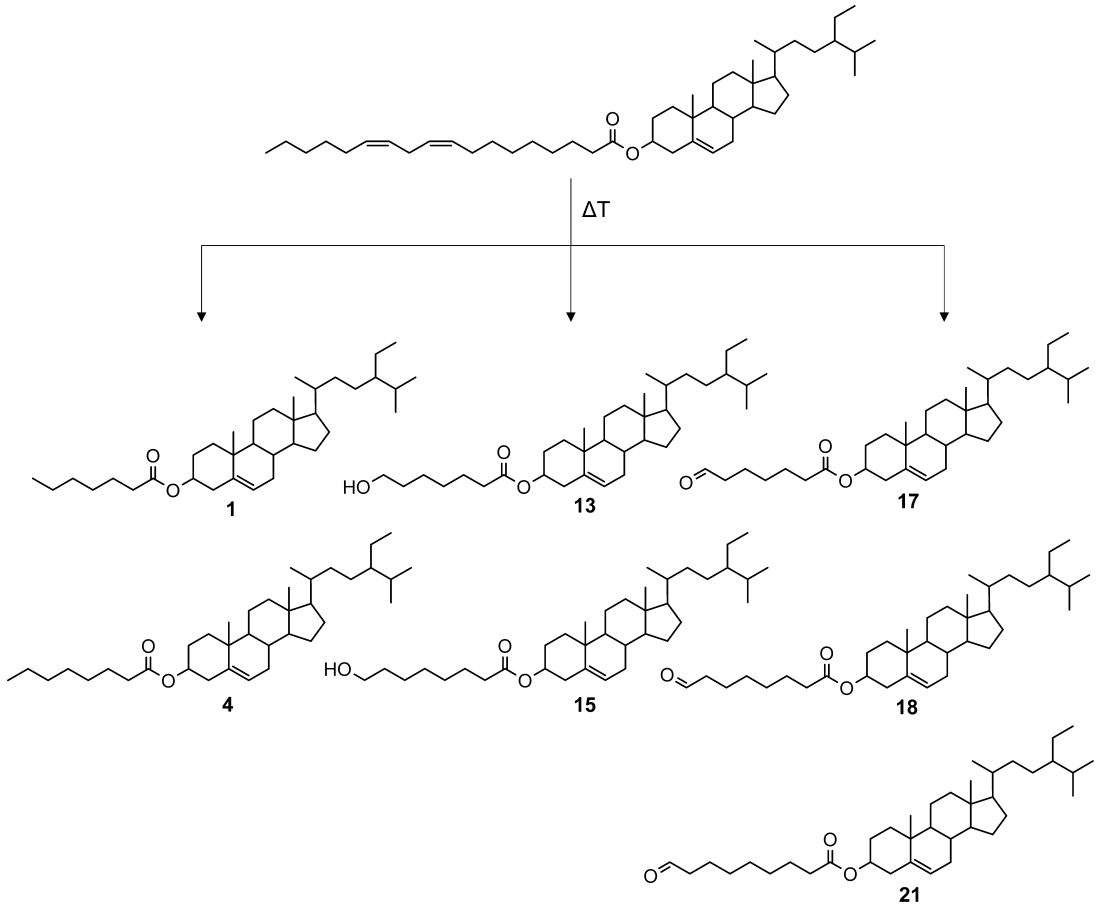

Figure 5. Classes of identified oxidation products in the fatty acid part upon heating of sitosteryl, campesteryl, and sitostanyl esters, exemplarily shown for sitosteryl linoleate. Class I (alkanoates): **1**, sitosteryl heptanoate; **4**, sitosteryl octanoate. Class II (hydroxyalkanoates): **13**, sitosteryl 7-hydroxyheptanoate; **15**, sitosteryl 8-hydroxyoctanoate. Class III (oxoalkanoates): **17**, sitosteryl 7-oxoheptanoate; **18**, sitosteryl 8-oxooctanoate; **21**, sitosteryl 9-oxononanoate. The numbering corresponds to that of Figure 2.

and further fragmentation involving the sterol side chain of short-chain sitostanyl esters.[21] This enabled the differentiation of steryl and stanyl esters; exemplary mass spectra are shown in Figure 3.

In a previous study[21] in which the same mass spectrometric conditions were used, weak but characteristic molecular ions could be detected for sitostanyl fatty acid esters. Therefore, on the basis of the oxidative modifications in the acyl part known from peroxidation of cholesteryl linoleate[14] and high-temperature degradation of methyl linoleate and triacylglycerides,[18,35,36] molecular masses of potential acyl chain oxidation products of sitostanyl linoleate, that is, oxo- and hydroxyalkanoates, were calculated and compared with the mass spectra determined in fraction 2. This screening gave first indications that the peaks 16, 19, and 22 corresponded to sitostanyl 8-hydroxyoctanote, 8-oxooctanoate, and 9-oxononanoate, respectively.

These preliminary assignments initiated the next phase of the study, namely, the synthesis and characterization of authentic reference compounds. First, potential candidates of oxidized fatty acid moieties were synthesized via established synthetic routes: 9-oxononanoic acid was obtained by oxidative diol cleavage of aleuritic acid;[22] 8-oxooctanoic acid and 7-oxoheptanoic acid were obtained by Baeyer−Villiger reaction of the corresponding cycloalkanones and subsequent oxidation with iodoxybenzoic acid.[24] These synthesized acids as well as heptanoic and octanoic acid were esterified with the same phytosteryl/-stanyl mixture as used for the esterification of linoleic acid. The corresponding hydroxyalkanoates were obtained by reduction of 7-oxoheptanoates and 8-oxooctanoates with sodium borohydride.

The identities of the synthesized reference compounds were confirmed via NMR. With a proportion of 75%, sitosterol was the major phytosterol in the synthesized phytosteryl/-stanyl ester mixtures; therefore, $^{1}H/^{13}C$ NMR signals of sitosteryl esters could be interpreted. By making use of a repertoire of two-dimensional techniques, it was possible to assign almost every carbon and proton signal to their respective atom number (Figure 1). By comparison of the ^{13}C spectrum of unesterified sitosterol with ^{13}C spectra of the sitosteryl ester reference compounds, the carbon signals of the acyl chains could be assigned. Each synthesized compound exhibited the same proton and carbon signals in the sterol moiety, thus only differing in their signals in the fatty acid part. Equally characteristic for each sitosteryl ester was the carbon atom FA-1 involved in the ester bond, showing a chemical shift of 173 ppm, and the carbon atom FA-2, with a chemical shift of 34.6 ppm and a well-resolved triplet proton signal pattern at 2.3 ppm. The signals of terminal carbon and proton atoms of the fatty acid part enabled discrimination between the synthesized reference classes. The terminal carbon atom of sitosteryl alkanoates exhibited a signal at 14.1 ppm, and the corresponding proton showed a triplet proton signal pattern at 0.9 ppm, whereas the terminal aldehyde function of core aldehyde esters had a chemical shift at

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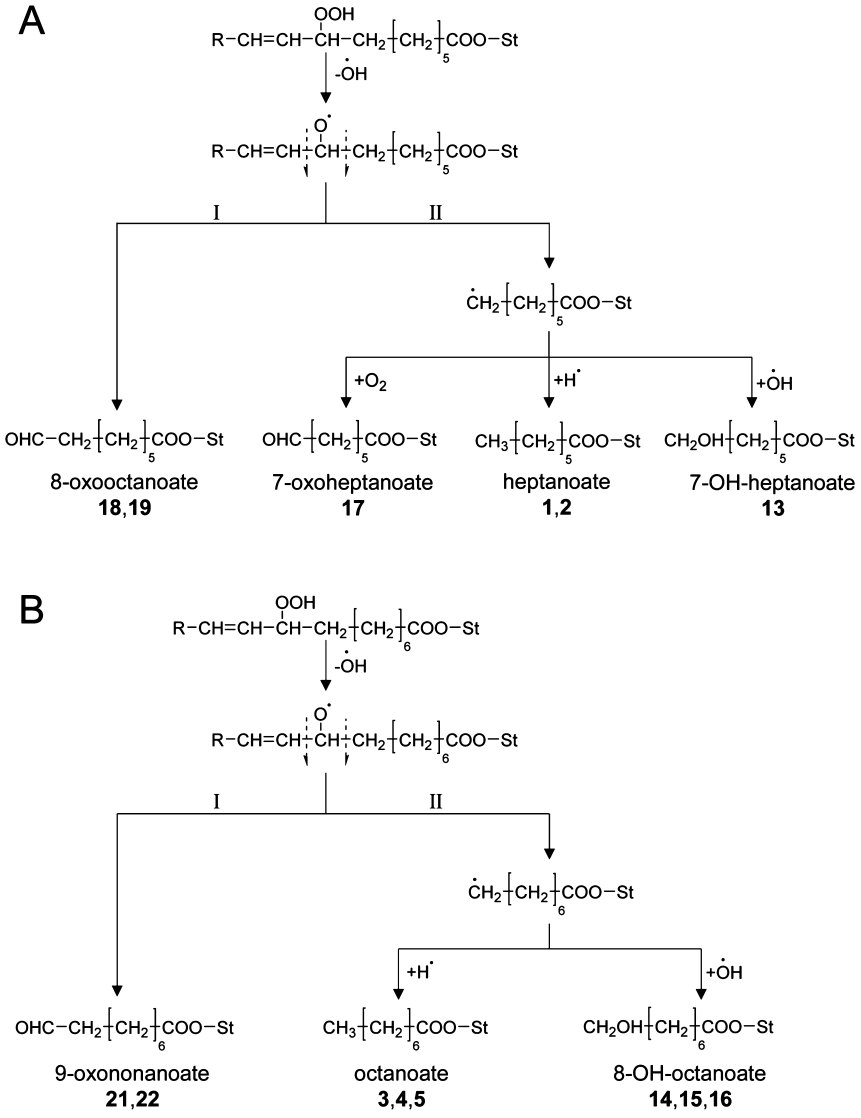

Figure 6. Formation pathways of steryl/stanyl ester oxidation products in the acyl chain by homolytic β-scission of primarily formed allylic hydroperoxides on positions (A) 8 and (B) 9. Adapted from Frankel.[37] Numbering corresponds to that in Figures 2 and 5. Only pathways leading to the compounds identified in this study are illustrated.

203 ppm and an intense triplet pattern was observed for the aldehyde proton at 9.8 ppm (Figure 4). The acquired signal pattern and peak assignments correlated with published ^{1}H and ^{13}C data of cholesteryl 9-oxononanoate.[23] The terminal carbon atom of sitosteryl hydroxyalkanoates showed a signal at 63 ppm and a well-resolved triplet pattern at 3.66 ppm of the terminal protons.

Final peak identifications were carried out by comparing retention times and mass spectra of sample peaks with those of the synthesized reference compounds (Table 1). Due to their slightly higher polarity compared to the respective linoleates, sitosteryl octanoate **4** and sitostanyl octanoate **5** were also present in fraction 2. In total, seven types of oxidation products of the fatty acid moiety belonging to three different classes, that is, alkanoates, hydroxyalkanoates, and oxoalkanoates, could be identified upon heating of phytosteryl/phytostanyl linoleates (Figure 5). These classes of acyl chain oxidation products were in line with those previously reported for cholesteryl esters, fatty acid methyl esters, and triacylglycerides.[14,17,18]

The formation of these acyl chain oxidation products may be explained analogously to the routes described for the oxidation of fatty acid methyl esters.[18,37,38] The primarily formed allylic hydroperoxides on positions 8 and 9, respectively, are degraded by homolytic β-scission according to route I (Figure 6). After interaction of the resulting alkyl radical with a hydrogen radical, heptanoates and octanoates, respectively, are obtained. Alternatively, 7-hydroxyheptanoates and 8-hydroxyoctanoates are formed by reaction of the alkyl radical with a hydroxyl radical. Repeated oxidation of the alkyl radical formed from the degradation of the 8-hydroperoxide with oxygen leads to the formation of 7-hydroperoxides, which can decompose to 7-oxoheptanoates via an alkoxyl radical. On the other hand, 8-oxooctanoates and 9-oxononanoates emerge directly upon β-scission of allylic hydroperoxides on positions 8 and 9 following route II. In addition, 9-oxononanoates may be formed from the allylic hydroperoxide in position 10 following route I and subsequent reaction with a hydroxyl radical.

In addition to the described acyl chain oxidation products, three representatives of linoleates of oxidized sterols were detected in fraction 2. Peaks 23 and 24 (Figure 2B) were assumed to be silylated α/β-7-hydroxysitosteryl linoleates, because their mass spectra (Table 2) exhibited a characteristic base peak of m/z 484 corresponding to $[M - FA]^{+}$, which was

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Table 2. Characteristic GC-MS Fragment Ions of Tentatively Assigned Sitosteryl Linoleates Oxidized in the Sterol Moiety

sitosteryl linoleate oxidation product[a]	RT[b] (min)	molecular ion $[M]^+$	characteristic fragments $[m/z]$
23, 7α/β-hydroxy sitosteryl linoleate[c,d]	20.955	765 (−)	486 (12), 485 (55), 484 (100), 483 (33)
24, 7α/β-hydroxysitosteryl linoleate[c,d]	23.038	765 (−)	486 (16), 485 (46), 484 (100), 483 (56)
25, 5,6α/β-epoxysitosteryl linoleate[d]	25.845	693 (−)	413 (44), 395 (100)

[a]Numbers correspond to those in the chromatogram shown in Figure 2B. [b]Retention times on Rtx-200MS. [c]As trimethylsilyl ether. [d]α/β-Stereoisomers could not be distinguished.

also described as the base peak of double-silylated 7-hydroxysitosterols in a previous study.[39] Peak 25 was tentatively assigned as α- or β-5,6-epoxysitosteryl linoleate, exhibiting characteristic fragment ions similar to those reported for silylated α/β-5,6-epoxysitosterols, with m/z 395 potentially corresponding to $[M - FA - 18]^+$ and m/z 413 resulting from $[M - FA]^+$.[40] As no reference compounds were available for these esters, their identifications are considered only tentative.

There are other representatives of potential acyl chain oxidation products that have not been detected in this study, for example, those deriving from the 13-hydroperoxides or dihydroxyacids, comparable to the previously reported 9,10-dihydroxystearates of sitosterol or of 7-ketositosterol.[20] It remains to be investigated whether they are not covered by the analytical procedure or whether they are not formed under the employed conditions.

In conclusion, the employed methodology, that is, a preconcentration of polar oxidation products via SPE, the establishment of capillary gas chromatographic conditions enabling the separation of various oxidation products, and the synthesis of authentic reference compounds, provided data on the formation and identities of previously unreported acyl chain oxidation products resulting from the thermal treatment of phytosteryl/-stanyl fatty acid esters. The observed classes of products correspond to those expected from the thermooxidation of cholesteryl fatty acid esters, unsaturated fatty acids, and triglycerides. However, the fact that the approach is not based on the formation of methyl esters via transesterification enabled for the first time the analysis of a spectrum of individual phytosteryl/-stanyl fatty acid esters oxidized in the acyl moiety. This will be a crucial cornerstone of subsequent quantitative studies on the contribution of these oxidation products to the decreases of phytosteryl/-stanyl fatty acid esters upon thermal treatment.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

POP, phytosterol oxidation products; SPE, solid-phase extraction; *m*-CPBA, 3-chloroperbenzoic acid; 4-TsOH, 4-toluenesulfonic acid monohydrate; DCC,, dicyclohexylcarbodiimide; DMAP,, 4-(dimethylamino)pyridine; MTBE, methyl *tert*-butyl ether

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