GC–MS Method for Characterization and Quantification of Sitostanol Oxidation Products

Laura Soupas*, Laura Juntunen, Suvi Säynäjoki, Anna-Maija Lampi, and Vieno Piironen

Department of Applied Chemistry and Microbiology, FIN-00014 University of Helsinki, Finland

ABSTRACT: A new GC–MS method for characterization and quantification of phytosterol oxidation products was developed. Applicability of this method was tested by characterizing sitostanol oxides formed in bulk and then quantifying selected oxides in purified rapeseed oil and tripalmitin matrices in which the complex matrix made oxide analysis difficult. In bulk, nine different sitostanol oxides were characterized, including epimers of 7- and 15-hydroxysitostanol and 6- and 7-ketositostanol. In both lipid matrices, the amounts of sitostanol oxides generated in thermo-oxidation were very low. According to statistical analyses, depending on the oxide, the GC–MS results were the same or slightly higher than those quantified by the more common GC–FID method. Thus, GC–MS provides a powerful alternative for characterization and quantification of phytostanol oxides found in low amounts in complex matrices and is a promising method for future phytosterol oxide studies.

Paper no. J10686 in *JAOCS 81*, 135–141 (February 2004).

KEY WORDS: GC–MS quantification, oxidative stability, phytosterol oxides, sitostanol.

Phytosterols, phytostanols, and their FA esters have found a place in the growing market of functional foods in many countries (1). Phytosterol- and phytostanol-enriched products have been sold as cholesterol-lowering foods for several years without any evidence of hazard (2). However, attention should be paid to those individuals with the inborn trait of impaired phytosterol metabolism (phytosterolemia) and to patients on cholesterol-lowering medication (3).

Owing to their structural similarity with cholesterol, phytosterols may be equally as susceptible to oxidation when exposed to air and heat, for example, during food processing (4). To date, data on the absorption and physiological effects of these oxidized phytosterol compounds are limited: Some phytosterol oxides may pass through the intestinal barrier of rats (5), some oxides show cytotoxic effects on mealworms comparable with those of cholesterol oxides (6), and some inhibit growth in cultured macrophage-derived cell lines, providing information on the toxic action of these oxides (7).

The presence of phytosterol oxides in foods has been studied much less intensively than that of cholesterol oxides. Studies have revealed small amounts of these compounds in wheat flour (8), fried potato products (9,10), and some vegetable oils (10). In potato chips fried in palm oil, sunflower oil, and high-oleic sunflower oil, the sterol oxide contents were 5, 46, and 35 mg/kg in lipids, respectively (9). French fries fried in a rapeseed oil/palm oil blend, sunflower oil, and high-oleic sunflower oil for 15 min at 200°C contained 32, 37, and 54 mg/kg, respectively, of phytosterol oxides in lipids. The above-mentioned oils contained 41, 40, and 47 mg/kg of phytosterol oxides in lipids before frying and 59, 57, and 56 mg/kg of oxides in lipids 2 d after frying (10). In these studies, the main phytosterol oxides identified were 7-hydroxysterols, 7-ketosterols, 5,6-epoxysterols, and triols. Because precise analysis of minor components as oxysterols is difficult, data in this field remain scarce. Furthermore, no information on the oxidation of saturated phytosterol compounds is available.

Separation and quantification of oxysterols in biological and food samples have mainly been performed using GC–FID methods (11). In the case of phytosterol oxides, the complexity of GC analysis is increased: Owing to complex sample matrices and mixtures of many phytosterol oxides, coelution may become a large problem. However, during the last few years the methods for analyzing oxysterols in foods have improved (11,12). As a result of solid-phase extraction (SPE) techniques, the purification process has become more effective (11). Moreover, by using capillary columns, the separation of different phytosterol oxides has become more effective and quantification thus more accurate (11,12). However, to improve these methods further, the possibility of separating and quantifying phytosterol oxides by GC–MS also should be examined (13).

Bearing in mind the complexity of phytosterol oxide extracts and thus the potential of having interfering substances, quantification by GC–MS in the selected ion monitoring (SIM) mode is an effective technique. In some studies, the SIM mode has already been used for quantification of cholesterol oxides and has proved to be a selective and sensitive method for oxysterols. This is possible, of course, only when the selection of characteristic target ions is adequate (14).

The main purpose of this work was to develop a new GC–MS method for the characterization and quantification of phytosterol oxides in foods. The applicability and specificity of this new method was tested by studying the oxidation of sitostanol in purified rapeseed oil and tripalmitin, i.e., having low levels of analytes in difficult matrices. This work contributes to the

*To whom correspondence should be addressed at the Dept. of Applied Chemistry and Microbiology, P.O. Box 27 (Latokartanonkaari 11), FIN-00014 University of Helsinki, Finland. E-mail: laura.soupas@helsinki.fi

TABLE 1
EI-MS (70 eV) Data on Sitostanol and Cholestanol Oxidation Products (TMS ethers)

	M^{+a}	$M^+ - CH_3{}^a$	$M^+ - TMSOH^a$	$M^+ - TMSOH - CH_3{}^a$	Others[a]
Sitostanol oxides					
7α-OH-Sitostanol	576 (1)	561 (6)	**486 (100)**	471 (14)	396 (12), 345 (7), 255 (14), 213 (12), 129 (17)
7β-OH-Sitostanol	576 (1)	561 (49)	**486 (100)**	471 (21)	396 (11), 345 (12), 255 (24), 213 (12), 129 (28)
15β-OH-Sitostanol	576 (—)	561 (12)	486 (75)	471 (17)	396 (15), 345 (35), **269 (100)**, 255 (46), 213 (14), 129 (54)
15α-OH-Sitostanol	576 (—)	561 (11)	486 (48)	471 (11)	396 (13), 345 (25), **269 (100)**, 255 (36), 213 (11), 129 (39)
6α-OH-Sitostanol	576 (29)	561 (32)	486 (78)	471 (26)	396 (67), 345 (7), 255 (22), 213 (29), **204 (100)**, 191 (80)
5β-OH-Sitostanol	576 (2)	561 (23)	486 (79)	471 (8)	396 (26), 345 (5), 255 (17), 213 (12), 147 (97), **143 (100)**
7-Ketositostanol	**502 (100)**	487 (24)	412 (6)	397 (—)	343 (16), 320 (37), 266 (68), 253 (20), 129 (11)
6-Ketositostanol	502 (17)	487 (57)	412 (4)	397 (2)	**473 (100)**, 159 (11), 129 (5)
25-OH-Sitostanol	576 (—)	561 (1)	486 (—)	471 (—)	215 (1), **131 (100)**
Unidentified (RRT 1.779)	576 (10)	561 (2)	486 (13)	471 (7)	445 (88), 397 (79), 357 (73), **147 (100)**, 129 (29)
Cholestanol oxides					
7α-OH-Cholestanol	548 (—)	533 (7)	**458 (100)**	443 (14)	368 (12), 345 (7), 255 (13), 213 (12), 129 (16)
7β-OH-Cholestanol	548 (2)	533 (51)	**458 (100)**	443 (22)	368 (12), 345 (11), 255 (22), 213 (13), 129 (30)
15β-OH-Cholestanol	548 (—)	533 (5)	458 (20)	443 (6)	368 (7), 345 (14), 255 (14), **241 (100)**, 213 (7), 129 (25)
15α-OH-Cholestanol	548 (—)	533 (6)	458 (36)	443 (8)	368 (6), 345 (14), 255 (22), **241 (100)**, 213 (5), 129 (34)
6α-OH-Cholestanol	548 (32)	533 (39)	458 (88)	443 (34)	368 (67), 345 (6), 255 (18), 213 (33), **204 (100)**, 191 (80)
5β-OH-Cholestanol	548 (2)	533 (22)	458 (63)	443 (8)	368 (25), 345 (3), 255 (11), 213 (11), **147 (100)**, 143 (84)
7-Ketocholestanol	**474 (100)**	459 (28)	384 (6)	369 (1)	343 (17), 320 (37), 266 (59), 215 (6), 129 (13)
6-Ketocholestanol	474 (17)	459 (57)	384 (4)	369 (2)	**445 (100)**, 159 (10), 129 (4)
25-OH-Cholestanol	548 (—)	533 (1)	458 (1)	443 (1)	215 (1), **131 (100)**
Unidentified (RRT 1.337)	548 (7)	533 (2)	458 (11)	443 (7)	417 (85), 369 (76), 329 (82), **147 (100)**, 129 (25)

[a]Relative abundances are reported in parentheses and the base peak is in boldface. A dash indicates that the fragment was not observed. TMS, trimethylsilyl; TMSOH, trimethylsilanol; RRT, relative retention time.

information available about the applicability of GC–MS in studies concerning phytosterols and, furthermore, about the oxidation products of sitostanol.

EXPERIMENTAL PROCEDURES

Chemicals and materials. 24α-Ethyl-5α-cholestan-3β-ol (sitostanol; 95%), 5α-cholestan-3β-ol (cholestanol; 95%), cholest-5-ene-3β,19-diol (19-OH-cholesterol), cholest-5-ene-3β,25-diol (25-OH-cholesterol), 5α,6α-epoxy-5α-cholestane-3β-ol (5α,6α-epoxycholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), and 5α-cholestane-3β,5,6β-triol (cholestanetriol) were purchased from Sigma (St. Louis, MO). Cholest-5-ene-3β,7α-diol (7α-OH-cholesterol) and cholest-5-ene-3β,7β-diol (7β-OH-cholesterol) were from Steraloids (Wilton, NH). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) (E. Merck, Darmstadt, Germany) and trimethylchlorosilane (TMCS) (Fluka Chemie, Buchs, Switzerland) were used as a 99:1 mixture for silylation. Pyridine (>99%) (Sigma), anhydrous Na_2SO_4 (E. Merck), diethyl ether (J.T.Baker, Deventer, The Netherlands), KOH (Eka Nobel, Surte, Sweden), hexane and acetone (Rathburn Chemicals, Walkerburn, Scotland), 99.5% ethanol (Primalco, Rajamäki, Finland), and water (purified by Milli-Q Plus, Millipore, Molsheim, France) were used; all were of analytical grade.

Tripalmitin (minimum 85%) (Sigma) and rapeseed oil (Raisio Group, Raisio, Finland) were used as matrices in the thermo-oxidation of sitostanol. For purifying the rapeseed oil, column chromatography was carried out on ASTM aluminum oxide 70–230 mesh (90 active neutral, activity stage I) purchased from E. Merck.

Analytical TLC plates (silica gel 60, 20 × 20 cm) were purchased from E. Merck. Heptane (Rathburn Chemicals) and ethyl acetate (E. Merck) were used as solvents. Spots were visualized by staining with 10% H_2SO_4 (E. Merck) in methanol (Rathburn Chemicals). Before silylation, stanol oxides generated in thermo-oxidation and isolated from TLC plates were filtered through a GH Polypro Acrodisc® 13-mm syringe filter (with a 0.45-μm GH Polypro membrane; Pall Gelman Laboratory, Ann Arbor, MI).

Bond Elut SiOH-SPE cartridges (500 mg; Varian, Harbor City, CA) were used to purify stanol oxides during the thermo-oxidation and TLC studies.

Sample preparation for characterization and preparative TLC studies of sitostanol oxides. For characterization, pure sitostanol (10 mg) was heated in a thermostated oven at 180°C for 3 h. The oxidized sample was dissolved in 5 mL of hexane/diethyl ether (9:1, vol/vol) and purified by the SiOH-SPE method described previously by Lampi *et al.* (13). In brief, the SIOH-SPE cartridge was activated with 5 mL of hexane, after which 1 mL of oxidized sample was applied. The cartridge was washed with 5 mL of hexane/diethyl ether (9:1 vol/vol) and 5 mL of hexane/diethyl ether (1:1 vol/vol) to remove apolar compounds and nonoxidized sterols, respectively. Stanol oxides were eluted with 5 mL of acetone. Cholestanol (10 μg) and 19-OH-cholesterol (10 μg) were

TABLE 2
TLC and GC Data on Sitostanol and Cholestanol Oxidation Products (TMS ethers)

	Sitostanol[a]		Cholestanol[a]		Cholesterol	
Oxidation product	TLC R_f[b]	GC RRT[c]	TLC R_f[b]	GC RRT[c]	TLC R_f[b]	GC RRT[c]
3β,5,6β-Triol					0.04	
7α-OH	F1: 0.07–0.20	1.14	F1: 0.07–0.20	0.93		0.91
15β-OH	F3: 0.40–0.55	1.24	F3: 0.40–0.55	0.99		
15α-OH	F2: 0.20–0.40	1.32	F2: 0.20–0.40	1.05		
7β-OH	F1: 0.07–0.20	1.38	F1: 0.07–0.20	1.09	0.20	1.04
6α-OH	F1: 0.07–0.20	1.41	F1: 0.07–0.20	1.10		
5β-OH	F1: 0.07–0.20	1.52	F1: 0.07–0.20	1.17		
7-Keto	F2: 0.20–0.40	1.65	F2: 0.20–0.40	1.25	0.35	1.34
5α,6α-Epoxy					0.40	1.11
25-OH	F3: 0.40–0.55	1.73	F3: 0.40–0.55	1.33	0.62	1.31
6-Keto	F2: 0.20–0.40	1.74	F2: 0.20–0.40	1.31		
Unidentified (RRT 1.779[d], RRT 1.337[e])	F1: 0.07–0.20	1.78	F1: 0.07–0.20	1.34		
Unoxidized stanol	F4: 0.74		F4: 0.74			

[a]TLC zones of sitostanol and cholestanol were divided into four bands (fractions F1–F4).
[b]Mobility value of oxidation products in relation to that of solvent front (heptane/ethyl acetate: 1:1, vol/vol).
[c]RRT of sitostanol, cholestanol, and cholesterol oxidation products (TMS ethers) in relation to that of 19-OH-cholesterol (TMS ether) (1.00). For abbreviations see Table 1.
[d]Unidentified oxide formed from sitostanol.
[e]Unidentified oxide formed from cholestanol.

added to these acetone extracts to act as references, and the extracts were then dried under nitrogen, dissolved in 100 μL of pyridine, and subjected to silylation by BSTFA/TMCS (100 μL) overnight at room temperature. The reagent was evaporated and trimethylsilyl (TMS) ether derivatives were dissolved in 200 μL of hexane before GC–MS analysis. Oxidized cholestanol, as a reference, was studied similarly (Table 1).

In addition to the aforementioned procedure, the SIOH-SPE oxide fraction of sitostanol was applied to preparative TLC studies. After SPE, this fraction was dried under nitrogen, dissolved in 300 μL of hexane/diethyl ether (9:1, vol/vol), and applied to the TLC plate. Cholestanol oxides were treated in the same way. Cholesterol oxides were also applied to the TLC plate to act as references. The plate was eluted with heptane/ethyl acetate (1:1, vol/vol) (15) and the spots were visualized. On the basis of the reference spots, the visualized stanol oxide zone was divided into four bands (fractions F1–F4) (Table 2), which were then scraped off the plate unstained. The bands were extracted with diethyl ether (2 × 2 mL). Finally, the solutions were filtered and prepared for GC–MS analysis as described above.

S*ample preparation for application to phytostanol-enriched tripalmitin and rapeseed oil matrices.* Tripalmitin and rapeseed oil matrices (1.0 g) added with sitostanol (1%) were heated in a thermostated oven at 180°C for 2 h. Sample preparation for analysis of oxidized sitostanol was based on a method described by Lampi *et al.* (13), which consisted of cold saponification, extraction of unsaponifiable material, purification of sitostanol oxides by SPE, and derivatization to TMS ethers. The following changes were made, however: The amount of 19-OH-cholesterol (internal standard, ISTD) was reduced to 1.5 μg, and before GC–MS analysis, the TMS ethers were dissolved in 100 μL of hexane.

To remove anti- and pro-oxidants, the rapeseed oil was purified. Purification was performed *via* adsorption chromatography using a glass column packed with activated aluminum oxide (16). Purified rapeseed oil contained no detectable amounts of α-, β-, γ-, or δ-tocopherol upon analysis of HPLC (17), in which the detection limits were 5, 5, 5, and 6 μg/g oil, respectively.

Characterization and quantification of sitostanol oxides. GC–MS measurements were performed on a Hewlett-Packard 6890 Series gas chromatograph coupled to an Agilent 5973 mass spectrometer. The injection technique used was on-column injection. GC conditions were as follows: column: Rtx-5MS w/ Integra Guard capillary column (crossbond 5% diphenyl–95% dimethyl polysiloxane; Restek, Bellefonte, PA), film thickness 0.10 μm, 60 m × 0.25 mm i.d.; carrier gas: helium (>99.996%), 1.2 mL/min (constant flow); temperature program: 70°C (1 min), 40°C/min to 280°C (35 min). MS conditions were as follows: interface temperature, 280°C; ion source, 230°C; ionization: electron impact (EI) 70 eV.

Samples were also analyzed by GC–FID using a Hewlett-Packard 5890 Series II gas chromatograph equipped with an automated on-column injection system and an FID. Conditions were as follows: Rtx-5 w/ Integra Guard capillary column (crossbond 5% diphenyl–95% dimethyl polysiloxane; Restek), film thickness 0.10 μm, 60 m × 0.32 mm i.d.; carrier gas: helium (>99.996%) at a constant flow of 1.4 mL/min; temperature program: 70°C (1 min), 60°C/min to 245°C (1 min), 3°C/min to 275°C (41 min); detector temperature: 300°C.

Identification of sitostanol oxides was done with GC–MS in full-scan mode (*m/z* 100–600) (2.94 scans/s). After identification, four compounds were selected based on preliminary tests to act as indicator compounds when studying the oxidation of sitostanol in lipid matrices. The SIM mode was used for quantification of these compounds. For each compound, one target ion and one qualifier ion were chosen on the basis

TABLE 3
Summary of Data on GC–MS Quantification of Sitostanol Oxides (TMS ethers)

Oxidation product	RT (min)[a]	Target ion	Qualifier ion	Time intervals for ion monitoring (min)	r^{2b}	LOD[c]
19-OH-Cholesterol[d]	18.76 ± 0.00	353.3	366.4	17.50–26.00	—	—
7α-OH-Sitostanol	21.31 ± 0.00	486.5	487.5	17.50–26.00	0.997	0.1
6α-OH-Sitostanol	26.38 ± 0.01	486.5	576.5	26.00–41.25	0.994	0.2
5β-OH-Sitostanol	28.31 ± 0.02	486.5	576.5	26.00–41.25	0.997	0.3
Unidentified (RRT 1.779)	33.16 ± 0.01	486.4	576.5	26.00–41.25	0.991	0.6

[a]Mean values and SD of five data points.
[b]Correlation coefficients for calibration curves.
[c]Limit of determination, μg/g matrix.
[d]Internal standard (ISTD). RT, retention time; see Table 1 for other abbreviations.

of their abundance and specificity for the compounds (Table 3). The limits of analyte confirmation were established by calculating ±20% of the qualifier/target ion ratio as measured for the standard. Selected target and qualifier ions were monitored at appropriate time intervals (Table 3).

Calibration curves had to be calculated indirectly, as no phytosterol or phytostanol oxidation products were available. For the calibration, 10 mg of sitostanol was heated (180°C/3 h) and purified as described above. After purification, stanol oxide fractions from each SiOH-SPE column were mixed and the following dilutions were made: 1:2, 1:5, 1:10, 1:25, and 1:75. A fixed amount (1.5 μg) of 19-OH-cholesterol (ISTD) was added to each dilution. Calibration samples were then subjected to silylation as described earlier. The reagent was evaporated, the TMS ethers were dissolved in 150 μL of hexane, and all of the samples were injected onto both the gas chromatograph with an FID and the gas chromatograph–mass spectrometer. The calibration curves for each indicator compound were constructed by plotting response ratios, taken from GC–MS analyses, vs. concentration ratios, calculated from GC–FID analyses. To calculate the sitostanol oxides determined with GC–FID, a general relative response factor of 1.00 was used (13).

GC–MS quantification for each indicator compound was accomplished by monitoring the ratios of the peak areas of the chosen target ions for the corresponding internal standards and by interpolation of these values on the respective calibration curves.

Statistical analyses. Statgraphics Plus 3.0 (Statistical Graphics Corp., Manugistics Inc., Rockville, MD) paired-sample comparison was used to analyze the success of GC–MS quantification compared with GC–FID quantification. In brief, compound concentrations quantified with GC–MS and GC–FID were paired and analyzed by calculating the difference between each value in each pair of observations. A confidence level of 95.0% was used.

RESULTS AND DISCUSSION

Characterization of sitostanol oxides. Characterization of sitostanol oxide TMS ether derivatives was based on their mass spectral and GC properties. To assure characterization, we also studied the mobility of oxides on the TLC plates and then compared all of our results to those of cholestanol and cholesterol oxides.

GC. Figure 1 shows a GC–MS total ion chromatogram of TMS ether derivatives of thermo-oxidized sitostanol. The chromatogram reveals that satisfactory resolutions were achieved with the exception of peaks 4 and 5, which eluted very close to some unidentified peaks, and peaks 10 and 11, which were not fully separated. The internal standard (19-OH-cholesterol) (peak 2) was well-separated. In addition to nine characterized sitostanol oxides, other polar compounds, including unidentified sitostanol oxides and some impurities, were observed.

Previous studies (13,18,19) enabled us to make the assumption that when using nonpolar GC columns the hydroxylation of the $C_{27/29}$-sterol side chain and the presence of oxo groups in the ring structure cause more retention in the GC column than the presence of the hydroxyl group in the ring structure. Moreover, the hydroxylation of the ring structure causes an increase in retention times: 5β-Hydroxylated oxides are retained the most, followed by 5α-, 6-, and 7-hydroxylated oxides. Although a large variation in retention times can exist, the order of elution here yielded tentative information about the structures of the sitostanol oxides.

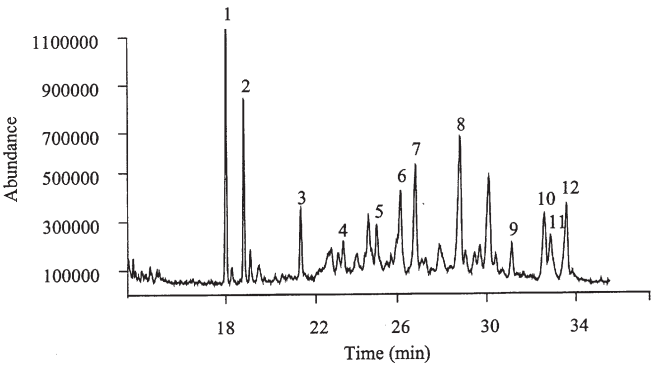

FIG. 1. Total ion chromatogram of GC–MS separation of trimethylsilyl ether derivatives of thermo-oxidized sitostanol (180°C/3 h) on an Rtx-5MS w/ Integra Guard capillary column (60 m × 0.25 mm i.d., 0.1 μm film; Restek, Bellefonte, PA): (1) cholestanol, (2) 19-OH-cholesterol (internal standard), (3) 7α-OH-sitostanol, (4) 15β-OH-sitostanol, (5) 15α-OH-sitostanol, (6) 7β-OH-sitostanol, (7) 6α-OH-sitostanol, (8), 5β-OH-sitostanol, (9) 7-ketositostanol, (10) 25-OH-sitostanol, (11) 6-ketositostanol, and (12) unidentified compound (relative retention time 1.779).

MS. Further information was obtained through EI mass spectra. The principal fragments and their relative abundances are listed in Table 1. The molecular ions (M^+) were observed for all sitostanol oxides characterized, excluding epimers of 15-hydroxysitostanol and 25-hydroxysitostanol. The M.W. of epimers of 15-hydroxysitostanol were deduced from the fragments at *m/z* 486 (loss of trimethylsilanol, M^+ – TMSOH). 25-Hydroxysitostanol was identified on the basis of the fragment at *m/z* 131 resulting from the α-cleavage at C_{24}–C_{25} (20).

In addition to the loss of TMSOH, a certain pattern was observed in the fragmentation of hydroxyl derivatives of sitostanol, i.e., the occurrence of fragments $M^+ - CH_3$, M^+ – TMSOH – CH_3, and M^+ – 2TMSOH. Also, ions at *m/z* 345, 255, and 213, originating from M^+ – side chain – TMSOH, M^+ – side chain – 2TMSOH, and M^+ – 2TMSOH – side chain – C_3H_6, respectively, were characteristic of these hydroxyl derivatives. Corresponding fragmentations have been observed for 5- and 7-hydroxy TMS ether derivatives of phytosterols (21).

The following fragmentations were also used to characterize sitostanol oxide TMS ether derivatives: fragments at *m/z* 143 for 5β-hydroxysitostanol, at *m/z* 191 and 204 for 6α-hydroxysitostanol, at *m/z* 269 for epimers of 15-hydroxysitostanol, at *m/z* 473 for 6-ketositostanol, and at *m/z* 266, 343, and 502 for 7-ketositostanol. As can be seen in Table 1, fragmentation patterns for the sitostanol oxides were similar to those obtained for the corresponding cholestanol derivatives, with sitostanol oxide fragments being 28 mass units higher. Some fragments were common to both sitostanol and cholestanol derivatives and were also recorded in similar relative abundances, indicating that these fragments contained no side chains. These observations are similar to those of Aringer and Nordström (18).

TLC. The R_f values of TLC bands of sitostanol oxides (F1–F4) are shown in Table 2. Comparable values of cholesterol and cholestanol oxides, as references, are also listed. The GC analyses of these roughly divided TLC bands gave supplementary structural information, although the presence of some unidentified oxidation products and impurities was obvious; in each band, at least four peaks were observed during GC–MS analyses. By comparing the R_f values on TLC and the elution order in GC, the following assumptions could be made: F1 consisted of the hydroxyl derivatives of the stanol ring structure (A,B ring); F2 consisted of the hydroxyl derivatives of the stanol ring structure (A,D ring) as well as the ketone derivatives of the stanol ring structure; F3 consisted of the hydroxyl derivatives of the stanol side chain as well as the hydroxyl derivatives of the stanol ring structure (A,D ring); and F4 consisted of unoxidized stanol. These results are in accord with previous studies of cholesterol oxides (22). Moreover, similar elution orders were observed for cholestanol oxides by Aringer and Nordström (18), although they developed the TLC plate using diethyl ether/cyclohexane (9:1, vol/vol).

The characterization of sitostanol oxides was accomplished by combining the results of TLC, GC, and MS and further comparing this data with literature (18). Using these pieces of information, we could also deduce whether the hydroxyl derivatives of stanols were in α- or β-form. These interpretations were strongly based on characteristic mass fragments and the premise that β-epimers are more mobile on TLC (18). A more conclusive evaluation of these epimers requires other analytical techniques.

Despite all of the information available, the structure of one interesting compound [unidentified, relative retention time (RRT) of 1.779] remained unresolved. Based on its mobility on TLC, it seemed to be a hydroxyl derivative of the stanol ring structure (A,B ring). In GC, it eluted just after 25-hydroxysitostanol, revealing that it was instead a ketone or hydroxyl derivative of the stanol side chain. MS fragmentation of this compound uncovered some characteristic fragments of hydroxyl derivatives, but three fragments (*m/z* 357, 397, 445) not characteristic of hydroxyl derivatives were also present. The same compound was also seen in oxidized cholestanol. Although this compound could have been impure, we chose to use it as a marker of stanol oxidation in the application described in the next section.

Application of GC–MS to phytostanol-enriched lipid matrices. To test the applicability of GC–MS for quantification of sitostanol oxidation products, the following compounds were selected: 7α-hydroxy-, 6α-hydroxy-, and 5β-hydroxysitostanol, and the unidentified oxide of RRT 1.779. Linear calibration curves were obtained for these compounds over the range of 0.1–6.3 (7α-hydroxysitostanol), 0.2–15.5 (6α-hydroxysitostanol), 0.3–12.7 (5β-hydroxysitostanol), and 0.6–10.7 μg/g (unidentified, RRT of 1.779) of matrix. Limits of determination were the lowest levels of these curves. More information on the calibration is presented in Table 3.

The results of applying this method to sitostanol oxides in tripalmitin and purified rapeseed oil are shown in Table 4. As expected, the amounts of sitostanol oxides generated in thermo-oxidation (180°C, 2 h) were very low, i.e., for 7α-hydroxysitostanol 4.0 μg/g matrix in tripalmitin and 2.0 μg/g matrix in purified rapeseed oil. In previous studies on the oxidation of phytosterols in thermo-oxidized (180°C, 2 h) natural oils, the amounts of 7α-hydroxysterols ranged from 17.8 to 55.7 μg/g oil (4). Factoring in the different levels of sterols (<1%) in these oils and stanols (1%) in our samples, the greater stability of sitostanol is obvious. Furthermore, the amount of 7α-hydroxysitostanol formed in these deep-frying conditions was smaller than, for example, the amount of 7α-hydroxysitosterol in refined vegetable oils (4).

The CV in triplicate experiments ($n = 6$) of 7α-hydroxysitostanol, 6α-hydroxysitostanol, and unidentified, RRT of 1.779, were 12.5, 14.1, and 5.8% in tripalmitin and 12.2, 8.3, and 8.5% in rapeseed oil, respectively. These values were in agreement with the Horwitz curve, illustrating that for analytes present in the μg/g range, CV values of up to 20% are acceptable (23). When comparing the results between GC–MS and GC–FID analyses, we noticed that the indirect construction of calibration curves had only a minor effect on quantification. *P*-values for the *t*-test evaluating differences

TABLE 4
Formation of Some Sitostanol Oxides (TMS ethers) in Tripalmitin and Purified Rapeseed Oil Matrices When Heated at 180°C (2 h)

	Tripalmitin matrix[a]		Purified rapeseed oil matrix[a]	
Oxidation product	GC–MS[b]	GC–FID[b]	GC–MS[b]	GC–FID[c]
7α-OH-Sitostanol	4.0 ± 0.5	3.3 ± 0.2	2.0 ± 0.2	—
6α-OH-Sitostanol	6.2 ± 0.9	5.8 ± 0.6	5.1 ± 0.4	—
5β-OH-Sitostanol[d]	—	2.9 ± 0.2	—	—
Unidentified (RRT 1.779)	2.3 ± 0.1	2.1 ± 0.1	8.0 ± 0.7	—

[a]Sitostanol added at a 1% level.
[b]μg/g matrix; mean values and SD of triplicate experiments (n = 6).
[c]19-OH-cholesterol (ISTD) overlapped with matrix. See Tables 1 and 3 for abbreviations.
[d]The selected target ion was not sufficiently specific in GC–MS quantification.

in each pair of observations (GC–MS vs. GC–FID, n = 6) in tripalmitin samples were P = 0.0025 for 7α-hydroxysitostanol, P = 0.0860 for 6α-hydroxysitostanol, and P = 0.0121 for unidentified (RRT of 1.779). Thus, the means were significantly different for 7α-OH and unidentified RRT of 1.779, but not for 6α-OH. Generally, the amounts of oxides determined by GC–MS were slightly higher than those obtained by GC–FID (Table 4). However, it is noteworthy that the above-mentioned statistical differences between these two methods of quantification could still be described as "insignificant" when compared with interlaboratory studies of cholesterol oxides having CV values of up to 240% (24).

We concluded that the overall performance of GC–MS in sitostanol oxide analyses was quite good, but a few points need to be highlighted. The selection of characteristic target ions should be done with care when setting up quantification. In this study, the selected target ion was not sufficiently specific for 5β-OH-sitostanol; the base peak at *m/z* 143 could have been better. The target ion used at *m/z* 486 (M^+ – TMSOH) was probably interfered with by background fragment ions, making quantification difficult. Moreover, the limits of analyte confirmation in the SIM procedure (qualifier/target ion, ±20%) sometimes extended beyond this arbitrarily established range—mainly because of the small oxide concentrations and difficult matrices.

While quantifying with GC–FID, an interesting analytical situation arose: Owing to overlapping of the internal standard with 7α-OH-campesterol in the purified rapeseed oil matrix, we could not quantify oxides of the added sitostanol in purified rapeseed oil. This emphasizes the specificity and selectivity of GC–MS in studies like this. An effective purification system is not always sufficient.

The GC–MS method presented herein proved to be a promising alternative for determination of a large number of compounds at low levels in complex matrices such as foods. Using this method, we were able to characterize and further quantify very low levels of sitostanol oxides in difficult lipid matrices both reliably and reproducibly. New information about the stability of sitostanol was uncovered, indicating that thermo-oxidation of sitostanol does not lead to significant oxidation reactions. Furthermore, this method can be readily applied to future phytosterol studies.

ACKNOWLEDGMENTS

The authors would like to thank Laura Holkeri for providing technical assistance. This work was supported by the National Technology Agency of Finland, some Finnish food companies, and the Finnish Graduate School on Applied Bioscience.

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[Received July 1, 2003; accepted November 21, 2003]