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Effects of Sterol Structure, Temperature, and Lipid Medium on Phytosterol Oxidation

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Factors contributing to the oxidative stability of phytosterols were studied. Unsaturated stigmasterol and saturated sitostanol were used as model compounds and were heated at different temperatures in different lipid matrices for various periods of time. Accumulations of the major secondary oxidation products were used as a marker of the stability of heated compounds, and the products were analyzed by gas chromatography−mass spectrometry. The results showed that both temperature and heating time, as well as sterol structure and lipid matrix composition, affected phytosterol oxidation. In particular, the interactions between different lipid matrices and temperatures had drastic effects on the total contents of the phytosterol oxides formed and also on the reaction pathways of oxidation. During heating at high temperatures for prolonged periods, >20% of stigmasterol was oxidized. At moderate temperatures the oxidation of stigmasterol was rather slow. Sitostanol oxide contents were low under all heating conditions studied.

KEYWORDS: Phytosterol oxides; phytostanol oxides; gas chromatography−mass spectrometry; thermo-oxidation; lipid medium

INTRODUCTION

Lipid oxidation is one of the principal deteriorating reactions in food products during processing and storage. The introduction of phytosterol-enriched foods to the market has given rise to questions about the oxidative stability of phytosterols as minor lipid components. As was recently shown, phytosterols may undergo oxidation in food-processing conditions, forming mainly such oxidation products as 7-hydroxysterols, 7-ketosterols, 5,6-epoxysterols, triols, and 25-hydroxysterols (*1*−*4*).

The possible adverse health effects of phytosterol oxides are under investigation. Adcox et al. (*5*) showed that phytosterol oxides exhibit patterns of toxicity similar to those of their structural counterparts, cholesterol oxides, in cultured macrophage-derived cell lines. However, the effects of phytosterol oxides were less severe than those of cholesterol oxides. Maguire et al. (*6*) also reported phytosterol oxides to exhibit toxicity similar to that of cholesterol oxides in cultured mammalian cells and further suspected that individual phytosterol oxides may behave differently from oxide mixtures. On the other hand, one recent toxicity study showed that phytosterol oxides did not possess genotoxic potential and no obvious evidence of toxicity when administered in the diet of rats at mean dietary phytosterol oxide concentrations up to 0.443−0.450% (w/w) (*7*).

As a part of the safety evaluation of phytosterol oxides, their absorption should also be studied. It was recently shown that phytosterol oxides were present in the plasma of healthy human subjects (*8*). However, the source of the oxides was not known: it is possible that they were absorbed and were thus of food origin, but they could also have originated from the in vivo oxidation of phytosterols in plasma. It was also observed that phytosterol oxides were present in markedly elevated concentrations in the serum of sitosterolemic patients (*9*). In this study the oxide concentrations in the serum of healthy control subjects were below the limit of detection.

Until more is known about the health effects and absorption of phytosterol oxides, there is a need to know the concentrations and distribution of different oxides in enriched foods and to study the factors affecting their stability. For example, in one commercial spread enriched with phytosterol esters, 0.1% of phytosterols were oxidized, allowing the ingestion of 2−4 mg of phytosterol oxides at a recommended daily intake of 2−4 g of phytosterols (*10*). In our previous study we observed minor phytosterol oxidation in phytosterol-enriched (1.5%) wheat bread; in bread crusts and toasted bread, 0.3 and 0.1% of sitosterol and 0.4 and 0.1% of campesterol were oxidized, respectively (*11*).

As phytosterol-enriched products are marketed for frying purposes, and as they may be exposed to many kinds of heat treatments during food processing, temperature is among the most important factors affecting their oxidative stability, as the rate of oxidation is related to temperature (*12*). The stability of phytosterols is also influenced by interactions between lipid components in foods (*4*, *13*) and/or their decomposition products, as was observed in cholesterol oxidation studies (*14*, *15*).

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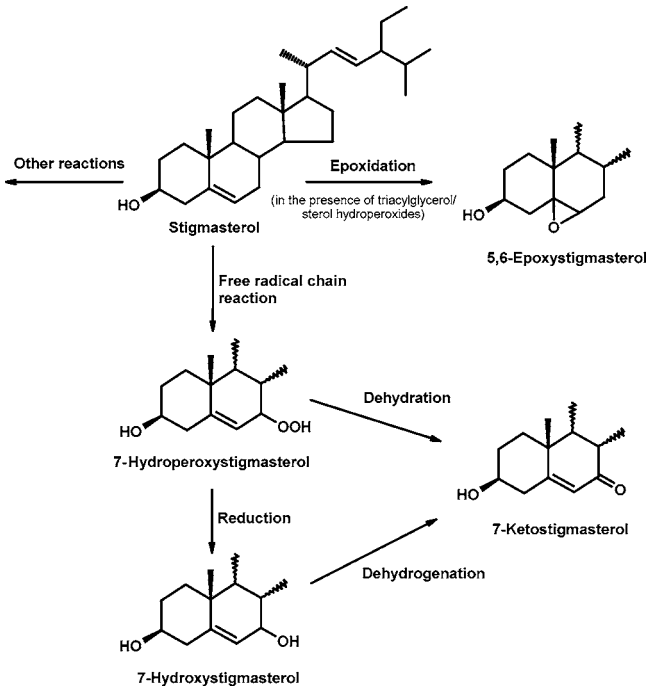

Figure 1. Reaction pathways for stigmasterol oxide formation. 7-Hydroperoxy-, 7-hydroxy-, and 5,6-epoxystigmasterols shown can undergo epimerization to form both α- and β-epimers. Sitostanol, which was also studied, is the saturated form of stigmasterol.

Although a number of studies concerning the oxidation of sterols, especially cholesterol, in different foodstuffs and processing conditions have been published (*1*−*4*, *14*−*17*), no systematic evaluation has been performed to demonstrate how the oxidation of sterols proceeds, for example, during heat treatments. Considering the fact that individual sterol oxides may have different biological activities, more needs to be known about their accumulation and possible changes in their distribution in different conditions. It is known that in lipid oxidation several different reaction pathways are active, with one or more pathways predominating (*18*). As an example, the studies of Chien et al. (*19*) have shown that in the early stage of cholesterol oxidation (at 150 °C) the rate constant is highest for 7-hydroperoxides, followed by epoxidation, dehydration, reduction, and dehydrogenation (**Figure 1**).

The aim of our study was to examine factors contributing to the stability of phytosterols in enriched foods. This was accomplished by analyzing the accumulation and distribution of the main secondary oxidation products of phytosterols in different conditions. Variables in this study were phytosterol structure (unsaturated stigmasterol and saturated sitostanol) (**Figure 1**), lipid matrix (purified rapeseed oil and tripalmitin), temperature (60−180 °C), and heating time (0.5 h−8 weeks). The heating conditions used were rather drastic because the experiment was also designed to find the critical conditions in which significant oxidation reactions may occur.

MATERIALS AND METHODS

Materials and Reagents. 3β-Hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol; 95%), 24α-ethyl-5α-cholestan-3β-ol (sitostanol; 96%), cholest-5-ene-3β,19-diol (19-OH-cholesterol), 3β-hydroxy-5α-cholestane (dihydrocholesterol; 95%), and tripalmitin (minimum 85%) were purchased from Sigma (St. Louis, MO), and rapeseed oil was from Raisio Group (Raisio, Finland). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from E. Merck (Darmstadt, Germany) and Fluka Chemie (Buchs, Switzerland), respectively, and were used as a 99:1 (v/v) mixture for silylation. Analytical grade pyridine (>99%) from Sigma and anhydrous Na_2SO_4 from E. Merck were also used. Bond Elut SiOH solid-phase extraction (SiOH-SPE) cartridges (500 mg), which were applied in the purification of phytosterol/-stanol oxides, were purchased from Varian (Harbor City, CA). Aluminum oxide 70−230 mesh ASTM (90 active neutral, activity stage I) from E. Merck was used in rapeseed oil purification.

Purification of Rapeseed Oil. Adsorption chromatography was used for purifying rapeseed oil from pro- and antioxidants as described by Lampi et al. (*20*) with minor changes: a glass column (48 cm × 3 cm i.d.) was packed with 210 g of aluminum oxide activated at 100 °C for 16 h and then at 200 °C for 8 h, suspended in hexane, and washed with 350 mL of hexane after sample introduction. The purified oil fraction did not contain detectable amounts of α-, β-, γ-, or δ-tocopherol based on HPLC analysis (*21*), in which the detection limits were 5, 5, 5, and 6 μg/g of oil, respectively.

Sample Treatment. Purified rapeseed oil and tripalmitin were enriched with stigmasterol and sitostanol at a level of 1%. Enriched samples (1.0 g) were heated in open 22 × 46 mm glass vials (Brown Chromatography Supplies Inc., Wertheim, Germany) in an oven for various periods of time at different temperatures. Samples were shaken several times during heating. Vials were taken from the oven at each sampling point and cooled in a desiccator before analyses. The heating experiments were performed three times, and each sample was analyzed in duplicate.

Analysis of Phytosterol/-stanol Oxides. Analysis of phytosterol/-stanol oxides from heated samples was carried out according to an internal standard (ISTD) method in which the main steps were cold saponification (overnight at 25 °C), extraction of unsaponifiable material with diethyl ether, purification of oxides by SiOH-SPE, derivatization of oxides to TMS ethers, and analysis by GC-MS (*1*, *22*). The following changes to this previously described method were made: the amount of 19-OH-cholesterol (ISTD) was reduced to 15.0, 3.0, or 1.5 μg depending on the amount of oxides formed in heating experiments, and before GC-MS analysis the TMS ethers were dissolved in 100−400 μL of hexane depending on the amount of oxides.

Analysis of Nonoxidized Phytosterols/-stanols. Phytosterol/-stanol contents of unheated enriched lipid samples were analyzed using a previously published direct hot saponification method (*1*, *23*) with minor modifications. In brief, lipid samples (0.25 g) were weighed into a 50-mL test tube. The internal standard, dihydrocholesterol (0.4 mg) was added, and the samples were subjected to hot saponification and extraction of unsaponifiables with 20 mL of hexane/diethyl ether (1:1, v/v) after the addition of 12 mL of water. All of the samples were analyzed as TMS derivatives by GC-FID.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS was used for the identification (full scan mode, *m*/*z* 100−600) and quantification (selected ion monitoring, SIM, mode) of phytosterol/-stanol oxide TMS ethers. Measurements were performed with a Hewlett-Packard 6890 series gas chromatograph (Wilmington, DE) coupled to an Agilent 5973 mass spectrometer (Palo Alto, CA). The injection technique used was on-column injection. GC conditions were as follows: column, 60 m × 0.25 mm i.d., 0.10 μm, Rtx-5MS w/Integra Guard (crossbond 5% diphenyl−95% dimethyl polysiloxane) capillary column (Restek, Bellefonte, PA); carrier gas, helium (>99.996%) at a constant flow of 1.2 mL/min; temperature program, 70 °C (1 min), 40 °C/min to 280 °C (35 min). The MS interface temperature was 280 °C and the ion source 230 °C. Electron ionization energy was 70 eV.

GC-MS calibration curves for sitostanol oxides were prepared indirectly by plotting response ratios, taken from GC-MS analyses, versus the concentration ratios, calculated from GC-FID analyses, as described previously (*22*). Sitostanol oxide TMS derivative quantification was achieved by SIM acquisition of the following target and qualifier ions: *m*/*z* 353.3 and 366.4 (19-OH-cholesterol, ISTD), *m*/*z* 486.5 and 487.5 (7α-OH-sitostanol), *m*/*z* 486.5 and 576.5 (6α-OH-sitostanol), and *m*/*z* 486.4 and 576.5 (unidentified compound of RRT 1.779), respectively (*22*). The same indirect method was used for calibration of stigmasterol oxides, but with the following modifications: to construct the calibration curves, stigmasterol was heated at 180 °C for 1 h and after SiOH-SPE purification, sample dilutions of 1:10, 1:25, 1:50, 1:75, 1:100, 1:200, and 1:500 were made using a fixed

Table 1. Comparison of Stigmasterol Oxide Concentrations (Micrograms per Gram of Matrix ± Standard Deviation) ($n = 6$) Quantified by GC-MS and GC-FID[a]

	7α-OH-stigmasterol		7β-OH-stigmasterol		5β,6β-epoxystigmasterol		5α,6α-epoxystigmasterol		7-ketostigmasterol	
treatment	GC-MS	GC-FID	GC-MS	GC-FID	GC-MS	GC-FID	GC-MS	GC-FID	GC-MS	GC-FID
					Rapeseed Oil					
180 °C, 0.5 h	19.4 ± 2.1 a	13.4 ± 1.4 b	26.3 ± 2.5 a	21.9 ± 3.9 b	15.7 ± 1.8 a	14.9 ± 2.4 a	14.6 ± 0.7 a	15.3 ± 1.4 a	20.6 ± 0.7 a	11.7 ± 0.6 b
1 h	45.6 ± 5.5 a	33.4 ± 3.1 b	68.4 ± 3.7 a	56.7 ± 3.3 b	31.2 ± 5.8 a	37.9 ± 5.5 b	29.4 ± 1.8 a	26.5 ± 3.0 b	24.1 ± 0.8 a	15.7 ± 1.1 b
2 h	108.3 ± 4.5 a	74.2 ± 2.3 b	161.9 ± 8.5 a	125.2 ± 5.6 b	76.1 ± 3.4 a	91.5 ± 4.1 b	66.6 ± 6.4 a	57.4 ± 2.3 b	32.7 ± 0.5 a	27.3 ± 0.6 b
3 h	161.5 ± 17.2 a	106.2 ± 8.5 b	240.8 ± 13.1 a	180.3 ± 9.4 b	119.5 ± 18.0 a	139.5 ± 16.8 b	111.9 ± 5.2 a	93.1 ± 4.1 b	52.9 ± 3.8 a	48.2 ± 4.6 b
					Tripalmitin					
100 °C, 3 h	0.8 ± 0.0 a	0.8 ± 0.4 a	1.2 ± 0.1 a	0.9 ± 0.3 a	1.2 ± 0.1 a	1.1 ± 0.3 a	1.3 ± 0.1 a	1.0 ± 0.2 b	1.9 ± 0.1 a	1.3 ± 0.0 b
6 h	0.9 ± 0.1 a	0.7 ± 0.2 a	1.3 ± 0.1 a	0.9 ± 0.0 b	1.3 ± 0.2 a	1.3 ± 0.3 a	1.4 ± 0.1 a	1.3 ± 0.1 a	2.0 ± 0.1 a	1.6 ± 0.1 b
24 h	1.3 ± 0.1 a	1.4 ± 0.5 a	1.7 ± 0.2 a	1.9 ± 0.5 a	1.5 ± 0.2 a	2.3 ± 0.5 b	2.5 ± 0.1 a	3.2 ± 0.3 b	2.8 ± 0.2 a	4.0 ± 0.5 b
48 h	3.4 ± 0.3 a	3.6 ± 0.4 b	3.9 ± 0.4 a	4.3 ± 0.5 b	4.2 ± 0.5 a	7.2 ± 1.1 b	7.8 ± 0.4 a	9.3 ± 0.6 b	10.6 ± 1.0 a	13.7 ± 1.0 b

[a] Results followed by the same letter were not significantly different between GC-MS and GC-FID analysis ($P > 0.05$) of that oxidation product for that time point of heating.

amount (1.5 μg) of 19-OH-cholesterol as an internal standard. Stigmasterol oxide TMS derivative quantification was achieved by SIM acquisition of the following target and qualifier ions: *m*/*z* 353.0 and 366.0 (19-OH-cholesterol, ISTD), *m*/*z* 482.0 and 483.0 (7α- and 7β-OH-stigmasterol), *m*/*z* 253.0 and 500.0 (5α,6α- and 5β,6β-epoxystigmasterol), and *m*/*z* 359.0 and 498.0 (7-ketostigmasterol), respectively. As the GC-FID peak of 5β,6β-epoxystigmasterol was impure, the calibration of its 5α,6α-isomer was applied.

Gas Chromatography (GC). GC-FID was used as a tool for the construction of GC-MS calibration curves of phytosterol/-stanol oxides as described above and for the quantification of enriched lipid samples to analyze their stigmasterol/sitostanol contents before heat treatments (*1*). Analyses were carried out using a Hewlett-Packard 5890 series II GC (Karlsruhe, Germany) equipped with an automated on-column injection system and a flame ionization detector. Conditions were as follows: column, 60 m × 0.32 mm i.d., 0.10 μm, Rtx-5 w/ Integra Guard (crossbond 5% diphenyl−95% dimethyl polysiloxane) capillary column (from Restek); 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 (32 min); detector temperature, 300 °C (*1*).

Statistical Analysis. Statgraphics Plus 4.0 (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 analyzed by calculating the difference between each value in each pair of observations.

To analyze the differences in phytosterol/-stanol oxide formation between different heat treatments, a nonparametric Kruskal−Wallis test and a notched box-and-whisker plot were used. The Kruskal−Wallis test, comparing the medians instead of the means, was selected because the data were not normally distributed. A confidence level of 95.0% was used in all statistical analyses.

RESULTS AND DISCUSSION

GC-MS Analysis. The main stigmasterol oxides formed during heating experiments were the epimers of 7-OH-stigmasterol, the epimers of 5,6-epoxystigmasterol, and 7-ketostigmasterol (**Figure 1**). Traces of 25-OH-stigmasterol were also present. During heating, saturated sitostanol also oxidized, and as reported before (*22*), 7α-OH-sitostanol, 6α-OH-sitostanol, and an unidentified compound, at relative retention time (RRT) of 1.779, were selected as markers of its oxidation. The unidentified compound of RRT 1.779 was studied because its abundance was relatively high (*22*).

Linear calibration curves for sitostanol oxides were obtained over the ranges of 0.1−6.3 μg/g (7α-OH-sitostanol), 0.2−15.5 μg/g (6α-OH-sitostanol), and 0.6−10.7 μg/g (unidentified compound of RRT 1.779) of lipid matrix. For stigmasterol oxides the linear ranges of calibration curves were dependent on the amount of internal standard (19-OH-cholesterol) added to the sample. In brief, by adding levels of 1.5−15 μg of internal standard, the following linear ranges were obtained: 0.3−189 μg/g (7α-OH-stigmasterol), 1.2−293 μg/g (7β-OH-stigmasterol), 1.0−212 μg/g (5α,6α-epoxy- and 5β,6β-epoxystigmasterol), and 1.6−283 μg/g (7-ketostigmasterol) of lipid matrix. The limits of determination used were always the lowest levels of these curves. Regression analyses for the linearity of the calibration curves showed good correlation for both sitostanol ($r^2 = 0.991-0.997$) and stigmasterol ($r^2 = 0.994-0.998$) oxides.

As GC-MS quantification is still infrequently applied as a technique in phytosterol oxide studies, all of the samples were also simultaneously analyzed by GC-FID. **Table 1** shows an example of the success of GC-MS quantification compared with GC-FID quantification. Generally, the amounts of oxides determined by GC-MS were slightly higher than those analyzed by GC-FID. The GC-MS quantification succeeded best for stigmasterol oxides in tripalmitin (**Table 1**) due to a more optimal internal standard (19-OH-cholesterol) amount in these samples. We observed that when using the indirect construction of calibration curves the amount of internal standard compared with analytes in the samples was critical for accurate quantification. As the prediction of the oxide formation in different heat treatments was difficult at the beginning of this study, the amount of internal standard was not always optimal.

When attention was paid to internal standard addition, the use of GC-MS in SIM mode was concluded to be a powerful quantification technique. Because of its selectivity, we were able to quantify low levels of stigmasterol and sitostanol oxides in difficult lipid matrices. Due to overlapping of 19-OH-cholesterol (ISTD) with purified rapeseed oil matrix, the GC-FID quantification was not possible in all of the samples. The specificity of the GC-MS-SIM technique allowed us to make this quantification.

Some examples of standard deviations in triplicate heating experiments are shown in **Table 1**. The coefficients of variation (CV) are not reported, but were typically in the range from 0 to 20%, being in agreement with the Horwitz curve (*24*). However, some CV values were >20%, especially in the case of 5β,6β-epoxystigmasterol, which was quantified using the calibration curve of 5α,6α-epoxystigmasterol. High CV values for sterol epoxides, quantified using the same calibration, were also observed in previous studies (*17*). In our study, high CV values were also obtained for sitostanol oxides quantified after heating for 4 weeks at 80 °C.

Table 2. Formation of Stigmasterol Oxides[a] (Micrograms per Gram of Matrix) during Different Heat Treatments in Stigmasterol-Enriched (1%) Tripalmitin

treatment	7α-OH	7β-OH	5β,6β-epoxy	5α,6α-epoxy	7-keto	total	%[b]
none[c]	1.1	1.5	1.5	1.3	2.1	7.6 a	0.1
80 °C, 1 week	1.9	2.4	2.9	3.5	4.4	15.2 b	0.2
2 weeks	11.1	14.0	18.4	24.6	47.6	115.7 c	1.2
3 weeks	74.2	96.7	158.5	201.0	360.1	890.5 d	9.5
4 weeks	173.5	221.7	427.0	607.5	721.9	2151.6 e	23.0
100 °C, 3 h	0.8	1.2	1.2	1.3	1.9	6.4 a	0.1
6 h	0.9	1.3	1.3	1.4	2.0	6.9 a	0.1
24 h	1.3	1.7	1.5	2.5	2.8	9.7 b	0.1
48 h	3.4	3.9	4.2	7.8	10.6	30.0 c	0.3
140 °C, 0.5 h	1.7	2.1	2.3	2.4	4.3	12.9 b	0.1
1 h	2.8	3.4	4.4	5.5	9.0	25.1 c	0.3
3 h	26.7	39.4	35.8	56.7	110.9	269.4 d	2.9
6 h	92.0	109.0	112.5	182.6	250.4	746.5 e	8.0
180 °C, 0.5 h	32.5	33.0	39.5	59.7	55.0	219.8 b	2.3
1 h	116.7	102.5	96.0	148.8	99.0	563.1 c	6.0
2 h	428.3	260.4	252.0	397.6	168.7	1507.0 d	16.1
3 h	652.3	323.6	407.0	612.4	272.4	2267.6 e	24.2

[a] For each temperature the contents of total oxides followed by different letters were significantly different ($P < 0.05$) ($n = 6$). For full compound names see **Table 1**. [b] Percentage of total oxides of the original nonoxidized stigmasterol. [c] The untreated sample was the same for all temperatures.

Table 3. Formation of Stigmasterol Oxides[a] (Micrograms per Gram of Matrix) during Different Heat Treatments in Stigmasterol-Enriched (1%) Purified Rapeseed Oil[b]

treatment	7α-OH	7β-OH	5β,6β-epoxy	5α,6α-epoxy	7-keto	total	%[c]
none[d]	0.8	1.4	1.4	1.2	1.9	6.6 a	0.1
60 °C, 1 day	44.4	38.8	8.9	5.4	36.2	133.8 b	1.4
2 days	138.2	136.0	24.8	15.1	78.2	392.3 c	4.2
3 days	251.2	268.9	49.3	37.0	118.6	725.1 d	7.7
7 days	648.6	853.7	226.7	217.6	220.1	2166.7 e	23.2
100 °C, 3 h	24.1	22.2	2.3	4.4	25.5	76.8 b	0.8
6 h	67.3	69.8	5.5	13.3	56.9	204.9 c	2.2
24 h	375.4	507.6	52.1	136.8	165.5	1237.5 d	13.2
48 h	697.4	889.7	135.8	401.3	359.9	2484.1 e	26.5
140 °C, 0.5 h	4.4	4.8	3.6	2.4	8.3	23.4 b	0.3
1 h	13.7	14.5	8.7	5.6	18.8	61.2 c	0.7
3 h	71.9	92.3	45.7	35.1	51.1	296.1 d	3.2
6 h	187.5	256.8	130.1	106.9	78.0	759.3 e	8.2
180 °C, 0.5 h	19.4	26.3	15.7	14.6	20.6	96.6 b	1.0
1 h	45.6	68.4	31.2	29.4	24.1	198.6 c	2.1
2 h	108.3	161.9	76.1	66.6	32.7	445.6 d	4.8
3 h	161.5	240.8	119.5	111.9	52.9	686.8 e	7.4

[a] For each temperature the contents of total oxides followed by different letters were significantly different ($P < 0.05$) ($n = 6$). For full compound names see **Table 1**. [b] Rapeseed oil was purified from pro- and antioxidants using adsorption chromatography. [c] Percentage of total oxides of the original nonoxidized stigmasterol. [d] The untreated sample was the same for all temperatures.

When evaluating the above-mentioned standard deviations and CV values, one must bear in mind that the experimental data came from three different heating experiments, all analyzed in duplicate, and were thus influenced both by the methodology used and by the nature of lipid oxidation. Such a complex phenomenon as lipid oxidation is difficult to control, especially under slow oxidation and during prolonged stability tests (*25*).

Effect of Heating Temperature and Time on Phytosterol/-stanol Oxidation. Changes in individual and total stigmasterol and sitostanol oxide contents during different heat treatments are shown in **Tables 2**−**5**. The initial stigmasterol and sitostanol oxide levels were low in both enriched lipid matrices (<2.1 μg/g). The total contents of stigmasterol and sitostanol oxides

Table 4. Formation of Sitostanol Oxides[a] (Micrograms per Gram of Matrix) during Different Heat Treatments in Sitostanol-Enriched (1%) Tripalmitin

treatment	7α-OH-sitostanol	6α-OH-sitostanol	RRT 1.779[b]	total	%[c]
none[d]	0.3	<0.2	<0.6	0.3 a	0.003
80 °C, 2 weeks	0.3	<0.2	<0.6	0.3 a	0.003
4 weeks	0.4	0.2	1.0	1.5 a	0.02
8 weeks	0.9	2.0	3.0	5.9 b	0.06
140 °C, 3 h	0.4	<0.2	0.7	1.1 b	0.01
6 h	0.7	0.9	1.4	3.0 c	0.03
24 h	4.4	4.8	4.6	13.8 d	0.1
180 °C, 2 h[e]	4.0	6.2	2.3	12.5 b	0.1
3 h	6.3	9.8	3.3	19.3 c	0.2
6 h	10.6	18.9	4.7	34.2 d	0.4

[a] For each temperature the contents of total oxides followed by different letters were significantly different ($P < 0.05$) ($n = 6$). [b] Unidentified oxide formed from sitostanol. [c] Percentage of total oxides of the original nonoxidized sitostanol. [d] The untreated sample was the same for all temperatures. [e] For comparison, results already published by Soupas et al. (*22*) are shown.

Table 5. Formation of Sitostanol Oxides[a] (Micrograms per Gram of Matrix) during Different Heat Treatments in Sitostanol-Enriched (1%) Purified Rapeseed Oil[b]

treatment	7α-OH-sitostanol	6α-OH-sitostanol	RRT 1.779[c]	total	%[d]
none[e]	0.4	<0.2	<0.6	0.4 a	0.004
60 °C, 1 day	0.5	0.2	6.1	6.9 b	0.1
2 days	0.7	0.8	12.4	13.9 c	0.1
3 days	1.2	2.3	22.1	25.7 d	0.3
7 days	3.7	10.3	53.6	67.6 e	0.7
140 °C, 3 h	0.9	2.0	8.3	11.2 b	0.1
6 h	1.9	4.4	13.7	19.9 c	0.2
24 h	5.3	11.8	58.3	75.4 d	0.8
180 °C, 2 h[f]	2.0	5.1	8.0	15.1 b	0.2
3 h	2.8	7.0	13.0	22.9 c	0.2
6 h	5.3	14.0	31.2	50.5 d	0.5

[a] For each temperature the contents of total oxides followed by different letters were significantly different ($P < 0.05$) ($n = 6$). [b] Rapeseed oil was purified from pro- and antioxidants using adsorption chromatography. [c] Unidentified oxide formed from sitostanol. [d] Percentage of total oxides of the original nonoxidized sitostanol. [e] The untreated sample was the same for all temperatures. [f] For comparison, results already published by Soupas et al. (*22*) are shown.

increased during heating in both matrices at all temperatures (60−180 °C) and time points (0.5 h−8 weeks) ($P < 0.05$). The only exceptions were the total contents of stigmasterol oxides in tripalmitin during heating at 100 °C for 0−6 h and of sitostanol oxides in tripalmitin during heating at 80 °C for 0−4 weeks.

In tripalmitin, the highest levels of total stigmasterol oxides were observed after heating for 3 h at 180 °C (2267.6 μg/g of matrix) and in purified rapeseed oil after heating for 48 h at 100 °C (2484.1 μg/g of matrix). Almost the same amounts of stigmasterol oxides were formed in tripalmitin after heating for 4 weeks (672 h) at 80 °C (2151.6 μg/g of matrix) and in purified rapeseed oil after heating for 7 days (168 h) at 60 °C (2166.7 μg/g of matrix). It can be concluded that significant oxidation reactions may also occur at rather low temperatures, when the heating time is long enough (**Tables 2** and **3**).

The comparison of the total stigmasterol oxide contents after heating for 3 h at 100, 140, and 180 °C reveals that phytosterols are oxidized at a much higher rate in conditions simulating deep-fat frying (180 °C) than in conditions to which foods might be subjected during other food processing (100/140 °C). In tripalmitin, the total stigmasterol oxide contents were 6.4, 269.4,

and 2267.6 μg/g of matrix and in purified rapeseed oil, 76.8, 296.1, and 686.8 μg/g of matrix at 100, 140, and 180 °C, respectively. These results are in accordance with the fact that temperature has a marked effect on the rate of lipid autoxidation. Increasing temperature accelerates the propagation reactions and also the lipid hydroperoxide decomposition, thus producing a greater concentration of free radicals available for the initiation and propagation of lipid autoxidation (*26*).

The formation of sitostanol oxides was low at all temperatures and time points. However, the stanol oxide contents increased during different heat treatments, the highest total amounts being observed in tripalmitin after heating for 6 h at 180 °C (34.2 μg/g of matrix) and in purified rapeseed oil after heating for 24 h at 140 °C (75.4 μg/g of matrix) (**Tables 4** and **5**). These results were in agreement with the literature (*27*, *28*) stating that although it is generally known that autoxidation is a problem for unsaturated fatty acids, the saturated compounds also undergo slow oxidation, particularly at or above 100 °C. However, as in the case of stigmasterol oxidation, we observed that sitostanol was also oxidized at lower temperatures when the heating time was long enough (i.e., 60 °C/7 days), although this phenomenon also depended on the lipid matrix as discussed below.

Effect of Sterol Structure on Phytosterol Oxidation. The effects of unsaturated (stigmasterol) and saturated (sitostanol) sterol structures on sterol/stanol oxidation are shown in **Tables 2**−**5**. By comparing the formation of the same kind of oxidation product (7α-hydroxy) from stigmasterol and sitostanol, it was observed that after heating for 7 days at 60 °C in purified rapeseed oil matrix, the amount of 7α-hydroxystigmasterol formed was ∼175 times higher than the amount of 7α-hydroxysitostanol (3.7 vs 648.6 μg/g of matrix). When heated for 6 h at 140 °C in tripalmitin matrix, the amount of 7α-hydroxystigmasterol was ∼131 times higher than the amount of 7α-hydroxysitostanol.

It was also observed that although sitostanol remained rather stable, a marked increase in the formation of 7α-hydroxystigmasterol occurred, for example, after 24 h heating at 60 °C in purified rapeseed oil matrix and after 3 h at 140 °C in tripalmitin matrix. Our results confirmed that the oxidation of sterol compounds follows the same chemistry as the oxidation of other lipids: the rate of oxidation increases with increasing number of double bonds (*29*).

When evaluating the above-mentioned differences between the stability of sitostanol and stigmasterol, one should bear in mind that the conditions in these studies were rather drastic and that the oxides formed and their profiles are different for these two compounds.

Effect of Lipid Matrix on Phytosterol/-stanol Oxidation. *Total Oxide Contents.* The results in **Tables 2** and **3** show the effect of lipid matrix composition on stigmasterol oxidation and those in **Tables 4** and **5** on sitostanol oxidation. The unsaturation degree of the lipid matrix, that is, purified rapeseed oil versus tripalmitin, had a marked effect on stigmasterol oxidation. Moreover, the effect of lipid matrix was dependent on temperature. The influence of lipid matrix on sterol oxidation was also observed in heating experiments with sitostanol.

As an example, after heating for 48 h at 100 °C, for 6 h at 140 °C, and for 3 h at 180 °C, the stigmasterol oxide contents, expressed as percentage of total oxides of the original nonoxidized stigmasterol, in tripalmitin were 0.3, 8.0, and 24.2% and in purified rapeseed oil, 26.5, 8.2, and 7.4%, respectively. At 60 °C, in purified rapeseed oil, the stigmasterol oxide content was 23.2% after heating for 7 days. By comparison, only 0.2% stigmasterol oxidation was observed in tripalmitin matrix at 80 °C after the same heating time. These results suggest that the oxidation of lipid matrix and sterol is coupled; that is, once the oxidative process has started with oxidation of the unsaturated lipids, it also spreads to the sterol compounds. However, closer examination indicates that the situation is even more complex. The interactions of matrix composition and temperature had major effects on sterol oxidation: at high temperatures (>140 °C) sterols were more stable in unsaturated than in saturated matrix, whereas at lower temperatures (<140 °C) this order was reversed. Our hypothesis is that at high temperatures the unsaturated lipid matrix (purified rapeseed oil) was more readily oxidized, protecting sterols from reacting. In saturated matrix (tripalmitin), the high temperature forced the more reactive lipid components, sterols, to react. At lower temperatures sterols reacted more rapidly in a matrix that was itself oxidized, as described above. At 140 °C, sterols oxidized almost at the same rate in both matrices, indicating that the reaction course of the sterol oxidation was again different compared with higher and lower temperatures.

Because previous sterol oxidation studies have been performed in various model systems, the comparison of the results is difficult. However, it has been observed that the percentage of altered sitosterol increased with the unsaturation of the lipid matrix after oxidation under the same conditions (120 °C/2 h) (*13*), and that during 32 days of autoxidation at 25 °C, cholesterol oxidation was accelerated by the autoxidation of coexisting unsaturated triacylglycerols (*30*). Furthermore, it has also been reported that at 130 °C, tristearin, triolein, trilinolein, and milk fat accelerated cholesterol decomposition so that, after 3 h of heating, tristearin was the least destructive and triolein the most destructive matrix (*15*). Our results were supported by these observations, although the above-mentioned studies were less extensive than ours with regard to the variables used in model systems.

In the case of sitostanol oxidation, the above-mentioned interactions between matrix composition and heating temperature were not observed. At all temperatures sitostanol was oxidized more in unsaturated lipid matrix (purified rapeseed oil) than in saturated tripalmitin matrix (**Tables 4** and **5**). This indicates that saturated fat (sterol compound) reacts with the free radicals generated from the more oxidizable fat, as also stated in the literature (*31*).

Oxidation Product Profiles. As shown in **Tables 2**−**5**, the product profiles of stigmasterol and sitostanol oxidation were also studied. As in the case of total oxide contents, lipid matrix and temperature and their interactions had major effects on the distribution of the main secondary oxidation products of sterol oxidation.

In tripalmitin matrix, the main oxidation product of stigmasterol at 80, 100, and 140 °C throughout the heating was 7-ketostigmasterol. At 180 °C, the main product in the beginning of the heating experiment was again 7-ketostigmasterol (28%), but after 0.5 h of heating its proportion started to decrease so that after 3 h of heating the production of 7-ketostigmasterol was the lowest (12%) and that of 7α-hydroxystigmasterol the highest (29%) (**Table 2**). In purified rapeseed oil, the proportion of 7-ketostigmasterol was always the highest in the beginning of heating (28%) at all temperatures (60−180 °C), but the lowest or the second lowest (8−15%) after the heating experiments, as in tripalmitin at 180 °C. When the proportion of 7-ketostigmasterol started to decrease, the proportions of 7-hydroxysterols were the first to increase in purified rapeseed oil and 5α,6α-epoxides in tripalmitin (**Table 3**).

The oxidation product profile of sitostanol also changed during heating experiments. In purified rapeseed oil, the proportion of unidentified compound (RRT 1.779) was the highest at all temperatures (60−180 °C) and time points (**Table 5**). In tripalmitin, at 80 °C the product profile was similar to that in purified rapeseed oil. However, during heating at 140 and 180 °C, the proportion of unidentified compound of RRT 1.779 decreased and the proportions of 6α-hydroxysitostanol and 7α-hydroxysitostanol increased (**Table 5**). It appeared that in tripalmitin at elevated temperatures, the product profile started to change when the total content of quantified oxides reached 0.1% (**Table 4**).

Some of our observations, for example, that at lower temperatures in unsaturated lipid matrix the proportion of 7-ketones decreases during heating but not in more saturated lipid matrix, were also supported by information from the literature. Li et al. (*30*) autoxidized lipid mixtures that contained sardine oil triacylglycerols or partially hydrogenated sardine oil triacylglycerols plus cholesterol for 0−39 days at 25 °C. In these samples 7-ketocholesterol was the predominant oxide during the induction period, but after the oxide content increased rapidly the main oxides were 7β-hydroxides and 5β,6β-epoxides. Li et al. (*32*) studied cholesterol oxidation in food oils during storage and heating. During 0−35 days of storage at room temperature 7-ketocholesterol was the most prevalent oxide. After 22 h of heating at 110 °C, the main oxides in highly unsaturated flax oil were 5β,6β-epoxides and in more saturated palm oil 7-ketones.

The different distributions of sterol-/stanol oxides in different heating experiments in this study reveal that the reaction pathways of sterol-/stanol oxidation are not uniform under these conditions, but when compared with the information from the literature, it can be concluded that many of these reactions are too complex to permit accurate evaluation of their course. It is well-known that a complex mixture of secondary oxidation products is formed during the decomposition reactions of lipid hydroperoxides. However, the exact mechanisms of their formation and the kinetic and thermodynamic factors governing their distribution are not yet well understood (*18*). In most cases, several different reaction pathways are active, with one or more pathways predominating (*18*). These pathways lead to the formation of monomeric, polymeric, and/or volatile oxidation products, the monomeric products including such functional groups (hydroxyl, ketone, and epoxy groups) as studied in this work (**Figure 1**).

We observed that the pathways for phytosterol oxide formation were different in tripalmitin at 80−140 °C and at 180 °C. In purified rapeseed oil these pathways appeared to be rather similar at all temperatures (60−180 °C) and, furthermore, similar to that at 180 °C in tripalmitin matrix. The product profile formed depends on the rate constants between such reactions as reduction and dehydration of 7-hydroperoxides, dehydrogenation of 7-hydroxysterols, and epoxidation of sterol structure (*19*) (**Figure 1**). However, on the basis of our earlier studies (*33*) (unpublished data), it can also be concluded that the distribution between ketone, hydroxy, and epoxy compounds and especially the changes in the proportions of ketone compounds are associated with the phase of oxidation, that is, whether it is in the lag or dynamic phase or reached a plateau, and how extensively other reaction pathways of oxidation, for example, polymerization, are involved.

This study showed that critical conditions with regard to phytosterol stability may be encountered during food processing. High processing temperatures or prolonged storage may lead to decreased shelf life of phytosterols in enriched foods. However, it should be borne in mind that oxidation studies in model systems are only tentative compared with foods, in which interactions between lipids, carbohydrates, and proteins at elevated temperatures produce materials acting as catalysts or inhibitors, thus affecting oxidation of lipid components.

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