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Determination of thermo-oxidation products of plant sterols

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Abstract

Plant sterols are subjected to oxidation when exposed to air and, especially, when heated at high temperatures. We developed a method to study thermo-oxidation of plant sterols. The method consisted of cold saponification, purification of oxides by solid-phase extraction and gas chromatography analysis. To compensate for losses during the procedure, an internal standard was added before saponification. The method showed good recovery of added cholesterol oxides, separation of plant sterol oxides and reproducibility in detecting thermo-oxidation products of stigmasterol and rapeseed oil. Based on this study, the major products are 7-hydroxy, 5,6-epoxy and 7-keto compounds and oxides are formed faster in bulk stigmasterol than in rapeseed oil.

Keywords: Thermo-oxidation; Sterols

1. Introduction

Many in vitro studies have shown that cholesterol oxides have cytotoxic, mutagenic and atherogenic activities, induce apoptosis and are potent regulators of cholesterol metabolism; thus they may have harmful effects on human health [1]. Oxidation products of cholesterol are present in cholesterol-containing foods up to μg/g levels, but the actual physiological effects of dietary oxides are poorly characterized [2,3]. Owing to structural similarities between plant sterols and cholesterol, plant sterols are also prone to oxidation. Thus oxidation might be a problem in foods rich in plant sterols, e.g., refined vegetable oils containing 0.5–10.6 g/kg and cereal grains 0.330–1.8 g/kg plant sterols [4] and especially in plant sterol-enriched foods such as margarines. Biological effects of individual oxidation products of both cholesterol and plant sterols should be better understood.

Plant sterols and cholesterol like other unsaturated lipids are subjected to oxidation when exposed to air. Oxidation is enhanced by heating, ionizing radiation, exposure to light or by chemical catalysts. In general, oxidation of sterols is a free radical chain reaction that begins with formation of a hydroperoxide that may in turn decompose to various compounds. The main oxidation products are hydroxy, keto and epoxy compounds from which the last ones may hydrate to triol compounds [1,2,5,6]. Oxidation may also begin enzymatically or by attack of reactive oxygen species. Since there are several mechanisms by which oxidation can proceed, it is understandable that more than 80 oxidation products of cholesterol have been identified [5]. In terms of oxidation risk, critical points in food production and consumption

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are processes where food is heated at high temperatures, e.g., frying and during long-term storage of dry foods, e.g., whole egg and milk powders.

There are only a few studies where oxidation products of plant sterols have been analyzed [2,7]. Most of these have focused on either characterization of as many oxidation products as possible of one plant sterol, or synthesis of specific oxidation products (e.g., Refs. [8–10]). Plant sterol oxides in foods have been measured to examine stability in wheat flour, soybean oil [11], deep-fat frying oils and fried products [12–14]. The major plant oxides detected were similar to those formed from cholesterol, i.e. 7-hydroxy, 7-keto and 5,6-epoxy compounds.

Methods used for analyzing plant sterol oxides are based on those developed for cholesterol oxides. Sample preparation may include extraction and saponification of lipids, various enrichment and purification steps and removal of non-oxidized cholesterol. Analyses by gas chromatography (GC) or high-performance liquid chromatography (HPLC) are most commonly used for final separation and quantitation of cholesterol oxidation products (e.g., Refs. [8,10–16]). Despite extensive efforts, problems in quantitative analysis of cholesterol oxides still exist [2,17]. One of the major risks during analysis of plant sterols is formation of artefacts because they may be produced from non-oxidized sterols or preformed oxides decomposed further during sample preparation. Since plant materials contain a variety of sterols and they are present both as free sterols and steryl conjugates, profiles of oxidation products are numerous, which makes sample preparation and chromatographic separation even more challenging than with animal-based materials containing only cholesterol. Although HPLC allows good separation of cholesterol oxides [15,16], it is not applicable to study plant sterols because it is not capable of separating many plant sterols from each other. It is necessary to separate oxidation products from each other and not only to measure total oxidized sterols because different species may have different physiological effects. Since commercial plant sterol oxides are not available, versatile chromatographic and spectrometric techniques are needed to identify the products. Moreover, quantitation of oxides and method performance studies have to be conducted with cholesterol oxidation products unless plant sterol oxides are synthesized.

The aim of this study was to develop and evaluate a routine method to reliably analyze oxidation products of plant sterols in foods. The main focus in method development was to avoid formation of artefacts and losses during the sample preparation procedure. Thus the method consisted of gentle cold saponification followed by solid-phase extraction (SPE) and on-column capillary GC of derivatized sterol oxides. Finally, the applicability of the method to study and compare thermo-oxidation of sterols as such and in vegetable oils was examined.

2. Experimental

2.1. Chemicals and materials

24α-Ethylcholesta-5,22-dien-3β-ol (stigmasterol; 95%), 24β-ethylcholest-5-en-3β-ol (sitosterol; ≈60%), 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, USA) and cholest-5-ene-3β,7β-diol (7β-OH-cholesterol) and cholest-5-ene-3β,7α-diol (7α-OH-cholesterol) from Steraloids (Wilton, NH, USA). *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA; >98%; E. Merck, Darmstadt, Germany) and trimethylcholorosilane (TMCS; >99%; Fluka Chemie, Buchs, Switzerland) were used as a 99:1 mixture for silylation. Analytical grade anhydrous pyridine, Na_2SO_4 (from E. Merck), diethyl ether (from J.T. Baker, Holland) and KOH (from Eka Nobel, Surte, Sweden), HPLC grade hexane and acetone (from Rathburn Chemicals, Walkerburn, Scotland), 99.5% ethanol (from Primalco, Finland) and water (purified by Milli-Q Plus, Molsheim, France) were used.

Bond Elut SiOH solid-phase extraction (SiOH-SPE) cartridges (500 mg, Varian, Harbor City, CA, USA) were used in purification of sterol oxides. For method validation and thermo-oxidation studies,

rapeseed oil (Raisio Group, Raisio, Finland) was obtained from a local grocery store.

2.2. Sample preparation for analysis of oxidized sterols

Native and thermo-oxidized oil samples (about 0.25 g) were weighed into a 100 ml erlenmeyer flask and dissolved in 9 ml ethanol with minimal heating. An aliquot of 1.0 ml 19-OH cholesterol (internal standard) in ethanol (20 μg/ml) and 0.5 ml saturated KOH in water were added. The flasks were purged with nitrogen and the samples were saponified overnight in a shaking water bath at 25 °C in the dark.

After saponification, the mixture was diluted with 10 ml water and the unsaponifiable lipids were extracted three times with 15 ml diethyl ether. The combined extract was washed once with 20 ml 0.5 *M* aqueous KOH and three times with 20 ml aqueous 0.235 *M* Na_2SO_4 according to Rose-Sallin et al. [18]. The extract was dried with anhydrous Na_2SO_4 and the solvent was evaporated under vacuum in a rotary evaporator at 30 °C. Finally, the lipid residue was dissolved in 1.0 ml hexane:diethyl ether (9:1, v/v).

Oxidized sterols were purified by SiOH-SPE. The procedure was based on a method to purify free sterol fraction from the unsaponifiable lipids [19] except for using an additional elution step to liberate sterol oxides from the cartridge. In brief, the cartridge was activated with 5 ml hexane after which the unsaponifiable lipids were applied in 1 ml hexane:diethyl ether (9:1, v/v). The cartridge was washed with 5 ml hexane:diethyl ether (9:1, v/v) and 5 ml hexane:diethyl ether (1:1, v/v) to remove apolar compounds and non-oxidized sterols, respectively. Finally, sterol oxides were eluted with 5 ml acetone into a test tube containing 40 μg of cholestanol (secondary internal standard) in 2 ml ethanol. The secondary internal standard was used for calculating recovery of the internal standard, 19-OH-cholesterol, during sample preparation. The amount of sterols and sterol oxides applied to a cartridge was adjusted to ≤3 mg to avoid overloading.

Sterol oxide extracts were dried under nitrogen flow at 30 °C and in general, half of the residues were subjected to silylation by BSTFA/TMCS (trimethylchlorosilane) (99:1, v/v) reagent in pyridine [20]. After silylation overnight at room temperature, the reagent was evaporated and the trimethylsilyl (TMS) ethers were dissolved in 200 μl hexane before GC analysis.

2.3. Sample preparation for analysis of non-oxidized plant sterols

For analysis of non-oxidized plant sterols, a direct hot saponification method was used [4]. Sample preparation was similar to that described above except for the following: cholestanol was used as an internal standard, the unsaponifiable lipids were extracted with hexane:diethyl ether (1:1, v/v) and washed with water while purification by SiOH-SPE was omitted.

2.4. Gas chromatography–flame ionization detection (GC–FID)

GC–FID analyses were used for quantitative analyses after the compounds had been identified by relative retention times and GC–mass spectrometry (MS). GC analyses were performed using an automated on-column instrument equipped with a flame ionization detector (Hewlett-Packard 5890 Series II GC and HP-7673 autosampler; Karlsruhe, Germany), ChemStation 3.1 software and using an RTX-5w/Integra fused-silica capillary column (60 m×0.32 mm I.D., 5% diphenyl–95% dimethyl polysiloxane, 0.1 μm film with 10 m Integra-Guard column; Restek, Bellefonte, PA, USA). Helium (>99.996%, AGA, Espoo, Finland) was used as the carrier gas at a constant flow (110 kPa at 200 °C). The initial temperature was 70 °C (1 min), then programmed with 60 °C/min to 245 °C (1 min) and then 3 °C/min to 275 °C (41 min). The detector temperature was 300 °C.

2.5. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was used for identification of sterol oxides and to check purity of eluted compounds. The analyses were performed on an Incos 50 Finnigan

Mat mass spectrometer (Finnigan, San Jose, CA, USA) with ionization energy of 70 eV in the positive electron impact mode. Samples were introduced to the MS via a Varian 3400 GC (Walnut Creek, CA, USA) working in the splitless mode. The operating conditions of GC were as follows: similar column as above except for having a 0.25 mm I.D., injection and oven temperatures 270 °C and 260 °C, respectively, and helium as carrier gas at 240 kPa. Spectra were scanned within the range of m/z 100–600 at scan and inter-scan times of 0.7 s and 0.3 s, respectively. The ion-source and transfer-line temperatures were 160 °C and 270 °C, respectively.

2.6. Method validation

Representative oxidation products of cholesterol (7β-OH-cholesterol, 5,6-epoxycholesterol, cholestanetriol, 7-ketocholesterol) and the internal standard (19-OH-cholesterol) were used to validate the method. GC–FID response factors of sterol oxides relative to cholestanol were determined. First, the efficiency of the SPE purification step was studied to separate native sterols from oxidized sterols with a cholesterol oxide mixture applied on the SiOH cartridge (19-OH-cholesterol, 5,6-epoxycholesterol, cholestanetriol, 7-ketocholesterol) at levels of 0.5–25 μg combined with sitosterol at levels of 0.2–1 mg in four combinations all conducted in triplicate. Thereafter recovery studies of the purification step and the performance of the whole method were evaluated by recovery studies of added sterol oxides. Recovery studies were conducted with three levels of cholesterol oxides added to rapeseed oil in the range of 10–200 μg in triplicate. Rapeseed oil was used as lipid matrix when recovery of cholesterol oxides and formation of artefacts during the whole procedure, including saponification, was studied. The determination limit of plant sterol oxides in oil was 1 μg/g by GC–FID. Contents of such oxidation products that were positively identified by GC–MS were reported.

2.7. Thermo-oxidation experiments

Stigmasterol (10 mg) and rapeseed oil (1.0 g) were heated in glass vials (1.7 cm, I.D.) at 180±5 °C in an electric oven for up to 6 and 24 h, respectively. Several thermo-oxidized sterol samples during the experiments were taken and analyzed in triplicate. When stigmasterol samples were analyzed, the saponification step in the procedure was omitted. Thermo-oxidation experiments of both materials were conducted in duplicate.

3. Results and discussion

3.1. GC analysis of sterol oxides

Sterol oxides were identified by their relative retention times (RRT) and mass spectral properties (Tables 1 and 2). By the GC–FID method used, both epimers of 7-OH and 5,6-epoxy products and 7-keto products of campesterol, stigmasterol and sitosterol were analyzed. In addition to these major oxidation products, one group of side chain oxidation products, i.e. 25-OH products, were also identified and analyzed. They showed a typical fragmentation ion at m/z 131 [21]. Elution orders of sterol oxides by the GC method used were similar to those analyzed earlier by capillary columns with dimethyl polysiloxane phases containing 5% diphenyl [22,23]. Specific ions of each plant sterol oxide detected in this study (Table 2) were similar to those analyzed from synthesized oxidation products [10,12,13].

Most major plant sterol oxidation products could be analyzed using the 56-min GC–FID analysis (Fig. 1). Both internal standards (19-OH-cholesterol and cholestanol) were well separated from the analytes. Total separation of all compounds could not, how-

Table 1
Relative retention times of cholesterol oxide TMS ethers in relation to that of cholestanol TMS ether

TMS ether	Relative retention time
7α-OH-cholesterol	0.955
Cholestanol	1.000
19-OH-cholesterol	1.063
7β-OH-cholesterol	1.117
5β,6β-Epoxycholesterol	1.150
5α,6α-Epoxycholesterol	1.188
Cholestantriol	1.329
25-OH-cholesterol	1.415
7-Ketocholesterol	1.442

RTX-5w/Integra 60 m×0.32 mm I.D., 0.1 μm film; for operating conditions, see Section 2.4.

Table 2
Specific mass spectral ions used in identification of campesterol, stigmasterol and sitosterol oxide TMS ethers

Sterol oxide TMS ethers	Specific ions	Mass spectral ions, m/z		
		Campesterol	Stigmasterol	Sitosterol
7α/β-OH	M^+-TMSOH	470	482	484
5,6α/β-epoxy	M^+, M^+-TMSOH	488, 398	500, 410	502, 412
Triol	M^+-TMSOH, M^+-A ring	560, 417	572, 429	574, 431
25-OH	M^+, α-cleavage between C_{24} and C_{25}	560, 131	572, 131	574, 131
7-Keto	M^+, M^+-TMSOH	486, 396	498, 408	500, 410

M^+, molecular ion; TMSOH, trimethyl silanol group.

ever, be achieved because some oxidation products coeluted and there was interference from non-oxidized sterols and matrix compounds from the oil. For example, if the sample contained oxidation products from stigmasterol and sitosterol, 5α,6α-epoxystigmasterol and 7β-OH-sitosterol coeluted (RRT= 1.395). If the sample contained both oxidized and non-oxidized sterols, sitosterol would coelute with 7β-OH-campesterol (RRT=1.251). Thus removal of intact sterols by SiOH-SPE was needed. Contamination of the oxide fraction with sitosterol could easily be detected, since other non-oxidized sterols would be concurrently present in the chromatograms. Native rapeseed oil contained four polar compounds that were collected in the sterol oxide fraction by SiOH-SPE and eluted after cholestanol with GC. One of these compounds coeluted with 5α,6α-epoxysitosterol (RRT=1.552) and another closely proceeding it (RRT=1.510; Fig. 1). The two other interfering compounds eluted before 7β-OH-campesterol (RRT=1.227) and 5α,6α-epoxycampesterol (RRT= 1.336), respectively. To quantitate 5α,6α-epoxysitosterol in rapeseed oil samples more accurately, detection by selective ion monitoring should be used.

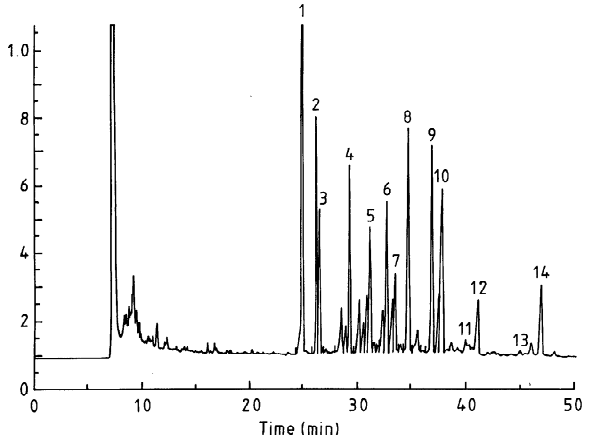

Fig. 1. GC separation of TMS ether derivatives of oxidized sterols from thermo-oxidized rapeseed oil (180 °C, 12 h) on a capillary column (RTX5w/Integra, 60 m×0.32 mm I.D., 0.1 μm film; GC parameters see text): (1) cholestanol, (2) 19-OH-cholesterol, (3) 7α-OH-campesterol, (4) 7α-OH-sitosterol, (5) 7β-OH-campesterol, (6) 5β,6β-epoxycampesterol, (7) 5α,6α-epoxycampesterol, (8) 7β-OH-sitosterol, (9) 5β,6β-epoxysitosterol, (10) 5α,6α-epoxysitosterol+unknown from rapeseed oil, (11) 25-OH-campesterol, (12) 7-ketocampesterol, (13) 25-OH-sitosterol and (14) 7-ketositosterol.

Relative GC–FID responses of commercial cholesterol oxidation products in relation to cholestanol ranged from 0.942 to 1.084 (Table 3). Hydroxy cholesterols had a slightly higher and 7-ketocholesterol lower responses than other oxidation products while the average was 1.00. Thus, a general relative response factor 1.00 was used. In earlier studies, relative response factors of cholesterol oxidation products in relation to cholestane and cholestanol were 0.98–1.13 [21] and 0.99–1.20 [22], respectively. Moreover, assumptions have been made that the GC–FID responses of plant sterol oxidation products are equal to that of cholestane [11]. Since we used

Table 3
Relative GC–FID response factors of selected cholesterol oxide TMS ethers in relation to cholestanol TMS ether

TMS ether	GC–FID response factors	
	N	Mean±SD
19-OH-cholesterol	10	1.037±0.015
7β-OH-cholesterol	10	1.084±0.024
5α,6α-Epoxycholesterol	9	0.954±0.026
Cholestantriol	10	1.002±0.032
7-Ketocholesterol	10	0.942±0.030

19-OH-cholesterol as the internal standard and related all peak areas to that, we slightly overestimated the contents of hydroxy compounds and underestimated those of the others. Despite these minor defects, the GC–FID method developed met the needs for routine analysis of plant sterol oxides.

3.2. Validation of the analytical procedure

Sample preparation procedure consisted of cold saponification and purification by single SiOH-SPE. First the performance of the SiOH-SPE method to separate native and oxidized sterols was studied with mixtures of cholesterol oxides (0.5–25 μg each) and sitosterol (0.2–1 mg). The 12 test purifications showed that no cholesterol oxides were eluted with 5 ml of hexane:diethyl ether (1:1, v/v). Moreover, non-oxidized sterols of rapeseed oil were quantitatively eluted from the cartridge by the hexane–diethyl ether (1:1, v/v) eluent and the sterol profile was unaltered by this purification step (data not shown). Only in four of the test purifications minor amounts (mean 0.4%) of sitosterol were carried over to the sterol oxide fractions. This carry-over of native sterols might be a problem if the food sample contained elevated levels of sterols, like in e.g., sterol-enriched margarines. To avoid this problem, sample size and especially the amount of non-saponifiable lipids applied on a cartridge should be reduced or the SiOH-SPE purification repeated. When reducing the sample size one should bear in mind that simultaneously the sample would be less representative and the sensitivity of the whole method would decrease.

The recovery of 19-OH-cholesterol from SiOH-SPE procedure relative to cholestanol that was added after elution from the cartridge was 99.0±1.6% indicating that sterol oxides were quantitatively removed from the SiOH cartridge by 5 ml of acetone. The SiOH-SPE procedure was relative consistent to all sterol oxides, since the relative recoveries of added cholesterol oxides (7β-OH-cholesterol, 5α,6α-epoxycholesterol, cholestanetriol, 7-ketocholesterol) at levels of 10–200 μg ranged from 82.9 to 104.4% (Table 4). Most of the oxides had a relative recovery >90%. The lowest recovery and the biggest variation were obtained for cholestanetriol, which has been found to readily retain on SiOH [22] and on aminopropyl phases [23], thereby being less efficiently eluted from the SiOH cartridge.

When rapeseed oil was spiked with cholesterol oxides similarly as described above and the spiked samples were subjected to the whole sample preparation procedure, the recovery of 19-OH-cholesterol in relation to cholestanol was 54.4±6.8%. Relative recoveries of 7β-OH-cholesterol, 5α,6α-epoxycholesterol and cholestanetriol during the whole procedure were comparable to those of the SiOH-SPE purification step only (Table 4). 19-OH-cholesterol seems to be a good internal standard for hydroxy and epoxy compounds since their relative recoveries ranged between 94.5 and 108.6%. There was some more loss of cholestanetriol during the saponification step, which might be due to its low recovery from the saponified mixture. Since triols are the most polar products they may prefer to retain with soap more than the other products [24]. Apparently, most of the losses of 19-OH-cholesterol, other hydroxy compounds and epoxy compounds occur during the saponification and the following extraction steps. It would be interesting to study, if these losses would be smaller by decreasing the amount of

Table 4
Recoveries of added cholesterol oxides of the solid-phase extraction (SPE) step and the whole procedure (saponification, extraction and washing of the unsaponifiable lipids and solid-phase extraction) compared to 19-OH-cholesterol at three levels (10–200 μg)

Cholesterol oxides	Recovery of the SPE step, %		Recovery of the whole procedure, %	
	N	Mean±SD	*N*	Mean±SD
7β-OH-cholesterol	9	104.4±3.2	9	108.6±4.1
5α,6α-Epoxycholesterol	9	91.5±4.0	9	94.5±5.3
Cholestanetriol	9	82.9±6.5	9	72.4±12.2
7-Ketocholesterol	9	92.7±2.3	9	54.1±2.8

For details, see Section 2.6.

saponifiable lipids because soap formation is known to withdraw some cholesterol oxidation products to the aqueous phase [24].

There was a marked decrease in the relative recovery of 7-ketocholesterol when it was subjected to saponification. It was 54.1±2.8% (Table 4). During hot saponification 7-ketocholesterol is converted to its dehydration product cholesta-3,5-dien-7-one [25,26]. Thus hot saponification cannot be used. Moreover, keto compounds are also known to be the most sensitive compounds under mild saponification conditions. In one study using overnight saponification at room temperature, as much as 60–89% of 7-ketocholesterol was destroyed [27]. Cholestanetriol was somewhat sensitive with 10–22% loss while hydroxy compounds were stable under these saponification conditions. However, in some experiments using cold saponification, 7-keto compounds and other oxidation products were equally stable with only minor losses (<5%) [24] or only 4% of 7-ketocholesterol was degraded [26]. When studying oxidation of plant sterols, saponification is a necessary step because plants contain remarkable proportions of esterified sterols in addition to free sterols. Without saponification or other hydrolysis procedures, oxidation products of esterified sterols could not be included in the analysis.

No oxidation products were detected in the native rapeseed oil used, which indicates that during the analytical procedure oxidation did not occur. It should be recognized that if less polar artefacts were formed, they would be removed during the SiOH-SPE. When each cholesterol oxide was separately added to rapeseed oil at a 50 μg level in a 0.25 g sample, no other oxidation products or unknown compounds were detected in the gas chromatograms. Our finding is similar to that of Park and Addis [24] who stated that sample preparation with cold saponification was gentle and no artefact formation occurred. On the contrary, Rose-Sallin et al. [18] found that 2% of deuteriated cholesterol was oxidized in milk powder during sample preparation including cold saponification and NH_2-SPE purification. They recommended that artefact formation should be taken into account when reporting data, which is, however, seldom done.

This study demonstrated that addition of the internal standard, 19-OH-cholesterol, at the beginning of the analytical procedure is important to appropriately compensate for the losses during the whole analytical procedure. When adding the secondary internal standard, cholestanol, to sterol oxide mixture prior to derivatization, we found that most of the oxidation product losses occur during the saponification step followed by the extraction of the unsaponifiable lipids.

3.3. Thermo-oxidation of stigmasterol and rapeseed oil

The analytical procedure developed and evaluated was applied to study thermo-oxidation of sterols in stigmasterol and rapeseed oil. The mean coefficient of variation of triplicate analysis of oxidation products of stigmasterol and rapeseed oil samples were 4 and 6%, respectively, which are similar to those measured earlier for the spiked cholesterol oxides. Likewise, recoveries of 19-OH-cholesterol from stigmasterol and rapeseed oil samples compared to cholestanol added prior to derivatization were at a similar level as in the method performance studies, being 97.0±3.3% and 65.7±5.1%, respectively. Reproducibility of the duplicate heating experiments was good, since the sterol oxide contents of the experiments deviated, on an average, from the mean values by 5 and 3%, respectively. Since the analytical and experimental variations were small, only mean values of duplicate experiments are given.

Plant sterols are labile when heated at 180 °C due to oxidative and thermolytic reactions during which a variety of oxidation products are formed [6,24,28]. When stigmasterol was thermo-oxidized, formation of six analyzed oxidation products began after 15 min (Table 5). Minor oxidation products were also formed but they contributed to less than 10% of total. The contents of 7α-, 7β- and 25-OH-stigmasterol and of 5α,6α- and 5β,6β-epoxystigmasterol reached their highest levels already during the first hour. After 1 h at 180 °C, the total amounts of oxidation products were 198 mg/g, which represents 21% of the amount of non-oxidized stigmasterol. After 1 h, the amounts of oxidation products leveled off and slightly decreased to 145 mg/g by the end of the 6-h experiment indicating that further reactions of the analyzed products occurred. Similarly, the amounts of cholesterol oxides decreased after 12 and

Table 5
Formation of oxides in stigmasterol when heated at 180 °C

Heating time	Oxidation products of stigmasterol[a]						
	7α-OH	7β-OH	5α,6α-Epoxy	5β,6β-Epoxy	7-Keto	25-OH	Total
0	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15 min	5	7	6	9	14	2	43
30 min	18	24	17	30	28	6	123
1 h	27	37	28	50	44	12	198
2 h	20	32	29	47	50	11	189
6 h	7	21	25	33	51	8	145

[a] mg/g, mean values of duplicate experiments.
[b] n.d., not detected.

1 h when a thin film of cholesterol had been heated at 150° and 200 °C, respectively [29]. During thermo-oxidation 7-ketostigmasterol became the major product after 2 h and its content did not decrease as those of the other oxides.

Thermo-oxidation of native plant sterols was studied in rapeseed oil. The total sterol content of rapeseed oil was 893 mg/100 g and the contents of the three major sterols were 416, 314 and 88 mg/100 g for sitosterol, campesterol and brassicasterol, respectively. Since some oxidation products of brassicasterol could not be identified by GC–MS, we followed only the formation of sitosterol and campesterol products. Thermo-oxidation of sterols was slower in rapeseed oil than in bulk stigmasterol. After 6 h of heating, only 266 μg/g of oxidation products of sitosterol and campesterol were detected (Table 6). The amounts of total and individual oxidation products increased steadily during the 24-h experiment. Thus after 24 h of heating, rapeseed oil contained 1098 μg/g of oxidation products. The amounts of sterol oxidation products found in this study are at the same level as found in cooking oil with 0.25% cholesterol when heated at 150 °C with a constant air flow of 5 ml/min [30].

Thermo-oxidation of sitosterol and campesterol in rapeseed oil proceeded similarly, since their share in the total oxidation products followed that of their ratio in the native rapeseed oil, i.e. 57 and 43%, respectively. This is an interesting finding indicating that the structural difference between the two plant sterols, i.e. one methyl group, has no effect on their

Table 6
Formation of plant sterol oxides in rapeseed oil when heated at 180 °C

Heating time	Oxidation products of sitosterol[a]						
	7α-OH	7β-OH	5α,6α-Epoxy	5β,6β-Epoxy	7-Keto	25-OH	Total
0	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6 h	31	52	27	38	12	2	162
12 h	71	105	58	92	42	4	372
24 h	109	154	107	172	102	2	646
Heating time	Oxidation products of campesterol[a]						
	7α-OH	7β-OH	5α,6α-Epoxy	5β,6β-Epoxy	7-Keto	25-OH	Total
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6 h	21	27	13	31	9	3	104
12 h	52	59	33	72	31	6	253
24 h	83	87	69	131	72	11	452

[a] μg/g, mean values of duplicate experiments.
[b] n.d., not detected.

thermo-oxidation. Moreover, since the difference between cholesterol and campesterol is also one methyl group at C_{24}, it may be suggested that their thermo-oxidations might proceed similarly and at least should be studied further. There were some minor deviations in the ratios of some individual sitosterol and campesterol products, but they could be accounted for by coelution in the GC analyses. Overestimation of 7β-OH-sitosterol and 7β-OH-campesterol contents due to coelution with 5α,6α-epoxystigmasterol and sitosterol may be neglected in this study because rapeseed oil contains only minor amounts of stigmasterol that could oxidize to 5α,6α-epoxystigmasterol and the non-oxidized sterols were efficiently removed from the sample by SPE, respectively. However, the values for 5α,6α-epoxysitosterol may be slightly bigger due to coelution with one polar compound present in rapeseed oil at RRT=1.552. To improve the analytical method further and to apply it to study complex sample mixtures, possibilities to separate and quantitate oxidation products on GC–MS should be examined and evaluated.

In both thermo-oxidized stigmasterol and rapeseed oil, more β- than α-epimer products were formed. For example, after 1 h of heating at 180 °C of stigmasterol, there were 37 and 27 mg/g 7-OH-stigmasterols and 50 and 28 mg/g 5,6-epoxystigmasterols of the β- and α-epimers, respectively. The same figures/values for sitosterol after 24 h of heating in rapeseed oil were 154 and 109 μg/g and 172 and 107 μg/g, respectively. Smith [5] has stated that formation of β-epimer of cholesterolepoxide is favored over the α-epimer. Triol compounds were not produced in any of the stigmasterol or rapeseed oils samples, which is reasonable because thermo-oxidation was carried out in anhydrous lipid matrix, where epoxy compounds are more stable than under aqueous acidic conditions where they hydrate to triol compounds [5]. Likewise no cholestanetriol was formed in cooking fats with 0.25% cholesterol when heated at 150 °C [30].

The lipid matrix of sterols remarkably affected their thermo-oxidation. Despite the amounts of sterols heated in both stigmasterol and rapeseed oil samples being relatively similar, i.e. 10 and 8.9 mg, respectively, the amounts of oxidation products in the stigmasterol samples were bigger than in the rapeseed oil samples. Also the proportions of converted sterols were higher in stigmasterol than in rapeseed oil. For example, it took less than 1 h at 180 °C to find 15% of stigmasterol as an oxidation product, while it took 24 h for rapeseed oil to reach the same level. This indicates that thermo-oxidation like autoxidation is significantly dependent on the sample area subjected to air and the area to volume ratio, which were much larger in the stigmasterol than in the rapeseed oil samples. Studies on the effects of co-oxidizing matrix lipids are controversial because some indicate that sterol oxidation is enhanced by unsaturated lipids [31], while others have shown that oxidation is more pronounced in saturated than in unsaturated lipid matrix [28]. Moreover, the profile of products was more stable in rapeseed oil than in stigmasterol where further reactions of oxidation products occurred.

The method developed was concluded to be applicable to study thermo-oxidation of plant sterols in lipid matrix. With this routine method, we could reliably and reproducibly identify and quantify the major oxidation products of stigmasterol, sitosterol and campesterol. Further possibilities to apply the method should be investigated, e.g., to study oxidation in more complex plant matrices.

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