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ORIGINAL PAPER

# Products Formed During Thermo-oxidative Degradation of Phytosterols

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**Abstract** Oxidative degradation of cholesterol has been extensively researched, however, not all products formed have been established. When a phytosterols standard was heated at 60, 120 and 180 °C for different period of time the following groups of components were detected: oxidized phytosterols, fragmented phytosterol molecules, volatile compounds and oligomers. Taking into account all the components formed, we were able to balance the amounts of disappearing sterols with components formed. We established that the amount and type of products formed during thermo-oxidative degradation is affected by temperature and time. The amount of intact phytosterols decreased when temperature and time increase. The amount of oxidized phytosterols was at the highest level when a temperature of 120 °C was applied, whereas the lowest amounts were observed when a temperature of 60 °C was used. At a temperature of 180 °C the amount of oxidized sterols was lower than at 120 °C and it decreased when the heating time was increased. This indicates that oxidized sterols were the main precursors involved in the formation of other components during thermo-oxidative degradation. The amount and type of volatile compounds formed increased when time and temperature increased. We observed diversified groups of volatile compounds formed and most of them are defined as off-flavor compounds for rancid oils.

**Keywords** Sitosterol · Campesterol · Campestanol · Sitostanol · Heating · Oxyphytosterols · Off-flavor compounds · Oligomers · GC/MS · HPSEC

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## Introduction

Phytosterols, or plant sterols, are compounds that occur naturally and bear close structural resemblance to cholesterol, but have different side chain configurations. Oxidative degradation of cholesterol have been extensively researched, mainly directed to the formation of oxidative derivatives, however, the formation of volatile flavor components and oligomers was neglected [1]. Since phytosterols have a similar chemical structure to cholesterol, they are involved in the same oxidation and degradation processes (Fig. 1). Oxysterols are the primary compounds formed during oxidation and due to their instability and chemical reactivity; they are the precursors for a variety of secondary products such as volatile compounds, fragmented sterol molecules and oligomers (Fig. 2).

Phytosterols are the main constituent of unsaponifiables present in all oils and fats, forming the main source of sterols in a consumer diet. Among commodity oils, canola and corn oils contain the highest amounts of phytosterols [2]. Currently, a large number of food products are fortified with phytosterols, particularly on the European market [3] and with it stability and potential degradation into health affecting products is of concern. The stability of phytosterols is affected by their chemical structure, such as unsaturation in the ring and side chain, temperature, time and composition of a matrix [4, 5]. During frying, sterols

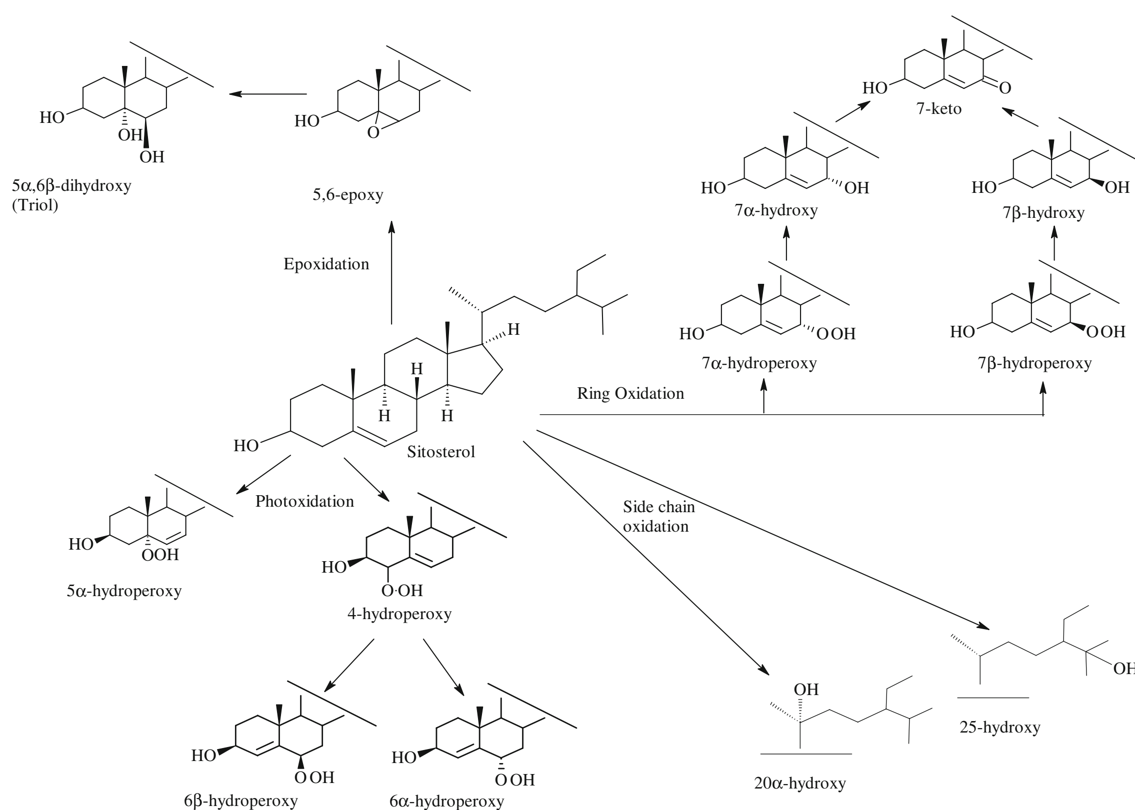


**Fig. 1** Sitosterol oxidation pathways. Adapted from Smith [1]

undergo thermo-oxidative degradation and a variety of oxidative derivatives, oxysterols, are formed [6–9]. Recently, oxyphytosterols have been detected in dried canola seeds, cold-pressed and refined oils, French fries, spreads, potato chips, infant formulas and coffee [10–15].

Phytosterols have been found to play an important role in fighting atherosclerosis [16]. It has been established that consumption of specific amounts of phytosterols negatively affects the absorption of cholesterol and reduces hypercholesterolemia [17]. Treatment of epithelial cells with $\beta$-sitosterol, the most common phytosterol, decreased the number of developed tumors in a colon, also a dose-dependent effect on the proliferation of tumor cells was established [18, 19].

Macrophage cells were used to show the cytotoxicity of oxyphytosterols [20]. Meyer et al. [21] showed cytotoxicity of oxyphytosterols by measuring the mortality of mealworms after injection of α- and $\beta$-epoxy- and triol derivatives of $\beta$-sitosterol and stigmasterol. The pro-atherogenic role of oxyphytosterols is still debated, but current research results suggesting that these compounds can play an important role in their pathogenesis [21, 22].

When thermo-oxidative changes of pure cholesterol were studied, compounds with a molecular mass higher than individual sterols were observed [23, 24]. The authors suggested that cholesterol and oxysterols at elevated temperatures might form oligomers. Lercker and Rodriguez-Estrada [25] identified 3,3′-dicholesterol ether when cholesterol standard was heated to 170 °C.

The main goal of this paper was to establish what compounds are formed during thermo-oxidative degradation of phytosterols when thermally treated at temperatures simulating food processing.

## Materials and Methods

### Materials

Sitosterol standard was purchased from Calbiochem (San Diego, California, USA) while 19-hydroxycholesterol from Steraloids (Newport, RI, USA). Solvents, 5α-cholestane, deuterated naphthalene-$d_8$ and anhydrous pyridine were supplied by Sigma-Aldrich (St. Louis, MO, USA). Tetrahydrofuran (THF) was obtained from VWR (Mississauga, ON, Canada). A silylation mixture of BSTFA (*N*,*O*-Bis (trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane) was received from Fluka Chemie (Buchs, Switzerland), while SEP-PAK amino cartridges came from Waters (Milford, USA). The divinylbenzene/

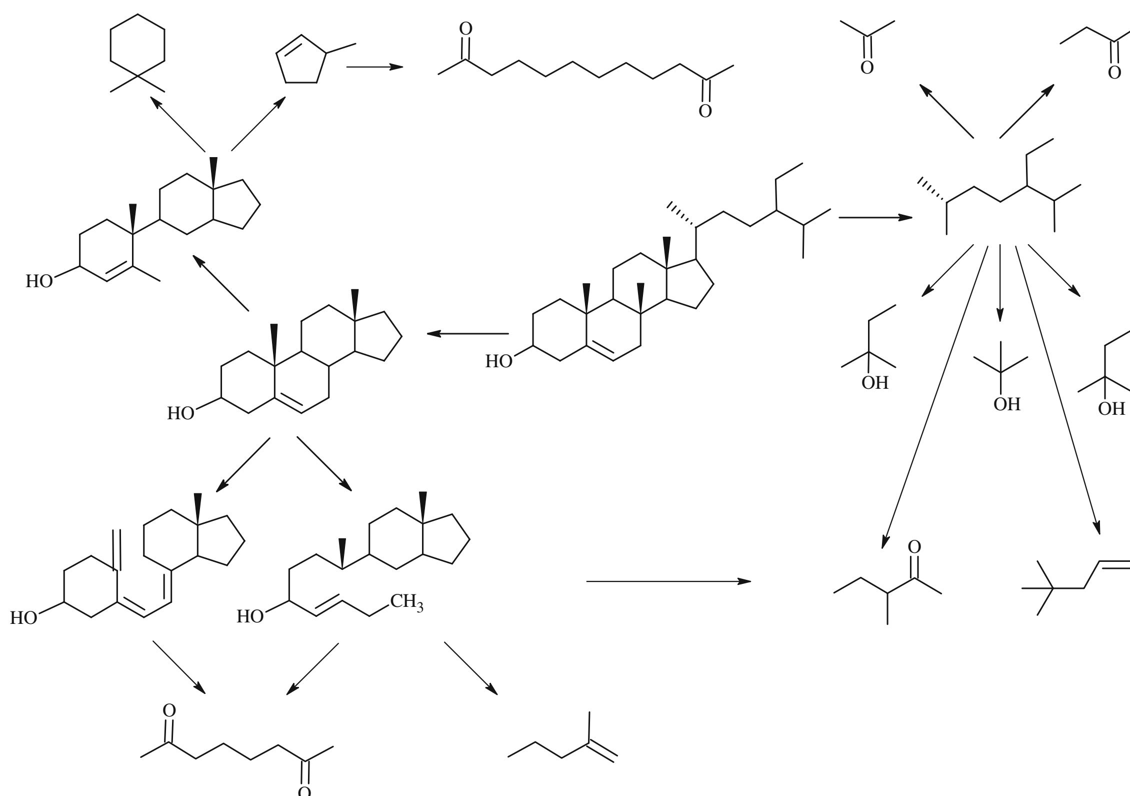


**Fig. 2** Formation of fragmented sterol molecules and volatile compounds during thermo-oxidative degradation of sitosterol. Adapted from Smith [1] and van Lier et al. [37]

carboxene/polydimethylsiloxane (DVB/CAR/PDMS) fibers were used to isolate volatile components and purchased from Supelco (Bellefonte, USA).

Sample Heating

Sitosterol standard, 500 mg, was placed in a glass ampule of 200 mL capacity and before sealing 100 mL of pure oxygen was slowly injected to provide a surplus of oxygen preventing oxygen starvation. Samples were heated at 60, 120 and 180 °C for up to 24 h. Heating experiments were run in triplicate and data presented are averages. A non-heated sample of sterol was used as a control.

Sterols

About 1 mg of heated phytosterols was silylated with BSTFA/TMCS and silyl derivatives were separated by gas chromatography. Hewlett-Packard 6890 gas chromatograph in split mode (1:25), with a FID detector and capillary column DB-5 (30 m × 0.25 mm × 0.25 μm, J&W, Folsom, CA) were used. Analytical parameters were as follows: isothermal oven temperature at 290 °C; injector and detector temperatures set at 310 °C; helium used as the carrier gas at a flow rate of 1.6 mL/min. For quantification, 5α-cholestane was used as an internal standard.

Oxysterols

Oxyphytosterols were isolated from heated samples using SPE. Briefly, 4 mg of heated phytosterols was dissolved in 250 μL of chloroform and applied to an amino SEP-PAK cartridge. Fractions were eluted using the following sequence of solvents: 10 mL of hexane, 5 mL hexane-MTBE (5:1; v/v), 5 mL hexane-MTBE (3:1; v/v) and 7 mL acetone to elute oxysterols. Solvent from the later fraction was evaporated under stream of nitrogen. Dry residue was dissolved in 100 μL of anhydrous pyridine and derivatized with 100 μL of BSTFA/TMCS for 4 h at room temperature. Oxidized phytosterol derivatives were analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with DB-5MS column (30 m × 0.25 mm × 0.25 μm; J&W, Folsom, CA). Samples were injected in a splitless mode and the column temperature was programmed as follows: initial temperature of 160 °C was held for 1 min, then programmed at 40 °C/min to 270 °C and held for 1 min; further programmed at 4 °C/min to 280 °C, the final temperature being held for 25 min. The helium carrier gas at a

flow rate of 1 mL/min was used. For quantification, 19-hydroxycholesterol was utilized as an internal standard.

Oxidized sitosterol and campesterol compounds were identified using a Finnigan TRACE 2000 gas chromatograph coupled to a Finnigan Polaris Q Quadrupole Ion Trap mass spectrometer and capillary column DB-5 (50 m × 0.2 mm × 0.32 μm; J&W, Folsom, CA). The column temperature was programmed as describe above. The helium carrier gas with a flow rate of 0.6 mL/min was used. All mass spectra were recorded using the electron impact mode with an ionization energy of 70 eV and masses were scanned from 100 to 650 Da. The ion source was held at 200 °C, and the injector at 300 °C. For identification of the compounds the combination of the NIST Mass Spectra Library, a laboratory library of collected spectra, and retention data of standards were utilized. Oxidation compounds formed from campestanol and sitostanol were only partially identified due to the lack of standards and spectra.

### Volatile Compounds

Headspace solid-phase micro-extraction (SPME) was used to isolate volatile compounds from heated phytosterol samples. Volatiles were absorbed on three-phase fiber (DVB/CAR/PDMS) as previously described [26] in triplicate. Briefly, the fiber was first conditioned in the GC injection port at 270 °C for 4 h. Conditioned fiber was placed in a vial with a sample equilibrated at room temperature and an absorption run for 5 min. Desorption of volatiles was run in the GC injection port for 5 min at 260 °C in splitless mode. Volatiles were identified on a Hewlett-Packard 5890 II GC coupled to a quadrupole mass detector HP 5971 MSD (Hewlett-Packard, Palo Alto, CA). For separation DB-5MS capillary column (25 m × 0.2 mm × 0.33 μm; J&W, Folsom, CA) was used with helium as the carrier gas at a flow rate of 0.6 mL/min. The injector and transfer line were held at 260 and 280 °C, respectively. The column temperature was programmed as follows: initial temperature of 40 °C held for 3 min, then programmed at 4 °C/min to 160 °C and further programmed at 10 °C/min to 280 °C, and the final temperature held for 3 min. Mass spectra were collected in an electron impact mode (70 eV) and masses scanned from 33 to 333 Da. For quantification deuterated naphthalene-$d_8$ was used as the internal standard.

### Oligomers Analysis

High performance size exclusion chromatography (HPSEC) according to ISO Method 16931-2009 was applied to assess the composition of the oligomers [27]. Separation was performed on a Finnigan Surveyor HPLC (Thermo Electron Corporation, Waltham, MA). Components were separated on three size exclusion columns coupled in series (Phenogel 500, 100 and 50 Å; 5 $\mu$, 300 × 4.60 mm; Phenomenex, Torrance, CA). THF was used as a mobile phase at a flow rate of 0.3 mL/min, and the column held at 30 °C. A sample of 10 μL was injected and components detected with a Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France), operated at 30 °C with an air pressure of 2.5 bar.

The amounts of the fractions were assessed using the external standard calibration method where stigmasterol was the calibration standard. The amounts of oligomers represent equivalents of stigmasterol per 1 g of phytosterol standard. For assessment of the fragmented phytosterols amounts, deconvolution software Peak-Fit was used (Systat Software Inc., San Jose). The molecular weights of the oligomers were assessed based on HPSEC calibration with a Phenomenex AL0-2761standard (Phenomenex, Torrance).

## Results and Discussion

### Changes in Phytosterols

Sitosterol standard, declared by supplier as 95% pure, contained 71% $\beta$-sitosterol, 19% sitostanol, 8% campesterol and 2% campestanol (Table 1). During thermal treatment, the total amounts of intact sterols decreased when the temperature and time increased. In samples heated at 60 °C for 1 and 24 h, 7 and 16% of the total phytosterols disappeared, whereas at 120 °C for the same times the amounts of phytosterols decreased by 14 and 63%, respectively. When the highest temperature was applied, 54–66% of the sterols was transformed into degradation products. Oehrl et al. [28] found that in canola oil heated at 100 °C for 20 h, 33% of the phytosterols were lost, when the temperature was increased to 150 °C only 5% of intact sterols were observed. The authors found higher losses of sterols in canola and soybean oils than in oils containing higher amounts of saturated fatty acids [28]. Rudzinska et al. [10] found that the amount of phytosterols decreased when canola seeds were dried at different temperature and higher losses were observed when seeds were dried at 120 °C. During simulated frying in canola and hydrogenated canola oils, 50 and 60% of the sterols disappeared, respectively [29]. Similar rate of sterols disappearance was observed during frying French fries in canola oil [13]. Soupas et al. [30] observed that 1–6% of phytosterol oxidized during 5–10 min pan-frying at 180 °C and the amount of degraded sterols increased when time was extended. Similar amounts of sterols degraded when canola oil and tripalmitin, both enriched in phytosterols, were used for baking for 2 h at 180 °C. When purified canola triacylglycerides and enriched in stigmasterol tripalmitin were heated for 3 h at 180 °C, 20% of the stigmasterol disappeared [31, 32].

**Table 1** Changes in the content of sitosterol standard during heating at different temperatures and times (mg/g)

| Temperature and time (h) | Campesterol | Campestanol | Sitosterol | Sitostanol | Total phytosterol content |
|---|---|---|---|---|---|
| Control | 75 ± 1 | 23 ± 1 | 691 ± 7 | 182 ± 2 | 971 ± 9 |
| 60 °C | | | | | |
| 1 | 71 ± 5 | 22 ± 1 | 651 ± 6 | 156 ± 1 | 900 ± 8 |
| 4 | 69 ± 6 | 20 ± 1 | 641 ± 28 | 148 ± 15 | 878 ± 7 |
| 8 | 66 ± 4 | 21 ± 1 | 631 ± 22 | 130 ± 7 | 848 ± 7 |
| 12 | 62 ± 1 | 17 ± 1 | 624 ± 22 | 132 ± 8 | 835 ± 6 |
| 24 | 63 ± 1 | 16 ± 1 | 604 ± 23 | 131 ± 2 | 814 ± 6 |
| 120 °C | | | | | |
| 1 | 73 ± 1 | 18 ± 3 | 599 ± 3 | 147 ± 5 | 837 ± 8 |
| 4 | 64 ± 2 | 17 ± 3 | 591 ± 3 | 139 ± 5 | 811 ± 7 |
| 8 | 35 ± 3 | 11 ± 1 | 323 ± 14 | 84 ± 6 | 453 ± 4 |
| 12 | 30 ± 1 | 7 ± 1 | 255 ± 3 | 63 ± 5 | 355 ± 3 |
| 24 | 30 ± 2 | 7 ± 1 | 256 ± 2 | 65 ± 5 | 358 ± 3 |
| 180 °C | | | | | |
| 1 | 38 ± 1 | 7 ± 1 | 328 ± 20 | 78 ± 5 | 451 ± 4 |
| 4 | 41 ± 1 | 8 ± 1 | 347 ± 19 | 75 ± 5 | 471 ± 4 |
| 8 | 32 ± 1 | 7 ± 1 | 278 ± 8 | 73 ± 5 | 390 ± 3 |
| 12 | 30 ± 2 | 7 ± 1 | 254 ± 15 | 66 ± 2 | 357 ± 3 |
| 24 | 29 ± 1 | 7 ± 1 | 237 ± 5 | 60 ± 5 | 333 ± 3 |

Results are averages of three replications ± SD

### Formation of Phytosterol Oxides

Eleven oxyphytosterols derivatives were positively identified in the analyzed samples, however, other compounds were observed on the chromatograms. Based on a specific isolation procedure we applied it could be deduced that no identified compounds ought to be oxidative derivatives of other sterols present in the sitosterol standard, and in presented results they are combined as a group of oxysterols (Table 2). The content of oxyphytosterols in the control sample, i.e., the unheated standard, was at 1.3 mg/g (Table 2). When phytosterols were heated at 60 and 120 °C for 24 h the amount of oxyphytosterols formed increased to 6.0 and 62.1 mg/g, respectively. When the temperature was increased to 180 °C, after 1 h the oxide content was 34.5 mg/g, whereas extending the heating time caused the the amounts of oxyphytosterols to decrease. After 4, 8, 12 and 24 h of heating at the highest temperature, the oxyphytosterols content dropped to 26.2, 30.1, 14.5 and 10.6 mg/g, respectively (Fig. 3).

Oxyphytosterols formed during heating of the standard consisted of 14–28% campesterol derivatives, 63–76% of sitosterol derivatives and 10% of stanols oxidation derivatives. The pathways of the oxyphytosterol formation are presented in Fig. 1. Oxysterols are formed as a result of ring and side chain free radical oxidation and epoxidation. Among the oxidized sterol derivatives, we were not able to detect specific oxides formed by photooxidation processes. The content of epoxy- and 7-hydroxysterols was affected by heating time. When a higher temperature was applied, we observed more 7-hydroxysterols formed than epoxy derivatives. This shift in hydroxy derivatives formation can be related to the accumulation of sterol oxidation products which are acceptors of radicals and affects oxidation [1]. It is also possible that sterol oxidation products further interact among themselves forming compounds with higher molecular weights [23, 24]. The level of phytosterol losses in the analyzed samples of the standard was much higher than the content of oxyphytosterols formed. The highest amounts of oxidative derivatives of phytosterols were formed during heating at 120 °C for 8, 12 and 24 h. Converting stoichiometrically the amount of oxyphytosterols formed into parent sterols, only 8 to 16% of the later were oxidized into these derivatives. When sterols were heated at 60 °C, 1–3% of phytosterols were transferred into relevant oxidation derivatives. At this temperature the amount of oxysterols increased slightly when the time of heating was extended. Whereas at the highest temperature applied, after 1 h, 6% of the phytosterols were transformed into oxysterols and the amount decreased when the time was extended. However this relation is not as simple as shown by calculation, mainly because under these conditions, we have to expect that a significant amount of oxysterols was transformed into oligomers.

The oxidative stability of the phytosterols in canola, coconut, peanut, and soybean oils was examined under

**Table 2** The composition of oxyphytosterols formed during thermal treatment of the sitosterol standard at different temperatures and times (mg/g of sterol)

| Time (h) | 7α-Hydroxy campesterol | 7β-Hydroxy campesterol | β-Epoxy campesterol | α-Epoxy campesterol | α-Epoxy sitosterol | β-Epoxy sitosterol | 7α-Hydroxy sitosterol | 7β-Hydroxy sitosterol | Triol sitosterol | 25-Hydroxy sitosterol | 7-Keto sitosterol | Others |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0.13 ± 0.01 | Nd | Nd | 0.22 ± 0.02 | 0.12 ± 0.01 | Nd | 0.04 ± 0.01 | 0.02 ± 0.01 | | 0.20 ± 0.02 | 0.44 ± 0.03 | 0.17 ± 0.02 |
| 60 °C | | | | | | | | | | | | |
| 1 | Nd | Nd | Nd | 0.35 ± 0.02 | 0.33 ± 0.03 | 0.07 ± 0.01 | 0.10 ± 0.01 | 0.26 ± 0.03 | 0.28 ± 0.03 | 0.07 ± 0.01 | 0.79 ± 0.05 | 0.23 ± 0.03 |
| 4 | Nd | Nd | Nd | 0.56 ± 0.04 | 0.51 ± 0.04 | 0.11 ± 0.01 | 0.22 ± 0.02 | 0.31 ± 0.03 | 0.26 ± 0.02 | 0.08 ± 0.01 | 0.67 ± 0.05 | 0.27 ± 0.03 |
| 8 | 0.22 ± 0.02 | Nd | Nd | 0.66 ± 0.04 | 0.47 ± 0.03 | 0.07 ± 0.01 | 0.15 ± 0.01 | 0.27 ± 0.01 | 0.15 ± 0.01 | 0.09 ± 0.01 | 0.91 ± 0.05 | 0.29 ± 0.04 |
| 12 | 0.37 ± 0.03 | Nd | Nd | 0.77 ± 0.05 | 0.63 ± 0.04 | 0.10 ± 0.01 | 0.13 ± 0.01 | 0.37 ± 0.02 | 0.24 ± 0.01 | 0.13 ± 0.01 | 0.92 ± 0.06 | 0.36 ± 0.04 |
| 24 | 0.60 ± 0.05 | 0.35 ± 0.02 | 0.70 ± 0.05 | 0.51 ± 0.04 | 0.22 ± 0.01 | 0.04 ± 0.01 | 1.15 ± 0.11 | 1.29 ± 0.12 | 0.12 ± 0.01 | 0.07 ± 0.02 | 0.64 ± 0.04 | 0.37 ± 0.04 |
| 120 °C | | | | | | | | | | | | |
| 1 | 0.16 ± 0.01 | 0.87 ± 0.07 | 0.07 ± 0.01 | 2.15 ± 0.15 | 0.69 ± 0.05 | 1.05 ± 0.08 | 1.24 ± 0.09 | 1.56 ± 0.09 | 0.28 ± 0.02 | 0.35 ± 0.03 | 2.20 ± 0.12 | 1.06 ± 0.09 |
| 4 | 0.22 ± 0.02 | 0.79 ± 0.06 | 0.18 ± 0.01 | 1.52 ± 0.11 | 1.01 ± 0.08 | 1.43 ± 0.12 | 1.74 ± 0.12 | 1.79 ± 0.13 | 0.56 ± 0.06 | 0.40 ± 0.03 | 2.26 ± 0.20 | 1.19 ± 0.10 |
| 8 | 0.96 ± 0.08 | 3.47 ± 0.28 | Nd | 5.62 ± 0.46 | 4.45 ± 0.38 | 1.98 ± 0.14 | 5.51 ± 0.35 | 9.35 ± 0.58 | 4.33 ± 0.37 | 1.24 ± 0.08 | 7.68 ± 0.59 | 4.46 ± 0.31 |
| 12 | 0.86 ± 0.07 | 1.90 ± 0.13 | Nd | 5.36 ± 0.56 | 5.07 ± 0.42 | 4.48 ± 0.41 | 6.78 ± 0.57 | 10.56 ± 0.92 | 3.98 ± 0.30 | 0.49 ± 0.04 | 8.91 ± 0.76 | 4.83 ± 0.42 |
| 24 | 2.97 ± 0.18 | 5.94 ± 0.51 | 2.82 ± 0.22 | 4.47 ± 0.40 | 3.89 ± 0.35 | 2.94 ± 0.23 | 9.66 ± 0.89 | 14.54 ± 1.02 | 1.94 ± 0.14 | 0.88 ± 0.06 | 6.41 ± 0.45 | 5.65 ± 0.51 |
| 18 °C | | | | | | | | | | | | |
| 1 | 0.61 ± 0.05 | 2.13 ± 0.09 | 0.34 ± 0.04 | 2.98 ± 0.28 | 2.55 ± 0.23 | 2.14 ± 0.18 | 4.30 ± 0.39 | 9.17 ± 0.87 | 1.80 ± 0.17 | 0.52 ± 0.04 | 4.81 ± 0.45 | 3.12 ± 0.22 |
| 4 | 0.28 ± 0.03 | 2.13 ± 0.09 | 1.73 ± 0.14 | 2.71 ± 0.25 | 2.20 ± 0.19 | 1.16 ± 0.11 | 2.09 ± 0.18 | 6.41 ± 0.56 | 1.13 ± 0.10 | 0.39 ± 0.03 | 3.49 ± 0.30 | 2.37 ± 0.21 |
| 8 | 0.16 ± 0.01 | 1.19 ± 0.10 | 2.48 ± 0.20 | 3.72 ± 0.36 | 3.05 ± 0.28 | 0.76 ± 0.08 | 1.06 ± 0.10 | 5.82 ± 0.55 | 1.51 ± 0.12 | 0.59 ± 0.05 | 6.97 ± 0.61 | 2.73 ± 0.27 |
| 12 | 0.07 ± 0.01 | Nd | 1.25 ± 0.10 | 1.45 ± 0.11 | 1.99 ± 0.15 | 0.40 ± 0.04 | 0.33 ± 0.03 | 2.37 ± 0.21 | 1.07 ± 0.10 | 0.48 ± 0.04 | 3.82 ± 0.31 | 1.32 ± 0.10 |
| 24 | 1.00 ± 0.08 | Nd | 0.39 ± 0.04 | 0.48 ± 0.04 | 1.30 ± 0.06 | 0.60 ± 0.01 | 1.24 ± 0.02 | 2.70 ± 0.05 | 0.37 ± 0.03 | 0.54 ± 0.04 | 1.06 ± 0.09 | 0.95 ± 0.10 |

Results are averages of three replications ± SD

*Nd* not detected, *others* not identified oxysterols

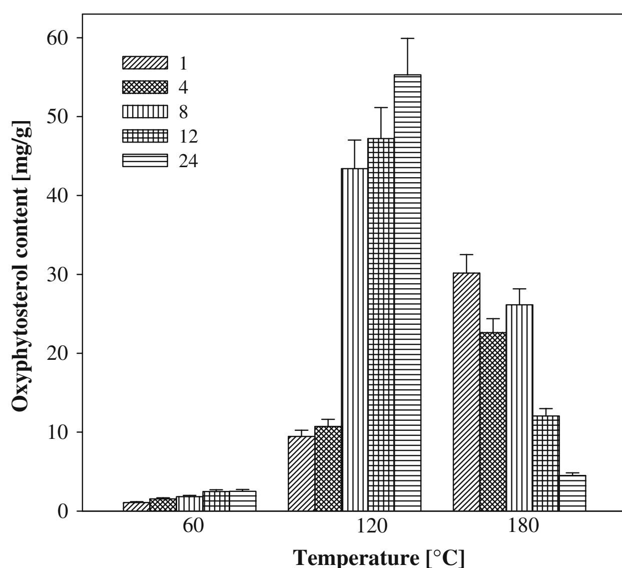


**Fig. 3** Formation of oxyphytosterols during thermal treatment of sitosterol at different temperatures and times.

simulated frying conditions when heated for 20 h at 100, 150 and 180 °C. A greater variety of sterol oxides was observed at lower temperatures compared to 180 °C [28]. The content of oxyphytosterols formed in olive oil was higher than in peanut and corn oils when heated for 2 h at 180 °C [14]. In the present study, lower amounts of oxysterols were observed when a temperature of 180 °C was used, this suggests that a further interaction of the oxysterols occurred leading to the formation of other components including oligomers.

## Volatile Compounds

Identified volatile compounds formed in the control and the heated phytosterol standard samples are presented in Tables 3, 4, 5 and 6. Hydrocarbons were the most abundant volatiles found in unheated phytosterols, where 3,5,5-trimethyl-1-hexene, heptane, 4-methyl-2-pentene and 2,4,4-trimethyl-1-pentene were observed as the highest contribution. Among alcohols, 2-methyl-1-propanol contributed mostly to the volatiles. When the standard was heated at 60 °C, four hydrocarbons, two ketones and an acetaldehyde were found. Acetone and 3-methyl-2-pentanone were the main volatile components formed and they contributed a total amount at 49% after 1 h and 70% after 24 h of heating. The amount of total volatiles formed during

**Table 3** Compositions of identified volatile compounds in the unheated sitosterol standard

| Compound | Percentage in the total amount |
|---|---|
| Hydrocarbons | |
| 2,4,4-Trimethyl-1-pentene | 10.91 |
| 2-Methyl-1-pentene | 7.12 |
| 4-Methyl-1-pentene | 4.79 |
| 2,4,4-Trimethyl -2-pentene | 6.65 |
| 4-Methyl-2-pentene | 10.91 |
| 2,2,4-Trimethyl-pentane | 6.40 |
| 2,3-Dimethyl-pentane | 4.65 |
| 3,5,5-Trimethyl-1-hexene | 13.63 |
| Heptane | 11.38 |
| Alcohols | |
| Ethanol | 2.25 |
| 2-Methyl-1-propanol | 12.72 |
| Ketones | |
| 3-Methyl-2-pentanone | 6.32 |
| Others | |
| Carbon dioxide | 2.28 |
| Total amount of volatiles (μg/g) | 0.17 ± 0.02 |

**Table 4** Contribution of identified volatile compounds formed during thermo-oxidative degradation of the sitosterol standard at 60 °C (Percentage of the total amount)

| Compound | Heating time (h) | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 8 | 12 | 24 |
| Hydrocarbons | | | | | |
| 4-Methyl-2-pentene | 22.98 | 9.33 | 8.35 | 10.38 | |
| 2,4,4-Trimethyl-1-pentene | 2.52 | 14.95 | 10.06 | | |
| 3-Ethyl-2-methyl-pentane | 5.89 | 5.44 | 4.44 | 9.86 | |
| Hexane | 2.72 | 6.91 | 15.66 | | |
| Others | | | | | |
| Acetone | 25.21 | 30.70 | 32.49 | 35.77 | 55.00 |
| 3-Methyl-2-pentanone | 23.75 | 14.25 | 21.70 | 23.63 | 15.33 |
| Acetaldehyde | | | | 11.47 | 15.31 |
| Ethylene oxide | 7.03 | 10.12 | 7.30 | 8.88 | 14.36 |
| Total amount of volatiles (μg/g) | 1.92 ± 0.19 | 1.26 ± 0.12 | 0.85 ± 0.10 | 0.47 ± 0.05 | 0.69 ± 0.06 |

**Table 5** Compositions of identified volatile compounds formed during thermo-oxidative degradation of the sitosterol standard at 120 °C (Percentage of the total amount)

| Compound | Heating time (h) | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 8 | 12 | 24 |
| Hydrocarbons | | | | | |
| 4-Methoxy-1-pentene | 2.59 | 3.49 | 1.53 | 2.14 | 1.85 |
| 4-Methyl-2-pentene | 0.66 | 1.91 | 7.22 | 2.74 | 3.69 |
| 3-Ethyl-1-pentene | 0.57 | 0.35 | 0.58 | 0.99 | 1.36 |
| 2,4,4-Trimethyl-2-pentene | 1.84 | 1.31 | 1.42 | 3.38 | 6.69 |
| 3-Methylene-pentane | 0.64 | | | 4.29 | 3.94 |
| 3-Ethyl-2-methyl-pentane | 0.75 | | 0.57 | | |
| 2-Methyl-2-hexene | 1.47 | 1.33 | 0.68 | 0.83 | 0.85 |
| 3,5,5-Trimethyl-1-hexene | 4.01 | | 1.64 | 3.01 | 8.63 |
| Hexane | 1.69 | 1.30 | 1.77 | 3.42 | 3.11 |
| 3-Methyl-hexane | | | 0.47 | 0.57 | 0.43 |
| Heptane | 2.49 | 0.91 | 0.35 | 3.50 | 9.95 |
| 3-Ethyl-2-methyl-heptane | 0.51 | 0.73 | 1.53 | 1.09 | 1.09 |
| 4-Octene | | | 0.66 | 1.98 | 3.26 |
| 2-Nonanone | | 0.26 | 0.49 | 1.10 | 1.79 |
| Benzene | 5.04 | 2.71 | 0.25 | 0.44 | 0.70 |
| Ketones | | | | | |
| Acetone | 32.98 | 33.35 | 20.47 | 20.04 | 14.96 |
| 2-Butanone | 1.23 | 2.12 | 0.99 | 0.97 | 1.02 |
| 3-Methyl-2-butanone | 1.59 | 0.22 | 4.50 | 12.94 | 4.48 |
| 3-Methyl-2-pentanone | 14.38 | 29.04 | 20.15 | 14.81 | 12.19 |
| 5-Methyl-2-hexanone | | 0.45 | 0.19 | | |
| 2,5-Hexanedione | | | 3.31 | 0.84 | 0.58 |
| 2-Heptanone | 1.73 | 1.95 | 2.70 | 0.97 | 0.43 |
| 2,7-Octanedione | 1.52 | 1.31 | 2.88 | 1.60 | 0.77 |
| 2,11-Dodecanedione | 14.08 | 9.75 | 17.81 | 7.39 | 3.56 |
| 4,4,5,5-Tetramethyl-dihydro-2-furanone | 0.80 | 0.75 | 0.65 | 1.72 | 3.17 |
| Aldehydes | | | | | |
| 2-Ethyl-3-methyl-butanal | 0.50 | 0.70 | 0.99 | 2.35 | 3.67 |
| 2-Ethyl-2-hexenal | 2.36 | | 0.73 | 1.70 | 2.77 |
| Alcohols | | | | | |
| Ethanol | 1.91 | 1.28 | 1.55 | 1.68 | 3.07 |
| 3,4-Dimethyl-2-hexanol | 1.60 | | 0.73 | | |
| Acids | | | | | |
| Formic acid | 0.42 | 0.63 | | 1.67 | 1.62 |
| Acetic acid | 2.61 | 4.14 | 3.19 | 1.86 | 0.38 |
| Total amount of volatiles (μg/g) | 7.09 ± 0.7 | 9.44 ± 0.8 | 16.6 ± 1.5 | 11.4 ± 1.0 | 7.87 ± 0.7 |

heating the phytosterol standard at 60 °C decreased with time from 1.92 to 0.69 μg/g after 1 and 24 h, respectively (Table 4). Ethylene oxide, the simplest epoxide, detected in all samples heated at 60 °C, is toxic when inhaled and is an irritant to the skin and tissues in the respiratory tract [33].

When cholesterol was aged at ambient temperature, 14 volatile compounds were identified and they were products of cholesterol hydroperoxide decomposition [34]. Since other sterols share the same chemical structure, formation of volatile components will follow the same mechanism. Presence of additional double bond(s) in the side chain may further stimulate degradation processes with volatile compounds being formed. Possible degradation of sterols into fragmented sterol molecules and volatile compounds in Fig. 2 is proposed.

**Table 6** Composition of identified volatile compounds formed during thermo-oxidative degradation of the sitosterol standard at 180 °C (Percentage of the total amount)

| Compound | Heating time (h) | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 8 | 12 | 24 |
| Hydrocarbons | | | | | |
| 2-Methyl-2-pentene | | 0.52 | 0.44 | 0.41 | |
| 3-Ethyl-2-methyl-pentane | 0.66 | 2.07 | 4.64 | 4.59 | 5.84 |
| 3-Methyl-3-hexene | | 7.43 | | 4.02 | |
| 3-Ethyl-2-methyl-heptane | | 0.66 | 0.95 | 0.82 | 1.15 |
| Cyclooctane | | 0.64 | 0.52 | 0.60 | 0.60 |
| 3,3-Dimethyl-1-octene | | | | | 6.23 |
| 2-Methyl-2-dodecene | 0.90 | 0.81 | 0.60 | 0.40 | 0.51 |
| 2,2,4,4-Tetramethyl- tetrahydro-furan | | 0.53 | 0.68 | 0.62 | 0.79 |
| Ketones | | | | | |
| Acetone | 2.56 | 3.43 | 9.65 | 9.26 | 8.73 |
| 2-Butanone | | | 0.41 | 0.72 | 0.59 |
| 2-Methyl-1-penten-1-one | | 0.33 | 0.42 | 0.46 | 0.40 |
| 2-Methyl-3-pentanone | 0.32 | 2.43 | 4.73 | 5.29 | 5.45 |
| 3-Methyl-2-pentanone | | 0.78 | 1.66 | 1.45 | 1.57 |
| 3-Methylcyclopentanone | 7.64 | | 4.81 | | |
| 2,5-Hexanedione | 1.07 | 0.47 | 0.56 | 0.26 | |
| 2-Cyclohexen-1-one | 2.17 | 2.27 | 1.87 | 1.72 | 2.43 |
| 2-Heptanone | 3.84 | 4.33 | 2.83 | 3.25 | 3.94 |
| 6-Methyl-2-heptanone | | 0.54 | 0.39 | 0.52 | 0.54 |
| 2-Octanone | | | 0.35 | 0.28 | 0.40 |
| 2,7-Octanedione | | 6.62 | 4.61 | 4.92 | 4.60 |
| 4-Octen-3-one | | | 0.42 | | |
| 2,11-Dodecanedione | 45.82 | 30.82 | 27.11 | 23.31 | 18.99 |
| Aldehydes | | | | | |
| Acetaldehyde | | 0.34 | 0.68 | 0.90 | 0.97 |
| 2-Methyl-benzaldehyde | | 1.67 | 1.28 | 1.63 | |
| 2-Ethyl-3-methyl-butanal | | 0.58 | 0.88 | 1.07 | |
| 3,3-Dimethyl-hexanal | 26.09 | 25.15 | 27.26 | 29.15 | 31.71 |
| Acids | | | | | |
| Acetic acid | 3.47 | 2.82 | 1.69 | 3.35 | 3.80 |
| Formic acid | 1.86 | 0.98 | | | |
| Others | | | | | |
| Carbon dioxide | 1.08 | 0.60 | | 0.38 | |
| 2-Methyl-3-pentanol | | 0.52 | 0.57 | 0.64 | 0.75 |
| Total amount of volatiles (μg/g) | 24.1 ± 2.1 | 38.1 ± 3.5 | 39.4 ± 3.5 | 45.3 ± 4.2 | 41.6 ± 4.0 |

When the temperature was increased, the amount of volatiles increased accordingly, this indicates that more energy is required to degrade oxyphytosterols and at the same time shows their thermal instability. In the sample of the phytosterols standard heated at 120 °C we identified 31 volatile compounds, where hydrocarbons and ketones were the most abundant (Table 5). The type of volatiles formed was affected by heating time, after heating for 1 h we observed the contribution of hydrocarbons and ketones at 22 and 68%, whereas after 4 h, it changed to 14 and 79%, respectively. When the heating time was further increased, the contribution of hydrocarbons and ketones changed to 47 and 41%, respectively. Acetone was the most abundant ketone and its contribution decreased from 38 to 15% when the heating time was increased (Table 5). The latter compound originated from the decomposition of

25-hydroperoxide, side chain peroxide, by scission of the C24/C25 bond (Fig. 2). However, we should not exclude the possibility of pyrolytic decomposition of the 24-hydroperoxides, where the terminal isopropyl group may also form acetone (Fig. 2) [34]. In all samples heated at 120 °C acetic acid was found, which possible originated from the scission of a bond in the side chain hydroperoxide [37]. Acetic acid represents a higher level of oxidation and it is possible that both ethanol and acetic acid may be formed from the same precursor [35]. The total amounts of volatile compounds formed during heating of the phytosterol standard at 120 °C increased from 7.1 to 16.6 µg/g and subsequently decreased to 7.9 µg/g after heating for 1, 8 and 24 h, respectively (Table 5). The decrease in the amount of volatiles can be explained by the possible formation of compounds with a higher molecular weight which are not absorbed on SPME due to their low volatility.

In samples of phytosterols heated at 180 °C, 30 major volatiles were identified, among them 8 hydrocarbons, 13 ketones, 4 aldehydes, and 2 acids (Table 6). During heating for 1 h, the major compound detected was diketone, 2,11-dodecanedione, which can be formed from the degradation of methylcyclopentenyl hydroperoxide (Fig. 2) [34]. A similar contribution of 3,3-dimethylhexanal in the range of 22–28% was observed for all samples heated at 180 °C (Table 6). Among the hydrocarbons, 3-methyl-3-hexene was the main compound and its contribution decreased with time. At the highest tested temperature, the total amounts of volatiles were 24.1, 45.3 and 41.6 µg/g after heating for 1, 12 and 24 h, respectively (Table 6).

## Oligomers

The amounts of oxyphytosterols and volatiles formed from phytosterol standard can be stoichiometrically balanced to 1–15% of the amount of the disappearing sterols, depending on the temperature and heating time applied. In the heated phytosterol standard, we found a significant amount of oligomers, and their amounts increased when higher temperatures and longer times were used. The oligomers found are products of oxysterol condensation/polymerization, and within this group we observed: dimers (MW 780–860 Da), trimers (MW 1,100–1,800 Da) and tetramers (MW 1,500–1,800 Da). We also found sterol monomers (MW 400–450 Da) and partially decomposed sterol molecules (MW 50–200 Da) (Fig. 4). The data on the amounts of oligomers are presented as equivalents of stigmasterol, which was used as the calibration standard.

In the control sample, oligomers were absent, however, in all heated samples partially decomposed sterol molecules (PS) were observed. The amounts of PS were 153, 155 and 162 mg/g after heating at 60, 120 and 180 °C for 24 h, respectively (Fig. 5). Dimers and trimers were found at the levels of 229 and 201 mg/g, and 167 and 180 mg/g in samples heated at 120 and 180 °C, respectively. The dimers were the dominant oligomer present in the sterol standard heated to the previously mentioned temperatures. Tetramers were detected in the phytosterols standard when heated at 180 °C for 12 and 24 h, and at the latter time, 86 mg/g was observed (Fig. 5).

Oligomers in frying oils are associated with the oxidative degradation of unsaturated fatty acids present in TG, where oxides are the precursors [36, 37]. Similarly, sterols became oxidized by a free radical mechanism and hydroperoxy radicals are formed which through polymerization and/or condensation form oligomers [36, 37].

By combining stoichiometrically amounts of all the groups of components formed during thermo-oxidative

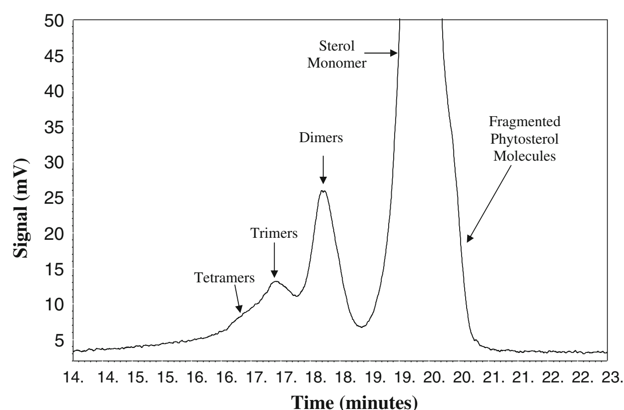


**Fig. 4** Separation of oligomers formed during thermo-oxidative degradation of the sitosterol standard

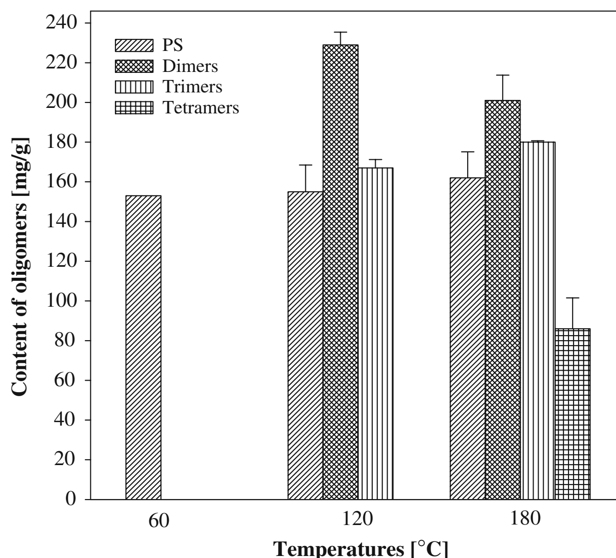


**Fig. 5** The amount of oligomers formed during heating sitosterol standard for 24 h. *PS* fragmented molecules of phytosterols standard

**Table 7** Percentage of sitosterol standard transformed into products during heating for 24 h at different temperatures

| Component | Contribution (%) | | |
|---|---|---|---|
| | 60 °C | 120 °C | 180 °C |
| Volatile | 0.0004 | 0.001 | 0.01 |
| PS | 97.0 | 25.4 | 25.4 |
| Oxyphytosterol | 3.0 | 9.9 | 1.5 |
| Dimer | Nd | 37.4 | 31.5 |
| Trimer | Nd | 27.3 | 28.2 |
| Tetramer | Nd | 0.0 | 13.5 |

*PS* fragmented molecule of sitosterol standards, *Nd* not detected

degradation of sterols, we were able to balance them with the amount of disappearing sterols (Table 7). Results in the Table 7 represent the contribution of specific groups of components as the part of the sterol standard losses. The losses of the phytosterols standard heated at 180 °C for 24 h were 638 mg/g and from it 9, 0.04, 162, 201, 180, and 86 mg/g were transformed into oxysterols, volatiles, PS, dimers, trimers, and tetramers, respectively. A similar balance was achieved for standards heated at 60 °C for 24 h, where 157.8 mg/g of sterols were transformed into 4.7 mg/g of oxyphytosterols, 0.0007 mg/g of volatiles, and 153 mg/g of PS. We were able to balance losses of sterol standard with the amounts of degradation compounds formed for all parameters used in this study.

In conclusion, we observed for the first time that during thermo-oxidative degradation of phytosterols, a diverse group of components is formed, and their presence and contribution is time and temperature dependent. At higher temperatures oligomers were the main products formed, with 60–74% of phytosterols transferred into these components. Volatile compounds are important from a sensory point of view and their formation can be a source of off-flavor formation in thermally treated oils. Additionally, formation of volatiles and other components may stimulate free radicals formation, directly stimulating or initiating oxidative degradation of other oil endogenous compounds. Further investigation is required to establish the health effects and the kinetics of all the components formed as an effect of sterol oxidative degradation.

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