Unsaturated lipid matrices protect plant sterols from degradation during heating treatment

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ABSTRACT

The interest in plant sterols enriched foods has recently enhanced due to their healthy properties. The influence of the unsaturation degree of different fatty acids methyl esters (FAME: stearate, oleate, linoletate and linolenate) on a mixture of three plant sterols (PS: campesterol, stigmasterol and β-sitosterol) was evaluated at 180 °C for up to 180 min. Sterols degraded slower in the presence of unsaturated FAME. Both PS and FAME degradation fit a first order kinetic model (R^2>0.9). Maximum oxysterols concentrations were achieved at 20 min in neat PS and 120 min in lipid mixtures and this maximum amount decreased with increasing their unsaturation degree. In conclusion, the presence of FAME delayed PS degradation and postponed oxysterols formation. This protective effect was further promoted by increasing the unsaturation degree of FAME. This evidence could help industries to optimize the formulation of sterol-enriched products, so that they could mantain their healthy properties during cooking or processing.

Keywords: phytosterols, sitosterol, oxidation, oxysterols, Sterol Oxidation Products, POPs

Chemical compounds studied in this article: phytosterol (PubChem CID: 12303662); β-sitosterol (PubChem CID: 222284); campesterol (PubChem CID: 173183); stigmasterol (PubChem CID: 5280794); methyl stearate (PubChem CID: 8201); methyl oleate (PubChem CID: 5364509); methyl linoleate (PubChem CID: 5284421); methyl linolenate (PubChem CID: 5319706)

1. Introduction

Plant sterols and stanols enriched products have experienced an increase in the last few years due to their demonstrated cholesterol-lowering effects at doses above 2 g per day (Katan, Grundy, Jones, Law, Miettinen, & Paoletti, 2003; Demonty et al., 2009; Shaghaghi, Harding, & Jones, 2014). Besides the well-established market of dairy products and yellow-fat spreads, a number of other foodstuffs have been approved by the European Commission to be enriched in plant-sterols-stanols, such as rye bread, vegetable oils and rice drinks (Eur-Lex, online). Among the several plant sterols specifically named as ingredients commonly added to functional products, as listed in the European legislation, sitosterol, campesterol and stigmasterol are those allowed to be used in a higher proportion of total plant sterols content.

Inappropriate food processing, storage conditions and cooking procedures can lead to oxidation of these plant sterols (Zhang et al., 2006; Menéndez-Carreño, Ansorena, & Astiasarán, 2008; Gawrysiak-Witulska, Rudzińska, Wawrzyniak, & Siger, 2012; Rudzińska, Przybylski, & Wąsowicz, 2014), reducing their presence in foods hence, the associated beneficial effects. Moreover, this oxidation process leads to the formation of phytosterol oxidation products (POPs), which have been related to atherosclerosis, citotoxity and inflammation (Otaegui-Arrazola, Menéndez-Carreño, Ansorena, & Astiasarán, 2010; O'Callaghan, McCarthy, & O'Brien, 2014; Alemany, Barbera, Alegría & Laparra, 2014). These deleterious compounds have extensively been found in vegetable foods and especially in enriched products, reaching values over 700 µg/g in spreads and 450 µg/100 mL in milk-type products (Menéndez-Carreño et al., 2008; Rudzińska et al., 2014). The hypothesis of their possible absorption into the organism

through the diet is supported by several studies (Bang, Arakawa, Takada, Sato, & Imaizumi, 2008; Liang et al., 2011). Moreover, recent evidence in a mice model fed with a mixture of POPs, reinforced this hypothesis (Plat et al., 2014).

Food matrix and its particular features are directly involved in phytosterol oxidation process. On the one hand, photosensitizers, metals or radical species have been shown to promote oxidation (Wanasundara, & Shahidi, 1998; Chien, Lu, Hu, & Chen, 2003; Derewiaka, & Obiedzinski, 2012; Yarnpakdee, Benjakul, & Kristinsson, 2014). On the other hand, phenolic compounds and tocopherols standout due to their widely demonstrated antioxidant effects towards phytosterols (Rudzińska, Korczak, Gramza, Wasowicz, & Dutta, 2004; Xu, Guan, Sun, & Chen, 2009; Kmiecik, Korczak, Rudzińska, Gramza-Michałowska, Hęś, & Kobus-Cisowska, 2015).

Interestingly enough, the role of unsaturated fatty acids and their esters and glycerides in this process leads to controversy, since opposite results concerning their protective or pro-oxidant effect have been observed by different authors, mainly using cholesterol in models systems and measuring its oxidation products (Chien et al., 2003; Lehtonen, Lampi, Riuttamaki, & Piironen, 2012). Nevertheless, certain unsaturated fatty acids have indeed been used as cholesterol oxidation protectors (Yen, Inbaraj, Chien, & Chen, 2010). Moreover, a recent study suggested that both the presence and the increasing unsaturation degree of triacylglycerides exhibited an inhibitory effect against cholesterol degradation and oxides formation (Ansorena, Barriuso, Cardenia, Astiasarán, Lercker, Rodríguez-Estrada, 2013a). However, the behavior of phytosterols under different degree of unsaturation of the surrounding lipids needs further investigation.

Over the last few years, the use of model systems in detriment of those involving direct work with foodstuffs, has risen up for the analysis of sterols oxidation (Xu, Sun, Liang, Yang, & Chen, 2011; Lehtonen et al., 2012; Kmiecik et al., 2015; Derewiaka, & Molińska, 2015). Model systems enable an easier selection and characterization of the elements affecting the process.

Taken together, all this evidence led us to evaluate the influence of the unsaturation degree of different lipid matrices (fatty acids methyl esters: Stearate (S), Oleate (O), Linoleate (L) and Linolenate (Ln)) on a mixture of three plant sterols (campesterol, stigmasterol and β-sitosterol) at 180 ºC for up to 180 min. The intensity and rate in which the degradation of sterols and formation of POPs take place in these model systems were assessed.

2. MATERIAL AND METHODS

2.1 Material and reagents

Fatty acid methyl esters (FAME) were purchased from Nu-Check (Elysian, MN, USA): Stearate, Oleate, Linoleate and Linolenate. Mixture of plant sterols (PS) (54 % sitosterol, 30 % campesterol, 15 % stigmasterol), 5α-cholestane, heptadecanoic acid and ammonium thyocianate were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). 19-hydroxycholesterol was obtained from Steraloids (Wilton, NH, USA). Tri-sil® reagent was obtained from Thermo-Scientific (Rockford, IL, USA). Hexane, heptane, acetone, chloroform, ethyl acetate, butanol, methanol, 2-propanol, hydrochloric acid, ammonium iron (II) sulfate and barium chloride, were obtained from Panreac (Barcelona, Spain).

Strata NH_2 (55 µm, 70 A) 500 mg / 3 mL Solid Phase Extraction cartridges were obtained from Phenomenex (Torrance, USA).

2.2 Heating treatment

For each type of FAME, a stock solution of FAME:phytosterols (100:1, w/w) was prepared in chloroform. Samples (240 mg of a commercial mixture of plant sterols) were put into open glass tubes (11 mm diameter, 90 mm height), dried under N_2 stream until constant weight. The unsealed tubes were then placed open in a termbloc (P Selecta, Barcelona, Spain) previously heated at 180 ºC. They were taken out from the termbloc after different heating times (0, 5, 10, 20, 30, 60, 120 and 180 min) and cooled down in an ice bath for 5 min. One mL of chloroform was added to each tube, and samples were shaken vigorously for 40 sec and kept under -20 ºC until analysis. The heating experiment was run in triplicate. A similar experimental set up was applied to the mixture of plant sterols without FAME (2.4 mg/tube). Samples were named as PS (plant sterols without FAME, used as control), PS+S (PS with stearate), PS+O (PS with oleate), PS+L (PS with linoleate) and PS+Ln (PS with linolenate). From each heated tube, approximately 1/20 part (in duplicate) was used for PV analysis, 1/20 part for sterols analysis, and the rest for FAME and POPs analysis.

2.3 Peroxides analysis

Peroxides Value (PV) was analyzed following the method of Shantha & Decker (1994) with slight modifications. Briefly, an aliquot (50 µL) of sample was transferred to a tube and chloroform was evaporated under a stream of N_2. The residue was solved in 5 mL of a mixture butanol:methanol, (2:1). $SCNNH_4$ (30 % in distilled water, 25 µL) was added

and tubes were vortexed for 4 s. Then, a solution of $FeCl_2$ (36 mM in HCl, 25 µL) was added and tubes were vortexed. After 15 min, absorbance was measured at 510 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). A calibration curve with Cumene hydroperoxide was done for quantification. Results were expressed as meq O_2 / Kg sample, being the data the average of 2 measurements per replicate.

2.4 Analysis of remaining plant sterols

An aliquot (50 µL) equivalent to approximately 10 mg of the heated sample was transferred to a test tube. The solvent was evaporated and the exact lipid weight was registered. 5α-cholestane (50 µL of a 2 mg/mL solution in chloroform) was added as internal standard, evaporated and sylanized (400 µL of Trisil® reagent were added) at 60 ºC for 45 min. Excess of sylanization reagent was evaporated and samples were re-solved in hexane (400 µL). 1 µL of sample was injected in a Gas Chromatograph coupled to a Mass Spectrometer (Agilent Technologies 6890N-5975), which was interfaced with a computerized system for data acquisition (Chemstation). A CP8947 Varian VF-5ms 5% phenylmethyl siloxane (50m x 250µm x 0.25µm) column was used. The oven temperature was programmed from 85 °C to 290 °C at 50 °C/min and then to 291 °C at 0.05 °C/min. The injector temperature was set at 250 °C, the ion source at 230 °C and the quadrupole at 150 °C. Helium was used as carrier gas. The acquisition and integration modes were Full Scan (TIC) and Single Ion Monitoring (SIM) of the characteristic ions of each sterol, respectively. The characteristic ions used for identification and quantification, as well as the retention times are detailed in Table 1S (Supplementary

Material). For quantification purposes, internal standard calibration curves were used. Results of each remaining sterol at every point of analysis were expressed as percentage over their initial amount in the mixture.

2.5 Purification

To determine FAME and POPs in the heated samples, it was first necessary to purify the samples by NH_2-SPE cartridges, as suggested by Rose-Sallin, Hugget, Bosset, Tabacchi and Fay (1995). An aliquot of sample (approximately 850 µL) was transferred to a test tube, evaporated and weighted accurately. 19-hydroxycholesterol (1 mL of 20 µg/mL in hexane:2-propanol, 3:2) and methyl heptadecanoate (1 mL of 10 mg/mL in heptane) were added to the tubes as internal standards, evaporated, re-diluted in hexane:ethyl acetate (95:5) and transferred to the cartridge. Then, three different solvents were applied to the cartridge: hexane/ethyl acetate (95/5, 8 mL), hexane/ ethyl acetate (90/10, 10 mL) and acetone (10 mL). The first eluted fraction was kept for FAME analysis and the third one for POPs analysis.

2.6 FAME analysis

As it was previously mentioned, the first SPE fraction contained the FAME and was used for their analysis. The solvent was evaporated and the residue was re-solved in heptane (2 mL). 1 µL was injected in a Gas Chromatograph coupled to a Flame Ionization Detector, as described in Ansorena, Echarte, Ollé, & Astiasarán (2013b). Briefly, identification was made comparing the retention times with those of pure standards. Quantification was carried out with internal calibration curves, with methyl heptanodecanoate as the internal standard.

2.7 POPs analysis

The solvent was evaporated and the residue was silylated (400 µL of the Trisil® reagent were added), dried under nitrogen stream and dissolved in 400 µL of hexane. One µL of the silylated POPs was analyzed by GC-MS (Agilent Technologies 6890N-5975). A CP8947 Varian VF-5ms 5% phenylmethyl siloxane (50m x 250µm x 0.25µm) was used. The temperature was programmed from 75 °C to 250 °C at 20 °C/min, then to 290 at 8 °C/min and finally to 292 °C at 0.05 °C/min. The injector temperature was set at 250 °C, the ion source at 230 °C and the quadrupole at 150 °C. Helium was used as the carrier gas. The injection was performed in the splitless mode. The electron energy was 70 eV. A mass range from *m/z* 50 to 600 was scanned at a rate of 2.66 scan/s.

The acquisition and integration modes were Full Scan (TIC) and Single Ion Monitoring (SIM) of the characteristic ions of each POP, respectively. The characteristic ions used for identification and quantification, as well as their retention times are detailed in Table 2S (Suplemmentary Material). For quantification purposes, calibration curves of COPs were used, as it has been demonstrated that the response factor obtained for cholesterol oxidation products are also valid for quantitative work regarding phytosterol oxidation products (Apprich & Ulberth, 2004). Six different POPs from each sterol were determined: 7α-hydroxy (7α-H), 7β-hydroxy (7β-H), 5β,6β-epoxy (5β,6β-E), 5α,6α-epoxy (5α,6α-E), 3,5,6-triol (Triol) and 7-keto (7-K).

2.8 Statistical analysis

For the statistical analysis of the data, Stata 12 program was used. Mean and standard deviation of data obtained from each replicate were calculated. One factor ANOVA, with

Tukey's post hoc multiple comparisons (p <0.05), was applied to evaluate the significant differences on phytosterols, POPs, PV and FAME amounts over time and among samples containing different FAME.

For the mathematical modelling of phytosterol and FAME degradation, the non-linear regression analysis in GraphPad Prism 6 was used.

3 RESULTS AND DISCUSSION

3.1 Fatty acids methyl esters and phytosterols degradation

Heating caused a progressive degradation of plant sterols, being the decrease modulated by the different fatty acids methyl esters (FAME). The remaining percentage of each phytosterol at the different sampling times is shown in Figure 1. Results showed a rapid and sharp decrease in the control sample (lacking FAME) followed by a less intense degradation of the plant sterols in presence of stearate. However, mixtures of PS+O, PS+L or PS+Ln presented a considerably lower degradation rate up to 30 min heating. The three plant sterols exhibited almost identical behavior, achieving around 82, 53, 21, 20 and 13% degradation of the initial amount after 30 min of heating in PS, PS+S, PS+O, PS+L and PS+Ln samples, respectively. At the end of the heating process, the lowest remaining values were for PS and PS+S samples (2-10%), followed by PS+O (25%) and finally the polyunsaturated FAME (40-43%). Less cholesterol oxidation has also been reported in samples containing conjugated linoleic acids compared to samples which were free of surrounding lipids (Yen et al., 2010).

In addition, the degradation of the three phytosterols clearly fitted a first order kinetic curve in the five types of samples, with R^2 values over 0.9 in all the cases (Table 1). The

kinetic constant (k) values progressively decreased along with the increase in the unsaturation degree of the lipid matrix, ranging from 0.0500 to 0.0553 min^{-1} among the three sterols in PS, and 0.0042 to 0.0049 min^{-1} in PS+L and PS+Ln. In a recent publication from our group, using 180 °C up to 180 min, similar k values were obtained when cholesterol and stigmasterol were heated in sunflower oil rich in polyunsaturated fatty acids (0.004 and 0.005 min^{-1}, respectively) (Barriuso, Ansorena, Poyato, & Astiasarán, 2015) and also when cholesterol was heated in different TAG (0.009, 0.005 and 0.004 $min^{-1,}$ for trioleate, trilinoleate and trilinolenate samples, respectively) (Ansorena et al., 2013a). In this last paper, k values also decreased along with the increase in the unsaturation degree of the lipid matrix, as well as in Hu & Chen's work (2002), where cholesterol photo-oxidation within different FAMEs was monitored. Hence, the more unsaturated the lipid matrix was, the less extent of phytosterols degradation was achieved. In other words, both the presence and the unsaturation degree of the surrounding lipids exhibited a protective effect against the degradation of plant sterols during heating.

A possible explanation for this behavior was the likelihood to oxidation of unsaturated lipids, and the consequent competition for oxygen. The adjusted first order kinetic curves for FAME degradation throughout the heating process (Table 1) showed that the kinetic constants significantly increased with the unsaturation degree of the FAMEs (0.0018, 0.0030, 0.0038 and 0.0046 min^{-1} for PS+S, PS+O, PS+L and PS+Ln, respectively). Thus, the association between FAME susceptibility to oxidation and their unsaturation degree is supported by our experimental data, and it could explain the observed trends in

phytosterols degradation observed in our model. Furthermore, physical protection or dilution of the sample could also be a mechanism by which, even the presence of lipids not prone to oxidation (such as methyl stearate, in the current study) was able to prevent sterols from degradation (Rodríguez-Estrada, Garcia-Llatas, & Lagarda, 2014).

3.2 Peroxides and POPs formation

The loss of phytosterol and FAME as a consequence of the oxidation process induced the formation of primary and secondary oxidation products in the media, which were assessed by Peroxides Value (PV) and POPs concentrations, respectively.

Figure 2a shows the evolution of PV in samples with the mixtures of plant sterols and FAME. As expected, formation of peroxides in PS+S sample was remarkably slower than in the unsaturated matrices at the beginning of the process. Maximum values (around 20 meq O_2/Kg) were achieved after 10-20 min heating for unsaturated mixtures and after 180 min (15 meq O_2/Kg) for PS+S. Regarding the unsaturated samples, a steady drop was noted from 60 min onwards, probably due to formation of secondary oxidation products. These results pointed out that, among the unsaturated FAME, the more unsaturated the FAME was, the higher PV degradation. Ansorena et al. (2013a) also found earlier and higher maximum PV for unsaturated TAGs compared to the saturated one.

The content on total oxidation products resulting from campesterol, stigmasterol and sitosterol, expressed as µg/mg each sterol, is reported in Figure 2b-d. As shown, very similar overall behavioral pattern was noted for derivatives from the three different sterols, showing in all cases that the formation of POPs was delayed by the presence of FAME in the samples. As shown in the figure, POPs content started to increase from the

beginning of the heating process and reached the apex at 20 min in the pure phytosterols sample, whereas its peak value was reached at 120 min in the FAME-containing ones. This maximum content was significantly higher in the PS samples than in the FAME-containing mixtures, except for the case of PS+S samples. Besides, among the FAME-containing samples, the one that yielded the highest POPs content was PS+S, followed by PS+O, whereas PS+L and PS+Ln samples resulted in the lowest total POPs levels. Thus, in accordance to the results from sterol degradation, both the presence and the unsaturation degree of FAME seemed to inhibit POPs formation, regardless of the sterol origin. Moreover, Lehtonen and coworkers (2012) also reported a protective effect of the surrounding lipid acyl moiety compared to the heating of free cholesterol, although in that study the increasing unsaturation degree of the acyl moiety promoted cholesterol oxidation, rather than slowing it down.

POPs values in the saturated matrix were up to 50 % higher than in the PS sample from 60 min onwards. These results could indicate a slow POPs degradation in this kind of matrix. In the PS sample, rapid POPs formation is noted, followed by a dramatic drop, which denoted that the degradation rate of POPs was higher than their formation rate. Nevertheless, in the case of the stearate matrix, the formation of oligomers and polymers characteristic of extended heating processes could have been hampered by the high viscosity of the mixture, compared to that of the unsaturated FAMEs (Knothe, & Steidley, 2005; Derewiaka, & Molińska, 2015). Hence, the overall balance yielded higher POPs values.

Oxyphytosterol distribution (Tables 2, 3 and 4) was, in general, similar among the different samples, although some slight differences were noticed. 7-keto derivatives were the most abundant oxides, representing over 70 % of total oxyphytosterols at certain points of the analysis, followed by 5,6-epoxides and 7-hydroxides. It was remarkable that, among campesterol oxidation products, 7-keto derivative accounted for around 44 %, whereas among stigmasterol and sitosterol oxides, this kind of derivative accounted for around 33 % of total POPs, when the maximum total POPs was achieved. Moreover, β-epoxides were expected to be at higher amounts than α-isomers due to the steric hindrance in position 3 (Gumulka, Pyrek, & Smith, 1982). However, although 5,6β-epoxides were higher than their α counterparts in PS+O, PS+L and PS+Ln samples, they were lower in PS and PS+S samples. This different distribution seemed to be somehow related to the unsaturation degree of the lipid matrix since the production of β-epimer was favoured in the presence of unsaturated lipids. Triol derivatives were, by far, the less abundant in all the heated samples, accounting for less than 6 % of total oxyphytosterols in most cases. These negligible levels were attributed to the lack of water in the medium, which is required for the generation of triol derivatives from epoxides (Iuliano, 2011).

When the amount of POPs was expressed as µg/mg total initial sterols (data not shown), those derived from sitosterol were the most abundant, followed by derivatives from campesterol and finally by those from stigmasterol, as it could be expected from the relative initial amounts of plant sterols in the analyzed mixture (54 % sitosterol, 30 % campesterol and 15 % stigmasterol). However, considering the extent of oxidation for each sterol (expressed as µg/mg initial sterol), as shown in Figures 2b-d, campesterol

oxidation products kept the highest values in all samples throughout the entire process, followed by sitosterol oxidation products, and finally stigmasterol oxidation products, which were the less abundant. In this sense, after 20 min in PS, and 120 min in FAME-containing samples, values ranged from 211 to 286 µg/mg sterol, 108 to 130 µg/mg sterol and 150 to 228 µg/mg sterol for POPs coming from campesterol, stigmasterol and sitosterol, respectively. Higher susceptibility to oxidation of campesterol compared to sitosterol and stigmasterol, accompanied by similar degradation patterns among them, has previously been reported, even in different mixtures of phytosterols (Barriuso, Otaegui-Arrazola, Menéndez-Carreño, Astiasarán, & Ansorena, 2012; Kmiecik et al., 2015). Hence, the differences on sterols likelihood to oxidation should be attributed to differences in their chemical structure. In this sense, González-Larena and coworkers (2015), based on a previous study (Cercaci, Rodriguez-Estrada, Lercker, & Decker, 2007), suggested that the different surface activity of campesterol and sitosterol could be responsible for their different oxidation levels. Consequently, further research would be required to understand this behavior properly, and to confirm that campesterol certainly yields higher amounts of oxysterols than other sterols. This issue, alongside the fact that campesterol is much less absorbed than sitosterol (Ostlund et al., 2002), would be of particular interest to the food industry in order to decide the proper profile of sterols to be added to a determined food product.

In conclusion, our results indicate that the presence of FAME delayed phytosterol degradation and postponed POPs formation during thermal treatment of plant sterols. This protective effect was further enhanced by the unsaturation degree of FAME.

Besides, campesterol was oxidated in a greater extent than stigmasterol and sitosterol. These data should be taken into account for the formulation of sterol-enriched products, in order to maintain their healthy properties during cooking and/or processing.

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Table 1. Kinetic parameters for remaining fatty acids methyl esters (FAME) and plant sterols, in plant sterols sample (PS), plant sterols with stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180°C for up to 180 min.

	FAME[a]		Campesterol[b]		Stigmasterol[b]		Sitosterol[b]	
	k (min^{-1})	R^2	k (min^{-1})	R^2	k (min^{-1})	R^2	k (min^{-1})	R^2
PS	-	-	0.0500 a	0.906	0.0553 a	0.943	0.0543 a	0.917
PS + S	0.0018 a	0.748	0.0237 b	0.980	0.0261 b	0.983	0.0253 b	0.980
PS + O	0.0030 b	0.955	0.0076 c	0.980	0.0078 c	0.980	0.0081 c	0.961
PS + L	0.0038 c	0.996	0.0046 d	0.918	0.0048 d	0.885	0.0042 d	0.865
PS + Ln	0.0046 d	0.972	0.0047 d	0.952	0.0049 d	0.947	0.0049 d	0.952

Different letters within the same column denote statistically different k values among samples.

[a] First order kinetic model corresponding to $ln\frac{FAME}{FAME_0} = -k.t$

[b] First order kinetic model corresponding to $ln\frac{sterol}{sterol_0} = -k.t$

Table 2. Concentration of campesterol oxidation products (μg/mg campesterol) in plant sterols sample (PS), plant sterols with stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180°C for up to 180 min.

For each sample, 6 different oxyderivatives were determined: 7α-hydroxy (7α-H), 7β-hydroxy (7β-H), 5β,6β-epoxy (5β,6β-E); 5α,6α-epoxy (5α,6α-E); 3,5,6-triol (Triol) and 7-keto (7-K).

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
PS								
7α-H-cam	1.55 b	10.79 d	13.36 e	6.11 c	3.39 b	2.12 b	0.10 a	1.55 a
7β-H-cam	1.19 b	13.70 d	23.14 e	14.97 d	11.53 c	11.93 c	0.69 a	1.19 a
5β,6β-E-cam	5.51 c	17.16 e	27.18 f	28.20 f	13.93 d	14.24 d	1.13 b	5.51 a
5α,6α-E-cam	1.07 a	13.29 c	28.10 d	46.69 f	28.13 d	31.65 e	9.21 b	1.07 b
cam-Triol	1.48 a	1.82 a	2.73 b	3.34 c	2.46 b	2.72 b	3.03 b	1.48 a
7-K-cam	6.36 a	33.59 b	59.72 c	111.7 2 e	88.81 d	53.70 c	39.95 b	6.36 c
Total cam	17.16 aA	90.34 dD	154.23 gD	211.03 hD	148.25 fB	116.36 eB	54.11 bA	17.16 cA
PS + S								
7α-H-cam	1.00 a	3.54 c	3.29 b	2.58 b	3.22 b	5.03 c	3.20 c	1.00 a
7β-H-cam	0.97 a	4.51 b	7.50 c	6.98 c	7.29 v	8.69 c	5.05 b	1.93 a
5β,6β-E-cam	5.32 a	12.24 ab	19.25 bc	23.13 c	25.76 v	38.18 d	40.90 d	24.16 c
5α,6α-E-cam	1.18 a	10.17 ab	20.31 b	35.21 c	51.53 d	79.17 f	89.38 f	69.36 e
cam-Triol	1.43 c	1.58 cd	1.92 de	1.69 de	1.98 e	1.62 c	1.03 b	0.71 a
7-K-cam	11.12 a	20.86 a	49.82 b	66.83 c	86.59 c	118.56 d	146.23 e	107.02 d
Total cam	21.02 aA	55.01 bC	102.09 cC	136.42 dC	176.39 eC	251.25 fC	285.79 fC	204.17 eC
PS + O								
7α-H-cam	1.18 a	2.62 a	5.83 b	12.14 c	15.52 d	15.40 d	19.33 e	14.15 d
7β-H-cam	0.92 a	2.59 b	6.20 c	12.66 e	15.75 f	13.10 ef	13.57 f	9.91 d
5β,6β-E-cam	5.41 a	8.30 b	10.21 c	24.35 d	25.38 d	25.56 d	34.67 e	26.91 d
5α,6α-E-cam	1.01 a	2.62 a	3.95 ab	12.33 cd	14.79 cd	16.71 d	25.56 e	21.19 e
cam-Triol	1.29 a	1.39 ab	1.31 bcd	1.82 d	1.92 d	1.40 ab	1.62 bcd	1.42 abc
7-K-cam	8.73 a	15.39 b	19.70 c	31.55 d	28.67 d	32.84 d	56.16 e	60.73 f
Total cam	18.54 aA	32.91 abB	54.53 cB	94.84 dB	102.04 dA	105.00 dB	150.92 fB	134.31 eB
PS + L								
7α-H-cam	1.34 a	1.96 b	3.01 c	4.73 d	5.57 e	10.54 f	15.45 g	18.10 h
7β-H-cam	1.01 a	1.78 a	2.91 b	4.88 c	6.09 d	10.57 e	14.67 f	17.94 g
5β,6β-E-cam	5.02 ab	5.95 b	7.08 b	10.03 c	10.84 c	17.93 d	16.00 d	2.83 a
5α,6α-E-cam	0.91 a	1.60 ab	2.56 ab	4.48 ab	5.63 ab	13.11 ab	17.98 b	5.23 ab
cam-Triol	1.21 a	1.30 ab	1.38 abc	1.57 abcd	1.78 bcd	1.90 cd	1.99 d	1.78 bcd
7-K-cam	7.08 a	8.25 a	9.14 a	12.21a	13.61 a	19.93 ab	36.57 b	33.93 b
Total cam	16.57 aA	20.83 abA	26.09 bA	37.91 cA	44.69 cA	73.98 dA	83.13 eA	79.80 deA
PS + Ln								
7α-H-cam	1.48 a	2.11 a	3.14 ab	4.66 b	7.00 c	11.33 d	11.56 d	14.01 e
7β-H-cam	1.19 a	2.06 ab	3.21 b	4.86 c	6.13 c	10.08 d	11.80 e	14.50 f
5β,6β-E-cam	4.85 a	5.77 ab	7.54 bc	9.44 c	12.01 d	19.08 e	11.96 d	5.52 ab
5α,6α-E-cam	1.00 a	1.87 ab	3.33 b	5.15 c	8.06 d	14.06 e	8.53 d	5.47 c
cam-Triol	1.32 a	1.36 a	1.43 a	1.48 ab	1.65 b	1.25 a	1.35 a	1.45 ab
7-K-cam	6.59 a	7.42 ab	9.09 ab	10.85 b	15.44 c	21.64 d	25.99 e	25.50 e
Total cam	16.44 aA	20.59 bA	27.75 bA	36.44 cA	49.76 dA	77.44 fA	71.19 efA	66.49 eA

Different small letters within the same row denote significant differences (p<0.05) among different heating times.

For total POPs, different capital letters within the same column denote significant differences (p<0.05) among different mixtures.

Table 3. Concentration of stigmasterol oxidation products (μg/mg stigmasterol) in plant sterols sample (PS), plant sterols with stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180°C for up to 180 min.

For each sample, 6 different oxyderivatives were determined: 7α-hydroxy (7α-H), 7β-hydroxy (7β-H), 5β,6β-epoxy (5β,6β-E); 5α,6α-epoxy (5α,6α-E); 3,5,6-triol (Triol) and 7-keto (7-K).

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
PS								
7α-H-stigma	1.19 b	10.03 e	11.77 f	5.92 d	3.33 c	2.08 b	0.10 a	0.13 a
7β-H-stigma	3.44 b	10.60 e	17.94 g	11.73 f	9.26 c	10.25 d	1.63 a	1.35 a
5β,6β-E-stigma	3.52 b	10.41d	15.99 e	15.56 e	8.51 c	8.39 c	1.16 a	1.31 a
5α,6α-E-stigma	0.75 a	7.24 b	14.99 cd	22.22 e	14.25 c	15.53 d	3.19 a	4.44 a
stigma-Triol	0.53 a	2.23 b	1.68 ab	2.30 b	2.25 b	2.50 b	2.71 b	1.98 a
7-K-stigma	2.67 a	16.38 b	29.35 d	50.82 f	41.25 e	26.30 d	17.97 c	26.85 b
Total stigma	12.10 aA	56.90 dD	91.73 eD	108.54 fD	78.84 eC	65.06 dBC	26.77 bA	36.07 cA
PS + S								
7α-H-stigma	0.71 a	2.81 bc	2.80 bc	2.05 b	2.53 bc	3.15 c	2.21 b	0.65 a
7β-H-stigma	1.09 a	2.71 ab	6.08 ce	6.10 e	5.90 e	7.17 e	3.90 bc	1.57 a
5β,6β-E-stigma	3.40 a	6.06 a	12.17 b	15.31 c	16.72 c	24.16 d	23.79 d	13.59 bc
5α,6α-E-stigma	0.60 a	4.41 ab	10.69 b	18.92 c	26.35 cd	38.94 e	40.60 e	28.04 d
stigma-Triol	0.96 a	0.97 a	1.57 ab	2.13 bc	1.45 abc	2.21 c	4.45 d	5.84 e
7-K-stigma	4.66 a	8.29 a	21.56 b	29.60 c	36.28 c	48.93 d	54.85 d	36.90 c
Total stigma	11.40 aA	25.26 aC	54.87 bC	74.12 cC	89.23 cD	124.55 dD	129.80 dD	86.58 cC
PS + O								
7α-H-stigma	0.74 a	1.91 a	4.60 b	10.02 c	12.66 e	12.30 de	14.93 f	10.94 cd
7β-H-stigma	0.99 a	2.25 b	5.09 c	10.83 e	13.77 f	11.23 e	12.01 e	8.23 d
5β,6β-E-stigma	3.29 a	5.32 a	8.83 b	16.84 c	18.03 c	18.34 c	24.04 d	17.97 c
5α,6α-E-stigma	0.49 a	1.49 a	3.02 a	6.54 b	8.36 bc	8.49 bc	13.23 d	9.98 c
stigma-Triol	0.84 a	0.83 a	1.15 ab	1.46 bc	1.91 c	1.17 ab	1.57 bc	1.76 c
7-K-stigma	3.27 a	6.54 b	9.96 c	13.97 d	13.78 d	15.52 d	25.67 e	27.05 e
Total stigma	9.62 aA	18.33 bB	32.47 cB	59.66 dB	68.51 dB	67.06 dC	91.45 fC	75.93 eC
PS + L								
7α-H-stigma	0.86 a	1.32 ab	2.10 abc	3.56 bc	4.17 c	8.32 d	13.45 e	14.51 e
7β-H-stigma	3.13 a	4.16 a	2.55 a	3.85 a	5.14 a	9.11 b	14.48 c	13.08 c
5β,6β-E-stigma	3.19 a	3.75 a	4.65 ab	6.27 b	7.22 b	11.91 c	11.64 c	4.01 a
5α,6α-E-stigma	0.49 a	0.67 a	1.19 a	2.28 abc	2.86 abc	6.61 bc	7.51 c	1.96 abc
stigma-Triol	0.88 a	0.88 a	0.96 a	1.20 ab	1.22 abc	1.21 abc	1.85 c	1.82 bc
7-K-stigma	2.93 a	3.60 ab	4.21 b	5.49 c	6.28 d	9.41 e	12.68 f	15.81 g
Total stigma	11.47 aA	14.37 aA	15.65 aB	22.64 bA	27.25 cA	46.57 dA	55.35 fB	51.20 eB
PS + Ln								
7α-H-stigma	0.98 a	1.39 a	2.32 ab	3.55 b	5.50 c	8.97 d	9.32 d	11.37 e
7β-H-stigma	1.17 a	1.74 a	2.60 ab	4.10 bc	4.91 c	8.61 d	10.22 e	12.05 f
5β,6β-E-stigma	3.11 a	3.77 ab	4.57 b	6.10 c	7.93 d	12.20 e	8.20 d	4.75 b
5α,6α-E-stigma	0.61 a	0.84 ab	1.57 b	2.51 c	4.19 d	6.98 e	2.61 c	3.52 d
stigma-Triol	0.88 a	0.00 a	0.00 a	0.57 a	1.90 c	1.23 bc	0.58 a	1.49 b
7-K-stigma	2.99 a	3.40 a	4.17 a	5.01 ab	7.17 b	10.39 c	12.30 cd	13.40 d
Total stigma	9.74 aA	11.01 abA	15.24 bA	21.83 cA	30.99 dA	48.37 fAB	43.23 eB	46.59 efB

Different small letters within the same row denote significant differences (p<0.05) among different heating times.

For total POPs, different capital letters within the same column denote significant differences (p<0.05) among different mixtures.

Table 4. Concentration of sitosterol oxidation products (μg/mg sitosterol) in plant sterols sample (PS), plant sterols with stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180°C for up to 180 min.

For each sample, 6 different oxyderivatives were determined: 7α-hydroxy (7α-H), 7β-hydroxy (7β-H), 5β,6β-epoxy (5β,6β-E); 5α,6α-epoxy (5α,6α-E); 3,5,6-triol (Triol) and 7-keto (7-K).

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
PS								
7α-H-sito	1.39 ab	9.66 e	11.41 f	5.21 d	2.96 c	1.82 b	0.10 a	0.14 a
7β-H-sito	1.09 a	11.64 c	19.11 d	12.44 c	9.10 b	9.47 b	0.49 a	0.47 a
5β,6β-E-sito	3.94 b	12.62 d	19.55 e	20.29 e	10.41 c	9.50 c	0.64 a	0.72 a
5α,6α-E-sito	1.12a	11.30 d	25.04 e	40.53 g	24.31 e	26.86 f	6.62 b	8.46 c
sito-Triol	2.37 a	3.81 ab	5.33 b	7.83 d	5.84 bc	5.97 bc	6.69 c	4.82 b
7-K-sito	3.06 a	20.25 b	34.22 c	64.08 e	50.67 d	30.56 c	34.90 c	34.01 c
Total sito	12.96 aA	69.28 dD	114.66 gD	150.37 hD	103.28 fC	84.18 eBC	49.44 cA	48.62 bA
PS + S								
7α-H-sito	0.89 a	3.17 bc	3.19 bc	2.42 b	3.00 bc	3.73 c	3.31 bc	0.88 a
7β-H-sito	0.80 a	4.21 b	7.45 bc	7.25 cd	7.23 cd	8.69 d	4.86 b	1.81 a
5β,6β-E-sito	3.67 a	9.45 b	16.02 b	19.70 c	21.58 c	32.62 d	34.84 d	19.69 c
5α,6α-E-sito	1.34 a	10.53 a	21.58 a	36.96 b	53.77 b	80.21 de	91.38 e	68.72 cd
sito-Triol	2.63 a	2.86 ab	4.38 bc	4.35 cd	4.57 cd	5.51 e	5.15 de	3.63 abc
7-K-sito	6.22 a	13.30 a	30.74 b	42.66 c	52.98 c	72.13 e	88.60 f	62.62 de
Total sito	15.55 aA	43.55 abC	83.36 bC	113.34 cC	143.13 cD	202.88 eD	228.14 eC	157.36 dC
PS + O								
7α-H-sito	1.01 a	2.43 a	5.55 b	11.76 c	14.57 d	14.39 d	18.03 e	12.46 c
7β-H-sito	0.78 a	2.52 a	5.73 b	13.31 b	15.07 c	13.10 b	13.91 bc	9.18 c
5β,6β-E-sito	3.58 a	6.17 b	10.42 c	19.91 d	20.63 d	21.36 d	28.72 e	18.94 d
5α,6α-E-sito	1.07 a	2.79 a	5.33 a	12.56 b	14.52 bc	16.29 bc	23.48 d	15.76 c
sito-Triol	2.25 a	2.51 a	3.10 b	3.84 cd	3.71 cd	3.27 cd	4.22 d	3.75 c
7-K-sito	4.57 a	9.01 b	12.55 c	18.93 d	16.92 d	19.78 d	34.43 e	36.21 e
Total sito	13.27 aA	25.42 bB	43.36 cB	80.33dB	85.42 dB	88.19 dB	122.79 gB	96.30 fB
PS + L								
7α-H-sito	1.07 a	1.60 a	2.43 b	3.88 c	4.69 d	8.69 e	12.37 f	14.69 g
7β-H-sito	0.78 a	1.44 b	2.40 c	4.08 d	5.12 e	9.19 f	13.11 g	15.40 h
5β,6β-E-sito	3.22 ab	3.90 ab	4.78 bc	6.82 cd	7.80 d	13.06 e	11.55 e	2.12 a
5α,6α-E-sito	0.93 a	1.48 a	2.21 ab	3.82 bc	4.58 c	10.17d	6.79 e	1.14 a
sito-Triol	1.96 a	2.10 a	2.18 ab	2.64 abc	3.02 bcd	3.32 cde	3.99 e	3.84 de
7-K-sito	3.61 a	4.33 ab	4.81 b	6.44 c	7.30 c	10.62 d	14.22 d	18.30 d
Total sito	11.57 aA	14.84 abA	18.82 bA	27.68 cA	33.02 dA	55.05 eA	61.58 fA	55.50 eA
PS + Ln								
7α-H-sito	1.21 a	1.72 a	2.59 ab	3.86 b	5.81 c	9.33 d	9.34 d	11.49 e
7β-H-sito	0.90 a	1.58 ab	2.69 b	4.06 c	5.26 c	8.53 d	9.98 e	12.28 f
5β,6β-E-sito	3.14 a	3.92 ab	5.05 b	6.56 c	9.09 d	13.45 e	7.92 cd	3.73 ab
5α,6α-E-sito	0.95 a	1.73 ab	2.87 bc	4.38 cd	6.76 e	11.18 f	6.02 de	3.53 bc
sito-Triol	1.94 ab	2.05 ab	2.02 a	2.24 abc	2.36 bcd	2.28 abcd	2.47 cd	2.67 d
7-K-sito	3.38 a	3.89 a	4.82 a	5.85 ab	8.24 b	11.79 c	14.38 cd	15.51 d
Total sito	11.53 aA	14.90 abA	20.05 bA	26.95 cA	37.12 cA	56.56 eAB	50.11 deA	49.20 dA

Different small letters within the same row denote significant differences (p<0.05) among different heating times.

For total POPs, different capital letters within the same column denote significant differences (p<0.05) among different mixtures.

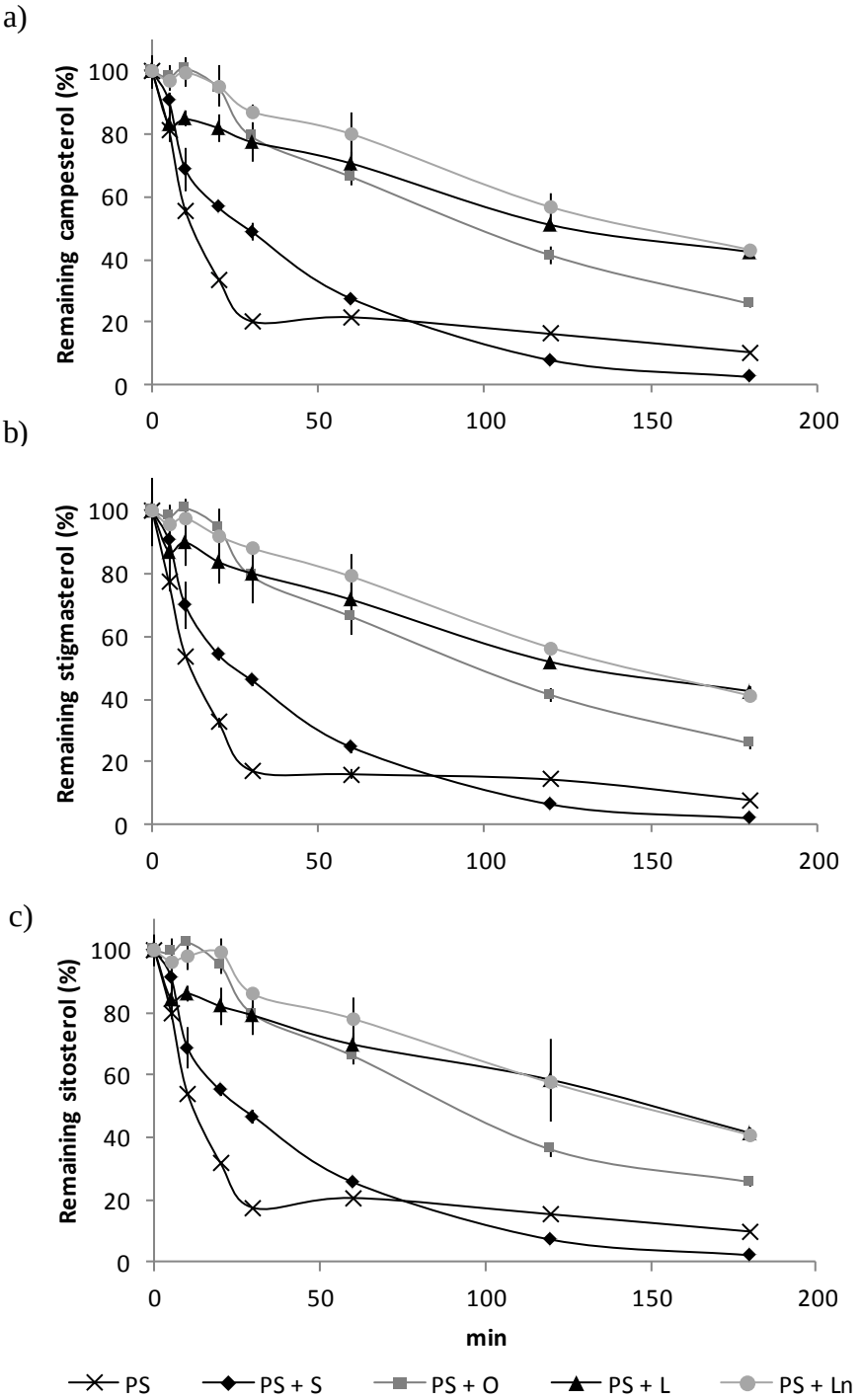

Figure 1. Remaining percentage of a) campesterol b) stigmasterol and c) sitosterol; in plant sterols sample (PS), plant sterols with stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180 °C for up to 180 min.

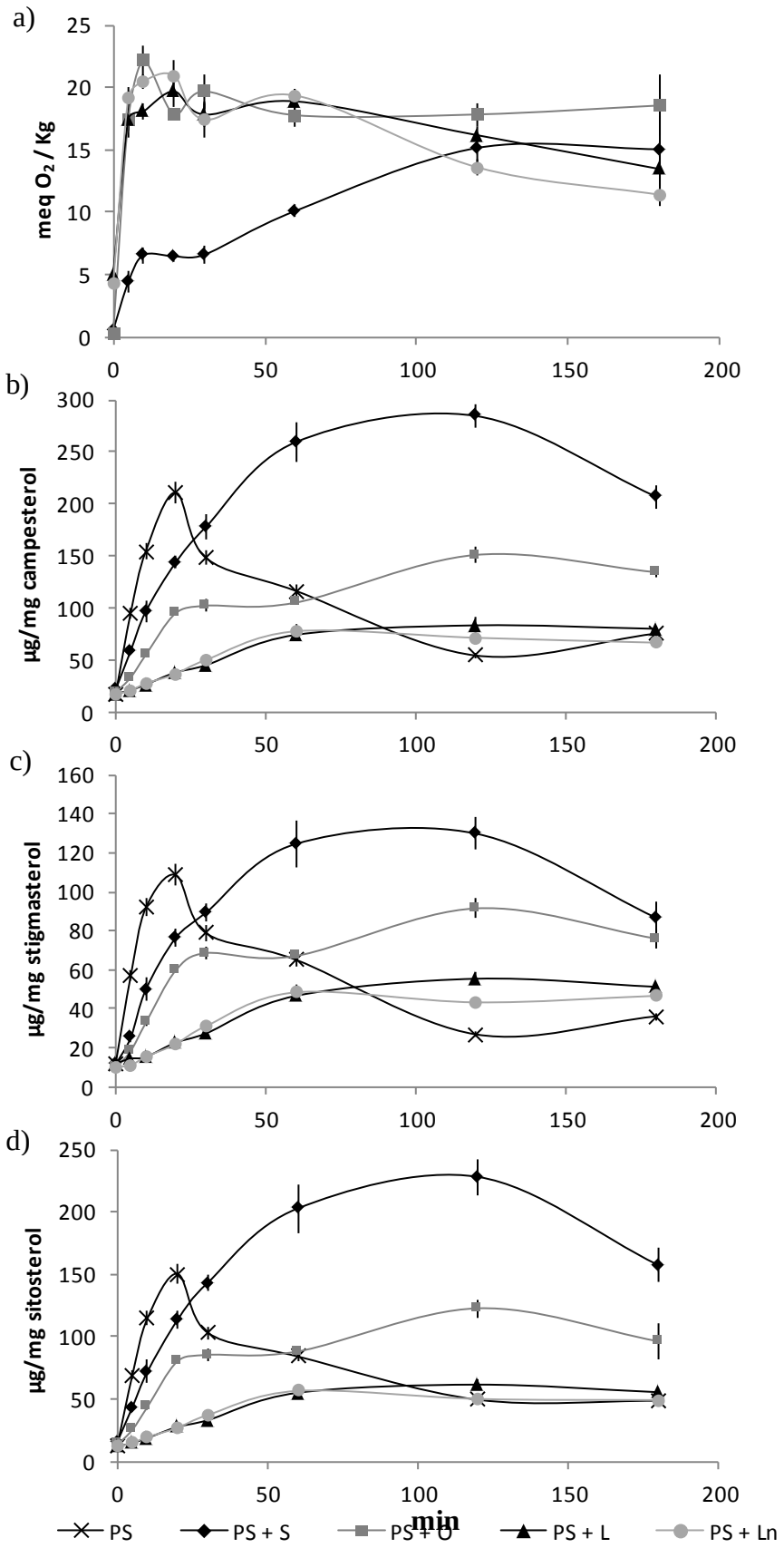

Figure 2. a) Peroxides Value (meq O_2 / Kg) and total plant sterol oxidation products from b) campesterol, c) stigmasterol and d) sitosterol ; in plant sterols sample (PS), plant sterols with

stearate (PS + S), plant sterols with oleate (PS + O), plant sterols with linoleate (PS + L) and plant sterols with linolenate (PS + Ln) mixtures, during heating at 180 °C for up to 180 min. For each sterol, results are expressed in µg of total POPs per mg of their corresponding sterol.