

Oxysterols formation: a review of a multifactorial process

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Highlights

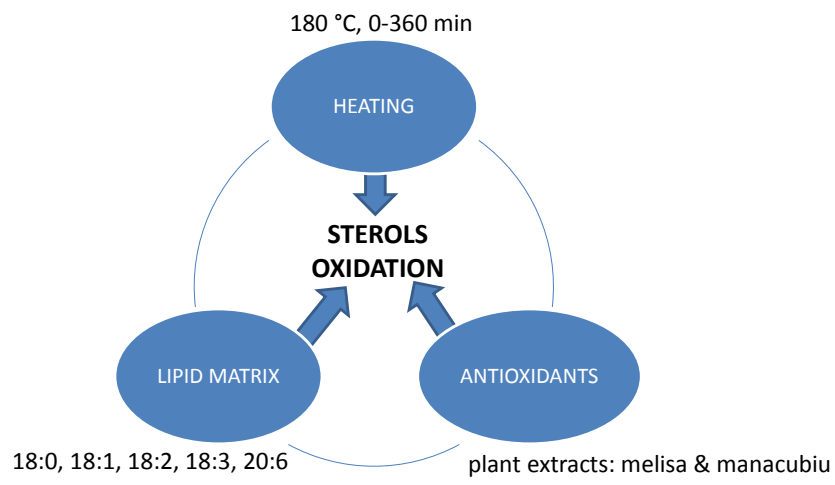
- 1- Heating sterols at 180 °C produced a high extent of oxidation.
- 2- The presence and unsaturation degree of the surrounding lipids protected sterols from oxidation.
- 3- Plant aqueous extracts protected cholesterol from oxidation.
- 4- Greater amounts of campesterol-derived oxidation products were observed, followed by sitosterol, cholesterol and stigmasterol.
- 5- In 5 out of 7 of our studies, 7-keto derivatives were the most abundant oxysterols formed.

Abstract

Dietary sterols are nutritionally interesting compounds which can suffer oxidation reactions. In the case of plant sterols, they are being widely used for food enrichment due to their hypocholesterolemic properties. Besides, cholesterol and plant sterols oxidation products are associated with the development of cardiovascular and neurodegenerative diseases, among others. Therefore, the evaluation of the particular factors affecting sterol degradation and oxysterols formation in foods is of major importance. The present work summarizes the main results obtained in experiments which aimed to study four aspects in this context: the effect of the heating treatment, the unsaturation degree of the surrounding lipids, the presence of antioxidants on sterols degradation, and at last, oxides formation. The use of model systems allowed the isolation of some of these effects resulting in more accurate data. Thus, these results could be applied in real conditions.

Keywords: cholesterol, plant sterols, Sterol Oxidation Products, thermo-oxidation, antioxidants

GRAPHICAL ABSTRACT



1. Introduction

The interest on dietary sterols and, specifically on their oxidation derivatives (SOPs- sterol oxidation products), has risen in the last years. High cholesterol intake is related to cardiovascular diseases. Moreover, its oxidation products (COPs) have been closely linked to toxicological effects (1-3). In the case of phytosterols, their beneficial effects, which are related to reductions in Low Density Lipoproteins (LDL) plasma levels (4), have promoted their incorporation into a variety of foodstuffs as functional foods (5-8). However, their presence in this kind of products could be threatened by oxidation reactions, which will lead to the formation of oxidation compounds. Interestingly enough, these compounds, as well as the COPs, have lately been associated with harmful effects in the organism (9- 11).

In this context, there is evidence that SOPs are actually absorbed from the diet (12, 13). Given their potentially harmful effects on human health, a deeper study of the factors affecting the formation of dietary SOPs is essential. The content and distribution of SOPs in foods depend on food composition, industrial processing, storage conditions and culinary procedures. All these processes share several particular factors which are key elements in sterols oxidation, such as temperature, oxygen, light exposure, the lipid surrounding matrix, and presence of antioxidants and water.

Foods are usually complex matrices where interferences among different components may hamper a clear view of the mechanisms involved in sterol oxidation. Therefore, model systems are a very useful tool to separately evaluate the factors exerting influence over this process, avoiding the ambiguity which results from the interferences among them. Thus, a deeper understanding of the underlying mechanisms is enabled and kinetic curves can be easily determined. Consequently, a diversity of experimental approaches can be found, from fully modelled studies where only chemical standards are used as components of the experiments

(14, 15), to intermediate model systems, where chemical standards are mixed within foods (16-18).

This paper aims to evaluate the effect of different factors involved in sterols oxidation, gathering recently published data from our research group (19-25). Four different sterols were evaluated in this report focusing on the effect of the heating treatment, the unsaturation degree of the surrounding lipids and also the presence of antioxidants in sterol oxidation. Model systems and some food applications are included in the review. Detailed information on experimental conditions can be found in the original papers. Briefly, regarding the model systems, sterols were dissolved in chloroform and added to the corresponding compounds in each case (fatty acids, triglycerides or plant extracts). For plant extracts addition, and due to their hydrophilic properties, they were dissolved in methanol before mixing with the chloroform solution. Then, the sample was evaporated and heated at 180 °C during times ranging from 0 to 360 min. Temperature of 180 °C was selected for the experimental procedure since it is common in cooking procedures and ensures full melting of sterols.

2. Effect of heating treatment

Heating is a well-known lipid oxidation inducer, since it causes the reduction of the activation energy for the hydrogen abstraction which leads to enhanced free radical formations. Thus, heat intensity and time are key factors affecting sterol oxidation. According to the results obtained in the different experimental designs, high sterol degradation and SOPs formation were observed in all the model systems analyzed. Figure 1a summarizes these findings based on the model systems containing sterols, as pure standards, without any other lipid matrix in the system.

All the experiments pointed out drastic sterols degradation already from the beginning of the heating treatment, exceeding 50% degradation after 10 min. Cholesterol degradation was below this value in the experimental setting where higher initial amounts of cholesterol were

placed in the tubes (20 mg in (21) compared to around 1-2 mg in the other experimental settings). As it has previously been described, higher amounts of sample hamper the heat transfer and reduce the area-to-volume ratio, slowing down the oxidation process (26).

One of the most variable experimental conditions in sterol degradation research is the heating temperature. A wide diversity of results has been noticed, even when comparing results from the same research group (15, 27, 28). Several studies performed sterol degradation at 150 °C using cholesterol samples. These works have roughly obtained only 50% degradation after 30-60 min heating, most likely due to the low temperature applied (28, 29). When higher temperature was applied (200 °C), up to 89% degradation was achieved in the first 10 minutes (30). However, at that same temperature (200 °C), Thanh et al. (31) obtained slower phytosterol degradations than those provided by our work. Regarding experiments attained using thermo-oxidation treatments similar to ours, Xu et al. (30) found 61% of cholesterol degradation after 10 min heating at 175 °C. Nevertheless, percentages of just 50% degradation of cholesterol, β -sitosterol and stigmasterol were detected after 60 min heating in some other works (17, 32).

As it can be observed in Figure 1b, SOPs started to form right at the beginning of the heating process, reached then maximum values and started to decrease after 5 to 20 min onwards in most cases. When 20 mg of cholesterol were initially heated, the maximum COPs value was delayed up to 30 min, which again could be attributed to the higher amount of sample used compared to the other experimental settings.

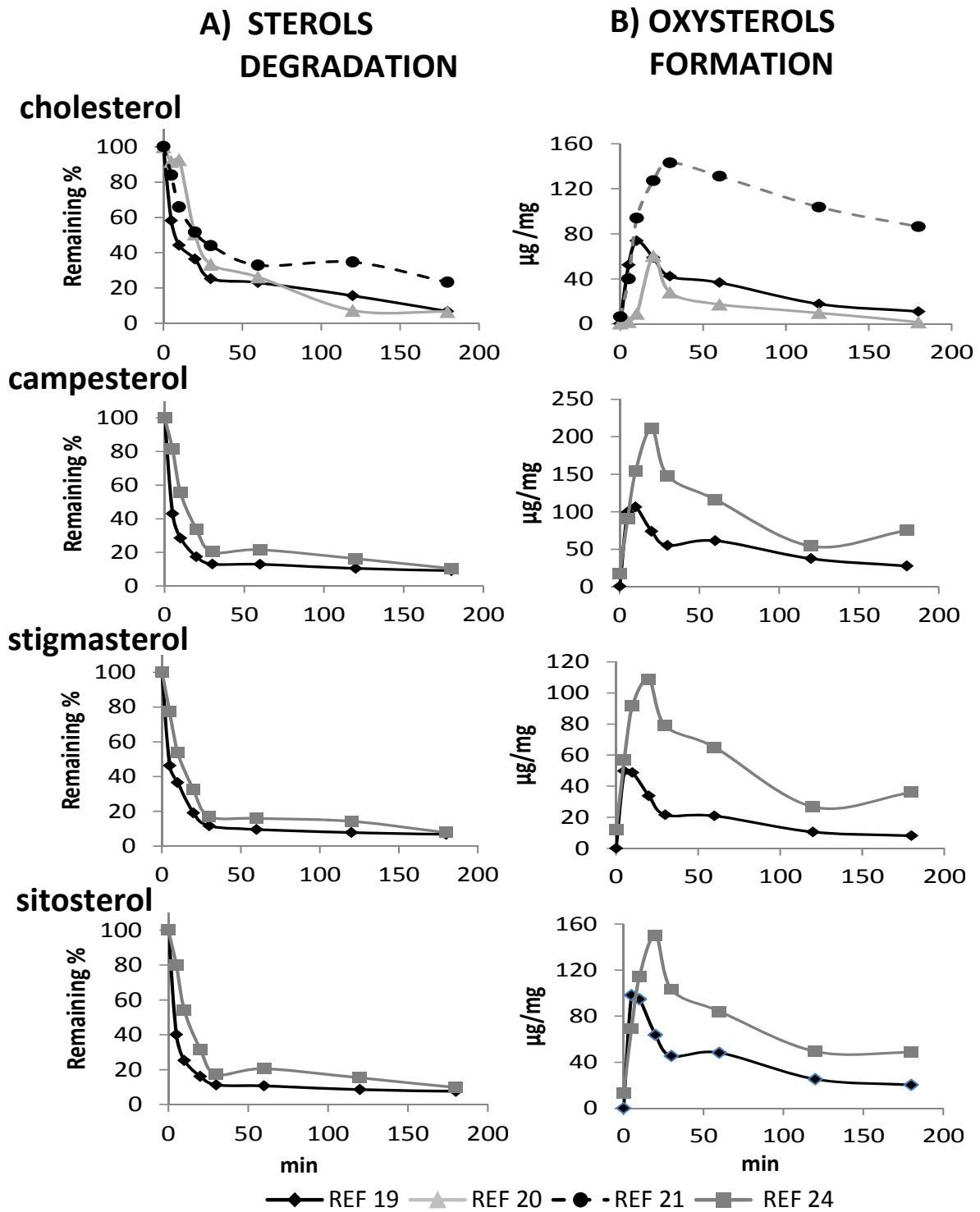


Figure 1. Sterols are degraded and oxysterols formed. A) Remaining sterol and B) oxysterol content during heating of the model system used for each sterol, in the different experimental settings.
 Remaining %= remaining sterol/initial sterol *100
 µg/mg= µg oxysterols / mg initial sterol

Seckin and Metin (33) and Derewiaka et al. (29) concluded that the changing of processing temperatures had an important significant effect on oxysterols content. Yen et al. (15), Zhang et al. (34) and Chien et al. (28) did not find any decrease in oxysterol levels at 150 °C even after 60 min heating, noticing constant levels or even increasing trends up to that heating time. However, at 200 °C, Zhang et al. (34) noted the drop in oxysterol much earlier (20 min). In this context, it has been hypothesized that only drastic heating treatments may induce oxysterol degradation (31), and 150 °C may not be enough to achieve it. In this sense, Soupas et al. (35) proposed 140 °C as the key temperature in the process, since sterols behaved differently below and above this temperature. At 175-180 °C, whereas Xu et al. (30) found the maximum amount of COPs at similar times as in our experiment, some other works, (14, 32, 36) obtained that SOPs decreased only after 40, 60 and 90 min, respectively.

It seems that the heat treatments applied in our previous work have been more destructive than those applied in other studies, since sterols degradation percentages were higher and formation of oxysterols occurred earlier. This suggests that other experimental conditions, besides temperature and time, could have influenced the results, such as the properties of the heating slopes, the higher initial sterol amount, the area-to-volume ratios, the different evaporation procedures, and the application of filtration methods or disposal of the tubes. In reference to the incidence of light, it should be highlighted that our samples were not capped neither covered during the heating procedure, which was performed in a room with natural light. Hence, a certain development of photo-oxidation could have occurred, which could explain the observed over-oxidation.

In contrast with the sterols, oxysterols showed to rapidly increase during the first 30 minutes in all the experiments. The apex in oxysterols levels was then followed by a decrease in all of our experiments, as shown in Figure 1. This fact could be explained by the formation of further oxidized compounds after a certain moment of the heating process. These compounds may

consist of high molecular weight compounds, such as di- and oligomers (of hydroxy, epoxy and keto derivatives) linked by ether bonds or by combining radicals (37-39). They can also consist on steradienes and steratrienes, compounds formed from sterols, 7-ketosterols or 7-hydroxysterols (40). Using a model system consisting on pure stigmasterol heated at 180 °C for 180 min, Menéndez-Carreño et al. (32), in agreement with Lampi et al. (37), reported that dimeric and polymeric products contributed to 30% of stigmasterol degradation. Menéndez-Carreño's group also described a strong relationship between non-polar/mid polar and the other indicators of oxidation reactions. This evidence was also supported by the formation of oligomeric species of campesterol, sitosterol and cholesterol during heating (29, 41).

3. Effect of unsaturation degree

The role played by surrounding lipids in sterol oxidation has been under debate during the last decade. In fact, the discussion is still in vogue, since opposite results have been reported by several research groups. Whereas some authors maintain the hypothesis that there is a protective effect of the surrounding lipid matrix over the sterols susceptibility to oxidation (42), some others state that it is the high unsaturation of this matrix what promotes sterol oxidation (43). Both trends are strongly supported by wide empiric evidence and powerful arguments. On the one hand, lipids can compete for oxygen with sterols and reduce the oxidation of the latter by selfoxidation. This way, unsaturated lipids would be more efficient than saturated ones in protecting sterols, because their double bonds enhance their likelihood to oxidation. On the other hand, radicals and oxygenated species derived from lipid oxidation can exert a pro-oxidant effect towards sterols. A balance between both mechanisms is the most probable situation. Therefore, the experimental conditions can displace the balance to one or other side.

In our studies, the effect of the presence of different surrounding lipids against sterols oxidation was analysed (20, 22, 24, 25). They were tested under different chemical forms:

18:0, 18:1, 18:2, and 18:3, as fatty acids methyl esters (FAME), triacylglycerides (TAG) standards, or within natural TAG from vegetable oils. In addition, free docosahexaenoic acid (DHA) was also tested. In general, the presence of lipids was protective against sterols oxidation; only in the case of cholesterol + free DHA model system showed an oxidative promoting effect by the fatty acids. The other lipid matrices (stearate, oleate, linoleate, linolenate and their TAG) inhibited both sterol degradation and SOPs formation.

In addition, we also tried to statistically describe sterol degradation during thermal treatment. Regression curves were built for every case (Table 1), obtaining kinetic models, whose k values can give an idea of the intensity and rate of sterol degradation: the higher the k value, the faster the sterol degradation occurs. According to the different experimental settings, k values obtained for isolated sterols were greater in comparison with any lipid medium. Furthermore, almost identical k values were observed for stigmasterol within linoleate and within sunflower oil (containing more than 60 % linoleic acid). Among the different lipid matrices, the more unsaturated ones produced lower k values for sterols degradation, except for DHA. This denoted slower sterols degradation in the presence of lipids of increasing unsaturation degree, according to previous explanations.

Table 1. First order kinetic model corresponding to $\ln(\text{sterol}/\text{sterol}_0) = -k.t$. For each sterol, the first column shows the kinetic constant (k) and the second one the level of adjustment (R^2). Equations modeled sterol degradation during heating treatment.

MATRIX	cholesterol		campesterol		stigmasterol		sitosterol		REFERENCE
	k (min ⁻¹)	R ²	k (min ⁻¹)	R ²	k (min ⁻¹)	R ²	k (min ⁻¹)	R ²	
Alone	0.0435	0.828	0.1199	0.883	0.0997	0.925	0.1376	0.909	19
Alone	0.0279	0.945	-	-	-	-	-	-	20
Tristearate	0.0146	0.972	-	-	-	-	-	-	20
Trioleate	0.0090	0.995	-	-	-	-	-	-	20
Trilinoleate	0.0064	0.891	-	-	-	-	-	-	20
Trilinolenate	0.0056	0.866	-	-	-	-	-	-	20
Alone	-	-	0.0500	0.906	0.0553	0.943	0.0543	0.917	24
Stearate	-	-	0.0237	0.980	0.0261	0.983	0.0253	0.980	24
Oleate	-	-	0.0076	0.980	0.0078	0.980	0.0081	0.961	24
Linoleate	-	-	0.0046	0.918	0.0048	0.885	0.0042	0.865	24
Linolenate	-	-	0.0047	0.952	0.0049	0.947	0.0049	0.952	24
Sunflower oil	0.0044	0.927	-	-	0.0050	0.878	-	-	22

As it has been previously discussed, a variety of results has been reported in respect to this issue. However, not all of them are in accordance with the present data. In an attempt to understand the causes of those differences, several hypotheses are suggested.

The type of interaction between the surrounding lipids and the sterols may be a key factor in the oxidation process, as well as the ratio sterol: lipid used. When sterols are esterified with fatty acids, the oxidation is promoted since the generated radicals are in close contact with the sterol oxidation sites. Hence, the more unsaturated the fatty acids are, the more sterol oxidation is achieved (43-44).

Nevertheless, free sterol, meaning not linked to the fatty acids, present a weaker, interaction with the lipid matrix. In these cases, the effect of the competition for oxygen by the lipids would be predominant. Moreover, increasing the unsaturation degree would imply a reduction in sterol oxidation (14, 42, 45). However, when double bonds are in high numbers, lipid susceptibility to oxidation is extremely high. Then, it is quickly degraded allowing the promotion of sterols oxidation by the large amount of generated pro-oxidant species. However, this promotion hampers the protection against sterols oxidation. This would be the case of the current work (Table 1), where free sterols are protected from oxidation in the presence of saturated (stearic) and 18C unsaturated lipids (oleic, linoleic and linolenic acids or their methyl esters), but not in the presence of highly unsaturated lipids (DHA).

Different routes of oxidation could also contribute to the divergence of the results. In this sense, some of works consider photo-oxidation at room temperature during several days, and some others induced oxidation by heat application. Among the thermo-oxidative experiments, different temperatures have been used. This factor has been shown to be critical in the protective/promoting effects of the lipids towards sterol oxidation (35). At the high temperature of our experimental settings (180 °C), our systems were at liquid phase in all

cases, which allowed a better solution of the sterols. This produced a certain steric hindrance around them, which hindered the access of oxygen, slowing down the oxidation rate.

Mathematical modelling appears to be very useful for sterol degradation studies, since it enables easy comparison of data from different experimental settings. Several authors have performed sterols kinetic studies (46), most of them using elegant approaches and complex mathematical calculations. Chien et al. (27) found cholesterol degradation and oxysterols formation to follow first order and second order reactions and calculated the corresponding equations. Their work, as well as others, efficiently contributed to elucidate the mechanisms by which sterol oxidation occurs. But for studies aiming to compare oxidative trends and general behaviors towards heating or storage, it is very useful, and easily approachable, to fit a trend curve, as we made in our works. To our knowledge, few authors give the mathematical fit of their sterol degradation curves, probably due to insufficient experimental data along time. Most of these studies show no more than 3 or 4 heating points (14, 29, 37, 47). The evaluation of 7-9 points, as made in our experimental conditions, enables obtaining useful information to build reliable regression curves.

4. Effect of antioxidants presence

Plant foods are rich in compounds with antioxidant properties. Among them, the most outstanding are vegetable oils, fresh fruits and fruit and seeds beverages, such as wine, tea and coffee. These natural antioxidant properties have been extensively reported to protect against lipid oxidation within foods, mainly during processing, cooking and storage (17, 48-50). Particular antioxidant compounds have been associated with these properties, mainly tocopherols, carotenoids and phenolic compounds.

Nowadays, the consumer's interest towards natural products has promoted the research on plant extracts due to their addition into foodstuffs (51-53). We selected two promising species for their potential use against cholesterol oxidation: *Melissa officinalis* (melisa) and *Solanum*

sessiliflorum (mana-cubiu) considering their antioxidant properties. The results obtained in the different experiments performed are summarized in Table 2.

Table 2. Effect of the presence of melisa and mana-cubiu aqueous extracts on cholesterol degradation and COPs formation.

Antioxidant	System	Dose	Cholesterol degradation	COPs formation	Reference
<i>M.officinalis</i> extract	Cholesterol	2 g/100 g chol	82% inh ¹	94% inh ¹	21
<i>M.officinalis</i> extract	Beef patty	150 µg/g patty	No effect	No effect	21
<i>S.sessiliflorum</i> extract	Cholesterol	1 g/2 g chol	59% inh ²	89% inh ²	25
<i>S.sessiliflorum</i> extract	Tuna patty	5000 µg/g patty	No effect	No effect	23

¹ Results after 10 min of heating treatment; ² Results after 10 min of heating treatment; inh (inhibition); COPs (cholesterol oxidation products)

As it can be observed, their aqueous extracts were strongly effective in isolated model systems containing only cholesterol. They drastically reduced cholesterol degradation and COPs formation. *M. officinalis* and *S. sessiliflorum* extracts were rich in phenolic acids, rosmarinic (123 mg/g) and 5α-caffeoylquinic acid (2.48 mg/g), respectively. Thus, the antioxidant capacity was attributed to the presence of these compounds in the aqueous extracts. In this sense, Kmiecik et al. (54) recently hypothesized about the stronger efficiency on cholesterol oxidation of phenolic acids compared to other phenolic compounds.

However, when tested in food systems, we found that phenolic acids were not that effective within beef or tuna patties. In beef patties, sensory aspects limited the acceptable dose of melisa to 65 µg/g in the non-emulsion containing patties and to 150 µg/g in the emulsion-containing ones. In these conditions, melisa was not effective in controlling sterol oxidation. In tuna patties, although a much higher concentration of mana-cubiu extract could be added (5000 µg/g), its antioxidant effect was probably devoted to other lipids within tuna, whose polyunsaturated fatty acids are easily oxidized. In this sense, when a pure DHA standard was heated in the presence of mana-cubiu, it degraded to a lesser extent than without the extract,

but there was no improvement on cholesterol oxidation. Thus, mana-cubiu exhibited an antioxidant effect towards DHA and not towards cholesterol, which would support the above-mentioned hypothesis. Moreover, hexanal formation (a marker of fatty acids oxidation) in tuna patties was drastically reduced in the presence of mana-cubiu, confirming its antioxidant effect over fatty acids.

When aiming to reduce COPs formation, the addition of antioxidant extracts to meat or fish patties should always take into account the sensory aspects, since their off-flavors commonly impair the applicable dose (52, 55, 56). This is of utmost importance in foodstuffs with few or no spices in their formulation, such as beef patties. If the extract flavour is not a limiting factor and high doses can be added to the foodstuff, then the properties of the product should be considered, such as its lipid profile. Foods rich in polyunsaturated fatty acids will require higher doses of antioxidant extracts. It is then likely that the antioxidant effect would be consumed by those fatty acids, lowering the effect towards cholesterol oxidation. Studies where the antioxidant efficiency has been demonstrated in polyunsaturated matrices are numerous, and the efficiency is usually higher towards these compounds than towards cholesterol (55, 57). Besides, when aiming to reduce COPs formation, antioxidant extracts have been mostly applied to moderately unsaturated foods, rather than highly unsaturated ones (49, 58). Other compounds, such as α -tocopherol, have previously been reported to exert protective effects towards sterols oxidation (17, 59).

5. Effect of the type of sterol

All sterols present a very similar chemical structure, only differing in the side chain. This high similarity implies a likely similar oxidative behavior. To tackle this question, a comparison among the oxidation patterns of cholesterol, campesterol, stigmasterol and sitosterol heated within sunflower, TAG, FAME or without any surroundings was carried out.

As we have previously observed (Fig 1), the four studied sterols showed similar degradation patterns among them. This similar degradation pattern accounts for their similar dissociation enthalpies in the sterol ring (60).

In contrast, SOPs presented some differences in their formation rate depending on their original sterol. Results from the different experimental settings are compiled in Table 3. As it can be observed, the behavior is uniform among the different experimental settings. Campesterol presents the highest oxidation rate, expressed as $\mu\text{g SOP/mg initial sterol}$, regardless the initial ratio within the mixture of sterols. After campesterol, cholesterol and sitosterol follow, being stigmasterol the least prone to oxidation.

Table 3. Oxidation rate ($\mu\text{g SOPs/mg initial sterol}$) of the four sterols within the different experimental settings, in decreasing order.

MATRIX	Oxidation rate ($\mu\text{g SOPs/mg initial sterol}$)	REFERENCE
Alone	campesterol > sitosterol > cholesterol > stigmasterol	19
Sunflower oil	cholesterol > stigmasterol	22
Alone	campesterol > sitosterol > stigmasterol	24
Stearate	campesterol > sitosterol > stigmasterol	24
Oleate	campesterol > sitosterol > stigmasterol	24
Linoleate	campesterol > sitosterol > stigmasterol	24
Linolenate	campesterol > sitosterol > stigmasterol	24

SOPs: Sterol Oxidation Products

Campesterol has previously been reported to yield more SOPs than other sterols (8, 54, 61). González-Larena et al. (61), postulated that the different surface activity of sterols could be behind this behavior, based on the results of Cercaci et al. (62). According to that work, differences in the surface activity of the sterols could influence their susceptibility to oxidation, since the higher the surface activity, the better the location of sterols in the oil-water interfaces. Thus, in our work, campesterol, due to its estimated surface activity higher than sitosterol, would locate on the interfaces of the medium, where higher contact with pro-oxidants is possible. However, this hypothesis would not support our data on relative oxidability of cholesterol and stigmasterol since their calculated surface activity is higher than

that of campesterol and sitosterol, whilst they yield less SOPs amounts, as it has previously been reported (32).

This behaviour could be attributed to lower efficiency in the oxidation reaction or slower kinetics. Besides, instead of the ones we analyzed, other oxides could have been formed, such as 6-hydroxy, 20-hydroxy, 22-hydroxy, dienes, trienes... (29, 36, 63). Moreover, SOPs derived from cholesterol and stigmasterol could have decomposed to form oligomers, polymers and other compounds characteristic of advanced stages of oxidation (29, 39), yielding an overall lower balance than for campesterol and sitosterol derived oxides.

Furthermore, we used different relative initial amounts of sterols within each experimental setting, which could also explain the different oxidation rate, as thermo-oxidation is significantly dependent on the sample area-to-volume ratio (26). In this sense, low amounts of sterols would be overexposed to oxygen since particles present a high area-to-volume ratio, showing a greater exhibition to pro-oxidation agents. Nevertheless, according to Cercaci et al. (62), higher sterol concentration would favour their presence at the interfaces and promote oxidation, contrary as the results obtained in the current work.

Regarding stigmasterol, the double bond present in C22 could lead to the idea of a greater oxidation of this compound, however, these bonds in the side chain do not affect its reactivity (60, 64). Therefore, it is not surprising that, in this work, stigmasterol was the less oxidized sterol.

Some authors (65) have recently attributed the different experimental conditions applied to sterols oxidation susceptibility.

As it has been reported, the absorption of campesterol (and its oxides) is higher than that of sitosterol (and its oxides)(12, 66). Nevertheless, oxysitosterol plasma levels are usually higher than campesterols levels, probably due to *in vivo* oxidation of sterols (67). Considering the higher campesterol oxidation rate compared to sitosterol, as reported in our work, higher

potential toxicity of campesterol-enriched foods could be hypothesized. In this regard, the European legislation establishes lower limits for campesterol addition to functional foods than for sitosterol (<40 and <80% of the sterols addition, respectively) (68).

6. Distribution of particular oxysterols

Seven different oxysterols derived from the four sterols analyzed were analyzed: 7 α -hydroxysterols; 7 β -hydroxysterols; 5,6 α -epoxysterols; 5,6 β -epoxysterols; 3,5,6-steroltrials; 25-hydroxysterols and 7-ketosterols. Table 4 summarizes the relative oxysterols distribution, excluding 25-hydroxysterol, at the moment of their maximum concentration for each experiment.

Table 4. Oxysterols distribution (derived from the four studied sterols) found at the moment of their maximum concentration in the different matrices, including the prevalence of alpha/beta epimer of 7-hydroxy and 5,6-epoxy derivatives.

MATRIX	distribution	REFERENCE
Alone	7-K > 7-H > 5,6-E > triol	19
Alone	7-K > 5,6-E > 7-H > triol	21
Alone	7-K > 7-H > 5,6-E > triol	20
Tristearate	7-K > 5,6-E > 7-H > triol	20
Trioleate	7-K > 7-H > 5,6-E > triol	20
Trilinoleate	7-K > 7-H > 5,6-E > triol	20
Trilinolenate	7-K > 7-H > 5,6-E > triol	20
Beef patty	7-K > 5,6-E > 7-H > triol	21
Alone	7-K > 5,6-E > 7-H	25
Tuna patty	7-H > 7-K > 5,6-E	23
Sunflower oil	7-H > 5,6-E > 7-K > triol	22
Alone	7-K > 5,6-E > 7-H > triol	24
Stearate	7-K > 5,6-E > 7-H > triol	24
Oleate	7-K > 5,6-E > 7-H > triol	24
Linoleate	7-K > 5,6-E > 7-H > triol	24
Linolenate	7-K > 7-H > 5,6-E > triol	24

7-K: 7-keto derivatives; 5,6-E: 5,6-epoxy derivatives; 7-H: hydroxyl derivatives

Among the different oxysterols, 7-keto derivatives were usually the most abundant ones, except for sunflower and tuna matrices. 7-ketosterols are commonly found as the main oxysterols both in model and food systems (14, 29). These compounds are moderately stable,

being formed either directly from hydroperoxides or through 7-hydroxysterols dehydration (69). Moreover, they are frequently the end molecules of the oxidation route. Hence, they have extensively been reported as good sterols oxidation markers (70).

Nevertheless, in the case of sunflower and tuna matrices, 7-hydroxy and 5,6-epoxy were the major compounds found. Given the different possible formation routes for these compounds, several hypotheses were proposed. First, the formation of 7-hydroxycholesterol could have been catalyzed by specific enzymes present in tuna. Second, the direct bimolecular addition of oxygen to the sterol double bond could have been favoured over the radical formation mechanism. This second hypothesis could be the result of the complex environmental feature of the sunflower oil, yielding higher 5,6-epoxy amounts.

A third hypothesis would involve photo-oxidation processes, which would enhance 7-hydroxysterols formation. Furthermore, the higher levels of 7-hydroxysterols found could indicate that first stages of oxidation are still ongoing, therefore, only low amounts of 7-keto would have been formed from them at that time. In addition, this last hypothesis is further supported by some works which reported this type of distribution profile for oxysterols (54Kmiecik et al., 2015; 61González-Larena et al., 2015).

At last, Triol and 25-hydroxy derivatives were, by far, the less abundant oxysterols. The low levels of triols found were expected since their formation from epoxides is favoured in aqueous acidic media (32, 69). As for the side-chain derivatives, 25-hydroxycholesterol was detected in small amounts only after long heating times, probably due to the lack of specific enzymes in the analyzed samples. They were not found among plant sterols' oxides, neither by other authors (71).

7. Conclusion

Sterols' thermo-oxidation is a multifactorial process which strongly depends on time-temperature combination, producing high sterol oxidation from the beginning of the process.

The presence of the lipid matrix in addition to its unsaturation degree, as well as the presence of phenolic compounds with antioxidant activity, significantly protected sterols from oxidation in model systems. The inclusion of plant extracts in foodstuffs to achieve this goal, appeared to be promising when sensory aspects and sample characteristics are taken into account.

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