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Sterols oxidation: effect of heating, unsaturation degree of the surrounding lipids and presence of antioxidants

Oxidación de esteroles: efecto del calentamiento, grado de insaturación de la matriz lipídica y presencia de antioxidantes

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Pamplona, Junio de 2015

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Memoria presentada por **Dña. Blanca Barriuso Esteban** para aspirar al grado de Doctor por la Universidad de Navarra

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Abstract

Dietary sterols are nutritionally interesting compounds which can undergo oxidation reactions during food manufacture and storage, as well as in the human body. Their oxidation products are associated with the development of highly prevalent non-infectious diseases. Therefore, it is relevant to evaluate the particular factors which affect sterol degradation and oxysterols formation in foods. In this context, the present work aimed to assess the effect of heating treatment, unsaturation degree of the surrounding lipids and also presence of antioxidants, on sterols degradation and oxides formation. Model systems (which are valuable tools to avoid the influence of interferences), as well as some food applications, were used in the experimental designs.

Our study concluded that: sterols thermo-oxidation is a multifactorial process which strongly depends on time-temperature combination, producing a high sterol oxidation already from the beginning of the process. Moreover, the presence and unsaturation degree of the lipid matrix, as well as the presence of phenolics and tocopherols, significantly protected sterols from oxidation in our model systems. The addition of plant extracts in foodstuffs to achieve this same goal appeared to be a promising strategy when the sensory aspects and characteristics of the sample are taken into account. In addition, we could establish that the monitoring of sterol oxidation through the measurement of the oxides generated is a complex issue, hence a scientific consensus to achieve a standardized methodology is still needed.

Resumen

Los esteroles dietéticos son compuestos interesantes desde el punto de vista nutricional, que pueden sufrir reacciones de oxidación durante el procesado y el almacenamiento de alimentos, así como en el organismo. Sus productos de oxidación están relacionados con el desarrollo de enfermedades no infecciosas de alta prevalencia. Por lo tanto, es importante estudiar los factores que afectan a la degradación de esteroles y a la formación de oxiesteroles en los alimentos. En este contexto, el presente trabajo trató de determinar el efecto del tratamiento térmico, del grado de insaturación de la matriz lipídica y de la presencia de antioxidantes, en la degradación de esteroles y la formación de óxidos. En los diseños experimentales se emplearon sistemas modelo (muy útiles para evitar la influencia de interferentes), y se llevaron a cabo algunas aplicaciones en alimentos.

Nuestro estudio concluyó que: la termooxidación de esteroles es un proceso multifactorial que depende en gran medida de la combinación tiempo-temperatura, produciendo una alta oxidación de esteroles desde el inicio del calentamiento. Además, la presencia y grado de insaturación de la matriz lipídica, así como la presencia de fenólicos y tocoferoles, protegió significativamente a los esteroles de la oxidación en nuestros sistemas modelo. La incorporación de extractos de plantas en alimentos para conseguir este mismo objetivo, resultó ser una estrategia prometedora si se tienen en cuenta los aspectos sensoriales y las características del alimento. Además, se constató que el seguimiento de la oxidación de esteroles por medio de la determinación de los óxidos generados es una cuestión compleja. Por tanto, sería esencial un consenso científico para alcanzar una metodología estandarizada.

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Introduction

1. LIPIDS

Lipids are defined as substances insoluble in water yet soluble in organic solvents. They comprise a wide variety of compounds, namely waxes, fatty acids (and their glycerides), phospholipids, sphingolipids, tocopherols and steroids, among others. Most of them are important components of animal and plant living cells, where their main biological functions include energy storage, maintaining the structure of cell membranes and cell signaling.

In relation to these important biological functions, lipids contained in foods play a key role in human nutrition and health.

Among fatty acids, an intake which is rich in saturated fatty acids (SFA) has been widely associated with harmful effects in the organism, mostly cardiovascular diseases (CVD) (Krauss et al., 2000). By contrast, a diet rich in monounsaturated fatty acids (MUFA) promotes a healthy lipid blood profile, improves blood pressure, modulates the sensitivity to insulin and glycemic levels and contributes to prevent obesity, hence improving the metabolic syndrome and reducing the risk of CVD (Ros, 2003; Gillingham et al., 2011). Oleic acid, as the main representative of this kind of fatty acids, is thus responsible of most of the beneficial effects attributed to olive oil consumption, characteristic of the Mediterranean Diet. Regarding omega-3 fatty acids (polyunsaturated fatty acids presenting the first double bond at position 3 from the end of the carbon chain), have been shown to reduce plasma triglycerides, heart rate and arterial pressure, as well as promoting anti-inflammatory effects and improving mental disorders, among other benefits (De Henauw et al., 2007; Sánchez-Villegas et al., 2007; Mozaffarian and Wu, 2011). The main omega-3 PUFA from plant origin is α-linolenic acid, and those most representative of marine origin are eicosapentaenoic acid and docosahexaenoic acid, both characteristic of algae and fish.

Dietary hydrophobic vitamins also have a beneficial impact on human health. First, vitamin A (and β-carotene, as provitamin A) consumption prevents vision problems and keratinization of the mucosae of several organic systems (Combs, 2001). Vegetables such as carrots and pumpkins, as well as liver or fish are the main sources of vitamin A. In addition, the intake of vitamin D contributes to the prevention of bone diseases by increasing calcium absorption, CVD, multiple sclerosis, diabetes and cancer (Zittermann, 2003; Bischoff-Ferrari et al., 2006; Vieth et al., 2007). Fatty fish, liver and dairy products are the main food sources of vitamin D, apart from solar exposure. Moreover, vitamin E consumption has been reported to counter the effects of effect CVD (Stampfer et al., 1993; Rimm et al., 1993), although its antioxidant properties in humans do not seem to be very effective (Roberts et al., 2007; Gaziano et al.,

2009). Nevertheless, an excessive intake of vitamins is toxic and can cause moderate to severe health problems (Ochoa and Mataix, 2009; Mataix and de la Higuera, 2009).

Steroids are another group of food lipids that has important biological effects in humans. Among this group, cholesterol is essential for maintaining membrane fluidity, although an excessive intake is again harmful, increasing the risk of CVD. On the other hand, phytosterols - which do not perform any specific function in human organisms - reduce cholesterol absorption in the intestine, decreasing plasma LDL levels (see section 2.3).

All these biological benefits, together with the increase in public interest in health issues, have promoted the development of lipid-enriched-functional products. In this sense, formulations increasing unsaturated fats in detriment of SFA are commonly applied in meat and dairy products (Berasategi et al., 2011; Rodríguez-Carpena et al., 2012a). Hydrophobic vitamins are also usually added into dairy products and fruit beverages (Petrogianni et al., 2013; Delavari et al., 2015) and the number of phytosterol-enriched products have also seen a sharp increase over the last decade (see section 2.2).

However, lipids can undergo oxidation reactions under favorable conditions characterized by oxygen (or any other oxidant) availability, incidence of light and temperature. This lipid oxidation can take place in foods before consumption or within the organism. Regardless of the environment where the oxidation takes place, the process itself is harmful not only because it damages cell functions by destroying the lipids, but also because it results in the production of hydroperoxides, alcohols and carbonyl compounds, which have been related to cytotoxicity and mutagenicity (Uchida et al., 2000; Del Rio et al., 2005; Gueraud et al., 2010; Otaegui et al., 2010), as well as spoiled organoleptic quality. Several types of lipids (fatty acids, phospholipids, vitamins and sterols) have been reported to be degraded through oxidation reactions and to produce the above-mentioned toxic substances.

Different approaches have been adopted to avoid or counteract lipid oxidation both in food and in vivo. First, oxygen availability, light exposure and temperature are controlled in foodstuffs by means of appropriate packaging conditions. Second, the control of cooking and other processes are critical points. And last, control over pro-oxidant environments both in food and biological tissues are exercised by means of the addition of antioxidants in the formulation and the incorporation of antioxidants in the diet, respectively. These strategies are sometimes applied to limit the oxidation of lipids in general, and occasionally to limit the oxidation of a particular kind of lipid, such as sterols.

Considering the widespread presence of lipids both in foodstuffs and in the human body, a large number of research studies has as their aim, the determination of lipids and, in particular, the determination of their oxidation products. However, the great diversity of oxidized compounds and the complexity of certain biological and food matrices, as well as the possibility of multiple technical approaches, make it difficult to establish universal methods for determining the status of lipid oxidation.

In particular, in the case of sterol oxidation products (SOPs), it is worth highlighting that their analysis is laborious and expensive, and hence the optimization of the methodology remains a central objective. Time and cost-efficiency are factors to be considered, without disregard for reliability of research results. In this sense, the formation of artefacts (oxysterols not present in the sample) during the laboratory preparation process is a major concern since both sterols and oxysterols are normally present in the samples and the former in relatively higher amounts. Besides, there are sensitivity difficulties associated with the determination of trace levels. Among the various methods available to analyze SOPs, the general procedure involves the following steps: lipid extraction, saponification, solid phase extraction, derivatization and chromatography. Furthermore, research groups have shown substantial variances in the execution of these steps. An inter-laboratory harmonization of the methodologies is an urgent issue since certain parameters (such as temperature, oxygen exposure or contact with alkaline solutions) play a crucial role in artefact generation and the sensitivity is notably affected by chromatographic conditions (Griffiths et al., 2013, Georgiou et al., 2014).

2. STEROLS

2.1 Chemical structure and properties

Sterols are unsaponifiable lipids, whose chemical structure is characterized by a cyclopentanophenanthrene ring with a hydroxyl group in position C-3 and a side chain in C-17, as illustrated in figure 1. Particular sterols differ from each other in terms of the substitutions of the side chain. Cholesterol is the main sterol of animal origin, in comparison to the more than 250 plant sterols (usually named as phytosterols) that have been identified. β-Sitosterol, campesterol and stigmasterol are the most abundant compounds, representing more than 95% of the whole phytosterols content in food.

The different substituents in the molecule provide a graduation of its hydrofobicity, and therefore, in the absorption properties of the sterol in organisms. Therefore, phytosterols are, in general, less polar than cholesterol. Sterols can be found as free or esterified molecules, with a fatty acid attached to their hydroxyl group.

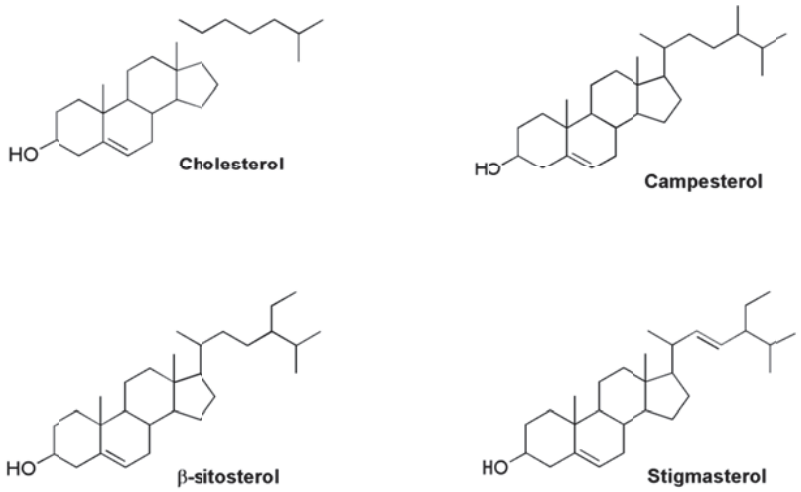


Figure 1. Chemical structures of cholesterol and some common phytosterols

2.2 Presence in foods

Cholesterol is a constituent of the cell membrane of animal tissues, and hence it is present in most foods derived from animals such as meat, eggs, fish and dairy products. Concentrations vary depending on the foodstuff, ranging from less than 10 mg/100g in fresh milk, to more than 400 mg/100g in animal entrails (Moreiras et al., 2011). Cholesterol usually manifests itself esterified with the fatty acids of the food matrix. While the American Heart Association recommends an intake of less than 300 mg/day of cholesterol, the European mean intake is 150-450 mg cholesterol per day (Andersson et al., 2004; Escurriol et al., 2010).

Phytosterols are the plant sterols counterparts, occurring naturally in fruits, nuts, cereals, vegetables and vegetable oils. Typical concentrations range from 1-50 mg/100g in fresh products to 70-1500 mg/ 100g in vegetable oils (Phillips et al., 2002; Marangoni et al., 2010; Gupta et al., 2011). β-Sitosterol is generally present in food at higher concentrations than those of campesterol and stigmasterol.

A Western diet provides between 100 and 400 mg phytosterols per day (Andersson et al., 2004; Kuhlmann et al., 2005; Escurriol et al., 2010). The required phytosterol intake to have any cholesterol-lowering effects (see section 2.3) is 2-3 g/day. Given the increase in consumer awareness regarding health and functional foods, the last decade has seen a considerable development of phytosterol-enriched food products. Since 1995, when the first such spread was introduced in Finland, the variety of foodstuffs on offer has grown significantly in the form of margarines, yoghurts, milk, salad dressings, vegetable oils, fruit juices, bakery products and snack bars (Kuhlmann et al., 2005; Ozer and Kirmaci, 2010; Alemany et al., 2012a; Botelho et

al., 2014;). The European Committee has authorized the distribution of most of these products in the European market (Eur-Lex, online). This enrichment in phytosterols has enabled the increase in their intake above the clinically important levels for its cholesterol-lowering properties to take effect. Moreover, the food matrix, the sterol form and their distribution in several servings throughout the day, may affect the magnitude of the LDL reduction achieved (Clifton et al., 2004; Gupta et al., 2011; Shaghaghi et al., 2014).

Phytosterols to be added as ingredients in enriched foods are generally extracted from byproducts from wood pulp in the paper industry or from vegetable oils. Plant stanols are mainly produced by hydrogenation of plant sterols (Brufau 2008). They can be found in powder, microencapsulated, emulsified or esterified. As they form crystals that are insoluble in water and difficult to disperse in fat (Sharma 2005), phytosterols are usually added to food products in their esterified form. This process makes them more soluble in dietary fat and enhances their dispersion in the intestine, thereby promoting their efficacy (Katan et al.,2003). However, hypocholesterolemic effectiveness has recently been found to be higher for water-dispersable-phytosterols than for esterified-phytosterols added to yogurts (Shaghaghi et al., 2014). Ratios of the phytosterol mixture which must be used for enrichment is determined by legislation, limiting the maximum amount for each sterol (Eur-Lex).

The development of new formulations of enriched products has been accompanied by the consequent legislation regarding scientific substantiation of their health effects and their safety. In 2009, the European Safety Association (Scientific Opinion, EFSA-Q-2009-00530 , EFSA-Q-2009-00718) accepted a claim related to plant sterols and lower/reduced blood cholesterol and reduced risk of heart disease in phytosterol-enriched food products. The Food and Drug Administration had previously (2000) authorized a claim on the same issue (Federal Registration of September 8, 2000-65FR 54686). As it happens with most functional foods, the proper scientific validation of functional claims still remains the critical issue.

2.3 Effects in the organism

Cholesterol

Cholesterol in blood and tissues comes mainly from endogenous formation and to a lesser extent from dietary cholesterol. Considering dietary cholesterol, around 50-80 % is absorbed in the intestine (Bosner et al., 1999) mainly through inclusion in mixed micelles (containing bile acids and phospholipids) but also by specific transporters (Altman et al., 2004). Several ABC-type transporters take control of the excretion of cholesterol back to the intestinal lumen. Once in the enterocyte, cholesterol is esterified by ACAT and transferred into Quilomicrons in

order to be sent to the blood torrent. Quilomicrons bring TG into the adipose tissue and become enriched in cholesterol. Similarly, VLDL which are also formed by combining cholesterol and TG, bring TG to muscular and skeletal tissue, giving rise to LDL, enriched in cholesterol. Cholesterol homeostasis is regulated by LXR (Liver X Receptor) and SREBPs (Sterol Regulatory Element Binding Proteins), by means of modulations in intestinal absorption, biosynthesis, HDL activity and cholesterol excretion (Fiévet amd Staels, 2009).

High cholesterol levels have been steadily associated with several chronic diseases, mainly cardiovascular diseases, such as metabolic syndrome (D' Adamo et al., 2014; Gilbert et al., 2014).

Phytosterols

Phytosterols cannot be synthetized endogenously and their limited presence in the organism (around 2 order of magnitude less than cholesterol) is completely of dietary origin. The absorption rate for these compounds is less than 5 %, which is reported to be much lower than that of cholesterol. This is primarily related to their lower aqueous solubility and slower transference to mixed micelles (Ostlund et al., 2002; Matsuoka et al., 2010; Alemany et al., 2013a).

Their cholesterol-lowering effect was firstly associated with phytosterols in the 1950s, and since then, the interest on the subject has grown over the last decades. Nowadays, due to the vast information provided by more than 100 clinical trials, an intake of 2-3 g/day of plant sterols is generally accepted to reduce plasma LDL cholesterol around 10 % (Law et al., 2000; Katan et al., 2003; Abumweis et al., 2008; Wu et al., 2009; Demonty et al., 2009; Talati et al., 2010). Current data suggest that triglyceride levels are also reduced although no effect is observed in HDL cholesterol (Theuwissen et al 2009; Baumgartner et al., 2013; Demonty et al., 2013; Langella et al., 2014). The structural similarity of phytosterols and cholesterol accounts for their similar metabolic pathways and explains their lipid-lowering effect (von Bergman et al., 2005). Several possible mechanisms have been suggested (Trautwein et al., 2003; Smet et al., 2012), mainly: 1) physical competition for space in mixed micelles between cholesterol and phytosterols (Matsuoka et al., 2010); 2) higher availability of phytosterols in the intestine due to better hydrolyzation by enzymes (Gupta et al., 2011); 3) Up-expression of ABC-type transporters as a result of the accumulation of phytosterols in the enterocyte due to their poor esterification by ACAT (Plat et al., 2005).

Besides their cholesterol-lowering effect, some *in* vitro and *in vivo* studies show promising results with respect to anti-inflammatory, antipyretic, antidiabetic, immunoregulator and anti-

carcinogenic properties of phytosterols (Woyengo et al., 2009; Brull et al., 2009; Cilla et al., 2015).

On the other hand, high phytosterol plasma levels have been associated with atherosclerosis and cardiovascular disease (Assman et al., 2006). Thus, patients with phytosterolemia - a disease characterized by high absorption and low excretion of phytosterols - present a higher risk of suffering this kind of pathologies (Weingartner et al., 2014). Furthermore, phytosterols may replace not only cholesterol from the core of the mixed micelles, but also other compounds present in the micelles, such as lipophilic vitamins (Katan et al., 2003).

3. Sterol oxidation products (SOPs)

3.1 Chemical properties

As any other lipid, sterols can undergo oxidation rendering other compounds. Sterol structure is susceptible of oxidation in the double bond of the sterol ring, as well as in other positions of its side chain (Ryan et al., 2009), classifying oxysterols into two categories: those oxygenated on the sterol ring (mainly at the position 7) and those oxygenated on the side chain (mainly at positions 24, 25 and 27). As a result, hydroperoxides are formed at first, and secondary

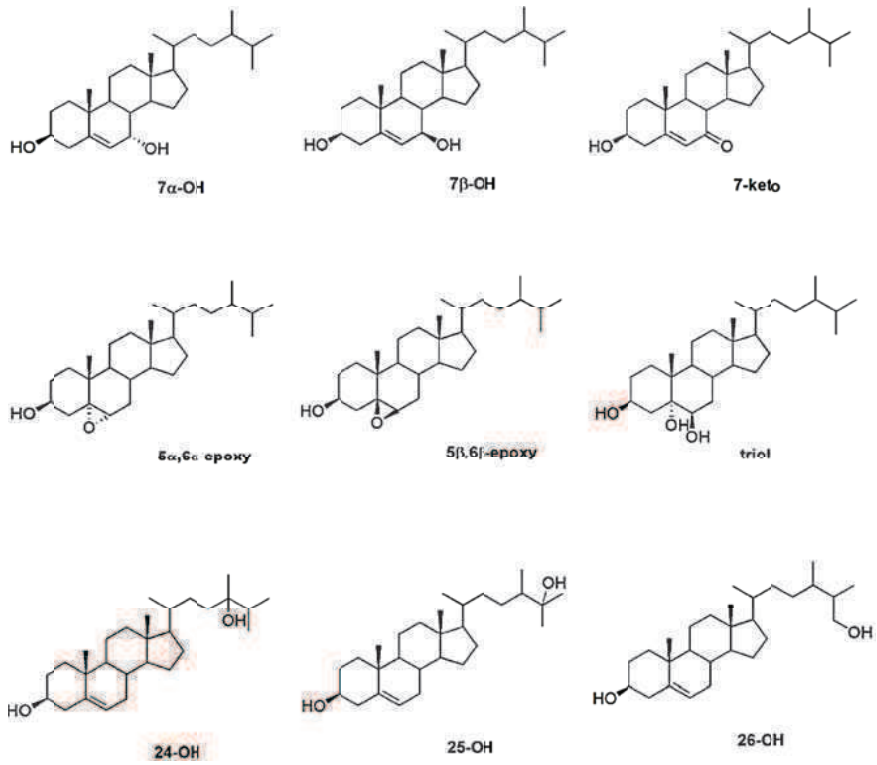


Figure 2. Chemical structures of common oxysterols (Hovenkamp et al., 2008)

oxidation products (alcohols, epoxides and carbonyls) later on. Dimers, oligomers and polymers of unoxidized and oxidized forms of sterols are also formed, mainly in advanced stages of oxidation. Sterol oxidation products are usually known as SOPs or oxysterols; when the oxidation products are derived from cholesterol their designation is COPs or oxycholesterols, and when derived from phytosterols, they are called POPs or oxyphytosterols.

3.2 Presence in food

Oxycholesterols are commonly found in animal foodstuffs containing notable amounts of cholesterol. Concentrations found in a selection of research articles range from 0.1 to 50 μg/g in meat, from 0.7 to 30 μg/g in fish, from 3 to 290 μg/g in egg and egg derived products and from 1 to 260 μg/g in dairy (Echarte et al., 2004; Otaegui et al., 2010; Derewiaka and Obiedzinski, 2012). The estimated common daily oxycholesterol intake is 3 mg/day (Hovenkamp et al., 2008).

Oxyphytosterols have been identified in a variety of vegetable foods, including vegetable oils, margarines, French fries, milk, coffee beans, wheat flour, fruit juices and infant formulas (Otaegui et al., 2010; Alemany et al., 2012a; Derewiaka and Obiedzinski, 2012). Concentrations range between 1 and 60 μg/g in vegetable oils. Considering a daily intake of 40 g of oil, a consumption of around 2 mg/day can be estimated.

Phytosterol-enriched foods may be an important dietary source of oxyphytosterols, compared to non-enriched products. Commercially available non-enriched and enriched-spreads contain up to 13 and 46 μg/g of these compounds, respectively (Conchillo et al., 2005). Considering an estimated daily intake of 15 g of spread, oxyphytosterol consumption would increase from 0.195 mg to 0.7 mg per day.

3.3 Effects in the organism

SOPs present in food are absorbed and incorporated into the organism through diet, as several *in vitro* and *in vivo* studies have assessed, using doses of around 100-500 ppm in the diet, mainly with rodents (Staprans 2000; Ando et al, 2002; Tomoyori et al., 2004, Soto-Rodríguez et al., 2009; Liang et al., 2011; Plat 2014). Non-human primates suffered adverse effects after consuming a diet containing oxidized cholesterol, compared to the control diet group, indicating a probable absorption of COPs (Deushi et al., 2011). In human studies, increases in plasma levels for up to 300-1600 μg/dL have been detected after intakes of 3-400 mg COPs within potato, salami, cheese and powdered eggs (Emanuel et al., 1991; Linseisen 1998; Staprans et al., 2003). The absorption, distribution and excretion are supposed to be accomplished through similar mechanisms as sterols (Hovenkamp et al., 2008; Brown and

Jessup, 2009; Terunuma et al., 2013). The absorption ratios range around 2-20% for COPs and 20-50% for POPs (Alemany et al., 2013a). Nevertheless, this absorption cannot always reflect an estimation of the plasma levels, since SOPs can also be generated within the organism (see section 4.1).

Regardless of their origin, the presence of both COPs and POPs in plasma and tissues has been extensively related to a number of biological effects (Poli et al., 2009; Sottero et al., 2009; Otaegui et al., 2010; O'Callaghan et al., 2014, Alemany et al., 2014). Whilst there is broad biological research on oxycholesterols, the amount of biological research on oxyphytosterols is more recent and limited, and the majority of studies compare COPs and POPs.

On the one hand, SOPs (mainly those generated from autooxidation) have been shown to up-regulate the expression of various pro-inflammatory molecules, including adhesion molecules, growth factors, cytokines and chemokines (Leonarduzzi et al., 2005; Lemaire-Ewing et al., 2005; Mascia et al., 2010; Alemany 2013b). Conversely, oxysterols originating from enzymatic sterol oxidation produce an anti-inflammatory signalling in macrophages (Olkkonen, 2012).

On the other hand, *in vitro* and *in vivo* cytotoxic effects have been widely reported for both COPs and POPs, although the former present much higher cytotoxicity levels (Adcox et al., 2001; Meynier et al., 2005; Maguire et al., 2003; Roussi et al., 2007; O'Callaghan 2010; Kenny et al., 2012; Vejux et al., 2012; Alemany et al., 2012b; Biasi et al., 2013). Nevertheless, recent studies have also supported the cytotoxic effects of campesterol, stigmasterol and β-sitosterol oxides (Koschutnig et al., 2009; O'Callaghan et al., 2010; O'Callaghan et al., 2013). Besides, COPs and POPs activate cell death signalling (including apoptosis) by different routes (Ryan et al., 2005; Roussi et al., 2005). Among the different SOPs studied, 7-hydroxy, 7-keto and triol derivatives are the most cytotoxic ones. The potential use of oxysterols as chemotherapeutic drugs is an emerging research line which deserves further attention since selective cytotoxicity has been found in some cases (Carvalho et al., 2011; Segala et al., 2013). Moreover, some genotoxic effects have been shown by COPs (Osada, 2002) but studies assessed with POPs failed to found mutagenesis (Maguire et al., 2003; Koschutnig et al., 2010).

SOPs in plasma and tissues are related to oxidative stress by two feed-back mechanisms. First, their presence contributes to the overall oxidative status in cells (Koschutnig et al., 2009; O'Callaghan et al., 2010). Second, an oxidant environment enhances *in situ* SOPs

formation (Vaya et al., 2013). But their cytotoxicity has not been counterbalanced by antioxidants in some studies (Ryan et al., 2005; O'Callaghan et al., 2010; Baumgartner 2013).

Taken together, all these SOP-induced effects suggest their potential importance in the onset of chronic diseases in which oxidative stress, inflammation and cell death appear to be involved, such as atherosclerosis and neurodegenerative diseases.

Menéndez-Carreño et al. (2011) found high correlation between COPs levels and CVD risk factors in humans. Special attention must be paid to the atherogenic effects. Wide research is available on the promotion of atheromatous plaque development by COPs in animal and human studies (Staprans et al., 2003; Larsson et al., 2006; Chen et al., 2009; Chalubinsky et al., 2013). Particularly, 7-hydroxy, 7-keto and triol derivatives are the main oxycholesterols involved. The first *in vivo* experiments searching for POPs pro-atherogenic activity reported no effect (Ando et al., 2002; Tomoyori et al., 2004) but recent studies point out to a certain pro-atherogenicity (Liang et al., 2011; Yang et al., 2013; Plat et al., 2014). Other hypercholesterolemic-related pathologies (such as diabetes and hyperlipidemia) also result in high oxycholesterol plasma levels (Abo et al., 2000; Arca et al., 2007). The involvement of SOPs with pathologies of the central nervous system includes optical, psycriatic and aged-related diseases. Particularly, patients with visual abnormalities, depression, fatigue, Alzheimer and Parkinson have shown elevated levels of oxysterols (mainly 24-hydroxycholesterol and 27-hydroxycholesterol) in certain tissues and fluids (Xu et al., 2012; Shichiri et al., 2013; Leoni et al., 2013; Björkhem et al., 2013; Freemantle et al., 2013). Besides, high 7β-hydroxycholesterol levels were found in samples from patients with lung cancer.

The use of SOPs as biomarkers in some of these pathologies is a promising strategy to allow an earlier diagnosis, as many of these studies suggest.

Finally, oxyphytosterols appear to improve cholesterol homeostasis. The mechanism involves up-regulation of the expression of ABC family genes through LXR activation, thus inhibiting intestinal cholesterol absorption (Engel and Schubert, 2005; Plat et al., 2005). Other lipid metabolism related parameters have shown to improve by dietary SOPs (Suzuki et al., 2002; Ikeda et al., 2006). They have also been related to modulation of the immune system (Kimura et al., 1995) and certain hormonal activity (Christianson-Heika et al., 2007).

4. Sterol oxidation process

4.1 Formation mechanisms

SOPs can occur both endogenously (*in vivo*) and exogenously (*ex vivo*). SOPs present in foods are undoubtedly formed exogenously. Conversely, SOPs in plasma can be attributed to both endogenous (*in vivo* oxidative transformation from sterols) and exogenous (oxidation in food and later absorption from the diet) sources. Whilst processes involved in cholesterol oxidation are well known, detailed knowledge is still lacking on phytosterol oxidation; however, current data suggest that both kinds of sterol oxidation products are formed following similar pathways. Two main mechanisms have been suggested: enzymatic and non-enzymatic. Generally, ring-oxygenated sterols tend to be formed non-enzymatically, whereas side-chain oxygenated sterols usually have an enzymatic origin. Nonetheless, 25-hydroxy and 7α-hydroxy can be formed by both pathways (Romer and Garti, 2006; Brown and Jessup, 2009). Non-enzymatic pathway comprises auto-oxidation and photo-oxidation and takes place both *in-vivo* and *ex-vivo*.

The initial reactions in sterol auto-oxidation process start when an allylic hydrogen at C7 is abstracted, generating a free radical. This one can react with molecular oxygen to form a 7-peroxyl radical, which is stabilized by hydrogen abstraction producing the more stable 7-hydroperoxides (Brown and Jessup, 2009; Iuliano et al., 2011). These compounds can decompose, yielding epimeric 7-hydroxysterols and 7-ketosterols. The epimers 5,6-epoxysterols are formed via a bi-molecular interaction of intact sterol and hydroperoxides. 5,6-epoxysterols can be further converted to 3β,5α,6β-triol through hydration in an acidic environment (Lampi et al., 2002; Saynajoki et al., 2003; Grandgirard et al., 2004; Ryan et al., 2009). Whereas the generation of side chain auto-oxidation products is not as common as that of the ring structure, 20/24/25/27-cholesterol hydroperoxides and their decomposition products have been reported. Reports on side-chain oxidation products of phytosterols are, on the contrary, limited; trace levels of 24-hydroxy and 25-hydroxy derivatives of phytosterols have been identified in heated vegetable oils (Smith, 1981; Lampi et al., 2002; Johnsson and Dutta, 2005).

Similar POPs may be generated through photo-oxidation (Synajoki et al., 2003; Zhang et al., 2006). The mechanism is not free-radical mediated and involves the incorporation of singlet oxygen species directly either on the 5-6 double bond or on the C7 position to give the corresponding hydroperoxides, which can further decompose to the previously named compounds.

Whereas auto-oxidation is enhanced at high temperatures due to the hydrogen-abstraction process, photo-oxidation is promoted by light or presence of photo-sensitizers since they favor singlet oxygen formation.

Side chain oxidation is believed to be due mainly to enzymatic reactions. Cytochrome P450 monooxygenases, dehydrogenases, epoxidases, hydroxylases and oxidases are involved in cholesterol oxidation. 24-, 25- and 27-hydroxycholesterols, among others, are generated by specific enzymes (Björkhem et al., 1998; Lund et al., 1998; Russel 2000; Bodin et al., 2001; Javitt 2002; Björkhem et al., 2007; Bretillon et al., 2007). The same enzymes and routes are presumably also involved in phytosterol oxidation. Alkyl groups at position C24 enable stereospecific 24S-hydroxylation and might limit the formation of 25- or 27-hydroxyphytosterols.

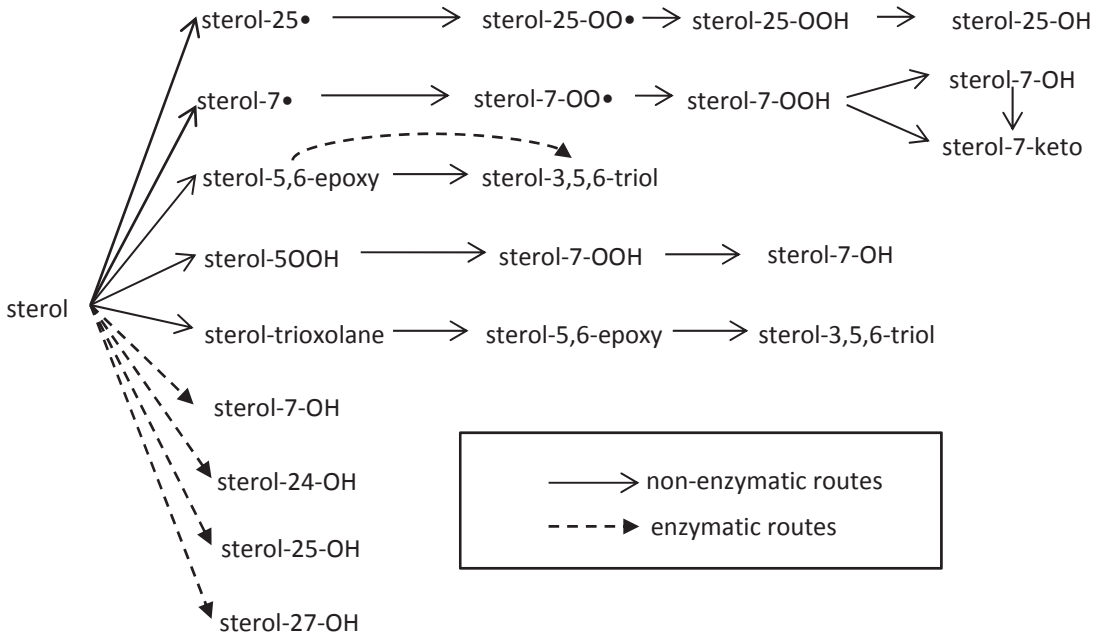


Figure 3. Main enzymatic and non-enzymatic routes of SOPs formation.

Extended degrees of oxidation (achieved at long-term and/or high temperature treatments) lead to degradation of SOPs and formation of oligomers and polymers (Struijs et al., 2010; Sosinska et al., 2014; Derewiaka et al., 2015).

4.2 Relevance of exogenous formation

Considering that the absorption of SOPs from the diet has been demonstrated, and given their potentially harmful effects for 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 process. Among them, particular factors could be identified as follows: heating, air, light, lipid surrounding matrix, antioxidants and water.

Heating

The sterol oxidation process is directly related to the temperature. The higher the temperature, the faster sterol degradation and SOPs formation, and higher concentrations are reached. Particular behaviors strongly depend on the conditions applied, but in the overall, it could be stated that temperatures below 120 °C hardly promote SOPs formation, whereas temperatures over 180 °C produce a very intense generation of SOPs (Zhang et al., 2005; Kemmo et al., 2005; Seckin et al., 2005; Soupas et al., 2007; Yen et al., 2010; Derewiaka et al., 2015). The influence of the heating time is undoubtedly important, too. After long-term heating treatments, oxidation is so high that SOPs may me degraded. This decrease in SOPs levels is observed at different times depending on the conditions applied. Cooking conditions and industrial processes have shown to induce SOPs formation and subsequent degradation through heating treatments (Menéndez-Carreño et al., 2008; Azadmard-Damirchi and Dutta, 2009; Broncano et al., 2009; Mazalli and Bragagnolo, 2009; Pikul et al., 2013; Lira et al., 2014; Zardetto et al., 2014).

Air

Oxidative reactions cannot occur unless sufficient elemental oxygen is available in the medium. The content on SOPS of several foodstuffs stored under different packaging conditions has been monitored, concluding that atmospheres poor in oxygen significantly improved the preservation of the products (Boselli et al., 2012; Penko et al., 2015). Storage temperature is also crucial for sterol oxidative stability (Gawrysiak-Witulska et al., 2012; Botelho et al., 2014; Rudzinska et al., 2014).

Light

On the other hand, light is known to be a free radical reactions inducer, as well as for singlet oxygen species. Thus, a significant increase in SOPs levels have been extensively found in vegetable oils, dairy products, eggs, meat and fish after exposure to natural or artificial light (Zhang et al., 2006; Boselli et al., 2012; Cardenia et al., 2013; Hernández-Becerra et al., 2014).

Photo-oxidation depends mainly on exposure duration, although very intense treatments could further oxidize POPs. Moreover, certain substances naturally occurring in foods such as rivoflavin, chlorophyll or porphyrin, can act as photo-sensitizers, increasing photo-oxidation (Wanasundara et al., 1998; Chien et al., 2003). Nevertheless, the use of alternative protective packaging and lighting conditions during commercial retail storage can efficiently prevent sterol photo-oxidation.

Water

The presence of water, either within the food or in the atmosphere, adversely affects sterols, as several studies with oils have recently shown (Cercaci et al., 2007; Gawrysiak-Witulska et al., 2012).

Unsaturation degree of the surrounding lipids

During the last 15 years, there has been considerable evidence of the influence of the lipid unsaturation degree on the intensity of sterol oxidative processes, despite no consensus on the matter has been achieved yet. Whereas some authors have found a protective effect (Chien et al., 2003), some others have observed a pro-oxidant effect of the unsaturated lipids surrounding the sterol (Lehtonen et al., 2012). Time and temperature conditions have also been proposed as possible critical factors on the behavior of sterols within unsaturated lipids (Soupas et al., 2004; Xu et al., 2011). Therefore, more research is needed to clarify this question.

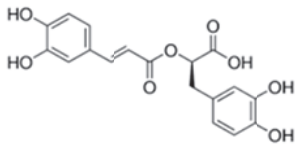
Antioxidants

Protection against sterol oxidation has been associated with the antioxidant capacity of foods (Xu et al., 2009; Tian et al., 2011). This protection is usually attributed to phenolic compounds and tocopherols, compounds naturally present in fruits, seeds and vegetable oils (Xu et al., 2001; Chien et al., 2006; Palozza et al., 2008). Hence, the addition of natural antioxidants is understood as an interesting tool to avoid plant sterol loss, as well as formation of toxic COPs and POPs. In this sense, plant extracts have been extensively tested in foods, achieving very successful results (Rodríguez-Carpena et al., 2012b; Das et al., 2012; Figueiredo et al., 2014).

Among the wide variety of plants containing antioxidant compounds and potentially applicable in foods, two of them have been selected in this work: *Melissa officinalis* and *Solanum sessiliflorum.*

Melissa officinalis (Lemon balm or melisa) is a medicinal plant usually consumed as infusion due to its recognized beneficial effects mainly towards the central nervous system and the digestive system. Recent studies have reported its antiproliferative effects upon colon cancer cells and its antioxidant effects towards lipid oxidation both in vivo and in foods (López et al., 2009; Encalada et al. 2011; Berasategi et al., 2011). Thus, it is a potentially interesting plant for the use against sterol oxidation. The major phenolic compound found in this plant is rosmarinic acid, followed by other phenolic acids such as caffeic, syringic and chlorogenic (Hoyos, 2009).

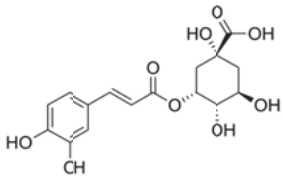
Melissa officinallis



Rosmarinic acid

Solanum sessiliflorum (mana-cubiu) is a fruit native to the Amazonian region and consumed mostly as salad, juice or jelly. It is traditionally used for medicinal purposes due to its hypoglycemic and hypocholesterolemic activity, and it also exhibits anti-genotoxic effects (Pardo, 2004; Hernandes et al., 2014). Besides, it shows high antioxidant activity (Rodrigues et al., 2013). Thus it is also a potentially interesting plant for its use against sterol oxidation. The major phenolic compound found in this fruit is 5-caffeoylquinic acid, and it also contains great amounts of carotenoids.

Solanum sessiliflorum



5-Caffeoylquinic acid

4.3 Model systems as a useful experimental tool

Foods are usually complex matrices where interferences among several components may hamper a clear view about the mechanisms of sterol oxidation. Therefore, model systems are a very useful tool to evaluate separately the factors that exert an influence in this process, avoiding the ambiguity from interferences among them. Thus, a deeper understanding of the underlying mechanisms is enabled and kinetic curves can be determined easily. The effect of several antioxidants and lipid matrices against sterol oxidation has been tested in model systems (Chien et al., 2006; Palozza et al., 2008; Xu et al., 2009; Yen et al., 2011; Kmiecik et al., 2011; Xu et al., 2011; Lehtonen et al., 2012; Ansorena et al., 2013). A diversity of experimental approaches can be found: from fully modelled studies where only chemical standards are used as components of the experiments, to intermediate model systems, where chemical standards are mixed within foods. Some mathematical models for sterols' degradation and oxysterols formation have been obtained from this kind of studies (Chien et al., 1998; Hu and Chen, 2002; Ansorena et al., 2013).

Justification and objectives

Taking into account the state of art in the field of lipid and sterol oxidation, it can be stated that:

a) There is overwhelming methodology for lipid oxidation analysis. Particularly, sterols and oxysterols determination results in complex, laborious and expensive procedures.
b) Dietary sterols are nutritionally interesting compounds which can undergo oxidation reactions during food manufacture and storage, as well as in the organism. Their oxidation products are associated with the development of highly prevalent non-infectious diseases. Therefore, it is relevant to evaluate the factors that affect sterol degradation and oxysterols formation in foods.
c) Model systems are valuable tools to separately evaluate factors exerting an influence in sterol oxidation, avoiding the ambiguity which normally results from interferences among them.

Consequently, in the present work, the following objectives were aimed:

1. To optimize the methodology for oxysterols analysis.
2. To monitor the behavior of cholesterol and three major plant sterols (sitosterol, campesterol and stigmasterol) during heating at 180 °C, assessing sterols degradation and oxysterols formation.
3. To evaluate the influence of the unsaturation degree of the surrounding lipids in sterols degradation and oxysterols formation under heating conditions.
4. To evaluate the potential protective effect of different natural antioxidants on sterol degradation and oxysterols formation under heating conditions, both in model and food systems.

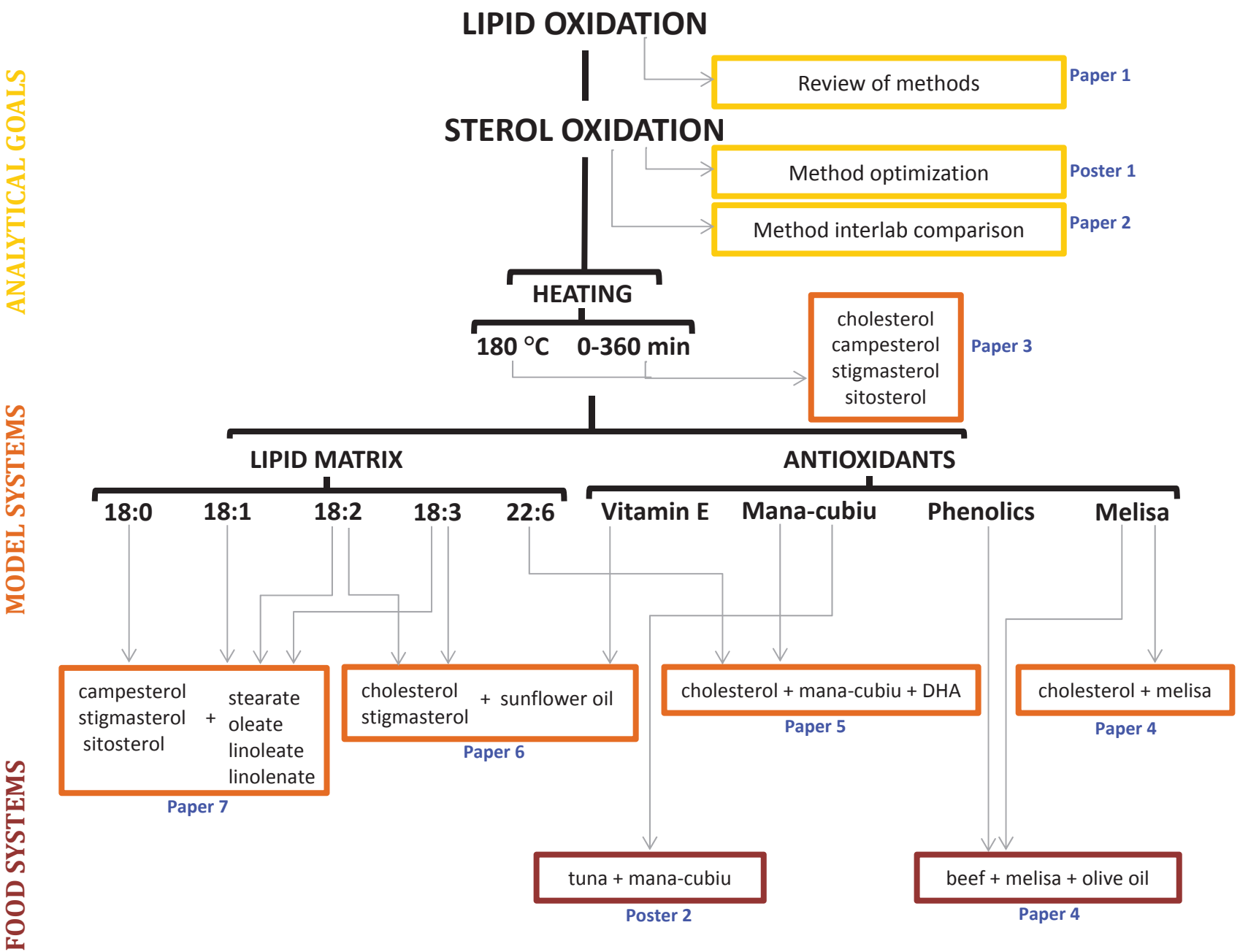
Teniendo en cuenta el conocimiento actual sobe oxidación de lípidos y de esteroles, se puede afirmar que:

a) La metodología de análisis de oxidación lipídica es muy variada. Particularmente, la determinación de esteroles y oxiesteroles requiere procedimientos complejos, laboriosos y caros.
b) Los esteroles dietéticos son compuestos interesantes desde el punto de vista nutricional, que pueden sufrir reacciones de oxidación durante el procesado y el almacenamiento de los alimentos, así como en el organismo. Sus productos de oxidación se relacionan con el desarrollo de enfermedades no infecciosas de alta prevalencia. Por lo tanto, es importante evaluar los factores que afectan a la degradación de esteroles y a la formación de oxisteroles en alimentos.
c) Los sistemas modelo son útiles para evaluar por separado los factores influyentes en la oxidación de esteroles, evitando la ambigüedad resultante de las interferencias entre ellos.

Por lo tanto, en el presente trabajo, los objetivos fueron los siguientes:

1. Optimizar la metodología de análisis de oxiesteroles.
2. Estudiar el comportamiento de colesterol y tres esteroles vegetales mayoritarios (sitosterol, campesterol y estigmasterol) durante el calentamiento a 180 °C, determinando la degradación de esteroles y la formación de oxiesteroles.
3. Evaluar la influencia del grado de insaturación de la matriz lipídica en la degradación de esteroles y la formación de oxiesteroles durante el calentamiento.
4. Evaluar el potencial efecto protector de diferentes antioxidantes naturales sobre la degradación de esteroles y la formación de oxiesteroles durante el calentamiento, tanto en sistemas modelo como en alimentos.

Experimental design



The following diagram represents the different items studied along the whole research period. The experimental sets are represented in colored boxes and the dissemination of results is highlighted in blue.

Material and methods

1. Sample preparation

1.1 Model systems

For each model system, the corresponding mixture of sterols, sunflower oil, FAME or plant extracts was solved in chloroform. Aliquots were transferred to open glass tubes (15 x 100 mm) and evaporated under a stream of N_2. Then, the tubes were placed in the termbloc, previously heated at 180 °C. After the corresponding heating time, they were cooled down in an ice bath, solved in chloroform and kept at -20 °C until analysis.

Table 1. Amounts (mg) of sterols, plant extracts and lipids used in the different model systems

Experiment	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Other compounds
Paper 3	2.5	0.08	1.34	0.93	---
Paper 4	20	---	---	---	Melisa extract (0.4)
Paper 5	1	---	---	---	Mana-cubiu extract (0.5) DHA (1)
Paper 6	1.2	---	1.2	---	Sunflower oil (240)
Paper 7	---	0.7	0.36	1.3	FAME (240)

1.2 Beef patties

Meat was conveniently double-minced and all patties weighed 80 g. Two types of patties were formulated: simple patties (without emulsion) and emulsion-containing ones (included an oil in water emulsion). In each case, patties with and without an aqueous extract of *Melissa officinallis* (melisa) were prepared. Simple patties contained 79.2 g meat and 0.8 g common salt. For “simple with melisa” patties, salt was substituted with enriched salt (previously prepared by mixture and homogenization with the *M. officinalis* extract: 16 g salt + 64, 80, 104, 200, 600 or 800 mg melisa extract). Formulation of the emulsion-containing patties consisted of 75.2 g of meat, 0.8 g salt and 4 g of an oil-in-water emulsion. To make the emulsion, 52.63 g of extra virgin olive oil was slowly added to 42.1 g water (containing 5.3 g soya protein), while continuously homogenizing with an ultra-turrax. For “emulsion with melisa patties”, melisa extract (250, 300 or 400 mg) was added to the water phase of the emulsion before mixing with oil.

Mixture of ingredients was compressed with a conventional burger maker until a compacted and homogenized patty was obtained (80 g, 8.6 cm diameter and 1.5 cm thickness).

For the different types of meat patties, four independent batches were prepared, each one containing 4 patties (two to keep raw and two for cooking). Patties were put in a pre-heated

oven at 185 °C for 12 min, reaching 65 °C of internal temperature. Just after the cooking process, they were cooled down for 10 min, weighted, minced, and stored at -20 °C under vacuum until the analysis.

The melisa extract was obtained by heating 50 g of leaves with 500 mL of distilled water at 100 °C during 30 min. The process was repeated twice and the solution was lyophilized (García-Íñiguez de Ciriano et al., 2010b).

1.3 Tuna patties

Tuna was minced with a conventional food mincer. All patties contained 50 g tuna, 0.5 g salt and 2.5 mL of an aqueous solution. This aqueous solution contained 0, 0.02 or 0.1 mg/mL of an aqueous *Solanum sessiliflorum* (mana-cubiu) extract. The ingredients were homogenized manually and introduced in a pre-heated griddle at 180 °C for 7 min (3.5 min each side of the patty), reaching 72 °C of internal temperature. Just after the cooking process, they were cooled down for 10 min and minced. Hexanal determination was carried out the same day of cooking. For cholesterol, COPs and lipid content determinations, samples were stored at -20 °C under vacuum until the analysis.

Mana-cubiu fruits were lyophilized before extraction. Fifty grams of lyophilized mana-cubiu fruit were homogenized with ultrapure water in a vortex for 5 min and centrifuged at 20000 g at 10 °C. The aqueous layer was lyophilized.

2. Moisture determination

The AOAC official method was used for moisture determination (AOAC, 2002a). 5 g of food sample were homogenized with sand and 5 mL ethanol. Samples were left at 100 °C until constant weight.

3. Lipid extraction

3.1 Lipid extraction (I) - Quantitative

Extraction with petroleum ether by the Soxhlet method was applied for a quantitative determination of the total fat content, according to the AOAC official method (AOAC, 2002b).

3.2 Lipid extraction (II) - Qualitative

Extraction with chloroform:methanol, as proposed by Folch et al. (1957) was followed with slight modifications. Samples (120 g) were homogenized with 300 mL of chloroform:methanol during 3 min, and centrifuged at 10000 rpm for 20 min at 0-10 °C . The solid residue was added with 100 mL chloroform and again homogenized, centrifuged and filtered. Both filtered

solutions were mixed and 100 mL of KCl 0.88% were added. The solution was shaken and then separation of phases was allowed. Chloroform phase was recovered, and evaporated in a rotavapor. This method was applied to beef patties.

3.3 Lipid extraction (III) - Qualitative

Extraction with chloroform:methanol, as proposed by Folch et al. (1957) was followed with slight modifications. Ten grams of sample were homogenized with 100 mL of chloroform:methanol during 2 min, and poured into a decantation funnel through filtration with paper. The solid residue was added with 50 mL of the solvents' mixture and again homogenized, and filtered. Finally, the solid residue was added with 25 mL of the solvents' mixture and again homogenized, and filtered. 40 mL of KCl 0.74% were added to the funnel. The solution was shaken and then separation of phases was allowed. After the recovery of the organic layer, 25 mL more of KCl 0.74% were added. Shaking and separation was carried out again and the solvent was evaporated in a rotavapor. This method was applied to tuna patties.

4. Fatty acids determination

For the determination in sunflower oil, an aliquot of sample (corresponding to 0.2 g of oil) was transferred to a round bottom flask and chloroform was evaporated under a stream of N_2. Fatty acid methyl esters (FAME) were prepared by derivatization with Boron trifluoride / Methanol, and their identification and quantitation was performed by CG-FID, as described in Ansorena et al. (2013b).

For FAME model systems no derivatization was required as the fatty acids used were already methylated. The first fraction recovered from NH_2-SPE purification of the samples (as detailed below) was evaporated, re-solved in heptane (2 mL) and injected (0.5 µL) in the GC-FID, as described in Ansorena et al. (2013b).

For the mana-cubiu model system, docosahexaenoic acid was converted into its methyl ester according to Joseph & Ackman (1992) and analyzed with a gas chromatograph (GC 2010 model, Shimadzu) equipped with a fused silica CP-SIL 88 capillary column 100 m x 0.25 mm, 0.20 µm and flame ionization detector. Chromatographic conditions were described in detail by Sancho et al. (2011).

Gas Chromatograph-Flame Ionization Detector (Perkin Elmer Clarus 500):

- Column: SP-2560 (100 m x 0.25 mm x 0.20 µm)
- Carrier gas: H_2, 2.15 mL/min
- Oven temperatures program:
  - 175 °C during 10 min
  - Slope 1: 10 °C/min up to 200 °C
  - Slope 2: 4 °C/min up to 220 °C
  - 220 °C during 15 min
- Injector temperature: 250 °C
- Volume of sample injected: 0.5 µL, split ratio = 120
- Detector temperature: 260 °C

Identification of the compounds was carried out by comparison with the retention times of their pure standards. Quantitation was performed by internal standard calibration curves, using methyl heptadecanoate as the internal standard.

Table 1. Retention times and calibration curves of the fatty acids methyl esters

Fatty Acid Methyl Esters	T_R (min)	Calibration curve
Palmitic	9,45	y=0,9805x-0,0204
t-Palmitoleic	10,22	y=0,9469x-0,0012
Palmitoleic	10,58	y=0,9497x-0,0019
Estearic	12,47	y=0,9983x-0,0002
Elaidic	13,10	y=0,9850x-0,0009
Oleic	13,40	y=1,0012x-0,0071
Vaccenic	13,45	y=1,0694x-0,0124
t-Linoleic	14,22	y=0,9538x-0,0019
c-t Linoleic	14,45	y=1,0241x-0,0028
t-c Linoleic	14,53	y=1,0758x-0,0062
Linoleic	14,75	y=0,9961x-0,0007
γ-linolenic	15,77	y=0,9260x-0,0005
Eicosaenoic	15,88	y=1,0522x-0,00004
α-linolenic	16,34	y=0,9200x-0,0004

Where: $y = \frac{A\ of\ FAME}{A_{is}}$; $x = \frac{mg_{FAME}}{mg_{is}}$

5. Sterols determination

• By gas chromatography

For model system samples, an aliquot (corresponding to approximately 0.2 mg of sterol) was transferred to a tube and 5α-cholestane (2 mg/mL in hexane) was added. The solvent was evaporated under gentle nitrogen stream. Samples were derivatized to trimethylsilyl (TMS) ethers according to a modified version of the method described by Dutta and Appelqvist (1997). Four hundred micro litres of Tri-Sil reagent were added to each sample and they were kept at 60 °C for 45 min in a water bath. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivatives were solved in hexane for gas chromatography. 5-10 mL of hexane were added when GC-MSD was aimed to be used, and 0.4 mL if GC-FID was the analyser. These solutions were filtrated with a syringe and a filter (0.45 µm) and poured to a glass vial, before the chromatographic analysis.

Beef patties samples required previous saponification and extraction according to Kovacs et al. (1979). Briefly, 3 g of sample were weigthed and added with 1 mL 5α-cholestane (2 mg/mL in chloroform). Then, 20 mL of ethanol (95 %) and 5 mL KOH (50%) were added and the mixture was heated to 50 °C during 1 h. When the sample was cooled down, 13 mL of distilled water were added and the extraction with hexane was performed (20 mL x 6 times). Finally, the solvent was evaporated in the rotavapor at 35°C. Derivatization to TMS-ethers was made as in the model system. GC-FID was used for the analysis.

For serum samples, the same saponification and extraction procedures as for beef patties were applied. Different volumes of sample were taken for cholesterol (0.05 mL) and for plant sterols (0.3 mL) determination. Derivatization to TMS-ethers was made as in the model system. And different final volumes of hexane were also added to the derivatised samples before chromatographic analysis: 5 mL for cholesterol and 0.4 mL for plant sterols. GC-MSD (6890-5973) was used for the analysis.

Gas chromatograpy-Mass spectrometer (Agilent 6890-5973):

- Column: 19091S-433 HP-5ms 5% Phenyl Methyl Siloxane (30 m x 250 µm x 0.25 µm)
- Carrier gas: He, 1 mL/min
- Oven temperatures program:
  - 85 °C during 0.5 min
  - Curve 1: 50 °C/min up to 290 °C
  - Curve 2: 0.5 °C/min up to 298 °C
- Injector temperature: 280 °C
- Volume of sample injected: 1 µL, splittless mode
- Transfer line to the detector: 280 °C
- Source temperature: 230 °C
- Electron impact: 70 eV
- Detector temperature: 300 °C
- Mass interval: 50.00-550.00 uma
- Detection mode: SCAN

Peak identification was based on comparison of their mass spectra with the spectra of the Wiley library and also with those obtained from the literature. A comparison of their retention time and MS fragments with those of standard pure compounds was also done.

An internal standard method was used for quantitation, with 5α-cholestane as the internal standard (is). Cholesterol and 5α-cholestane quantitation was made using total ion chromatograms, while plant sterols were quantified using extract ion chromatograms, on the basis of the amount of a specific ion for each peak (343, 484, 357, for campesterol, stigmasterol and β-sitosterol, respectively), and taking into account the relative abundance of each ion within each compound (Berasategi et al., 2012).

Table 3. Retention times, characteristic ions and mode of quantitation of sterols in chromatographic analysis

Compound	t_R (min)	Characteristic ions (m/z)	Quantitation
5α-cholestane (is)	7.80	217, 357, 372	Total area (A)
cholesterol	9.95	329, 353, 368, 458	Total area
campesterol	11.07	343, 367, 382	343 (6.62%)*
stigmasterol	11.56	355, 394, 484	484 (3.05%)
β-sitosterol	12.21	357, 381, 396, 486	357 (6.56%)

* ion used for integration (abundance of the ion)

Total area of each plant sterol was calculated as follows: $total\ A = \frac{A\ of\ ion}{abundance} * 100$

Table 4. Internal standard calibration curves of sterols

Compound	Calibration curve	R^2
cholesterol	y = 1.1903x - 0.0288	0.9937
campesterol	y = 1.1348x - 0.008	0.9923
stigmasterol	y = 1.0346x - 0.0156	0.9905
β-sitosterol	y = 1.1257x - 0.0324	0.9974

Where: $y = \frac{total\ \ A\ of\ \ sterol}{A_{is}}$; $x = \frac{mg_{sterol}}{mg_{is}}$

Gas chromatograph-Flame Ionization Detector (Autosystem Perkin Elmer):

- Column: HP1 (30 m x 0.25 mm x 0.1 μm)
- Carrier gas: H_2, 10 mL/min ,
- Oven temperatures program:

  265 °C during 8 min
- Injector temperature: 300 °C
- Volume of sample injected: 0.5 μL, split ratio = 20
- Detector temperature: 300 °C

Only some of the cholesterol samples were analysed with this equipment. Identification was performed by comparison of the retention time of the pure standard. Quantification was performed by integration of the areas of the peaks obtained, using an internal standard calibration curve, with 5α-cholestane as the internal standard (y = 1.0817x-0.0285 ; R^2=0.9993).

Gas chromatograph-Mass spectrometer (Agilent 6890N-5975):

- Column: VF-5ms CP8947 5% Phenyl Methyl Siloxane (50 m x 250 µm x 0.25 µm)
- Carrier gas: He, 1 mL/min
- Oven temperatures program:

  85 °C during 0.5 min

  Curve 1: 50 °C/min up to 290 °C

  Curve 2: 0.05 °C/min up to 291 °C
- Injector temperature: 250 °C
- Volume of sample injected: 1 µL, splittless mode
- Source temperature: 230 °C
- Electron impact: 70 eV
- Detector temperature: 150 °C
- Mass interval: 50.00-550.00 uma
- Detection mode: SCAN and SIM

Peak identification was based on comparison of their mass spectra with the spectra of the Wiley library and also with those obtained from the literature. A comparison of their retention time and MS fragments with those of standard pure compounds was also done.

An internal standard method was used for quantitation, with 5α-cholestane as the internal standard (is). Sterol quantitation was performed by selected ion monitoring (SIM) analysis: for each stage of time, a different ion was selectively detected and quantitated.

Table 5. Retention times and characteristic ions of sterols in chromatographic analysis

Compound	t_R (min)	Characteristic ions (m/z)
5α-cholestane (is)	13.0	**217***, 357, 372
cholesterol	17.7	**329**, 353, 368, 458
campesterol	20.5	**343**, 367, 382
stigmasterol	21.1	355, 394, **484**
β-sitosterol	23.2	**357**, 381, 396, 486

*Ions in bold denote the ion used for integration

Table 6. Internal standard calibration curves of sterols

Compound	Calibration curve	R^2
cholesterol	y = 0.3928x - 0.005	0.9972
campesterol	y = 0.3188x + 0.0253	0.9961
stigmasterol	y = 0.1033x + 0.0037	0.9995
β-sitosterol	y = 0.2977x - 0.0415	0.9993

Where: $y = \frac{Aion_{sterol}}{Aion_{is}}$ $x = \frac{mg_{sterol}}{mg_{is}}$

• By HPLC

For the mana-cubiu model system, each sample was dissolved with 1 mL hexane:2-propanol (97:3, v:v), filtered through a 22 µm filter, before the chromatographic analysis.

For tuna patties, previous saponification and extraction were required, which were performed as in Saldanha et al. (2008). Ten mL of KOH 20% in ethanol (90%) were added to 1 g of sample, and kept 22 h in absence of light under agitation. Then, 5 mL of water were added to the samples, and extraction with hexane was carried out (10 mL x 4 times). Afterwards, the samples were washed with water (5 mL x 3 times), dried with Na_2SO_4, and the solvent was evaporated in the rotavapor. Chromatographic analysis was performed as in the model system.

HPLC-UV-RI (Shimadzu)

- Column: Nova-Pack CN HP (300 mm x 3.9 mm, 4 µm)
- Mobile phase: hexane:2-propanol (97:3, v:v) at a flow rate of 1 mL/min, 30 min
- Injector temperature: 280 °C
- Volume of sample injected: 60 µL (loop = 20 µL)

Cholesterol identification was made by comparison of its retention time with that of the pure standard.

Quantitation was done by external standardization, using the areas from the refractive index detector, as in Mariutti et al. (2008), ($y = 183186x-4595$; $R^2=0.9948$).

6. SOPs determination

• By gas chromatography

For the model systems, an aliquot of the sample (see section 1.1) was transferred to a tube (corresponding to approximately 2 mg sterol), and added with 19-hydroxycholesterol as internal standard (1 mL of a 20 μg/mL hexane:isopropanol solution).

For the beef patties, previous lipid extraction, cold saponification and extraction was required. Approximately 0.5 g of the previously extracted fat (as reported by Folch et al., 1957) was weighted in a flask containing 10 mL of KOH 1M in methanol and 1 mL 19-hydroxycholesterol (20 μg /mL in hexane:isopropanol 3:2) and kept at room temperature for 20 h. Three extractions with diethyl ether (10 mL) were performed. The whole organic extract was washed with water (3 x 5 mL) and filtered through anhydrous sodium sulphate. Then it was recovered in a round-bottom flask, and the solvent was evaporated under a stream of nitrogen. Purification by NH_2-SPE, derivatization to trimethylsilyl ethers and analysis by GC-MS were performed following the same procedure as in the model system (Rosesallin et al., 1995; Menéndez-Carreño et al., 2008).

For serum samples, a simpler lipid extraction was carried out. Chloroform/methanol 2:1 (9 mL) was added to a tube containing 1 mL of serum and 0.1 mL of 19-hydroxycholesterol (0.02mg/ml in hexane:isopropanol 3:2). Shake for 1 min, centrifuge at 4000 rpm for 15 min and separate in a decantation funnel. Subsequent saponification, extraction, purification, derivatization and analysis were performed as previously detailed for beef patties.

Two different SPE purification procedures were used:

a) As described in detail in Guardiola et al. (1995), the samples diluted in 5 mL of hexane were applied to a SPE silica cartridge, previously equilibrated with 5 mL of hexane. The cartridge was subsequently treated with 10 mL of hexane:diethyl ether (95:5, v/v), 30 mL of hexane:diethyl ether (90:10, v/v), and 10 mL of hexane:diethyl ether (80:20, v/v). Sterol oxidation products were finally eluted from the SPE cartridge with 10 mL of a mixture of acetone/methanol (60:20, v/v). The solvent was evaporated in rotary evaporator under warm water bath (35 °C).
b) As described in detail in Rose-Sallin et al. (1995), samples were diluted in 400 µL hexane:ethyl acetate (95:5, v/v) and transferred to NH_2-SPE cartridge. The cartridge was subsequently treated with 8 mL of hexane: ethyl acetate (95:5, v/v) and 10 mL of hexane: ethyl acetate (90:10, v/v). Sterol oxidation products were finally eluted from the SPE cartridge with 10 mL of acetone/methanol. The solvent was evaporated under a stream of N_2.

The sample solutions of sterol oxidation products were derivatized to trimethylsilyl (TMS) ethers as previously described for sterols, before the chromatographic analysis.

GC-MS (Agilent 6890N-5975):

- Column: VF-5ms CP8947 5% Phenyl Methyl Siloxane (50 m x 250 µm x 0.25 µm)
- Carrier gas: He, 1 mL/min
- Oven temperatures program:
  - 75 °C during 0.5 min
  - Slope 1: 30 °C/min (or 20 °C/min $^{\Phi}$) up to 250 °C
  - Slope 2: 8 °C/min up to 290 °C
  - Slope 3: 0.05 °C/min up to 292 °C
- Injector temperature: 250 °C
- Volume of sample injected: 1 µL, splittless mode
- Transfer line temperature: 280 °C
- Source temperature: 230 °C
- Electron impact: 70 eV
- Quadrupole temperature: 150 °C
- Mass interval: 50.00-600.00 uma
- Detection mode: SCAN and SIM

$^{\Phi}$ the slower slope (20 °C/min) was applied in some experiments

Table 7. Retention times and characteristic ions of SOPs

Compound	t_R (min)	Characteristic ions (m/z)	Abundance (%)
7α-hydroxycholesterol	22.7 (23.6) $^{\phi}$	456 **457*** 458	12.0
19-hydroxycholesterol (is)	22.8 (25.61)	**353** 366	10.5
7α-hydroxycampesterol	(25.73)	470 **471** 472	11.0
7α-hydroxystigmasterol	(26.10)	482 **483** 484	12.0
7β-hydroxycholesterol	23.9 (26.9)	456 457 **458**	3.4
7α-hydroxysitosterol	(27.94)	484 **485** 486	11.0
5,6β-epoxycholesterol	25.4 (28.4)	384 **474** 445 357	2.0
5,6α-epoxycholesterol	25.8 (28.9)	384 474 445 **366**	1.5
7β-hydroxycampesterol	(29.74)	470 471 **472**	3.1
cholestanetriol	27.9 (30.94)	**403** 456 471 546	6.7
7β-hydroxystigmasterol	(29.88)	482 483 **484**	3.5
5β,6β-epoxycampesterol	(31.74)	370 383 398 **488**	1.3
5α,6α-epoxycampesterol	(32.28)	398 **380** 488	11.0
7β-hydroxysitosterol	(32.54)	484 485 **486**	3.0
5β,6β-epoxystigmasterol	(32.59)	253 382 410 **500**	1.5
25-hydroxycholesterol	30.0 (33.1)	131 456 546 **271**	2.7
5α,6α-epoxystigmasterol	(33.13)	253 **392** 410 500	0.8
campestanetriol	(34.84)	**417** 418 470 560	3.6
7-ketocholesterol	31.1 (34.14)	367 416 **472**	9.0
5β,6β-epoxysitosterol	(35.14)	384 394 412 **502**	0.14
stigmastanetriol	(35.62)	**429** 253 482 572	3.0
5α,6α-epoxysitosterol	(35.78)	**394** 397 412 502	0.9
7-ketocampesterol	(38.74)	**486** 381 487 396	4.8
sitostanetriol	(38.75)	**431** 432 484 574	1.4
7-ketostigmasterol	(39.96)	357 359 **498** 347	4.3
7-ketositosterol	(43.70)	395 **500** 510 410	6.9

* Ions in bold denote the ion used for integration

$^{\phi}$ Retention times in parenthesis are those from the method with the slower slope

Peak identification was based on comparison of their mass spectra with those obtained from the literature and, only in the case of COPs, their retention times and MS fragments with those of standard pure compounds.

Total area of each plant sterol was calculated as follows: $total\ A = \frac{A\ of\ ion}{abundance} * 100$

Quantitation was based on an internal standard method (19-hydroxycholesterol). It was performed using selected ion monitoring (SIM) analysis. For each stage of time, different ions were selectively detected and, consequently, extract ion chromatogram was used to integrate the corresponding peak areas.

Table 8. Internal standard calibration curves of COPs

Compound	Calibration curve	R^2
7α-hydroxycholesterol	y = 0.999x - 0.0256	0.9994
7β-hydroxycholesterol	y = 1.2394x - 0.0134	0.9996
5,6β-epoxycholesterol	y = 0.4132x-0.005	0.9982
5,6α-epoxycholesterol	y = 0.4256x+0.0022	0.9976
Cholestanetriol	y =1.4217x-0.0123	0.9996
25-hydroxycholesterol	y = 1.1149x-0.0044	0.9996
7-ketocholesterol	y = 0.2687-0.0133	0.9997

Where: $y = \frac{total\ A\ of\ COP}{A_{is}}$; $x = \frac{mg_{COP}}{mg_{is}}$

Given the lack of available POPs standards and their demonstrated similarity to COPs response, COPs calibration curves were also used to determine POPs content.

• **By HPLC**

For both the mana-cubiu model system and the tuna patties, COPs determination was made following the same procedure as for sterols determination.

Quantification in HPLC-UV-RI was done by external standardization, as in Mariutti et al. (2008).

Table 9. Retention times and detector used for COPs determination

Compound	t_R (min)	Detector
5,6α-epoxycholesterol	9.0	RI
5,6β-epoxycholesterol	10.2	RI
7-ketocholesterol	14.8	UV
7β-hydroxycholesterol	20.7	UV
7α-hydroxycholesterol	21.8	UV

Table 10. External standard calibration curves of COPs

Compound	Calibration curve	R^2
5,6α-epoxycholesterol	y = 97.299x-78.639	0.9988
5,6β-epoxycholesterol	y = 65.814x-99.497	0.9988
7-ketocholesterol	y = 8531.1x-858.1	0.9999
7β-hydroxycholesterol	y = 5876.2x-820.48	0.9999
7α-hydroxycholesterol	y = 4627.6x-1378.3	0.9999

Where: $y = A\ of\ COP \quad ; \quad x = mg\ COP$

The identification of COPs was confirmed by HPLC-APCI-MS/MS using the chromatographic conditions described in detail by Zardetto et al. (2014) and the MS conditions previously optimized by Mariutti et al. (2008).

Table 11. Retention times and mass spectrometry data of COPs identification.

Compound	t_R (min)	$[M+H]^+$ *(m/z)*	Fragment ions *(m/z)*
7α-hydroxycholesterol	5.5	nd	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
7-ketocholesterol	5.7	401	383 $[M+H-18]^+$, 365 $[M+H-18-18]^+$
7β-hydroxycholesterol	5.8	nd	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6β-epoxycholesterol	7.0	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6α-epoxycholesterol	7.6	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$

7. Other oxidation parameters

7.1 TBARS

TBARS values were determined in sunflower oil according to the method described by Poyato et al. (2013). Briefly, an aliquot of sample (corresponding to approximately 0.25 g of oil) was transferred to a tube and the solvent was evaporated under a stream of N_2. Distilled water (0.5 mL), BHT (20 µL, 1%) and the TBARS reagent (2 mL) were added to the sample and vortexed, placed in a boiling water bath for 15 min and then cooled down in an ice bath to room temperature. Cyclohexanone (4 mL) and ammonium sulphate (1 mL, 4M) were added to the mixture and vortexed. The mixture was centrifuged at 1300 g for 10 minutes. The absorbance was measured at 532 nm in a FLUOStar Omega spectrofluorometric analyzer. For beef patties, previous lipid extraction was performed, according to Folch et al (1957), as explained previously. A calibration curve was made with tetraethylpropane as external standard (y = 938.82x+0.0037; R^2=0.9991). Results were expressed in mg of malondialdehyde (MDA) / Kg sample.

7.2 PV

Peroxides Value (PV) was analysed in sunflower oil and FAME model system following the method of Shanta and Decker (1994) with slight modifications. Briefly, an aliquot of sample (corresponding to approximately 10 mg of fat) was transferred to a tube and the solvent was evaporated under a stream of N_2. The residue was dissolved 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. A calibration curve with cumene hydroperoxide was done for quantification (y =5.878x+0.0322; R^2=0.9963). Results were expressed as meq O_2 / Kg sample, being the data the average of 2 measurements per replicate.

7.3 Hexanal content

Hexanal determination was carried out in tuna patties according to Souza et al. (2014). Briefly, an aqueous dilution was performed in 10 g sample, extracted by SPME and injected in a GC-MS (GCMS-QP2010 Ultra Shimadzu).

8. Antioxidant capacity and specific bioactive compounds

8.1 Total phenolic compounds

Total Phenolic Content (TPC) was determined in melisa aqueous extract as described in Poyato et al. (2013). A 12 mg extract sample was solved in 10 mL water. Reagents were mixed: 237 µL distilled water, 3 µL sample solution, 15 µL of Folin-Ciocalteu's reagent, and 45 µL of 20 % sodium carbonate anhydrous solution. After 2 h in the dark, the absorbance was measured at 765 nm in a FLUOStar Omega spectrofluorometric analyzer. For oil samples the procedure was the same but previous phenol extraction was performed, as described in Poyato et al. (2013). A calibration curve with gallic acid was done for quantification ($y = 0.3318x+0.0053$; $R^2=0.9995$). TPC was expressed as µg gallic acid / mg sample (extract or oil).

8.2 ORAC

Antioxidant capacity in the melisa model system was assessed by means of the ORAC method, according to the procedure described in Ou et al. (2001), with slight modifications. An aliquot of sample (corresponding to approximately 0.25 mg cholesterol) was evaporated under a stream of nitrogen. Phosphate buffer (1 mL) and chloroform (300 µL) were added. Then, the samples were vortexed for 20 s and centrifuged at 4000 rpm for 10 min. A total of 0.5 mL of the aqueous layer was taken and kept in the dark until analysis. A 0.5 M stock solution of Trolox was prepared in 10 mM phosphate buffer, and divided into 1 mL aliquots, which were stored at -20 ºC until use. A new set of stock Trolox vials was taken from the freezer daily for the preparation of the calibration curve and the quality controls (12.5 and 50 µM). The phosphate buffer solution was used as blank, to dissolve the Trolox quality controls and to prepare the samples. To conduct the ORAC assay, an aliquot of the sample (40 µL) and 120 µL of the fluorescein solution (132.5 nM) were added to the 96 well black plate. The microplate was equilibrated (5 min, 37 ºC), and then the reaction was initiated by the addition of AAPH (40 µL, 300 mM); readings were obtained immediately, in a FLUOStar Omega spectrofluorometric analyzer. A calibration curve with trolox was done for quantification ($y = 0.7293x+5.4373$; $R^2=0.9923$). The results were expressed as mg trolox equivalent/g sample.

8.3 Rosmarinic acid

Rosmarinic acid content was determinted in the melisa model system. An aliquot of sample (corresponding to approximately 0.1 mg melisa extract) was evaporated under a stream of nitrogen. Ultrapure water (1 mL) and hexane (1 mL) were added. The sample was vortexed for 20 s and centrifuged at 1300 g for 6 min. The upper layer was discarded and the process was repeated two more times. The aqueous layer was filtered through a 0.20 µL membrane filter and analyzed using the chromatographic conditions described in García-Iñiguez de Ciriano et al. (2010b). Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA was used. Briefly, in a C18 column, and at a flow rate of 0.8 mL / min, a gradient of acidified water : acetonitrile was applied (starting at 90:10; changing to 70:30 for 20 min; and returning to 90:10 in 7 min). The profiles were recorded at 280 nm. A calibration curve was done for quantification (y =10000000x; R^2=0.9977). The results were expressed as mg rosmarinic acid / g sample.

8.4 5α-caffeoylquinic acid and other phenolic compounds

The identification and quantification of the phenolic compounds of the mana-cubiu extract (MCE) was carried out according to Rodrigues et al. (2013).

8.5 Vitamin E

The α-tocopherol content was determined in sunflower oil by HPLC-UV analysis according to the method described by Berasategi et al. (2012). Briefly, an aliquot of sample (corresponding to approximately 0.2 g oil) was transferred to a volumetric flask and chloroform was evaporated under a stream of N_2. α-tocopherol acetate (0.1 mL, 10 mg/mL solved in methanol) was added as internal standard and the flask was filled up to 10 mL with previously warmed (30 ºC) supergradient HPLC grade methanol. Dilution was vortexed for 30 sec and filtered with 0.20 µm filter. The sample (10 µL) was injected into the HPLC system and an isocratic elution with methanol/water (97:3) at 1.5 mL/min flow was performed. UV spectra were recorded at 295 nm on a Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA, using an analytical precolumn (3.8 mm x 8 mm with 4 mm x 3 mm of C18 cartridges; *Phenomenex,*) and a LC18 column (150 mm x 3.9 mm, 4 µm; Waters). Identification of α-tocopherol was done using the retention time of the pure standard compound and its characteristic UV spectra. The quantification was performed using an internal calibration curve previously prepared with tocopherol acetate as the internal standard (y = 7.3925x – 0.0261; R^2=0.9932). The results were expressed as mg vitamin E / 100 g sample.

9. Sensory analysis

Triangle analysis was carried out in the experiment in which beef patties were elaborated with varying amounts of melisa extract and olive oil emulsion. Each panellist was presented with three samples, two of which were identical, and asked to indicate which one differed from the others. This process was repeated several times, once for each different concentration of extract tested. The number of correct answers for each type of comparison was determined. According to ISO 4120:2004, for a 9 member-panel, the difference between samples was significant if the number of correct answers was 6 ($p < 0.05$).

10. Statistical analysis

SPSS 15.0, Stata 12 and StatGraphics were used for the statistical analysis. To evaluate the statistical differences between two samples, student-t and Kruskal-Wallis tests were used. To evaluate the statistical differences among several samples, ANOVA and Mann-Whitney U tests were used, with a statistical level of significance of 0.05. Tukey-b and Bonferroni post-hoc comparisons tests were also applied. Pearson and Spearman's coefficients were calculated to determine the correlation between two variables. The mathematical models were adjusted using non-linear regressions.

Results

Results I

Paper 1

A review of analytical methods measuring lipid oxidation status in foods: a challenging task

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A review of analytical methods measuring lipid oxidation status in foods: a challenging task

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Abstract

Lipid oxidation analysis in food samples is a relevant topic since compounds generated in the process are related to undesirable sensory and biological effects. As the process is complex and depends on the type of lipid substrate, oxidation agents and environmental factors, proper measurement of lipid oxidation remains a challenging task. A great variety of methodologies have been developed and implemented so far, both for determining primary oxidation products and secondary oxidation products. Most common methods and classical procedures are described, including peroxide value, TBARS analysis and chromatography. Some other methodologies such as chemiluminescence, fluorescence emission, Raman spectroscopy, infrared spectroscopy or magnetic resonance, provide interesting and promising results, so attention must be paid to these alternative techniques in the area of food lipid oxidation analysis.

Keywords: Fat oxidation; Hydroperoxides; Secondary lipid oxidation products; TBA; Hexanal

1. Introduction

Lipid oxidation in foods constitute a complex chain of reactions that firstly yields primary products (peroxides), that, when exposed to extended oxidation conditions, give rise to secondary oxidation products, including aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. Most of them produce undesirable sensory and biological effects (Márquez-Ruiz et al., 2007; Kanner, 2007). Therefore, its control is of great importance.

Lipid oxidation occurs via different pathways: radical mechanism (known as autoxidation), singlet oxygen mediated mechanism (known as photooxidation) and also the enzymatic oxidation has been described, catalyzed by lipoxigenases. This review will be focused on the non-enzymatic routes. Both autoxidation and photoxidation give rise to identical or similar peroxides, differing just sometimes in position and stereoisomerism. The first mechanism requires an initial activation energy for the removal of a hydrogen atom, so it is enhanced by high temperatures and presence of double bonds. The latter is triggered by the highly reactive singlet oxygen specie, which is formed by excitation of triplet molecular oxygen, under light exposure and presence of photosensitizers (Choe and Min, 2006; Min and Boff, 2002).

The first compounds formed during oxidation process are peroxides, especially hydroperoxides; hence they are called primary oxidation products. Despite being intermediate compounds of lipid oxidation process, they are relatively stable (depending on the lipid structure), and can be used to assess lipid oxidation status in food samples, providing not too advanced autoxidation is developed in the sample. Because of this intermediate characteristic, temperature conditions during analysis must be controlled to avoid hydroperoxide decomposition, and addition of antioxidant is often required.

Hydroperoxides usually suffer further oxidation to give secondary oxidation products. Silvagni et al. (2010) proposed an alternative kinetic model where the aldehydes are generated not only via direct degradation of hydroperoxides but from peroxyl radicals through an independent pathway. This mechanism involves a bimolecular reaction to form intermediate tetraoxides, which are unstable at high temperatures and decompose to give alkoxyl radicals.

The wide variety of secondary oxidation products to which oxidation gives rise includes aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. Among them, both volatile and non-volatile compounds can be found, such as hexanal or malondialdehyde (MDA), respectively, as main representatives.

Evaluating lipid oxidation status is a challenging task due to a number of reasons. Firstly, different compounds are formed depending on the time, extent of oxidation and mechanism involved. Therefore, choosing just one parameter to analyse the oxidative status is rather difficult and it is frequently more convenient to combine different methods. Besides, as stated by Eymard et al. (2009), not only nature and composition of lipid as the substrate of the reaction have an impact on lipid oxidation process, but also type and concentration of proteins, antioxidants and prooxidants present in the food matrix, as well as its physicochemical characteristics. In meat samples, Richards and Dettmann (2003) suggested that rates of lipid oxidation may depend on the relative ability of haemoglobins from different animal species to promote it. Chen et al. (2010) proposed that colloidal structures formed by phospholipids in vegetable oils could have an impact on the oxidative stability of food oils. Lipid oxidation was observed to be delayed in fish sausages after the addition of several antioxidants (Maqsood et al., 2012). Milk samples oxidation has been recently studied in the presence of catechins and ascorbic acid (Mun, 2011). On the other hand, each method allows a number of different experimental conditions, and this, together with the lack of uniformity among laboratories, leads to (at least for the moment unavoidable) dissimilar results. Finally, most of the oxidation compounds are prone to be further degraded, which provides an added source of divergence. Therefore, a precise control of the experimental procedure must be kept.

Related to lipid oxidation in food samples, other assessments can be also performed. On the one hand, determination of parameters highly indicative of lipid deterioration and subsequent enhanced susceptibility to oxidation (such as hydrolysis of triglycerides) is very common. On the other hand, measuring the time required by a sample to achieve a certain oxidative level through artificially promoting oxidation is another valid procedure to evaluate lipid susceptibility to oxidation (and/or oxidation stability). However, this review will only focus on methods determining the actual and current lipid oxidation of a sample, discarding procedures assessing hydrolytic status and those involving induction of oxidative degradation, since they are not properly indicators of oxidation status but of oxidative susceptibility and stability, respectively.

This review will describe traditional methods to determine both primary and secondary lipid oxidation products in foods, from spectroscopic to chromatographic techniques. Their characteristics, advantages and limitations will be pointed out. Then, alternative methodologies developed during last decades will also be revised in order to provide the

complete oversight of possible options. Table 1 summarizes the main characteristics of the methods described in this review.

2. Primary oxidation products

2.1 Peroxides

Hydroperoxides redox properties are the base of some of the key methods applied in their determination. A number of reagents can be oxidized by hydroperoxides, including simple inorganic ions, such as iodide or ferrous ion. These methods usually require subsequent complexation to improve the sensitivity.

2.1.1 Volumetric method

Among the different methods proposed for the analysis of peroxides, the iodometry has been the most conventional and widespread method mainly due to the simplicity of the experimental procedure. Although the procedure requires prior lipid extraction, rapid and easily understandable results are provided.

In acidic medium, hydroperoxides and other peroxides react with the iodide ion to generate iodine, which is tittered using a sodium thiosulfate solution, in the presence of starch. The AOAC offers an official method since 1965 (AOAC, 2000). According to this method, Peroxide Value (PV) is considered to represent the quantity of active oxygen (in meq) contained in 1 kg of lipid and which could oxidize potassium iodide.

It shows however some drawbacks, mainly derived from the iodide high susceptibility to oxidation in the presence of molecular oxygen and accelerated by light exposure. Also spontaneous hydroperoxide formation can occur (which would lead to overestimation) and absorption of iodine by unsaturated fatty acids (which would lead to underestimation) (Sun et al., 2011). Moreover, it requires anhydrous systems to avoid interference problems, for what lipid extraction is required, and this procedure stage increases the contact with oxygen. In addition, the Peroxide Value determination does not give a real measure of the oxidative degradation, since peroxides are usually further degraded, so simultaneous measurement of secondary products would be appropriate.

2.1.2 VIS-UV spectroscopic methods

As well as the volumetric method, spectroscopic ones are rather simple and are moderately sensitive, reliable, and reproducible when carried out under standardized conditions. However, they are highly empirical as they measure complex mixtures of oxidized molecules. In addition, they are generally work-intensive and use large amounts of solvents and reagents that might be hazardous (Kamal-Eldin and Min, 2010).

Ferrous oxidation method

The ferrous oxidation method for determination of peroxide content is simpler to use than iodometry. The main reason is the lower sensitivity of ferrous ion to spontaneous oxidation by oxygen in air, as compared to high susceptibility to oxidation of iodide solutions. It consists of oxidation of Fe(II) to Fe(III), mediated by hydroperoxide reduction in acidic conditions and in the presence of thiocyanate or xylenol orange (in this later case, method is known as FOX). These two compounds provide the spectrophotometric properties, as they form complexes with the ferric ion, giving maximum absorbance peaks at 500 nm and 560 nm respectively, which can be measured with a UV-Vis Spectrophotometer (Eymard et al., 2009; Shantha and Decker, 1994; Bou et al., 2008; Verardo et al., 2009; Chotimarkon et al., 2009; Sorensen et al., 2010). However, neither of the methods is free from complications (Nielsen et al., 2003). The thiocyanate method requires large amounts of solvent, and as for the FOX, it detects in a small range of peroxides concentrations and molar absorptivity of the ferrilxylenol orange complex varies with different procedures of making the dye. Nuchi et al. (2009) concluded that FOX results (from degradation of fat for feed uses) correlated better with other oxidation parameters than traditional iodometry.

Iodide oxidation method

A spectrophotometric iodide-dependant method has also been set to determine hydroperoxide content. In this methodology, not so commonly used (Watanabe et al., 2010), the lipid sample is placed in an acidic solution, which is then merged with iodide. The lipid hydroperoxide oxidises iodide to iodine. Then, generated iodine and iodide (in excess) react to give triiodide anion, which is detected spectrophotometrically at 350 nm. Bloomfield (1999) used Fe (II) as a catalyst. The closed conditions prevent interference from atmospheric oxygen and the short reaction time minimises interference from side reactions.

2.1.3 Chromatography

Methodologies explained up to here are in general quite simple regarding theory base, implementation of the procedure and ulterior interpretation of the data, presenting low to moderate selectivity and sensitivity, though. On the other hand, chromatographic techniques are far more accurate, sensible and specific for the compound in interest, allowing better identification of individual products. Indeed, their implementation for hydroperoxides determination instead of that of volumetric and spectroscopic measurements is growing up more and more over the last years. As an unavoidable consequence, chromatographic methods usually require long or meticulous experimental work, precise control of the experimental conditions and the data processing presents some complexity.

Liquid chromatography

High Performance Liquid Chromatography (HPLC) is being recently used to determine hydroperoxides. This method is highly sensitive and pretty versatile considering both column and detector properties, allowing to analyze compounds with different characteristics of volatility, molecular weight or polarity. On the other hand, sample preparation is frequently tedious and usually requires lipid extraction. Zeb and Murkovic (2010) found the isocratic HPLC-ESI-MS a useful method for the identification and characterization of oxidized species of triacylglycerols (TAGs), i.e. mono- and bis-hydroperoxides. Gotoh et al. (2011) developed a method for measuring the peroxide value in colored lipids on the basis of the reaction with triphenylphosphine, forming a compound which absorbs at 260nm. Sample then underwent HPLC separation and UV detection. Ferrous oxidation mediated methods have also been adapted to HPLC separation (Sugino, 1999). Specific hydroperoxides generated from sterols can also be assessed by liquid chromatography. Saynajoki et al. (2003) determined stigmasterol hydroperoxides by means of a normal-phase column and two types of detectors (UV and fluorescence).

Gas chromatography

Gas chromatography coupled to mass spectrometry (GC-MS) can also be used for the analysis of lipid hydroperoxides, but due to their thermo-lability, previous reduction is needed. This, along with the prior lipid extraction and subsequent derivatization step, makes it a cumbersome and time consuming method (Lagarda et al., 2003).

2.2 Conjugated dienes/trienes

Hydroperoxide formation from polyunsaturated fatty acids is generally (over 90% of the cases) accompanied by stabilization of the radical state via double-bond rearrangement (electron delocalization), which gives rise to conjugated dienes and trienes. These relatively stable compounds absorb in the UV range (235 nm and 270 nm respectively) and this absorption can be measured by spectrophotometric techniques to assess oxidation level (Laguerre et al., 2007; Shahidi and Zhong, 2005). This technique is simple and rapid but not as widespread as determination of peroxides determinations, probably because it can lead to underestimation since oleic acid hydroperoxides, containing less than two double bonds, cannot be detected. On the other hand, overestimation is possible if conjugated double bonds are present in the original fatty acid. Furthermore, it is not suitable for oils that have been heated under conditions that decompose hydroperoxides because interference may occur with absorption of carbonyl compounds (Frankel, 1998). Even so, a number of studies have used them for the monitoring of lipid oxidation during heating treatments, especially in vegetable oils (Maggio et al., 2011; Karoui et al., 2011; Morales et al., 2003). Correlation between 235nm absorption values and peroxide values has been reported (Wanasundura et al., 1995).

3. Secondary Oxidation Products

Lipid primary oxidation products can generate, if submitted to further oxidation conditions, secondary oxidation products, including aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. These compounds show a wide variety of physico-chemical properties, differing mainly in volatility, polarity and molecular weight. Most relevant groups of compounds will be commented (aldehydes, volatiles and polymers), as well as a particular molecule very frequently used as oxidation marker (malondialdehyde).

3.1 Malondialdehyde

Malondialdehyde (MDA) is one of the most abundantly generated aldehydes during secondary lipid oxidation and it is probably the most commonly used as oxidation marker, too.

3.1.1 UV-Vis Spectroscopy

The most widely employed method for determination of MDA is the spectrophotometric determination of the red fluorescent MDA-thiobarbituric acid (MDA-TBA) complex.

Reaction occurs by attack of the monoenolic form of MDA on the active methylene groups of TBA, at low pH and high temperature, giving the mentioned chromophore which offers a maximum absorbance peak at 532nm. Reaction kinetics depends on the concentration of TBA

solution, temperature and pH (Fernández et al., 1997). Several variations of MDA-TBA method exist, with different procedures currently performed in food analysis: direct heating of the sample, sample distillation, lipid extraction with organic solvents or aqueous acid extraction, followed by acid reaction with TBA. General procedure usually consists of homogenization and centrifugation at acidic medium (usually provided by trichloroacetic acid) and posterior reaction with TBA at high temperatures (around 90-100 °C). Nevertheless, there is quite a lot of variability in reaction conditions, such as heat treatment exposure time; to illustrate it: Berasategi et al., Peiretti et al., Jung et al. and Jongberg et al. (2012; 2011; 2011; 2011) left mixture react at boiling water bath for 15, 20, 30 and 40 minutes, respectively. On the other hand, trichloroacetic solution concentrations have also been reported to be different (from 3% to 15% w/v) among works (Maqsood et al., 2012; Leygonie et al., 2011).

Traditional spectrophotometric TBA test has been criticised for some reasons. Firstly, TBA is not selective to MDA, since it also reacts with many other compounds, such as other aldehydes, carbohydrates, amino acids and nucleic acids (Salih et al., 1987), interfering in the TBA assay and resulting in considerable overestimation, as well as variability in the results. This is why it is also known as TBA reactive substances method (TBARS). There is also a risk of underestimating the response since malondialdehyde can, under *in vivo* conditions, form linear or cyclical Schiff bases, or even crosslinked bonds, with lysine and arginine from proteins. So poor quantification sensitivity and poor molecular specificity and selectivity can be attributed to this method. Furthermore, the high temperatures (95–100 °C), extended incubation times and strong acidic conditions commonly required for the reaction of MDA with TBA may cause an artifactual peroxidation of sample constituents even in the presence of added antioxidants. Note finally that malondialdehyde, which is mainly formed from linolenic acid oxidation, does not occur in other oxidized lipids (especially when only one double bond is present, i.e., oleic acid). So, it is often a minor secondary oxidation product, spoiling the role of lipid oxidation marker role usually assumed for this compound.

Despite the mentioned limitations, conventional spectrophotometric MDA-TBA methods are preferred because of their simplicity. In fact, it has been recently suggested as a more accurate and sensitive parameter in assessment of oxidative deterioration than p-anisidine test and hexanal determination (Nuchi et al., 2009; Pignoli et al., 2009).

3.1.2 Chromatography

To overcome some of these limitations, more advanced chromatographic determinations have been developed. These techniques provide, as in the case of hydroperoxides measurement

(section 2.1.3) more accuracy, sensitivity and specificity for MDA. Harder experimental work, and certain level of complexity in data processing are the drawbacks.

Some of them (Stalikas and Konidari, 2001; Jardine et al., 2002; de las Heras et al., 2003; Cesa, 2004; Seljeskog et al., 2006; Mendes et al., 2009) involve the formation of MDA-TBA complex, purification by chromatography (GC or HPLC) and subsequent detection by MS, UV-Vis or fluorometric detector. And some others use derivatization of MDA instead of reaction with TBA, in order to obtain a detectable compound. Reaction with 2,4-dinitrophenylhydrazine (DNPH) or pentafluorophenylhydrazine and conversion into pyrazole and hydrazone derivatives are the most commonly used procedures with HPLC separation and spectrophotometric/fluorometric detection (Mendes et al., 2009; Mateos et al., 2005; Ichinose et al., 1989). On the other hand, conversion into tetramethylacetal or methylpyrazole is more common with GC separation, with Flame Ionization Detector (FID) or Nitrogen/Phosphorus specific detector (Ichinose et al., 1989). Mendes et al. (2009) and Marcincak et al. (2006) compared two HPLC separation methods for MDA determination (MDA-TBA and MDA-DNPH adduct) with the traditional spectrophotometric MDA-TBA test, in samples of chilled fish and pork. The methods were fast, simple, sensitive and stable and presented overall better performance (based on accuracy, specificity and recovery levels) than the traditional spectrophotometric MDA-TBA test, although MDA-DNPH showed a relatively high limit of detection and a lower reproducibility at lower MDA contents in standards and samples.

3.2 Other secondary oxidation compounds

3.2.1 UV-Vis Spectroscopy

A number of other aldehydes apart from MDA are generated during lipid secondary oxidation. The spectroscopic method used the p-anisidine value (PAV) to detect their presence even when it is one of the oldest methods for evaluating secondary lipid oxidation, especially in the analysis of animal fats and vegetable oils. It provides useful information on carbonyl compounds, especially non-volatile α-unsaturated aldehydes (such as 2-alkenals and 2,4-dienals) because it is based on the reactivity of the aldehyde carbonyl bond on the p-anisidine amine group, leading to the formation of a Schiff base that absorbs at 350 nm. The p-anisidine value is defined as 100 times the absorbance of a solution containing 1 g of fat in 100 mL of solvent. It is considered a very simple and rapid methodology. PV and PAV allow calculating total oxidation. This parameter (total oxidation) combines evidence about the past history and present state of an oil, so it allows to estimate the overall extent of oxidation in the food (Sun et al., 2011).

PAV has been recommended as a good control parameter for secondary oxidation control since it correlates well with peroxides content (FOX and PV), TBA and volatile aldehydes analysis (Nuchi et al., 2009; Tompkins and Perkins, 1999). In the research field, it has remained a little backward, in favour of other techniques (Poulli et al., 2009).

It is well known that the colorimetric response with p-anisidine varies according to the extent of aldehyde unsaturation. Hence, at identical concentrations, the response is more intense with di-unsaturated aldehydes than with mono-unsaturated aldehydes, which in turn are more sensitive than saturated aldehydes. Moreover, p-anisidine reacts with all aldehydes, irrespective of their origin. This is especially the case for some phenol compounds of virgin olive oil, such as decarboxymethyloleuropeine dialdehyde, which could interfere in the assessment. Finally, studies on correlations between PAV and the organoleptic quality highlighted the efficacy of this test for measuring oxidation in many different lipids. However, these correlations may vary markedly between lipids and also according to the prevailing oxidation conditions. Caution is thus required when interpreting this index (Laguerre, 2007).

3.2.2 Chromatography

A number of other compounds apart from carbonyls are generated during lipid secondary oxidation.

Concerning fatty acids, they can suffer oxidation as free form, within triacylglycerols or bonded to phospholipids). Their secondary oxidation products can be assessed by HPLC (Rovellini and Cortesi, 2004). However, while this technique may be useful to obtain a fingerprint of the oxidation status of the sample, only a minority of signals can be attributed unequivocally to a specific compound because separation is not good enough. Better quantitative analysis can be carried out by means of GC-FID and GC-MS after derivatization into methyl esters (Aguirre et al., 2010). Development of LDI-TOF-MS and ESI-MS (Schiller et al., 2002; Calvano et al., 2005; Simas et al., 2010) has meant a great step forward in this field.

Even though Sterol Oxidation Products (generally known as SOPs) present low levels in foods, they show a number of harmful effects in the organism (Otaegui-Arrazola et al., 2010), so a significant number of studies have focused their attention in their analysis. Experimental procedure involves lipid extraction, saponification, purification, derivatization and chromatographic analysis. That determination is challenging in many ways: artifact generation, very low concentrations, matrix effects, incomplete identification and reporting, to note a few (Guardiola et al., 2004; Busch and King, 2009). GC-MS is the most accurate and commonly applied quantification method for this kind of compounds (Johnsson and Dutta, 2006;

Menéndez-Carreño et al., 2008b; Ubhayasekera and Dutta, 2009; Derewiaka and Obiedzinski, 2010; Xu et al., 2011). Clariana et al. (2011) found this technique better than GC-FID in a study performed with pork meat. Due to the necessity of a derivatization process and the impossibility of analysing thermolabile molecules, some liquid chromatography methods have been recently developed (Kemmo et al., 2008; Mazalli and Bragagnolo, 2009; Matsunaga et al., 2009). However, liquid chromatography shows lower resolution than gas chromatography, and the best way to overcome this problem is coupling it to a mass spectrometer detector, which in this case is quite complex and still has not been well solved. A new fast GC-MS method has been recently developed and applied to cholesterol oxidation products analysis, giving highly promising results (Cardenia et al., 2012). Satisfactory resolution, good repeatability and sensitivity, together with the consequent reduction of the time of analysis and consumables make it a valid alternative to conventional GC-MS.

3.3 Volatiles

Under this group of secondary oxidation products a great diversity of compounds has been included, presenting very different functional groups: aldehydes, ketones, alcohols, short carboxylic acids and hydrocarbons. They all share the property of giving from moderate to high smells and are related to rancidity in sensorial tests. Measurement of these secondary oxidation products is of great importance, since their formation closely relates to the deterioration of flavour. Some of these volatile compounds are highly specific to the oxidative degradation of a particular polyunsaturated fatty acid family: propanal is the main marker of oxidation of n-3 fatty acids, while hexanal and pentanal are markers of oxidation of n-6 fatty acids. Both propanal and hexanal are often used as indicators of lipid oxidation in foods because they can be measured in the sample headspace and their lack of double bonds makes them more stable towards oxidation than unsaturated aldehydes. Nevertheless, hexanal is more frequently measured as its formation is higher than that of most secondary oxidation products, apart from a few exceptions. However, measuring the extent of oxidation with just one or two markers is a rather coarse approach, so methods involving assessment of large set of compounds should be promoted (Laguerre, 2007).

Gas chromatography is the preferred method to quantify volatile molecules and mass spectrometry detection contributes to identify them. Different methods may be used to recover volatile oxidation compounds before chromatographic analysis, including: (a) solvent extraction and (b) headspace (HS) techniques.

(a) Although liquid-liquid extractions are not very suitable to recover the volatile content (because they are long, laborious and require a solvent evaporation step, which leads to substantial volatile compound degradation), novel variants have been recently proposed to overcome some of these limitations. Note especially simultaneous distillation extraction (SDE) and reduced pressure steam distillation extraction (RPDE). Both allow to obtain compounds of relatively high boiling point, but with RPDE evaporation is reached with lower temperatures, avoiding possible artefact formation (Varlet et al., 2007). SDE and RPDE show the advantage of being able to extract high quantities of target compounds since the volatile fractions generally have high solubility in organic solvents (Liu et al., 2010; Ning et al., 2011). Moreover, Ferhat et al. (2007) developed a microwave energy-mediated extraction method. Liquid-liquid extractions are the preferred recovering methods whenever the samples require derivatization step previous to chromatographic analysis (HPLC and GC). DNPH, benzyloxime and thiazolidine derivatives are the most frequently used compounds to improve stability and/or detection by visible-ultraviolet spectrometry, flame-ionization, nitrogen-phosphorous and mass spectrometry detection (Varlet et al., 2007b).

(b) HS analysis can be performed by static headspace (SHS), dynamic purge-and-trap headspace (DHS) or headspace-solid phase microextraction (HS-SPME) techniques. All of them are prior to gas chromatography analysis.

In SHS method, the sample is placed in an airtight vial. Most compounds that are volatile at the analysis temperature evaporate from the liquid or solid fraction and pass into the overhead gas HS. At equilibrium, an aliquot is harvested and injected on the GC column. This method is relatively inexpensive and easy to use, it does not require solvent extraction and can be automated. However, as equilibrium is established between the volatile compounds in the HS and those remaining in the sample, only low quantities of compounds are actually recovered, which limits the sensitivity. The increase in the extraction temperature could increase the volatilization of the target compounds and thus increase the quantities recovered, but the temperature must be kept as low as possible in order to minimize generation of new oxidation products and/or thermal degradation of oxidation markers. A number of authors (Joaquin et al., 2008; Vieira et al., 2012) have applied this method in food samples analysis.

On the contrary, DHS technique does not require the establishment of equilibrium: the sample is continually purged by inert gas to extract volatile compounds. Then, the gas effluent passes through a porous polymer trap that collects volatile analytes. Among all available trap materials, tenax is the most commonly used. As volatiles contained in the sample are

constantly released and trapped, a high concentration of compounds are injected on the GC column. Despite its high sensitivity, the instrumentation is complex and expensive, thus increasing the sources of error (trap drying, trap transfer, purging efficiency, etc.) and it is in general terms slower than SHS. Nevertheless, several studies have highlighted the efficacy of DHS-GC in assessing the oxidative status of different food matrix (Nielsen and Jacobsen, 2009; Haar and Jacobsen, 2008).

In SPME analysis, volatile compounds make a first equilibrium between sample and HS, followed by a second one between the HS and the contact fibre (which is coated with a highly adsorbant polymeric film). Finally, the fibre is introduced in the GC injector. This method provides many advantages over other ones, including easy manipulation and experimental set up, short sampling times, easy automation and high sensitivity (Iglesias et al., 2007). A number of authors have applied this method for food lipid oxidation determinations (Haar and Jacobsen, 2008; Iglesias and Medina, 2008). Its main drawback is that fibre degradation and contamination occurs quite rapidly, thus replacement is required periodically.

Recent comparative studies performed with all these methods for capture of volatile content lead to the conclusion that each one presents its shortcomings and advantages (Shu et al., 2010; Prosen et al., 2010), but HS-SPME is being used to an increasing extent on account of its most promising results.

3.4 Oligomers/Polymers

During extended oxidation, a lipidic compound can be linked together with other one or several ones, giving rise to dimers, oligomers or polymers. Simultaneous analysis of oxidized forms of triacylglycerols and their oligo/polymers is very common to assess lipid oxidation progress. Monomers are very reactive and highly correlate with peroxide value, so they could give information about the primary oxidation level of a sample. On the contrary, triacylglycerols oligopolymers are rather stable compounds, being considered as good indicators of secondary oxidation status (Bilancia et al., 2007; Gomes et al., 2012).

High Performance Size Exclusion Chromatography (HPSEC) has demonstrated to provide satisfactory results in the analysis of this kind of oxidation products. It allows separation and subsequent identification and quantification of molecules according to their molecular weight. It is usually performed on polar compounds, so it requires a previous purification of the polar lipid fraction, which is usually done by silica gel column chromatography. Some studies (Marquez-Ruiz et al., 2007; Summo et al., 2010; Caponio et al., 2011) have demonstrated the usefulness of HPSEC in the determination of the levels of the oxidative degradation of a variety

of food samples, and particularly that of refined vegetable oils, whose technological process involves quality deterioration. Morales et al. (2010) applied it for the determination of advanced oxidation in vegetable oils through the detection of fatty acids polymers. Oligomers formation during thermo-oxidation of phytosterols has also been reported (Struijs et al., 2010; Menéndez-Carreño et al., 2010; Rudzinska et al., 2009; Rudzinska et al., 2010) by means of HPSEC analysis.

4. Alternative methodologies

The previous techniques are either too empirical or highly dependant on several experimental factors, such as technician skill, light exposure and atmospheric oxygen, apart from the fact of being time-consuming. To avoid these limitations, various methodologies have been proposed as good alternatives in analysis of both primary and secondary oxidation products. They are based on direct spectroscopic analyses of samples, such as magnetic resonance, fluorescence and vibrational spectroscopy, and on chemiluminescent properties. As general good points, preliminary treatment is minimal or unnecessary, low amount of sample is required and highly specific results are obtained.

4.1 Chemiluminescence

Certain chemcal reactions generate electromagnetic radiation. This emission of energy is known as chemiluminescence (CL) and it can be applied to detect and quantify compounds of interest. However, light intensity is very low (ultraweak CL is accompanied during oxidation of hydrocarbons and lipids (Navas and Jimenez, 1996)), so light amplifiers should be introduced to increase it. One of the most commonly used one is the luminol. The luminol-enhanced chemiluminescence involves oxidation of luminol in basic solution generating a free radical intermediate which reacts with flux of oxidizing agents (active free radicals) present in the system, e.g. lipid hydroperoxides. This leads to formation of luminol derived product in excited state, which eventually returns to ground state emitting strong blue light at 430 nm (Roginsky and Lissi, 2005). Different versions of this method differ in the type of active free radical produced and the way of free radical production as well as in details of the procedure. Robinson et al. (1997) suggested the addition of *p*-iodophenol to provide more intensive, prolonged, and stable light emission as compared to the traditional luminol system. More recently, a new chemiluminescence method in non aqueous medium CL was developed to detect lipid peroxides in vegetable oils (Szterk and Lewicki, 2010), presenting good correlation with spectrophotometric PV analysis.

Baj et al. (2009) discovered that partial exclusion of oxygen from the reaction medium strongly influenced the light intensity of the luminol reaction, and the effect is dependent on the oxidant analyzed, so an alternative mechanism was suggested for some oxidant species. Besides, they stated that the oxygen concentration always affects the reproducibility of the results, so equilibrating the working solutions with oxygen or air should always lead to improved results.

The attractive features of CL methods are their higher quickness (taking only a few minutes), sensitivity (picomol levels have been assessed), low sample requirements, low cost and simplicity as compared with other methods (Rolewski et al., 2009). As for shortcomings of this kind of methods, first of all, the kinetic theory and mechanism for chemical processes resulting in CL is not known in detail. This may mean problems with data interpretation. Furthermore, this method is not specific to the lipids (other oxidizing agents also give signal); but this opportunity can be seized to estimate the overall total oxidant status of the sample.

Bunting and Gray (2003) developed an automated flow injection chemiluminescence system for measuring lipid hydroperoxide concentrations in oils and found good agreement with a traditional iodometric titration assay, what could denote the usefulness of CL methods to assess lipid primary oxidation; and also in vegetable oils, Yang et al. (2010) found a similar trend for TBARs and CL measurements during oxidation.

4.2 Fluorescence spectroscopy

When a compound is irradiated with an electromagnetic energy source, some of their electrons promote from their fundamental state to an excited one, and subsequently they return to their original state, re-emitting the energy previously absorbed. Nevertheless, certain compounds can lose some of that energy as heat, what allows their electrons to return to a higher level than the original one, so emitted light is in this case lower than the absorbed one. This phenomenon is named as fluorescence, and compounds presenting this property, fluorescents. Beam of light is usually from the UV range and emitted energy is typically, but not necessarily, from the visible range. It can be used in analytical chemistry for both qualitative and quantitative determinations, as well as in isolated and coupled to chromatography equipments.

Regarding food field, its implementation is growing up more and more (Karoui and Blecker, 2011). The free amino groups of proteins can react with aldehydes from lipid peroxidation or reducing sugars to give Schiff bases. These compounds present a high colour intensity (browning) and characteristic fluorescence spectra (excitation and emission wavelengths, and

fluorescence intensity) according to the type of protein and adduct. Although its sensitivity is high, excitation and emission wavelength maxima vary depending on the food sample and the procedure followed. They range from 250 nm to 500 nm for excitation, and from 280 nm to 600 nm for emission (Poulli et al., 2009: Tironi et al., 2009; Elmnasser et al., 2008; Gatellier et al., 2009). Many authors have used the ability of these Schiff bases to emit fluorescence to monitor thermal oxidative processes, especially in dairy products (Schamberger and Labuza, 2007; Dalsgaards et al., 2011), meat (Gatellier et al., 2007; Chelh et al., 2007), fish (Naseri et al., 2011; Nguyen et al., 2012) and oils (Barrett et al., 2011), but fluorescence methodologies are still poorly documented in food lipid oxidation analysis. Both Gatellier et al. (2009) and Nguyen et al. (2012) found a high correlation between fluorescent pigments and TBARS of meat and fish products, which demonstrated that the interaction between proteins and aldehyde products of lipid oxidation is mainly involved in the production of fluorescent pigments and these are good markers of lipid oxidation.

A different implementation of fluorescent properties was developed by Andersen et al. (2008) with a cheese sample. They measured the fluorescence of the photosensitizers involved in the lipid oxidation mechanism of the cheese and used the spectra to successfully predict the content of volatile compounds.

4.3 Infrared spectroscopy

Infrared (IR) spectroscopy is also known as a very helpful way to study lipid degradation under oxidative conditions (Kong and Singh, 2011), particularly since it is an easy, rapid, economical and non-destructive technology. It is based on the determination of fundamental vibrational transitions of a particular compound and involves the absorption of discrete energy levels from the IR region. These discrete energy levels are characteristic of each of atom-atom linkage, so studying the IR spectrum can provide enough information to find out the nature of the analyzed compound. Mathematical tools, such as Fourier Transform (FT) or chemometric methods, permit data processing. Continuous ageing monitoring can be carried out with this methodology, although for the moment, most of the works have been assessed in discontinuous way. Some advances have recently been performed regarding technological devices (García-González and van de Voort, 2009).

IR has been applied to measure the peroxide value in oxidized lipids (Guillén et al., 2007) and differences were found in the IR spectra of fresh and aged oils (Christy et al., 2003; Rusak et al., 2003); so IR spectra can be used to characterize the aging of various edible oils (Yang et al., 2005; Muik et al., 2007; le Dreau et al., 2009; Wang et al., 2011; Beltrán et al., 2011). The

investigation of the FTIR spectra of the treated oils revealed that the microwave heating of oils (Moharam and Abbas, 2010) caused significant changes in the intensities of their absorption bands and produced no shifts in the position of the bands. These changes were attributed to the reduction in 18:2 and 18:3 fatty acids content due to the oxidation.

It has also been used for the analysis of edible oils (Belhaj et al., 2010), horse mackerel patties (Giménez et al., 2011) and canned tomato juice (Rubio et al., 2010), in combination with other analytical methods which lead to similar conclusions, and therefore providing marker bands to improve the understanding of chemical changes taking place during processing and storage.

4.4 Raman spectroscopy

Raman spectroscopy also detects fundamental vibrational transitions although (contrary to infrared spectroscopy) not by means of direct energy absorption, but through an energy (originated from a UV, visible or IR laser) scattering: promotion to a virtual vibrational state and subsequent relaxation to a fundamental vibrational state different from the original one. Therefore, Raman and IR spectroscopy are complementary techniques and provide complementary structural information about molecules. Actually, only some molecules show Raman scattering properties, and most of them at a very small intensity, so quite sophisticated and expensive optical detection equipments are required. This reduces its practical use to a few cases. Indeed, it is still very sparingly used in the food field, in spite of its interesting characteristics, which include being non-destructive, fast, relatively inexpensive, non-involving chemical products, requiring very little sample preparation, being highly sensitive to unsaturations and poorly sensitive to water (Reid et al., 2003; Herrero, 2008). Two instrumental methods can be employed with Raman spectroscopy: confocal Raman spectroscopy with a powerful laser in visible range and Fourier Transform Raman spectroscopy. Most of the applications on oils have been performed by the later (Korifi et al., 2011). However, a portable Raman spectrometer has been recently developed (Guzmán et al., 2011), which, on the other hand, shows lower resolution than classic ones. Zhang et al. (2010) reported the first proof-of-concept study of surface-enhanced Raman detection of a TBA-MDA adduct using silver nanoparticles as the SERS substrate

Raman spectroscopy results and oxidation levels were related in lipids extracted from several meat and fish products (Herrero, 2008; Sarkardei and Howell, 2007). In line with peroxide values rises, Raman spectra data showed an increase in particular bands and regions of the spectra of oils extracted which could be attributed to alterations in lipids structure. Furthermore, Raman spectroscopy could be an alternative to gas chromatographic fatty acids

analysis, since it successfully predicted total unsaturation and individual compounds several meat products (Beattie et al., 2006). Salmon Raman spectra (Herrero et al., 2009) indicated differences in the fat fraction (as well as in protein fraction) in cold-smoked products. Regarding vegetable oils studies, Muik et al. (2005) detected formation of aldehydes and conjugated double bond systems, as well as isomerization of *cis* to *trans* double bonds. The time dependent intensity changes in certain Raman bands were compared to conventional parameters used to determine the extent of oxidation in oils, such as anisidine value and K_{270}, and showed good correlation. El-Abassy et al. (2009) assessed fatty acid content in olive oil. Zhang et al. (2010) developed a method to determine MDA in a model system by means of this technique. They found that it was selective and specific for MDA-TBA adducts- in terms of differential spectra and high response- versus adducts formed by TBA and other TBARS different from MDA. Besides, they achieved better sensitivity than in works using UV-Vis or fluorescence detectors. Sometimes, reduction of carotenoids content measured by Raman spectroscopy has been used to monitor lipid oxidation process (Kathrivel et al., 2008).

Simultaneous analysis of the oxidation of edible oils has been also performed by Infrared and Raman techniques (Muik et al., 2007). These techniques led to improved information compared to isolated analysis concerning assignment of peaks, and therefore, compounds formed during oxidation.

4.5 Magnetic Resonance

The basis of Nuclear Magnetic Resonance (NMR) relies on the property of certain atoms of absorbing and re-emitting energy in the presence of a strong magnetic field due to the excitation of their atomic nuclei. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and on the magnetic properties of the particular isotope of the atom in study. The energy absorptions of the atomic nuclei are affected by the nuclei of surrounding molecules, which cause small local modifications to the external magnetic field. Promising results are obtained by this alternative methodology considering reliability and specificity of the data since they provide an accurate fingerprint of the sample. It does not require extensive manipulation of the sample, thus preserving molecular integrity, and allowing detection of all the substances present in the sample at the same time. This, in addition to its high sensitivity even in complex matrices, highlights the necessity of improving and spreading its use. However, that is a very expensive methodology and requires special skills to interpret the spectra. The use of ^{1}H and ^{13}C NMR spectroscopy in food, applied by different research groups (Guillén and Ruiz, 2008; El Hajjouji et al., 2008; Dybvik et al., 2008;

Tyl et al., 2008; Colzato et al., 2011; Scano et al., 2011; Guillén and Uriarte, 2012a; Guillén and Uriarte, 2012b; Alonso-Salces et al., 2011), has proved to be very useful in evaluating the oxidative status of the lipid fraction, as well as in providing information on the nature (main functional groups) and concentration of the compounds found (i.e. hydroperoxides, carbonyl compounds and dienes). It is considered a valuable tool for quantification of oxidation of food lipids (Namal et al., 2007), and good correlation with conventional analysis such as TBA has been reported (de Oliveira et al., 2011).

Several multi-dimensional NMR techniques have been developed in last years (correlational spectroscopy, nuclear overhauser effect spectroscopy, diffusion-ordered spectroscopy…). They allow a better assignment than the one-dimensional spectra, improving the characterization of food lipid samples (Scano et al., 2011; Hatzakis et al., 2011). However, the main difficulty derived from the application of these tools is the high time required for the acquisition.

The basis of Electron Paramagnetic Resonance (EPR) is the same as that of NMR but in this case, energy excites spins of single electrons. So, only molecules presenting single electrons (that is, radicals) have EPR spectra. It has been used to detect oxidant intermediate species in food matrices (Szterk et al., 2011; Huvaere et al., 2011). However, these radicals show quite short lives unless very low temperatures are guaranteed (Huvaere et al., 2011; Geoffroy et al., 2000; Kamal-Eldin and Min, 2010). In an attempt to avoid this problem, some recently developed methodologies deal with the detection of unstable free radicals. Among them, spin trapping techniques allow the indirect detection of lipid-derived radicals by formation of stable spin adducts that can accumulate in detectable concentrations. This way, both identification and quantification of these intermediates is possible.Traps are not radical-specific, nevertheless particular traps are considered more or less useful for trapping particular radicals. Compounds such as PBN (α-phenyl-tert-butylnitrone) and DMPO (5,5-dimethyl-1-pyrroline-N-oxide) are frequently used for that purpose (Szterk et al., 2011; Papadimitriou et al., 2006).

Combined application of both methodologies (NMR and EPR) is of great interest. In this sense, Silvagni et al. (2010) used them in a study investigating the kinetics of thermally induced lipid peroxidation of peanut oil. The use of EPR allowed them to determine the primary alkyl radicals, and provided an estimation of the radical generation rate; whereas by means of NMR, simultaneously detection of primary and secondary oxidation products was performed, thus allowing a more detailed kinetic investigation.

5. Conclusion

Different kind of compounds can be used as lipid oxidation markers in food samples, among which hydroperoxides and a variety of aldehydes are the most common ones. Each one of them is indicative of a particular state of oxidation, so choosing just one parameter to analyse the oxidative status is rather difficult and it is frequently more convenient to combine different methods. Therefore, analyst must choose carefully the most adequate for his purpose, taking into consideration the most suitable molecules and experimental conditions required in each case. First general decision is whether determining primary or secondary oxidation compounds, considering mainly the extent of oxidation. Afterwards, precision required and characteristics of the food matrix must be considered to follow one methodology or another. A variety of conventional and alternative methodologies have been developed and implemented. Considering the later, they have been proven to provide interesting and promising results, so attention must be paid to these alternative techniques in the area of food lipid oxidation.

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Table 1. Characteristics of the different methods for analysis of lipid oxidation in foods reviewed in this article.

Method	Analyte	Sample preparation	Amount of sample	Sensitivity	Specificity	Cost	Limitations	Most relevant references
Titration	Peroxides	Medium-Short	1 g	Medium-low	Medium-low	Low	Reagents susceptible to oxidation Absorption by UFA Dryness required	AOAC, 2000
Uv-Vis[a] spectroscopy	Peroxides, *Conjugated dienes/trienes, *MDA, aldehydes	Medium	500 mg	Medium	Medium	Low	High amount of solvents Low concentration range Variability depending on the dye *Insensitive to oleic acid	Bou, 2008; Maggio, 2011; Berasategi, 2012; Nuchi, 2009
Chromatography	Peroxides, MDA, SOPs, volatiles, oligomers	Long	1-100 mg	High-very high (depending on the detector)	High-very high (depending on the detector)	High	Laborious experimental procedure and data processing	Márquez-Ruiz, 2007; Zeb, 2010; Mendes, 2009; Derewiaka, 2010
Chemiluminiscence	Peroxides	Short	1-200 mg	High	Medium	Low	Unknown mechanisms Light amplifiers required	Rolewski, 2009
Fluorescence	Aldehydes and volatiles	Very short	10-50 mm^2	Very high	High	Medium	Variability in wavelenghts	Gatellier, 2007
IR[b] spectroscopy	Peroxides, unsaturations, MDA	Very short-none	2-40 mg	Medium-high	High	Medium	Non-aqueous solutions required	Yang, 2005
Raman scattering	Peroxides, unsaturations, MDA	Very short-none	10-50 mm^2	Medium-high	High	Low	Some molecules are inactive	Muik, 2005
Nuclear magnetic resonance	Peroxides, aldehydes, dienes	Very short-none	10-200 mg	High	Very high	Very high	Complex data interpretation	Tyl, 2008; Namal, 2007
Electron paramagnetic resonance	Radicals	Very short-none	100-900 mg	High	High	Very high	Complex data interpretation	Szterk, 2011; Geoffroy, 2000

[a] Ultraviolet-visible
[b] Infrared

Results II

Poster 1

Determination of cholesterol oxidation products in foods: improvement of cost-time efficiency

28th EFFoST International Conference
Innovations in attractive and sustainable food for health

DETERMINATION OF CHOLESTEROL OXIDATION PRODUCTS (COPs) IN FOODS: IMPROVEMENT OF COST-TIME EFFICIENCY

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INTRODUCTION

Cholesterol oxidation products are an important cardiovascular risk, so the determination of their presence in foods and human plasma is highly relevant. However, this usually requires a laborious, time-consuming and expensive analythical procedure.

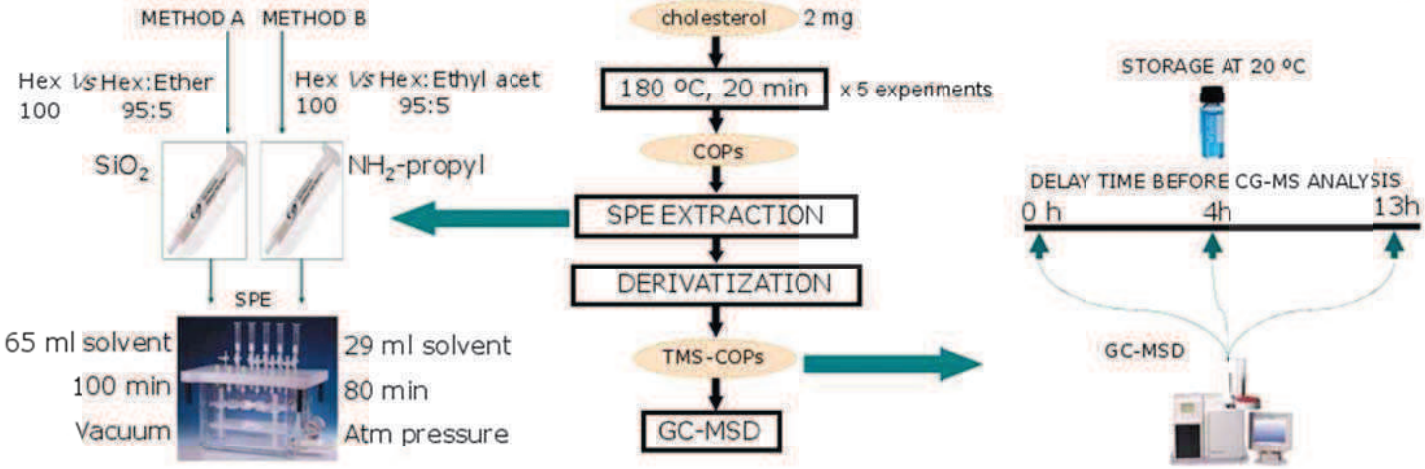
OBJECTIVE

To improve the cost-time efficiency of COPs determination. The target points to be assessed were:

1) Purification by solid phase extraction (SPE) with varying loading solvents and cartridge compositions.

2) Preservation of trimethylsylil-COPs (TMS-COPs) before chromatography analysis.

EXPERIMENTAL DESIGN



RESULTS

Figure 1. COPs content (mg / g cholesterol) obtained with different SPE cartridges (SiO_2 and NH_2-propyl) and solvent polarities (hexane and slightly polar mixture).

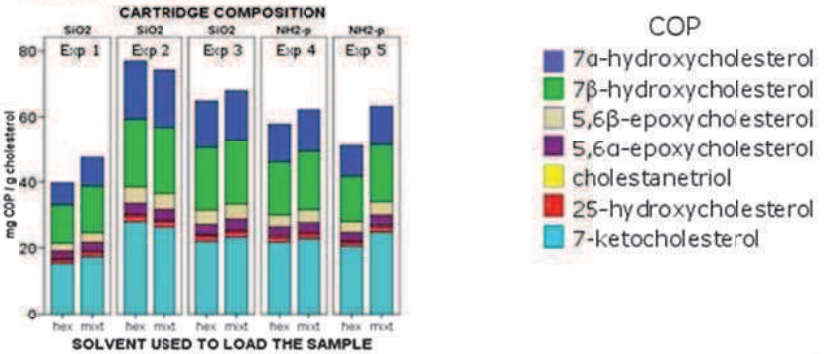


Figure 2. COPs content (% of initial concentration) obtained after different delay times (0, 4 and 13h) before injection in the GC-MSD.

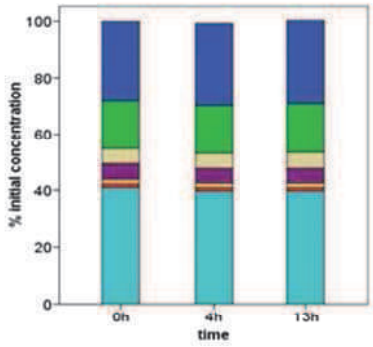


Table 1. Comparative data obtained from both methods

Type of cartidge	Solvents volume	Vacuum needed	Time required	Best solvent for sample loading	Preservation after 13h storage
SiO_2	65 mL	YES	100 min	Hex:Ether 95:5	97-101%
NH_2	29 mL	NO	80 min	Hex:Et acet 95:5	97-101%

REFERENCES

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- Menéndez-Carreño et al., 2008. J. Chromatogr. B, 864, 61-68
- Guardiola et al., 1995. J. Chromatogr. A, 705, 289

ACKNOWLEDGEMENTS

Consolider-CARNISENUSA CSD2007-00016 MCYTC. Asociación de Amigos de la Universidad de Navarra.

CONCLUSIONS

1) The use of a slightly polar solvent (hexane:ethyl acetate) with an amino-propyl cartidge was the best combination for SPE to minimize solvents waste and time of analysis.

2) Stability of TMS-COPs at 20 ºC was guaranteed for 13h, before injection in GC-MS.

Results III

Paper 2

Interlaboratory harmonization trial

(Under preparation)

Interlaboratory harmonization trial

Other authors, Blanca Barriuso[1], Diana Ansorena[1], Iciar Astiasarán[1], other authors

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Introduction

Sterol and SOPs analysis are complex procedures, usually involving the following steps: lipid extraction, saponification, purification by solid phase extraction, derivatization and chromatographic analysis. A great variability in the accomplishment of these steps is nowadays found among the different research groups. An inter-laboratory harmonization of the methodologies was then considered of interest. Thus, the aim of this work was to compare the analytical results obtained by up to 17 different European laboratories in the determination of sterols and oxysterols. Four sterols (cholesterol, campesterol, β-sitosterol, sitostanol) and two oxycholesterols (7α-HC and 7β-HC) were determined in two serum samples (A and B). Samples were prepared by the Reference Institute for Bioanalytics (RfB), in Bonn (Germany), and shipped at room temperature to the laboratories included in the study.

Results and preliminary discussion

Figure 1 shows the different concentrations obtained for each of the compounds analysed by the groups included in the study. Different colours for the spots indicate the application of different methodologies. A great dispersion of the data could be observed for all the compounds, as the high coefficients of variance confirmed (Table 1). This dispersion was not dependent on the methodology applied since data were not grouped according to the different methodologies applied. This could indicate that particular steps of the experimental procedures are key factors and should be taken into account to classify the results. Thus, further information was required and the detailed protocols of each laboratory were necessary to be collected.

Moreover, oxycholesterols levels in B serum sample were, for some laboratories, out of order compared to the other laboratories results. This was attributed to a possible oxidation of the sample during the transport, as a consequence of poor preservation conditions. Some authors highlighted the liophylization process to which samples were subjected before the delivery, as a likely cause of the sample deterioration.

All these questions are now being considered and a deeper discussion, pointing out final conclusions, is still under preparation by the coordinator of the study.

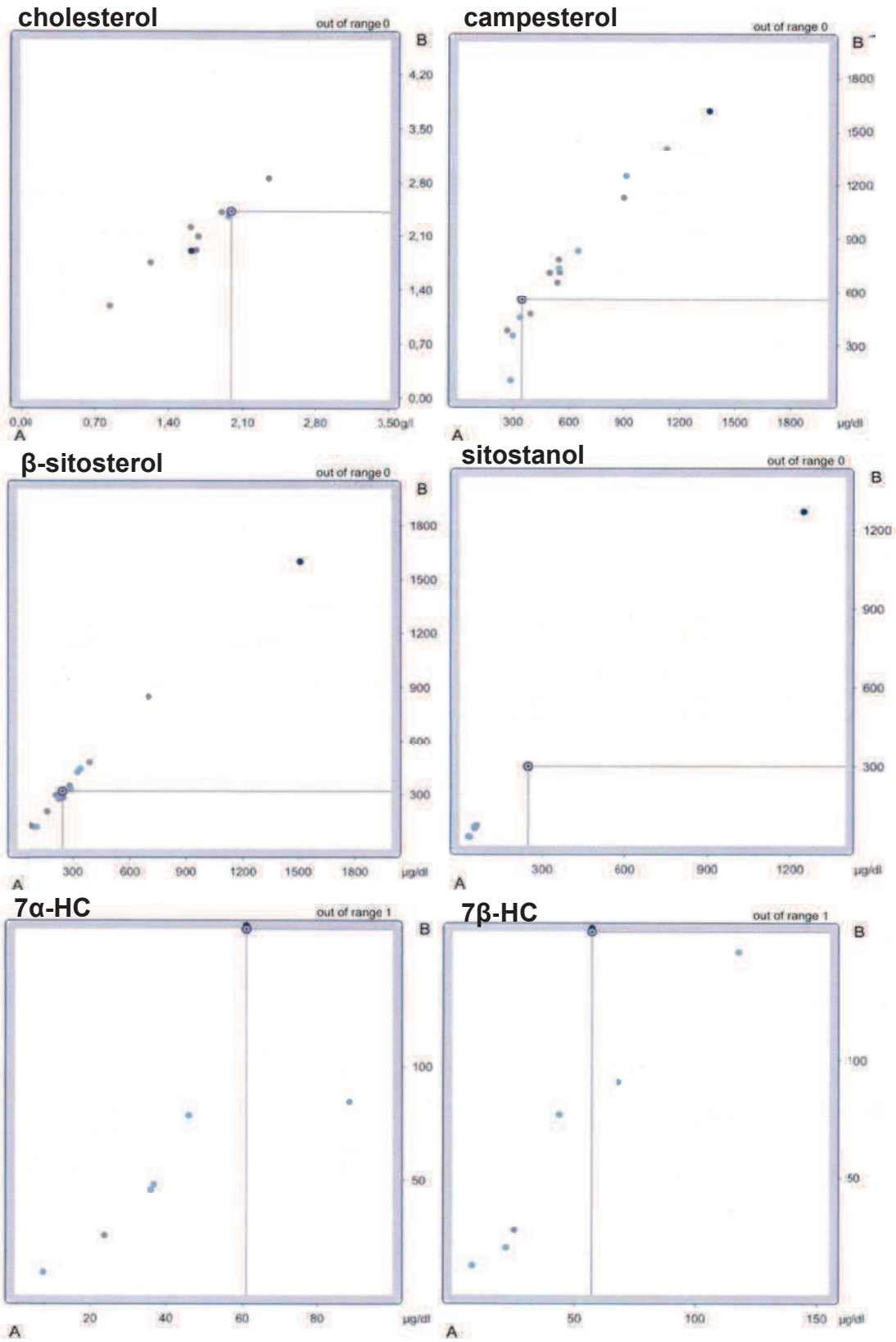

Figure 1. Concentration of cholesterol, campesterol, β-sitosterol, sitostanol, 7α-HC and 7β-HC, obtained for serum samples A and B, in different research laboratories. Different spot color denotes different methodologies applied.

Table 1. Statistical parameters for the determination of a) cholesterol, b) campesterol, c) β-sitosterol, d) sitostanol, e) 7α-HC and f) 7β-HC, obtained for serum samples A and B in all the research laboratories.

Compound	Number of participants	Sample A			Sample B		
		Mean	Sd	Cv	Mean	Sd	Cv
Cholesterol (g/L)	10	1.68	0.422	25.1	2.11	0.455	21.5
Campesterol (µg/dL)	16	597	321	53.8	760	410	54.0
β-sitosterol (µg/dL)	17	334	335	100	401	353	88.0
Sitostanol (µg/dL)	8	223	421	489	240	425	177
7α-HC (µg/dL)	7	39.7	27.2	68.5	48.8	28.9	59.2
7β-HC (µg/dL)	7	47.6	40.3	84.7	62.3	52.1	83.6

Results IV

Paper 3

Sterols heating: Degradation and formation of their ring-structure polar oxidation products

Food Chemistry (2012), 135, 706-712

Sterols heating: Degradation and formation of their ring-structure polar oxidation products

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Abstract

Cholesterol and phytosterols can suffer oxidation under heating conditions to give sterol oxidation products (SOPs), known by their toxic effects. This paper studied the degradation of cholesterol and three plant sterols during a 360 min heating treatment (180 °C). The formation and further degradation of SOPs was also analysed by GC-MS. Results revealed a sterol susceptibility to degradation according to the following decreasing order: campesterol ≈ β-sitosterol ≥ stigmasterol > cholesterol. Their degradation curve fit (R^2 = 0.907-0.979) a logarithmic model. Sterol Oxidation Products increased their concentration during the first 5-10 min and thereafter, their degradation rate was higher than their formation rate, resulting in a decrease over time. Irrespective of the sterol from which they had derived, 7-keto derivatives presented the highest levels throughout the entire process, and also SOPs with the same type of oxidation followed a similar degradation pattern (R = 0.90-0.99).

Keywords: Cholesterol; Plant sterols; Heating stability; Degradation; Kinetic models

Highlights

1. Sterol degradation at 180 °C was: campesterol ≈ β-sitosterol ≥ stigmasterol > cholesterol.
2. Sterols degradation curve fit (R^2 = 0.907 - 0.979) a logarithmic model.
3. The highest SOPs concentration was achieved at 5-10 min.
4. 7-keto derivatives presented the highest levels throughout the 360 min treatment.
5. SOPs with the same type of oxidation followed a similar degradation pattern.

1. Introduction

Cholesterol and plant sterols can suffer autoxidation to give sterol oxidation products (SOPs), named COPs (cholesterol) and POPs (phytosterols), respectively. Some of these compounds have been demonstrated to exert harmful effects in the organism, including atherosclerosis, cytotoxicity and mutagenesis (Larsson et al., 2006; Roussi et al., 2007; O'Callaghan et al.,2010). The presence of polar SOPs has been widely reported in a great variety of foods, from vegetable oils to pork meat (Otaegui-Arrazola et al., 2010). Particular attention has been paid to the development of phytosterol-enriched functional foods due to their much higher sterol content compared to conventional foods. As a consequence, final oxysterol are also increased, reaching up to 3-5-fold compared to the non-enriched foods (Conchillo et al., 2005; Menéndez-Carreño et al., 2008a). Assessing the perspectives of plant sterols-enriched food, particularly focusing on the occurrence of plant sterol oxidation products is of great interest (García-Llatas and Rodríguez-Estrada, 2011). Not only their formation but also their degradation patterns should be studied in depth to predict their levels in foods. In order to avoid interferences of the food matrix and identify their net influence on the process, model systems are commonly used so that a better comprehension of the factors governing this issue can be obtained and a better control of certain food processing conditions might be consequently proposed. Furthermore, there is also wide evidence of the capability of these compounds to be absorbed from the diet (Staprans et al., 2005). Given the above-mentioned harmful effects in human cell lines, their formation should be minimised and the responsible factors should be studied in detail.

In this sense, factors such as high temperature and exposure to oxygen or light are responsible for oxysterols formation. Although 7-keto oxiderivatives are normally the most abundant ones as a consequence of heat treatments, 7α-hydroxy, 7β-hydroxy, 5,6α-epoxy, 5,6β-epoxy and 5,6,7-triol derivatives are usually analysed, too (Lampi et al., 2002; Xu et al., 2011). Degradation of both sterols and oxysterols occurs over 150 °C, giving rise to fragmented phytosterol molecules, volatile compounds and oligomers (Rudzinska et al., 2009; Struijs et al., 2010). One hundred eighty degree celsius is the temperature commonly chosen in most of the studies to evaluate sterol thermal susceptibility as it represents the more usual temperature applied in frying culinary processes. At this temperature, Menéndez-Carreño et al. (2010) found a progressive decrease in stigmasterol content and formation and subsequent degradation of SOPs after one hour of heating. Nevertheless, this SOPs degradation has been reported to start at different moments of the heating process (Kemmo et al., 2005; Xu et al., 2011) depending on the experimental procedure, even when same temperature is applied.

Comparison between oxysterols derived from different initial sterols is a matter of great interest, which could contribute to complete a general overview. Some studies involving parallel monitoring of several sterols have been carried out (Grandgirard et al., 2004; Menéndez-Carreño, et al., 2012; González-Larena et al., 2011). However, few of them assess a discussed comparison of relative degradation data (Cercaci et al., 2006; Menéndez-Carreño et al., 2008a) and the majority used a low number of sterols.

There are some studies concerning modelling with regression equations of cholesterol degradation as well as COPs formation(Chien et al., 1998; Chien et al.,2006; Yen et al.,2010). However, to our knowledge, no study has dealt with plant sterols or SOPs degradation modelling and this would provide relevant information to estimate their actual levels.

Therefore, the aim of this work was to study the behaviour of three phytosterols (β-sitosterol, stigmasterol and campesterol), as well as the formation and degradation pattern of six ring-structure polar oxidation products at 180 °C, comparing them to those of cholesterol. Non-linear regression models for the degradation curves of both sterols and oxysterols were designed.

2. Material and methods

2.1 Reagents

Cholesterol, 5α-cholestane and commercial mixtures of β-sitosterol, campesterol and stigmasterol were purchased from Sigma-Aldrich Chemical (Steinhei, Germany). 19-hydroxycholesterol was purchased from Steraloids (Wilton, NH, USA). Tri-sil reagent was obtained from Pierce (Rockford, IL, USA). Acetone, chloroform, diethyl ether, methanol, hexane and 2-propanol were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography and dichloromethane for gas chromatography were from Merck (Whitehouse Station, NJ, USA). Sep-pack Vac 6cc silica 1g cartridges were obtained from Waters (Milford, USA).

2.2 Heating of sterol samples

Thermo-oxidation of sterol standards was done at 180 °C for various time durations: 0, 5, 10, 20, 30, 60, 90, 120, 180 and 360 min. For the thermo-oxidation, 0.5 mL of cholesterol standard solution (5 mg/mL) was added into 20 open glass vials (15 x 100 mm). Half of the samples were used for the analysis of sterols and half of the samples for the determination of SOPs. The solvent was evaporated under gentle nitrogen stream. Subsequently, the vials were placed open (allowing enough oxygen disposal) in the Tembloc (P Selecta, Spain) previously stabilized at 180 °C. After the corresponding time, vials were taken out from heat. Same procedure was

applied to the phytosterol standards solution (5 mg/mL; 3.29% campesterol, 0.40% campestanol, 53.81 % stigmasterol, 37.33% β-sitosterol and 5.18% sitostanol). Then, the samples were maintained at room temperature for 20 min, except for 5 and 10 min samples, which were cooled in ice for 5 min before acclimatisation. After heating, samples presented an oily appearance. The experiment was performed in quadruplicate.

2.3 Sterol analysis

In order to get a similar concentration than that of phytosterols mixture samples, cholesterol heated samples were redissolved with 5 mL hexane/2-propanol (3:2, v/v) and 1 mL was transferred into a new tube. Subsequently, 0.1 mL of 5α-cholestane (2 mg/mL) was added to each cholesterol and phytosterol heated sample. The solvent was evaporated under gentle nitrogen stream.

Both cholesterol and phytosterol heated samples were derivatized to trimethylsilyl (TMS) ethers according to a modified version of the method described by Dutta and Appelqvist (1997). Four hundred micro litres of Tri-Sil reagent were added to each sample and they were kept at 60 °C for 45 min in a water bath. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivates were solved in 10 mL of hexane for gas chromatography. Four hundred micro litres of this solution were filtrated with a syringe and a filter (0.45 µm) and poured to a glass vial prior to GC-MS analysis.

Gas chromatography-Mass spectrometry (GC-MS) analysis was performed on a HP Hewlett Packard 6890 GC coupled to a HP 5973 Mass Selective Detector. The TMS-ether derivatives of cholesterol and phytosterol standards were separated on a capillary column Agilent 19091S-433 HP-5ms 5% Phenyl Methyl Siloxane (30 m x 250 µm x 0.25 µm film thickness) (Agilent, CA, USA). Oven temperature conditions had previously been optimised in order to achieve proper separation of the individual compounds. The programme started at temperature of 85 °C, heated to 290 °C at a rate of 50 °C/min and, finally, increased to 298 °C at rate of 0.5 °C/min. High purity helium was used as a carrier gas at a flow rate of 1 mL/min. The inlet pressure used was 9.64 psi. The injector temperature was 280°C and the samples were injected (1µL) in a splitless mode.

Peak identification was based on comparison of their mass spectra with the spectra of the Wiley library (HPCHEM, Wiley, 275, 6th ed.) and also with those obtained from the literature. In some cases, a comparison of their retention time and MS fragments with those of standard pure compounds was also done. An internal standard method was used for quantification, with 5α-cholestane as the internal standard. Cholesterol and 5α-cholestane quantification was

made in SCAN, while plant sterols were quantified using selected ion monitoring (SIM) analysis on the basis of the amount of a specific ion for each peak (343, 484, 357, for campesterol, stigmasterol and β-sitosterol, respectively), and taking into account the relative abundance of each ion (Berasategi et al., 2012). Calibration curves were previously built. For the integration Agilent MSD Productivity ChemStation for GC and GC/MS Systems Data Analysis Application were used.

2.4 Sterol oxidation products analysis

The identification and quantification of sterol oxidation products was performed according to the validated method of Menéndez-Carreño et al. (2008b).

Firstly, 1 mL of internal standard (20 µg/mL of 19-hydroxycholesterol) was added to the heated samples. SPE was used to separate SOPs from non-polar and mid-polar products. The purification of oxysterols allows obtaining clear chromatograms. The SPE was made according to the procedures described in detail in Guardiola et al. (1995). The test tubes containing the samples diluted in 5 mL of hexane were applied to a SPE silica cartridge, previously equilibrated with 5 mL of hexane. The cartridge was subsequently treated with 10 mL of hexane:diethyl ether (95:5, v/v), 30 mL of hexane:diethyl ether (90:10, v/v), and with 10 mL of hexane:diethyl ether (80:20, v/v). Sterol oxidation products were finally eluted from the SPE cartridge with 10 mL of a mixture of acetone/methanol (60:20, v/v). The solvent was evaporated in rotary evaporator under warm water bath (35 °C).

The sample solutions of sterol oxidation products were derivatized to trimethylsilyl (TMS) ethers as previously described for sterols.

GC-MS analysis was performed on a Hewlett Packard 6890N GC coupled to a 5975 Mass Selective Detector. The TMS-ether derivatives of sterol oxides were separated on a capillary column Agilent CP8947 Varian VF-5ms 5% Phenyl Methyl Siloxane (50 m x 250 µm x 0.25 µm film thickness). Oven temperature conditions were as follows: initial temperature of 75 °C, heated to 250 °C at a rate of 30 °C/min, increased to 290 °C at rate of 8 °C/min, and finally, it was raised to 292 °C at a rate of 0.05 °C/min. High purity helium was used as a carrier gas at a flow rate of 1 mL/min. The inlet pressure used was 9.08 psi. The injector temperature was 250 °C and the transfer line to detector 280 °C. The samples (1µL) were injected in splitless mode.

Peak identification was made following the same procedure as for sterols. SOPs quantification was also based on an internal standard method (19-hydroxycholesterol). It was performed using selected ion monitoring (SIM) analysis. For each stage of time, different ions (33) were selectively quantified and, consequently, extract ion chromatogram had to be used to

integrate the corresponding peak areas. Given the lack of available POPs standards and their demonstrated similar to COPs response, COPs calibration curves assessed by Menendez-Carreño et al. (2008b) were also used to determine POPs content. For the integration Agilent MSD Productivity ChemStation for GC and GC/MS Systems Data Analysis Application (Agilent Technologies, Inc., CA, U.S.A.) were used.

2.5 Statistical analysis

For the statistical analysis of the data, SPSS 15.0 programme (SPSS, Inc., Chicago, IL, U.S.A.) was used. Mean and standard deviation of data obtained from each replicate were calculated. For the mathematical modelling of the degradation of sterols and their oxides, the nonlinear regression analysis was used (from beginning in the case of sterols and from the moment of the maximum achieved in the case of SOPs). For the evaluation of the significant differences of the amounts of sterols and sterol oxides along time and among different sterols, one factor ANOVA with Tuckey post hoc multiple comparisons ($p < 0.05$) was applied. Finally, correlations between oxysterols of the same oxidation pattern (concerning functional group and position) but from different sterol origin were assessed by means of Pearson´s correlation test.

3. Results

3.1 Sterol study

Amounts of remaining cholesterol and phytosterols after the different heating times (0-360 min) are shown in Table 1. Significant differences were observed among every heating time during the first 20-30 min, when a drastic drop was detected. Thereafter, small differences were found. Degradation reached around 55-60% of the initial sterol content -except for cholesterol, 41.80%- during the first 5 min of treatment (Table 2). After 30 min heating, around 88% and 74.71% of the initial sterols had already been degraded, for phytosterols (mean value) and cholesterol, respectively, and around 90% and 79.64 % respectively after 90 min. At the end of the heat treatment all sterols were degraded up to around 95% of their initial level.

Different nonlinear regression models were assayed to predict the loss of sterols (logarithmic, inverse and exponential), with logarithmic model showing the highest R^2 for all cases (0.907, 0.972, 0.953 and 0.979 for cholesterol, campesterol, stigmasterol and β-sitosterol, respectively). The plots and corresponding equations are shown in Figure 1. Cholesterol showed the highest first constant - named as α - (48.720) compared to the other equations (39.786 - 41.474).

3.2 Sterol Oxidation Products

The evolution of 24 different SOPs was followed in this study. 7α-hydroxy, 7β-hydroxy, 5,6β-epoxy, 5,6α-epoxy, triol and 7-keto derivatives of each sterol were analysed (Tables 3-6). The total amount of compounds derived from each sterol was also calculated (Figure 2). Maximum levels for each sterol total oxides were 73.79, 106.53, 49.75 and 98.38 μg/mg, for cholesterol (10 min), campesterol (10 min), stigmasterol (5 min) and β-sitosterol (5 min), respectively. After this time, there was a significant and progressive decrease for total oxysterols, reaching minimal amounrs, around 6% of the maximum concentration at the end of the process. Higher levels of total campesterol and β-sitosterol oxides were detected compared to those achieved by cholesterol and stigmasterol derivatives throughout the whole heating process.

The most abundant SOP formed was, by far, 7-ketosterol (irrespective of the type of sterol), reaching 64.28%, 65.93%, 53.85% and 67.68% of the total amount of oxides derived from cholesterol, campesterol, stigmasterol and β-sitosterol, respectively, at the moment of the maximum total SOPs concentration, which was set at 5-10 min treatment. A trend to increase in all 7-keto plant sterol derivatives was observed at minute 60, although not statistically significant. 7α-Hydroxy- and 7β-hydroxy- were the next derivatives formed from the quantitative point of view. While 7α-hydroxy- derivatives were more abundant than β-hydroxy- ones at the initial points of analysis, as the treatment progressed, β-hydroxy-formation is favoured over alpha's. Sterol epoxides level is much smaller than 7-hydroxysterols' at the beginning (especially in the case of campesterol and stigmasterol), but higher at the end, ie, 8.09 and 21.11 μg/mg of 5,6α/β-epoxycampesterol and 7α/β-hydroxycampesterol respectively at 5 min, compared to 5.89 and 0.52 μg/mg at 90 min. Triol derivatives were the ones formed in a lower amount, even finding no campestanetriol.

As a similar behaviour could be observed among the same kind of oxysterols of different sterol origin (Table 1 and Figure 2), correlation was also studied among them, showing Pearson's Coefficients between 0.90 and 0.99 in most cases.

Among the different nonlinear regression models assayed (Table 7), and considering the R^2 values, the ones which best fit the degradation were for 7α and 7β-hydroxy, an inverse model; for 5,6β-epoxy, a logarithmic model; and for the rest of compounds an exponential model – except for 5,6α-epoxycholesterol, which fit a logarithmic model-. In the overall view, total chol-ox, total cam-ox, total stigma-ox and total sito-ox followed an exponential-type degradation.

Concerning the degradation rate constants, 7α-hydroxy compounds showed a degradation rate constant around twice that of 7β-hydroxy, except for cholesterol samples. Among

exponential regression adjustments, rate constants were in all cases ranging between -0.010 and -0.004. Values were rather uniform within the same type of oxidation mechanism, regardless of the sterol origin -except for 5,6α-epoxycholesterol-, the constants of triol being the lowest (in absolute value) and that of 5,6α-epoxy the highest.

4. Discussion

Compared to other studies performed both in model or real systems (Xu et al., 2009; Yen et al., 2010; Menéndez-Carreño et al., 2010), the heat treatment conditions applied in our study seemed to be more destructive, since the degradation percentages of sterols were, in general, higher. Around 55% degradation of cholesterol and β-sitosterol standards and 38% stigmasterol standard have been previously reported after 1h heating at 180 °C (Xu et al., 2009; Menéndez-Carreño et al., 2010), compared to more than 70 % noted for the present study. Different heating temperature/exposure time and other experimental conditions (such as initial sterol amount) could be behind this diversity of results. In their work, Menéndez-Carreño et al. (2010) heated the stigmasterol standard samples into glass vials placing them into an oven where the energy transfer takes place via convection heating. In contrast, sterol standards were heated in the present study by using an electronic heating device. Thus, the transfer of energy here occurs between two objects that are in physical contact (conduction heating). Then, as it has been suggested in several previous works, the sterol degradation pattern can differ significantly after the application of different heating technologies (Chien et al., 1998).

The comparison among the different degradation percentages of sterols showed a greater susceptibility to oxidation for phytosterols than for cholesterol, during first 120 min. Moreover, non-linear regression equation parameters for remaining sterol content also suggested a rather lower degradation intensity of cholesterol compared to plant sterols, attending to the value of the first constant, higher for cholesterol than for plant sterols As different initial amounts of sterols were used, interferences during oxidation could have occurred since thermo-oxidation is significantly dependent on the sample and to volume ratio (Lampi et al., 2002). In this sense, cholesterol particles would be less exposed to oxidation because of the highest initial amounts used (2.5 mg). In this case, some controversial data can be observed in different works: Menéndez et al. (2008a) and Cercaci et al. (2006) found cholesterol to be more prone to oxidative degradation than β-sitosterol, whereas Xu et al. (2009) reported no differences in degradation rates among sterols. On basis on a kinetic study, Yen et al. (2010) and Chien et al. (1998) proposed first order equations for cholesterol degradation. Lower heating temperature (150 °C instead of 180 °C) resulted in a slower

degradation process, which could have led to the adjustment of a first order equation with these parameters instead of a logarithmic one.

Regarding the accumulation of SOPs, maximum levels achieved were in accordance to the values found in the literature for cholesterol and β-sitosterol oxides, but slightly lower for campesterol and stigmasterol oxides (Xu et al., 2011; Yen et al, 2010; Kemmo et al., 2008; Lampi et al., 2009). It is important to note that the maximum SOPs levels were reached at 5-10 min, whereas other studies found maximum levels at 60 min or longer times at the same temperature (180 °C) (Xu et al., 2011; Menéndez et al., 2010). These data pointed out that our degradation started earlier and higher concentrations could not have been achieved. The subsequent drastic drop was in accordance to what Xu, Zhang, Prinyawiwatkul and Godber (2005) found, when heating cholesterol at 175 °C. Nevertheless, compared to other studies (Kemmo et al., 2005; Xu et al., 2011; Chien et al., 2006; Yen et al., 2010; Menéndez-Carreño et al., 2010), our degradation occurs much earlier, probably due to the different experimental conditions applied mentioned above. Changing processing temperatures have an important effect on the formation of oxysterols, but other experimental conditions are likely to contribute to differential results, ie different initial sample amounts, purification procedures or chromatographic techniques applied. Polymeric products, steradiens and both non-polar and mid-polar compounds have been reported as possible degradation products formed during extreme heating conditions (Menéndez-Carreño et al., 2010; Rudzinska et al., 2009; Lampi et al., 2009; Lercker and Estrada, 2002).

7-Hydroxy and 7-keto oxides are usually expected to be the major SOPs (Grandgirard et al., 2004; González-Larena et al., 2011), at least during the first stages of oxidation. Dominancy of β-epimer among 7-hydroxy compounds is possibly due to stearic hindrance of the hydroxyl group at position 3 (Kemmo et al., 2005; Smith, 1987); other authors have also observed this trend (Lampi et al., 2002; Xu et al., 2005; Soupas et al., 2007). The trend to increase in all 7-keto plant sterol oxides observed at minute 60 (Tables 3-6), might be attributed to the conversion from 7-hydroxy derivatives (Rudzinska et al., 2009; Kemmo et al., 2008). Negligible levels of triol compounds have previously been reported (Conchillo et al., 2005; Lampi et al., 2002), only by heating treatments.

Regarding the higher levels of campesterol and β-sitosterol derivatives compared to that of cholesterol and stigmasterol ones, this behaviour could be attributed either to a faster oxidation of the campesterol and β-sitosterol to give rise to the corresponding oxides either to a slower degradation of these oxides. The lower initial amounts of campesterol (0.081 mg) and β-sitosterol (0.916 mg) could be behind this behaviour (Lampi et al., 2002), since it would have

been overexposed to oxygen. In addition, the presence of an extra double bound in the side chain of stigmasterol at position 22 may possibly have affected to the rate of oxidation of this compound. Thus, stigmasterol presented the lowest total maximum amount of oxidation products especially affecting 7-ketostigmasterol formation in comparison to the other sterols. Nevertheless, further research would be necessary to confirm this hypothesis.

Considering comparative graphs and kinetic curves, it can be stated that oxysterols followed a tendency according to the type of oxidation, and regardless of the sterol origin. However, a particular case can be highlighted: 5,6α-epoxycholesterol presented its best adjustment for a logarithmic model, whereas plant sterols analogous oxides were adjusted to an exponential one. Different side chain could be behind this behaviour, although further studies would be required to make more accurate conclusions. Drastic drop of 7-hydroxy derivatives levels (Tables 3-6) could be related to their adjustment to an inverse model, instead of an exponential one, as in the other cases.

In conclusion, our results revealed a sterol susceptibility to degradation following this decreasing order: campesterol ≈ β-sitosterol ≥ stigmasterol > cholesterol. Regarding sterol oxidation products, the levels increased during the first 5-10 min and thereafter, their degradation rate was higher than their formation rate. SOPs degradation seemed to depend on the molecular structure of the oxidised compound, irrespective of the sterol from which they were derived.

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Table 1. Degradation of sterol standards during 360-minute thermo oxidation at 180°C. Mean and standard deviation (n=4) are represented with the statistical analysis. Different letters within each column denote significant differences among heating times ($p < 0.05$).

Time (min)	Remaining sterols (mg)			
	cholesterol	campesterol	stigmasterol	sitosterol
0	2.41 ± 0.05 ^{g}	0.08 ± 0.01 ^{e}	1.32 ± 0.10 ^{d}	0.92 ± 0.07 ^{f}
5	1.40 ± 0.16 ^{f}	0.03 ± 0.01 ^{d}	0.58 ± 0.07 ^{c}	0.35 ± 0.03 ^{e}
10	1.07 ± 0.21 ^{e}	0.02 ± 0.003 ^{c}	0.48 ± 0.06 ^{c}	0.23 ± 0.03 ^{d}
20	0.88 ± 0.02 ^{d}	0.01 ± 0.001 ^{b}	0.25 ± 0.02 ^{b}	0.15 ± 0.02 ^{c}
30	0.61 ± 0.01 ^{c}	0.01 ± 0.001 ^{ab}	0.16 ± 0.02 ^{ab}	0.10 ± 0.01 ^{bc}
60	0.55 ± 0.02 ^{bc}	0.01 ± 0.001 ^{ab}	0.13 ± 0.003 ^{a}	0.10 ± 0.01 ^{bc}
90	0.49 ± 0.02 ^{bc}	0.01 ± 0.001 ^{ab}	0.12 ± 0.003 ^{a}	0.09 ± 0.01 ^{bc}
120	0.38 ± 0.01 ^{b}	0.01 ± 0.001 ^{ab}	0.10 ± 0.004 ^{a}	0.08 ± 0.00 ^{ab}
180	0.16 ± 0.01 ^{a}	0.01 ± 0.001 ^{a}	0.09 ± 0.002 ^{a}	0.07 ± 0.001 ^{ab}
360	0.13 ± 0.01 ^{a}	0.01 ± 0.001 ^{a}	0.05 ± 0.002 ^{a}	0.04 ± 0.001 ^{a}

Table 2. Degradation percentages of cholesterol, campesterol, stigmasterol and sitosterol during 360-minute thermo oxidation at 180°C. Within each row, different letters denote significant differences ($p < 0.05$) among sterols.

Time (min)	Degradation percentages (%)			
	cholesterol	campesterol	stigmasterol	sitosterol
5	41.80 ± 7.23 ^{a}	58.06 ± 5.83 ^{b}	56.12 ± 5.84 ^{ab}	61.45 ± 3.41 ^{b}
10	55.74 ± 9.75 ^{a}	71.56 ± 389 ^{b}	63.47 ± 4.71 ^{ab}	74.68 ± 3.65 ^{b}
20	63.58 ± 0.75 ^{a}	82.68 ± 1.83 ^{b}	80.87 ± 1.78 ^{b}	83.89 ± 1.73 ^{b}
30	74.71 ± 0.66 ^{a}	87.06 ± 1.31 ^{b}	88.30 ± 1.38 ^{b}	88.72 ± 1.14 ^{b}
60	76.99 ± 0.75 ^{a}	87.16 ± 1.22 ^{b}	90.40 ± 0.26 ^{c}	89.31 ± 0.79 ^{c}
90	79.64 ± 0.76 ^{a}	88.14 ± 0.40 ^{b}	90.87 ± 0.26 ^{d}	89.73 ± 0.53 ^{c}
120	84.37 ± 0.35 ^{a}	89.60 ± 0.27 ^{b}	92.20 ± 0.27 ^{d}	91.42 ± 0.28 ^{c}
180	93.25 ± 0.53 ^{c}	90.89 ± 0.35 ^{a}	93.22 ± 0.17 ^{c}	92.40 ± 0.15 ^{b}
360	94.51 ± 0.54 ^{a}	94.34 ± 0.20 ^{a}	96.39 ± 0.14 ^{b}	95.79 ± 0.14 ^{b}

Table 3. Concentration of cholesterol oxidation products (µg/mg).Within each column, different letters denote significant differences among heating times ($p < 0.05$).

Time (min)	cholesterol oxides (µg/mg cholesterol)					
	7α-hydroxy	7β-hydroxy	5,6β-epoxy	5,6α-epoxy	triol	7-keto
0	nd	nd	0.08 ± 0.01 ^{a}	0.25± 0.01 ^{a}	nd	nd
5	5.26 ± 0.35 ^{c}	3.60 ± 0.36 ^{c}	3.85 ± 0.30 ^{c}	3.62± 0.36 ^{c}	0.26 ± 0.05 ^{de}	35.83 ± 5.57 ^{ef}
10	7.05 ± 0.83 ^{d}	6.83 ± 0.74 ^{d}	5.42 ± 0.77 ^{d}	6.79± 1.12 ^{e}	0.26 ± 0.05 ^{de}	47.43 ± 6.80 ^{g}
20	2.76 ± 0,39 ^{b}	5.64 ± 1.01 ^{e}	3.21 ± 0.37 ^{c}	5.14± 0.20 ^{d}	0.28 ± 0.04 ^{de}	41.86 ± 1.77 ^{fg}
30	0.78 ± 0.15 ^{a}	1.49 ± 0.11 ^{b}	1.46 ± 0.25 ^{b}	3.88± 0.45 ^{c}	0.25 ± 0.02 ^{de}	34.40 ± 2.65 ^{ef}
60	0.55 ± 0.02 ^{a}	0.93 ± 0.01 ^{ab}	0.72 ± 0.08 ^{ab}	3.03± 0.23 ^{b}	0.31 ± 0.04 ^{e}	31.18 ± 4.63 ^{e}
90	nd	nd	0.09 ± 0.02 ^{a}	0.68± 0.13 ^{a}	0.24 ± 0.01 ^{de}	21.22 ± 2.80 ^{d}
120	nd	nd	0.04 ± 0.01 ^{a}	0.40± 0.08 ^{a}	0.20 ± 0.03 ^{cd}	17.07 ± 2.55 ^{cd}
180	nd	nd	0.02 ± 0.001 ^{a}	0.23± 0.02 ^{a}	0.12 ± 0.004 ^{bc}	10.76 ± 0.64 ^{bc}
360	nd	nd	0.01 ± 0.001 ^{a}	0.12± 0.01 ^{a}	0.05 ± 0.01 ^{ab}	3.32 ± 0.41 ^{ab}

Table 4. Concentration of campesterol oxidation products (μg/mg). Within each column, different letters denote significant differences among heating times (p <0.05).

Time (min)	campesterol oxides (μg/mg campesterol)					
	7α-hydroxy	7β-hydroxy	5,6β-epoxy	5,6α-epoxy	triol	7-keto
0	nd	nd	0.38 ± 003 ^{a}	0.001 ± 0.000 ^{a}	nd	0.09 ± 0.03 ^{a}
5	14.06 ± 2.32 ^{c}	7.05 ± 0.25 ^{d}	0.79 ± 0.11 ^{ab}	7.30 ± 0.28 ^{ef}	nd	70.23 ± 7.12 ^{e}
10	16.85 ± 1.59 ^{c}	7.19 ± 1.24 ^{d}	1.49 ± 0.20 ^{c}	10.13 ± 0.94 ^{g}	nd	70.87 ± 8.74 ^{e}
20	8.30 ± 0.71 ^{b}	4.66 ± 0.95 ^{c}	1.47 ± 0.03 ^{bc}	7.61 ± 0.80 ^{f}	nd	52.01 ± 6.41 ^{d}
30	1.89 ± 0.31 ^{a}	2.52 ± 0.27 ^{b}	124 ± 0.17 ^{bc}	6.23 ± 0.66 ^{de}	nd	43.25 ± 0.27 ^{cd}
60	0.89 ± 0.14 ^{a}	1.11 ± 0.03 ^{ab}	1.54 ± 0.26 ^{c}	4.91 ± 0.57 ^{cd}	nd	52.96 ± 2.60 ^{d}
90	0.20 ± 0.01 ^{a}	0.33 ± 0.06 ^{a}	1.62 ± 0.30 ^{c}	4.27 ± 0.61 ^{c}	nd	38.71 ± 2.32 ^{c}
120	nd	0.12 ± 0.02 ^{a}	1.47 ± 0.30 ^{bc}	2.65 ± 0.37 ^{b}	nd	33.19 ± 4.17 ^{bc}
180	nd	0.11 ± 0.02 ^{a}	1.33 ± 0.17 ^{bc}	2.09 ± 0.26 ^{b}	nd	24.18 ± 4.00 ^{b}
360	nd	0.06 ± 0.005 ^{a}	0.44 ± 0.05 ^{a}	0.59 ± 0.10 ^{a}	nd	7.90 ± 0.50 ^{a}

Table 5. Concentration of stigmasterol oxidation products (μg/mg). Within each column, different letters denote significant differences among heating times (p<0.05).

Time (min)	stigmasterol oxides (μg/mg stigmasterol)					
	7α-hydroxy	7β-hydroxy	5,6β-epoxy	5,6α-epoxy	triol	7-keto
0	0.08 ± 0.01 ^{a}	0.03 ± 0.004 ^{a}	0.01 ± 0.001 ^{a}	nd	nd	0.03 ± 0.01 ^{a}
5	9.96 ± 1.46 ^{c}	5.01 ± 0.25 ^{d}	4.77 ± 0.08 ^{d}	2.79 ± 0.55 ^{de}	0.44 ± 0.08 ^{bc}	26.79 ± 4.33 ^{f}
10	11.15 ± 1.92 ^{c}	5.02 ± 0.76 ^{d}	4.69 ± 0.85 ^{d}	3.44 ± 0.40 ^{e}	0.87 ± 0.13 ^{f}	23.61 ± 2.74 ^{f}
20	6.20 ± 0.75 ^{b}	3.37 ± 0.63 ^{c}	3.94 ± 0.67 ^{d}	2.44 ± 0.29 ^{cd}	0.81 ± 0.07 ^{ef}	17.09 ± 2.59 ^{e}
30	1.33 ± 0.23 ^{a}	1.74 ± 0.11 ^{b}	2.13 ± 0.36 ^{c}	1.88 ± 0.19 ^{bc}	0.70 ± 0.03 ^{def}	13.83 ± 0.59 ^{de}
60	0.51 ± 0.06 ^{a}	0.68 ± 0.03 ^{ab}	1.44 ± 0.11 ^{bc}	1.53 ± 0.14 ^{b}	0.65 ± 0.11 ^{de}	16.16 ± 2.42 ^{e}
90	0.15 ± 0.02 ^{a}	0.25 ± 0.05 ^{a}	1.05 ± 0.10 ^{abc}	1.38 ± 0.26 ^{b}	0.61 ± 0.11 ^{cd}	13.04 ± 1.22 ^{de}
120	0.06 ± 0.01 ^{a}	0.09 ± 0.02 ^{a}	0.59 ± 0.07 ^{ab}	0.57 ± 0.07 ^{a}	0.46 ± 0.02 ^{bc}	8.85 ± 0.15 ^{cd}
180	0.06 ± 0.01 ^{a}	0.07 ± 0.008 ^{a}	0.45 ± 0.09 ^{ab}	0.45 ± 0.09 ^{a}	0.31 ± 0.05 ^{b}	6.86 ± 0.75 ^{bc}
360	0.01 ± 0.00 ^{a}	0.02 ± 0.002 ^{a}	0.07 ± 0.01 ^{a}	0.10 ± 0.02 ^{a}	0.05 ± 0.01 ^{a}	2.51 ± 0.11 ^{ab}

Table 6. Concentration of β-sitosterol oxidation products (μg/mg). Within each column, different letters denote significant differences among heating times (p<0.05).

Time (min)	sitosterol oxides (μg/mg sitosterol)					
	7α-hydroxy	7β-hydroxy	5,6β-epoxy	5,6-α-epoxy	triol	7-keto
0	0.06 ± 0.01 ^{a}	0.02 ± 0.00 ^{a}	nd	0.001 ± 0.003 ^{a}	nd	0.04 ± 0.01 ^{a}
5	12.27 ± 1.69 ^{c}	6.86 ± 0.25 ^{d}	5.80 ± 0.56 ^{d}	6.05 ± 0.58 ^{e}	0.82 ± 0.13 ^{e}	66.58 ± 12.73 ^{f}
10	13.94 ± 2.44 ^{c}	7.04 ± 1.10 ^{d}	5.58 ± 0.67 ^{d}	8.36 ± 0.64 ^{d}	0.56 ± 0.06 ^{d}	59.17 ± 11.12 ^{ef}
20	6.02 ± 1.14 ^{b}	4.74 ± 0.86 ^{c}	4.17 ± 0.83 ^{c}	5.88 ± 0.68 ^{d}	0.42 ± 0.06 ^{c}	42.53 ± 6.59 ^{de}
30	1.31 ± 0.09 ^{a}	2.37 ± 0.17 ^{b}	1.87 ± 0.20 ^{b}	4.55 ± 0.40 ^{c}	0.38 ± 0.03 ^{bc}	34.95 ± 0.48 ^{cd}
60	0.60 ± 0.08 ^{a}	1.01 ± 0.03 ^{ab}	0.67 ± 0.07 ^{ab}	3.79 ± 0.29 ^{c}	0.43 ± 0.03 ^{c}	41.76 ± 6.28 ^{d}
90	0.18 ± 0.08 ^{a}	0.52 ± 0.18 ^{a}	0.30 ± 0.03 ^{a}	3.31 ± 0.61 ^{c}	0.43 ± 0.05 ^{c}	33.39 ± 3.23 ^{cd}
120	0.07 ± 0.008 ^{a}	0.17 ± 0.02 ^{a}	0.08 ± 0.004 ^{a}	1.39 ± 0.17 ^{b}	0.34 ± 0.02 ^{bc}	23.35 ± 0.49 ^{c}
180	0.08 ± 0.004 ^{a}	0.14 ± 0.02 ^{a}	0.06 ± 0.01 ^{a}	0.87 ± 0.05 ^{ab}	0.28 ± 0.03 ^{b}	18.94 ± 2.93 ^{bc}
360	0.01 ± 0.001 ^{a}	0.02 ± 0.00 ^{a}	0.01 ± 0.001 ^{a}	0.23 ± 0.03 ^{a}	0.11 ± 0.02 ^{a}	6.35 ± 0.25 ^{ab}

Table 7. Regression model and parameters of the kinetic equations of all SOPs.

Compound	Regression model	R^2	*a*	*k*
7α-hydroxy-chol	inverse [a]	0.956	-0.672	73.678
7α-hydroxy-cam	inverse	0.965	-1.439	179.080
7α-hydroxy-stig	inverse	0.947	-0.993	121.065
7α-hydroxy-sito	inverse	0.946	-1.363	149.086
7β-hydroxy-chol	inverse	0.868	-0.410	81.721
7β-hydroxy-cam	inverse	0.937	-0.204	77.478
7β-hydroxy-stig	inverse	0.945	-0.163	54.600
7β-hydroxy-sito	inverse	0.941	-0.170	75.919
5,6β-epoxy-chol	logarithmic [b]	0.772	7.275	-1.419
5,6β-epoxy-cam	-	-	-	-
5,6β-epoxy-stig	logarithmic	0.912	7.146	-1.296
5,6β-epoxy-sito	logarithmic	0.890	8.486	-1.635
5,6α-epoxy-chol	logarithmic	0.890	10.824	-1.997
5,6α-epoxy-cam	exponential [c]	0.960	8.296	-0.008
5,6α-epoxy-stig	exponential	0.949	2.790	-0.010
5,6α-epoxy-sito	exponential	0.954	6.856	-0.010
triol-chol	exponential	0.918	0.350	-0.005
triol-cam	exponential	-	-	-
triol-stig	exponential	0.947	1.043	-0.008
triol-sito	exponential	0.846	0.551	-0.004
7-ketochol	exponential	0.976	45.397	-0.007
7-ketocam	exponential	0.953	64.773	-0.006
7-ketositg	exponential	0.935	21.658	-0.006
7-ketosito	exponential	0.912	6.493	-0.007
totalchol	exponential	0.936	58.094	-0.008
totalcam	exponential	0.959	135.203	-0.007
totalstig	exponential	0.926	39.901	-0.008
totalsito	exponential	0.928	81.567	-0.007

[a] $y = a + k/t$

[b] $y = a + k.\log(t)$

[c] $y = a \,.\, e^{kt}$

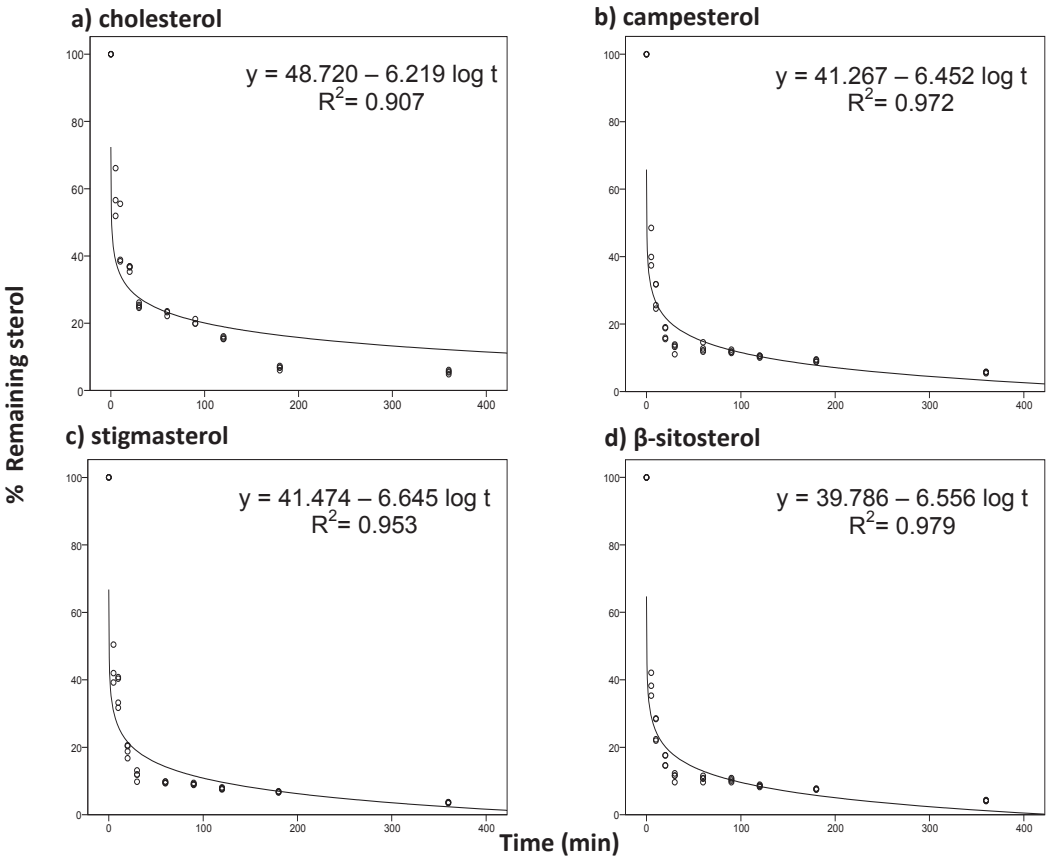

Figure 1. Mathematical modelling of the degradation kinetic of sterol standards during 360-minute thermo-oxidation at 180°C.Remaining percentages of a) Cholesterol b) Campesterol c) Stigmasterol d) β-sitosterol

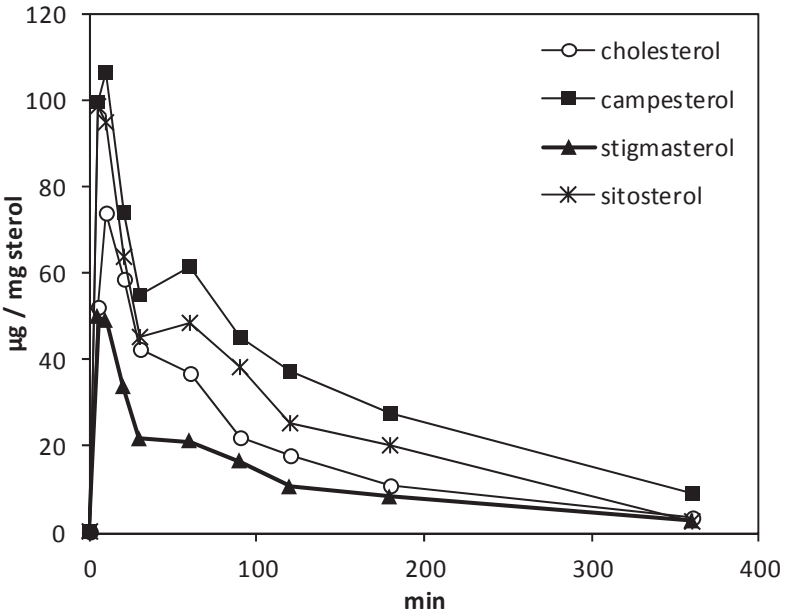

Figure 2. Graphic representations of total SOPs during thermo-oxidation up to 360min for different sterol origin

Results V

Paper 4

Role of *Melissa officinalis* in cholesterol oxidation: Antioxidant effect in model systems and application in beef patties

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Role of *Melissa officinalis* in cholesterol oxidation: antioxidant effect in model systems and application in beef patties

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Abstract

Cholesterol oxidation products (COPs) constitute a known health risk factor. The antioxidant effect of a lyophilized aqueous *Melissa officinalis* extract against cholesterol degradation and COPs formation during a heating treatment was evaluated in a model system (180°C, 0-180 min) at a ratio of 2 mg extract / 100 mg cholesterol. Furthermore, the plant extract was subsequently added to beef patties alone or incorporated within an oil-in-water olive oil emulsion to assess its effectiveness during cooking. Melisa extract protected cholesterol from thermal degradation in the model system, yielding higher remaining cholesterol and lower COPs values throughout the whole heating process. Maximum total COPs were achieved after 30 and 120 min of heating for control and melisa-containing samples, respectively. In cooked beef patties, even though the olive oil emulsion was used as flavor-masking approach, melisa extract off-flavor limited the maximum dose which could be added. At these doses (65 μg/g and 150 μg/g without and with the emulsion, respectively), no additional protective effect of melisa over the use of the emulsion was found. Addition of natural extracts into functional foods should definitively take into account sensory aspects.

Keywords: cholesterol oxidation, antioxidant, model system, lemon balm, oddflavor, beef patties

Highlights

1 - A lyophilized *Melissa officinalis* extract protected cholesterol from thermal degradation.

2 - Melisa extract inhibited COPs formation at 180 °C up to 180 min.

3 - Melisa extract off-flavor restricted the viable dose to be added into beef patties.

4 - No protective effect was found in meat patties at sensory acceptable doses**.**

5 - An olive oil in water emulsion exerted antioxidant effect in beef patties**.**

1. Introduction

Cholesterol Oxidation Products (COPs) have been related to several diseases (atherosclerosis, neurodegenerative diseases, mutagenic and carcinogenic effects, etc) (Otaegui-Arrazola et al., 2010; Biasi et al., 2013). They can be formed endogenously and also absorbed from the diet. As cholesterol is present in a variety of animal food samples, thermal oxidation, photooxidation and autooxidation can take place, compromising the safety of food. Thus, minimizing the formation of COPs leads to safer food.

The incorporation of antioxidants has been proposed as a good strategy for preventing sterol oxidation. Promising results have been reported in model systems (Xu et al., 2009; Yen et al., 2010; Kmiecik et al., 2011), using direct application of antioxidants on cholesterol and also on fat matrices, such as triglycerides and lard. Butylhydroxytoluene, Conjugated Linoleic Acid, tocopherol, quercetin, green tea catechins and rosemary extracts, among others, are some of the tested antioxidants. A higher interest on natural antioxidants than on synthetic ones is nowadays increasing among industries and researchers, given their similar or even higher activity (Xu et al., 2009; Kmiecik et al., 2011) and their assumed safer and healthier properties.

Melissa officinalis is a medicinal plant, usually taken as infusion, with a variety of beneficial effects, i.e. anti-depressive, anxiolytic, anti-tumoral, neurobiological and it has also been involved in the regulation of lipidemic disorders and in the prevention of oxidative damage (Encalada et al., 2011; Fazli et al., 2012; Jun et al., 2012; Taiwo et al., 2012; López et al., 2009). Its high antioxidant capacity, due to the presence of phenolic compounds, mainly rosmarinic acid (Barros et al., 2013), has induced its addition, mainly as extracts, in foods to prevent lipid oxidation for both functional and technological purposes (Fazli et al., 2012, Petrovic at el., 2012, Berasategi et al., 2011; García-Iñiguez de Ciriano et al., 2010a; Poyato et al., 2013). However, to our knowledge, the potential inhibitory effect of this plant against cholesterol oxidation and formation of cholesterol oxidation products has not been evaluated yet.

When selecting a natural antioxidant and the concentration to be added to foodstuffs, sensory impact on the product (such as flavor or color) should be considered to achieve desired traits (Karre et al., 2013). These attributes are determinants of whether a consumer will purchase a specific type of meat or not (Goodson et al., 2002). When using melisa, sensory aspects have been evaluated on different meat derivatives, giving rise to products in which no sensory problems were noticed when using up to 686 μg/g in the case of dry fermented sausages (García-Iñiguez de Ciriano et al., 2010a) and up to 965 μg/g in the case of cooked pork sausages (Berasategi et al., 2011). It is worth noting that these are meat derivatives with a high

content of sensory potent spices, that might mask its contribution to potential negative effects.

Considering all this, the aim of this study was to evaluate the antioxidant protective effect of a lyophilized aqueous *M. officinalis* extract against cholesterol degradation and cholesterol oxidation products formation in a model system. Once the effectiveness of melisa in the model system was probed, the application of this extract to a food system (beef patties) was carried out in order to assess its effectiveness as antioxidant at doses that were sensory acceptable.

2. Material and methods

2.1 Reagents

Cholesterol, 5α-cholestane, thiobarbituric acid, trolox, AAPH and fluorescein sodium salt were purchased from Sigma-Aldrich Chemical (Steinhei, Germany). 19-hydroxycholesterol was obtained from Steraloids (Wilton, NH, USA). Tri-sil reagent was obtained from Pierce (Rockford, IL, USA). Acetone, chloroform, ethyl acetate, methanol, hexane, 2-propanol, hydrochloric acid, cyclohexanone, trichloroacetic acid, potassium chloride, potassium hydroxide, anhydrous sodium sulfate and sodium phosphate were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography and dichloromethane for gas chromatography were from Merck (Whitehouse Station, NJ, USA). Strata NH_2 (55 µm, 70 A) 500 mg / 3 mL Solid Phase Extraction cartridges were obtained from Phenomenex (Torrance, USA). *M. officinalis* dried leaves were purchased from Plantaron S.L. (Barcelona, Spain). Beef meat was purchased in a minor local distributor, and showed *Ternera de Navarra* PGI (ES/PGI/0005/0130).

2.2 Preparation and characterization of *M. officinalis* extract

Aqueous extract of *M. officinalis* was prepared as described in García-Íñiguez de Ciriano et al. (2010b). Briefly, 50 g of dried leaves were weighted and added to 500 mL of distilled water, preheated at 100 °C. The mixture was subjected to reflux for 30 min at the temperature above. Extraction process was repeated with 500 mL of distilled water and both extracts were joined, filtered and lyophilized. Determination of its rosmarinic acid content was performed by HPLC-UV as described in García-Íñiguez de Ciriano et al. (2010b). Results were expressed as mg rosmarinic acid/g lyophilized melisa extract. Total Phenolic Content (TPC) was determined as described in Poyato et al. (2013). A 12-mg extract sample was solved in 10 mL water. Reagents were mixed: 237 µL distilled water, 3 µL sample solution, 15 µL of Folin-Ciocalteu's reagent, and 45 µL of 20 % sodium carbonate anhydrous solution. After 2 h in the dark, the absorbance was measured at 765 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG

Labtechnologies, Offenburg, Germany). TPC was expressed as μg gallic acid / mg sample (extract or oil).

2.3 Model system

2.3.1 Heating of samples

Thermo-oxidation of cholesterol was done at 180 °C for various time durations: 0, 10, 20, 30, 60, 120 and 180 min. For the thermo-oxidation, 4 mL of cholesterol standard solution (5 mg/mL hexane) was added into open glass vials (15 x 100 mm). The solvent was evaporated under gentle nitrogen stream. Subsequently, the vials were placed open (allowing enough oxygen disposal) in the Tembloc (P Selecta, Spain) previously stabilized at 180°C. After the corresponding times, vials were removed from the Tembloc and placed in ice for 10 min. The residue was solved in 4 mL hexane and stored at -20 °C until analysis. The same procedure was applied to the *M. officinalis*-containing samples with the following differences: 2 mL of standard solution (10 mg/mL cholesterol and 0.2 mg/mL melisa extract in a trichloromethane:methanol (2:1) mixture) was aliquoted, dried and heated as previously described. The experiment was performed in quadruplicate, with heating treatments done in four different days.

2.3.2 Cholesterol determination

50 μL was aliquoted from each sample (cholesterol or cholesterol+melisa) and 100 μL of the internal standard, 5α-cholestane (2 mg/mL, hexane:2-propanol, 3:2), was added. Chromatographic analysis, identification and quantification were performed according to Conchillo et al. (2005).

2.3.3 Cholesterol Oxidation Products determination

Firstly, 250 μL was aliquoted from each sample and 1 mL of 19-hydroxycholesterol (20 μg/mL, hexane:2-propanol, 3:2) as internal standard was added to the each aliquot. NH_2-SPE was used to separate COPs from non-polar and mid-polar products, as suggested by Rose-Sallin et al. (1995). COPs were finally eluted in acetone, which was further evaporated under a stream of nitrogen (35 °C). Samples were then derivatized to trimethylsilyl (TMS) ethers. Chromatographic analysis, identification and quantification were performed according to the validated method of Menéndez-Carreño et al. (2008b). Seven different COPs were determined: 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 5,6β-cholesterol epoxide (5,6β-CE), 5,6α-cholesterol epoxide (5,6α-CE), 3,5,6-cholestanetriol (CT), 25-hydroxycholesterol (25-HC), and 7-ketocholesterol (7-KC).

2.3.4 Antioxidant capacity along the heating process

Antioxidant capacity was assessed by means of the ORAC method, according to the procedure described in Ou et al., 2001, with slight modifications. Cholesterol and melisa extract containing sample was aliquoted (50 µL) and evaporated under a stream of nitrogen. Phosphate buffer (1 mL) and chloroform (300 µL) were added. Then, the samples were vortexed for 20 s and centrifuged at 4000 rpm for 10 min. A total of 0.5 mL of the aqueous layer was taken and kept in the dark until analysis. A 0.5 M stock solution of Trolox was prepared in 10 mM phosphate buffer, and divided into 1 mL aliquots, which were stored at -20 °C until use. A new set of stock Trolox vials was taken from the freezer daily for the preparation of the calibration curve and the quality controls (12.5 and 50 µM). The phosphate buffer solution was used as blank, to dissolve the Trolox quality controls and to prepare the samples. To conduct the ORAC assay, an aliquot of the sample (40 µL) and 120 µL of the fluorescein solution (132.5 nM) were added to the 96 well black plate. The microplate was equilibrated (5 min, 37 °C), and then the reaction was initiated by the addition of AAPH (40 µL, 300 mM); readings were obtained immediately, in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The results were expressed as mg trolox equivalent/g sample.

2.3.5 Rosmarinic acid content along the heating process

Cholesterol and melisa containing sample was aliquoted (1 mL) and evaporated under a stream of nitrogen. Ultrapure water (1 mL) and hexane (1 mL) were added. The sample was vortexed for 20 s and centrifuged at 1300 g for 6 min. The upper layer was discarded and the process was repeated two more times. The aqueous layer was filtered through a 0.20 µL membrane filter (Millipore, USA) and analyzed using the chromatographic conditions described in García-Iñiguez de Ciriano et al. (2010b). Briefly, in a C18 column, and at a flow rate of 0.8 mL / min, a gradient of acidified water : acetonitrile was applied (starting at 90:10; changing to 70:30 for 20 min; and returning to 90:10 in 7 min). The profiles were recorded at 280 nm. The results were expressed as mg rosmarinic acid / g sample.

2.4 Food system

The experimental design applied to this part of the work is presented in Figure 1. Four different meat patty formulations were assessed, namely simple (S), simple+melisa (SM), emulsion-containing (E) and emulsion-containing+melisa (EM).

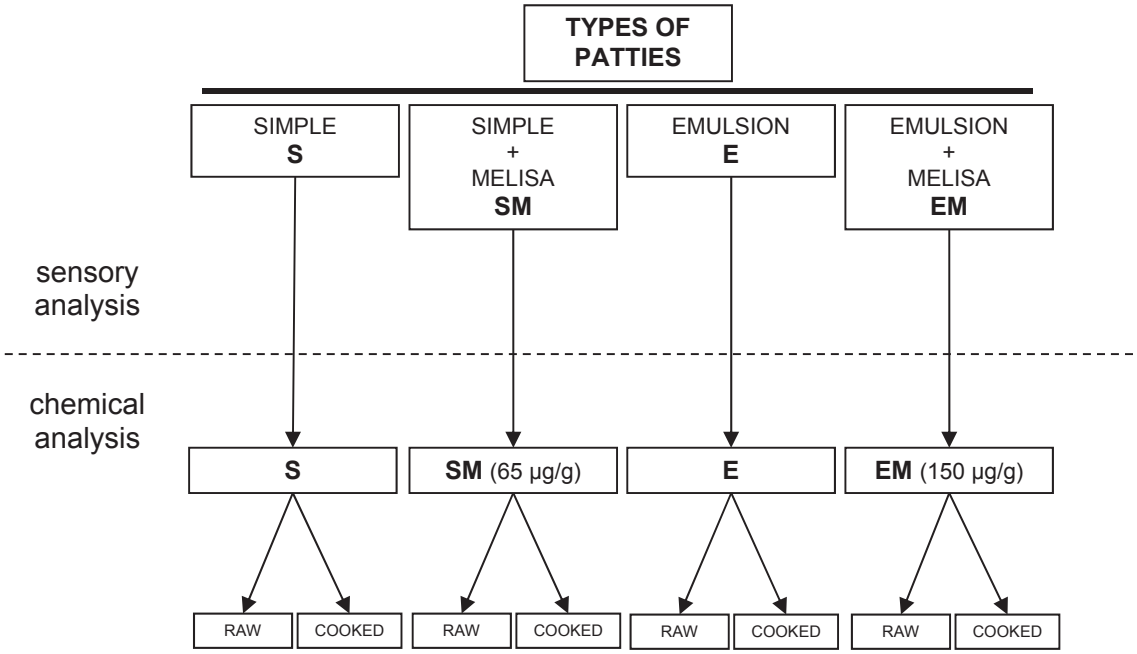

Figure 1. Experimental design for beef patties study.

2.4.1 Total phenolic content in extra virgin olive oil

The procedure was the same as for TPC in the melisa extract but previous phenol extraction was performed, as described in Poyato et al. (2013).

2.4.2 Meat patty preparation

All the patties contained lean beef meat (*Ternera de Navarra* PGI, ES/PGI/0005/0130). Meat was conveniently double-minced and all patties weighed 80 g. "S" patties contained 79.2 g meat and 0.8 g common salt. For "SM" patties, salt was substituted with enriched salt (previously prepared by mixture and homogenization with the *M. officinalis* extract: 16 g salt + 64, 80, 104, 200, 600 or 800 mg melisa extract). Formulation of "E" patties consisted of 75.2 g of meat, 0.8 g salt and 4 g of an oil-in-water emulsion. To make the emulsion, 52.63 g of extra virgin olive oil was slowly added to 42.1 g water (containing 5.3 g soya protein), while continuously homogenizing with an ultra-turrax. For "EM" patties, melisa extract (250, 300 or 400 mg) was added to the water phase of the emulsion before mixing with oil.

Mixture of ingredients was compressed with a conventional burger maker until a compacted and homogenized patty was obtained (80 g, 8.6 cm diameter and 1.5 cm thickness).

2.4.3 Cooking procedure

For the different types of meat patties, four independent batches were prepared, each one containing 4 patties (two to keep raw and two for cooking). Patties were put in a pre-heated

oven at 185 °C for 12 min, reaching 65 °C of internal temperature. Just after the cooking process, they were cooled down for 10 min, weighted, minced, and stored at -20 °C under vacuum until the analysis.

2.4.4 M. officinalis extract addition: sensory evaluation

The determination of the adequate quantity of melisa extract to be added to the meat patties (SM and EM) was done through sensory analysis with 9 semi-trained panelists, by means of a triangle sensory analysis on cooked samples. Panelists were trained by allowing them to taste beef patties in which different doses of melisa were added, in order to help in the identification of its taste. The comparisons made were: S vs SM and E vs EM. In each case, different amounts of *M. officinalis* extract were added to the respective type of patty (500, 375, 125, 65, 50 and 40 μg/g patty for SM and 200, 150 and 125 μg/g patty for EM). Additionally, differences between S and E were analyzed to take into account the potential effect of the emulsion, even without the extract, over the sensory evaluation of the product.

For every comparison, each panelist was presented with three samples, of which two were identical, and asked to indicate which one differed from the others. This process was repeated several times, once for each different concentration of extract tested. The number of correct answers for each type of comparison was determined. According to ISO 4120:2004, for a 9 member-panel the difference between samples was significant if the number of correct answers was 6 ($p< 0.05$).

2.4.5 Moisture, fat and cholesterol content

AOAC official methods were used for moisture and total fat quantitative determination (AOAC, 2002a, b). The determination of cholesterol was similar to that of the model system samples, but previous extraction was made according to Kovacs et al.(1979).

2.4.6 TBARS determination

TBARS values were determined on previously extracted fat according to the method described by Poyato el al. (2013). The absorbance was measured at 532 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany).

2.4.7 Cholesterol Oxidation Products determination

Approximately 0.5g of the previously extracted fat (as reported by Folch, J., Lees, M., Stanley, G.H.S., 1957) was weighted in a flask containing 1M KOH in methanol and 1 mL 19-hydroxycholesterol (20 μg /mL in hexane:isopropanol 3:2) and kept at room temperature for 20 h to complete the cold saponification. The unsaponificable material was extracted with

diethyl ether (3 x 10 mL). The whole organic extract was washed with water (3 x 5 mL) and filtered through anhydrous sodium sulfate. Then it was recovered in a round-bottom flask, and the solvent was evaporated under a stream of nitrogen. Purification by NH_2-SPE, derivatization to trimethylsilyl ethers and analysis by GC-MS were performed following the same procedure as in the model system (Rosesallin et al., 1995; Menéndez-Carreño et al., 2008b).

2.5 Statistical analysis

For the statistical analysis of the data, Stata 12 program (SataCorp LP, Texas, U.S.A.) was used. Mean and standard deviation of data obtained from each replicate were calculated. For the evaluation of the significant differences of the amounts of cholesterol, cholesterol oxides and TBARS along time and among different samples, one factor ANOVA with Bonferroni post hoc multiple comparisons ($p < 0.05$) was applied.

3. Results and discussion

3.1. Model system

3.1.1. Effect of *M. officinalis* extract on cholesterol degradation

Figure 2 shows the percentage of the remaining cholesterol throughout the heating process of cholesterol heated with and without melisa (2 mg melisa / 100 mg cholesterol). A significant drop was noticed for samples without melisa extract (control) after 10 min of heating, when the percentage of remaining cholesterol was 66 %, whereas with melisa it remained at 93 %. Degradation continued for 50 more minutes. As it has been previously found in studies dealing with neat cholesterol thermal stability (Barriuso et al.,2012; Ansorena et al., 2013a), the first stages of heating (10-20 min) were also critical at 180 °C. Throughout the whole process, the values were always lower ($p < 0.05$) for the control than for the treated samples, reaching 23 and 69 % at the end of heating (180 min), respectively. So it can be stated that *M. officinalis* extract, at the dose applied in this study, protected cholesterol from thermal degradation.

Known antioxidants such as green tea catechins and quercetin (200 ppm) have previously demonstrated their effectiveness during cholesterol heating at 180 °C, where, after 30 min, around 60 and 95 % of initial cholesterol were found in control and antioxidant-treated samples respectively (Xu et al., 2009). Yen et al. (2010), using 5 % Conjugated Linolenic Acid in cholesterol, also found a significant decrease in cholesterol degradation (54 vs 67 %, for control and treatment). However, a study using rosemary extract showed no significant differences in campesterol degradation after 4h at 180 °C, although differences among total sterol oxidation products content were detected (Kmiecik et al., 2011).

The cholesterol degradation curve presented a much higher slope for cholesterol alone than for melisa containing samples during the first 10 min, but very similar slopes could be observed thereafter for both samples. This could denote a high protective effect of the melisa during the first 10 min and slower protection thereafter. Accordingly, the antioxidant capacity values (ORAC) found for the model system that included the melisa extract were reduced after the first 10 min. Figure 3 shows that approximately half of the antioxidant capacity initially noticed in the model system was lost after 10 min of heating, decreasing from 43.11 to 23.71 mg trolox / g sample.

The high content of phenolic compounds in the extract matrix (TPC was 356 µg gallic acid / mg extract) could explain its antioxidant effect. As the major antioxidative compound in this water melisa extract was rosmarinic acid (123 mg / g extract), monitorization of its remaining concentration during the heating process was also done (Figure 3). A similar decreasing curve as that of ORAC determination was observed, with a decrease of around 50 % after the first 10 min. Thus, a high correlation between the antioxidant capacity loss and rosmarinic acid loss was noticed (Pearson R = 0.9517). No antioxidant capacity was noticed when cholesterol was heated alone, except after 180 min, where 0.60 mg trolox / g sample was detected, meaning a 5.1 % of total ORAC value at this point. Therefore, the protective effect observed for melisa extract in the current study was mainly attributed to its high rosmarinic acid content, which is a compound known by its antioxidant capacity (Erkan et al., 2008). Nevertheless, even if its contribution should be very important, other compounds found in the extract (showing peaks much smaller but not quantitated) could be also responsible for the antioxidant properties owing to synergistic effects, as it is stated in Miron et al. (2013).

3.1.2. Effect of *M. officinalis* extract on cholesterol oxidation products formation

COPs were progressively formed during heating until they achieved a maximum, and then their concentration started to decrease, following a different pattern depending on the type of COP and sample (Figure 4). Formation of COPs was quick and high in the control sample. At 10 min, 94 µg of total COPs per mg initial cholesterol was formed in the control sample, whereas practically no COPs were formed in the melisa-treated sample. This behavior is in accordance with data from cholesterol degradation, where the best antioxidant effectiveness was recorded during the first 10min of treatment.

The melisa-containing sample continued yielding COPs for a longer time, since the process was retarded in respect to the control. Consequently, the maximum COPs level was achieved at 30 and 120 min for control and treated samples, respectively, yielding 142.97 and 93.03 µg/mg in

control and in melisa-treated samples. Similar times (10 and 20 min) were required in previous studies to reach maximum COPs levels in neat cholesterol samples at 180 °C (Barriuso et al., 2012; Ansorena et al., 2013a). Therefore, it can be stated that *M. officinalis* extracts inhibited cholesterol oxidation products formation by both delaying their appearance and decreasing their formation rate.

COPs formation has been previously reported to be depleted in the presence of phenolic compounds such as green tea catechins and quercetin (Xu et al., 2009) from around 12 % to less than 5 % with respect to the initial cholesterol content after 30min at 180 °C. In the current study, at the same temperature-time conditions, similar reduction was found: from 31 to 11 % cholesterol oxidation. In general, better results have been observed for natural antioxidants than for synthetic ones regarding sterol oxidation products in model systems (Xu et al., 2009; Kmiecik et al., 2011).

Total COPs behavior was significantly affected by 7-ketocholesterol (Fig 4g), which was the most abundant COP among those analyzed. It was followed by epoxy and hydroxyl compounds, with triol at negligible levels (Fig 4e), as expected, giving the lack of water in the medium (Lampi et al., 2002). 25-hydroxycholesterol only suffered a small increase (Fig 4f) which was also expected due to the sterol chain lower likelihood to oxidize in the absence of enzymes.

3.2. Food system: meat patties

3.2.1. Incorporation of the *M. officinalis* extract into meat patties and sensory evaluation

The melisa extract dose used in the model system was 2 mg melisa / 100 mg cholesterol. To extrapolate this concentration to the food matrix (meat patty) it has to be considered that cholesterol is not the only lipid compound susceptible to oxidation in this foodstuff. Taking into account this fact, the concentration chosen was 2 mg melisa / 100 mg lipid fraction, which corresponded to 500 μg melisa /g meat patty. When the sensory evaluation was performed on these meat patties, an unpleasant taste was clearly detected by panelists. Therefore, sensory evaluation of meat patty samples containing decreasing levels of melisa extract (SM) was carried out until a non-detectable concentration of melisa was noticed. The comparison between the control patty (S) and the different melisa-containing patties (SM) in the triangle sensory test (Table 1a) revealed that panellists were able to detect significant differences with doses over 65 μg/g patty.

In order to compare the antioxidant efficiency of melisa extract with that of a recognized potent antioxidant in meat patties (Rodríguez-Carpena et al., 2012b), beef patties containing

extra virgin olive oil (E) were prepared. Besides, patties containing both extra virgin olive oil and melisa extract (EM) were also prepared to check for possible additional or synergistic effects of melisa extract and olive oil. The tasty and flavorful properties of olive oil would efficiently mask melisa oddflavor and would permit to enhance melisa dose in patties. Olive oil was applied through oil-in-water emulsion, where melisa extract was solved within the water phase. This technology has been successfully applied previously by our group in other meat products (García-Íñiguez de Ciriano et al., 2010b; Berasategi et al., 2011) and it has also been used by other authors (López-López et al., 2010) for improving the nutritional properties of lipid fraction of new meat product formulations. In this case, the emulsion was a good system to include higher amounts of the antioxidant, since direct contact with taste buds and melisa is avoided.

It has to be pointed out that the percentage at which the emulsion was present in the formulation (5 %) did not modify the typical sensory properties of beef patties, as panelists were not able to discriminate between S and E samples ($p < 0.05$).

Then, increasing concentrations of melisa vehiculized through the emulsion (EM) were added and sensory tests were performed facing them to E samples (Table 1b). As it was hypothesized, results led to the conclusion that the level of undetectable melisa extract was able to be increased up to 150 μg/g in emulsion-containing patties (EM), compared to 65 μg/g in patties where melisa was not vehiculized within an emulsion (SM).

Higher doses of *M. officinalis* extracts (965 and 686 μg/g) than those used in the current study have been previously added to meat products without noticing sensory problems - Berasategi et al. (2011) with bologna-type products and García-Iñiguez de Ciriano et al. (2010a) with fermented products-. Nevertheless, all these studies dealt with samples rich in aromas and flavors from garlic or red pepper, which can easily mask the sensory oddflavor melisa notes. This was not the case of our fresh beef patties (containing a quite simple formulation: beef meat, water, olive oil, soya protein and salt).

3.2.2. Effect of *M. officinalis* extract on lipid oxidation

Lipid oxidation in raw and cooked conditions was assessed for the following four types of patties: simple, without and with 65 μg/g melisa extract (S and SM) and emulsion-containing samples without and with 150 μg/g melisa extract (E and EM). Overall lipid oxidation results are shown in Figure 5 and cholesterol oxidation was monitored through COPs determination (Table 2). Raw samples did not show significant differences among the four types of patties,

presenting mean values around 0.1 mg MDA / Kg and from 538 to 913 µg COPs/ 00 g dry sample.

Cooking significantly increased the TBARS in simple patties but no efficient protection of the melisa extract was detected (S ≈ SM). The same behavior was observed for COPs: their values significantly increased after cooking in all four types of patties, but similar values were reported for cooked simple patties with and without melisa extract addition (S ≈ SM).

Comparison between simple patties and those that were incorporated with olive oil emulsion allowed us to conclude that the emulsion protected from lipid oxidation, probably due to the high phenolic content of olive oil (143 ppm gallic acid). Potential antioxidant properties of soy protein contained in the emulsion (Bloukas et al., 1997) could also be behind this behavior.

When the higher dose of melisa (150 ppm) was used within the olive oil emulsion, no additional protective effect of melisa was observed over that of the olive oil neither for TBARS nor for COPs values (0.18 vs 0.18 mg MDA/kg and 1030 vs 972 µg COPs/100 g dry sample for E vs EM, respectively).

The concentration was as high as the sensory quality allowed guaranteeing good flavor, so it had to be concluded that melisa extract was not efficient in these conditions. These unfavorable results were probably caused by the low dose of melisa used in the current experiment: sensory requirements have forced the decrease of the concentration of melisa extract below the level at which antioxidant effects can be observed in the meat system. Besides, rosmarinic acid could have disappeared during cooking, decreasing the extract antioxidant capacity, as it occurs in the model system (Figure 3).

A number of studies have reported more successful results in different meat patties enriched with high-phenolic extracts. Rodríguez-Caprena et al., (2012a); Sampaio et al., (2012) and Duthie et al., (2013) obtained from 20 to 85 % of reduction on TBARS values, and detected up to 1.8 mg MDA/kg. In chicken and pork patties, Mariutti et al. (2011), Rodríguez-Carpena et al. (2012a) and Karwowska et al. (2014) reported reductions from 2 to 7-fold as a consequence of antioxidant enrichment (from sage, mustard and avocado), and the COPs contents ranged between 90 and 1350 µg/100g, probably due to the higher doses applied. However, not all of them assessed sensory evaluation of the products, which is critical for acceptability. Karwowska and Dolatowski (2014), using pork meat added with mustard seed, did not find any protection against MDA formation at the end of the cooking procedure (as in the current study), but only after 12 days of storage.

In the current study, *M. officinalis* was much more effective in the model system than in meat patties. This is frequently reported in comparative studies: green tea catechins, tocopherol and quercetin were much more effective when cholesterol was heated alone compared to when the experiment was made within lard (Xu et al., 2009). This lower efficiency found in food-like systems can be related to the presence of other surrounding lipids, which can act as protective factors themselves (Yen et al., 2010; Yen et al., 2011; Ansorena et al., 2013).

In conclusion, *M. officinalis* aqueous extract protected cholesterol from oxidation in the model system, but no protective effect was found in meat patties at sensory-acceptable doses. Therefore, attention should be paid to sensory considerations in the evaluation of natural extracts as a source of bioactive compounds in foods. Besides, new technologies for the incorporation of these possible ingredients should be developed, such as the use of encapsulated structures or gelled emulsions.

4. Acknowledgements

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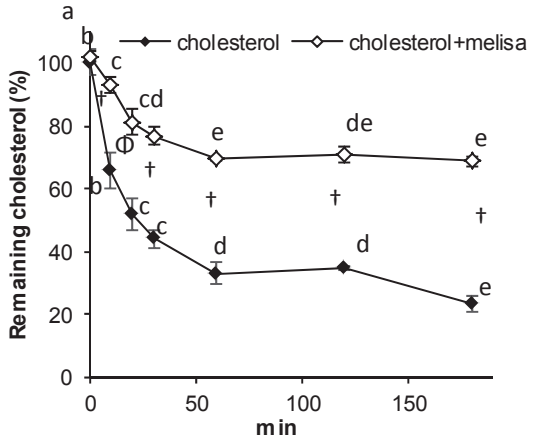

Figure 2. Remaining cholesterol (%) during the heating process in model system. Different letters for each sample denote statistical differences along time ($p < 0.05$). At every time of analysis, Student t test compared both types of samples (* $p < 0.05$; Φ $p < 0.01$; † $p < 0.001$)

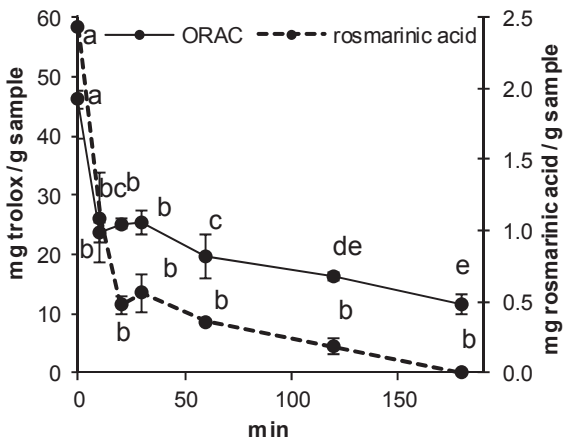

Figure 3. Antioxidant capacity (ORAC determination) and rosmarinic acid content during the heating process in the model system (cholesterol + melisa sample). Different letters for each sample denote statistical differences along time ($p < 0.05$).

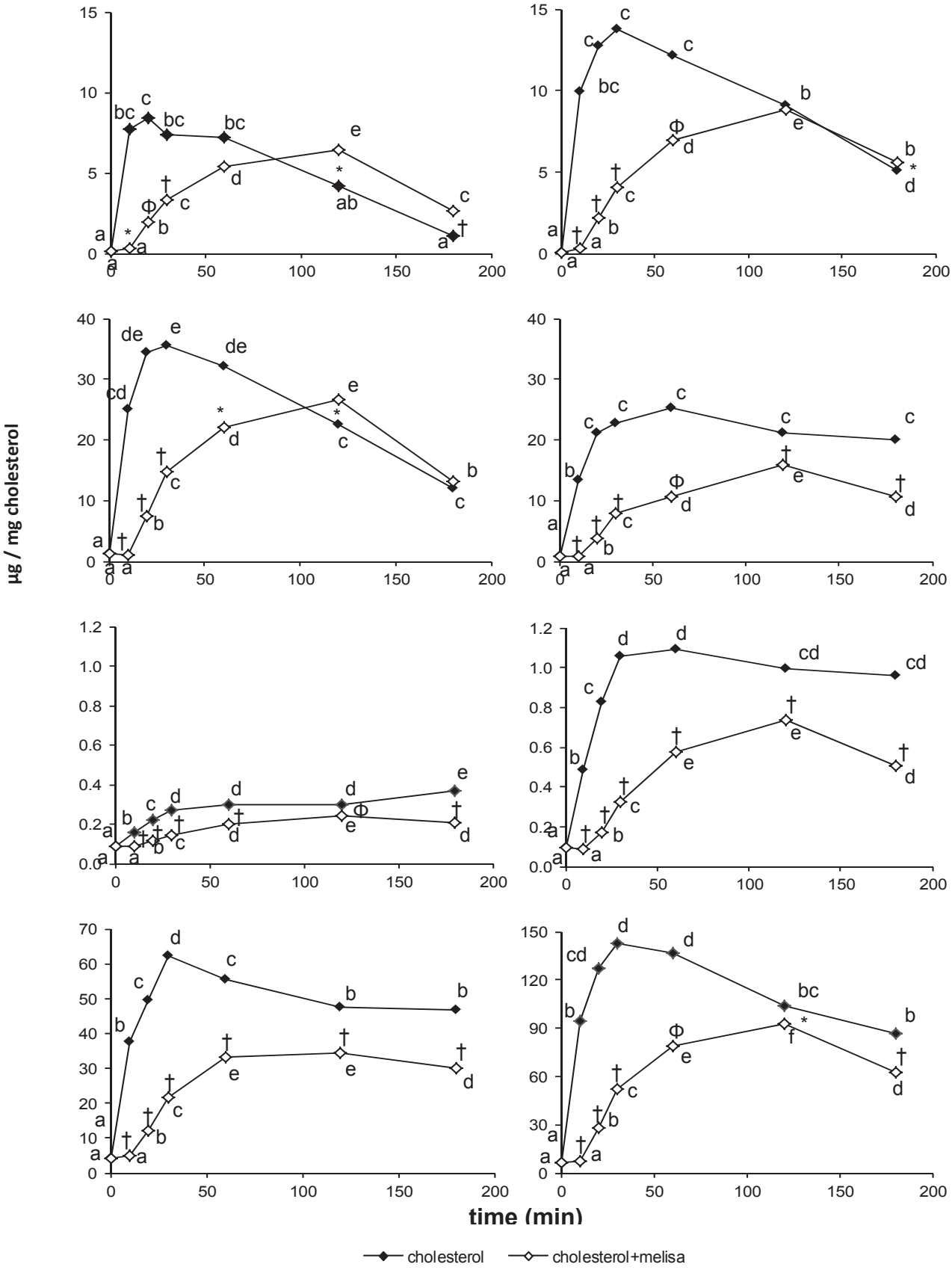

Figure 4. COPs in the model system during the heating process a) 7α-HC, b) 7β-HC, c) 5,6β-EC, d) 5,6α-EC, e) CT, f) 25-HC, g) 7-KC, h) total COPs. Different letters for each sample denote statistical differences along time ($p<0.05$). At every time of analysis, Student t test compared both types of samples (* $p<0.05$; Φ $p<0.01$; † $p<0.001$).

Table 1. Scores of triangle sensory analysis. Comparisons between a) S and SM patties; b) E and EM patties.

a)	**S *vs* SM**					
	500 µg/g	375 µg/g	125 µg/g	65 µg/g	50 µg/g	40 µg/g
Correct replies	9***	9***	7*	4ns	2ns	0
Incorrect replies	0	0	2	5	7	9

b)	**E *vs* EM**		
	200 µg/g	150 µg/g	125 µg/g
Correct replies	9*	4ns	1ns
Incorrect replies	7	11	6

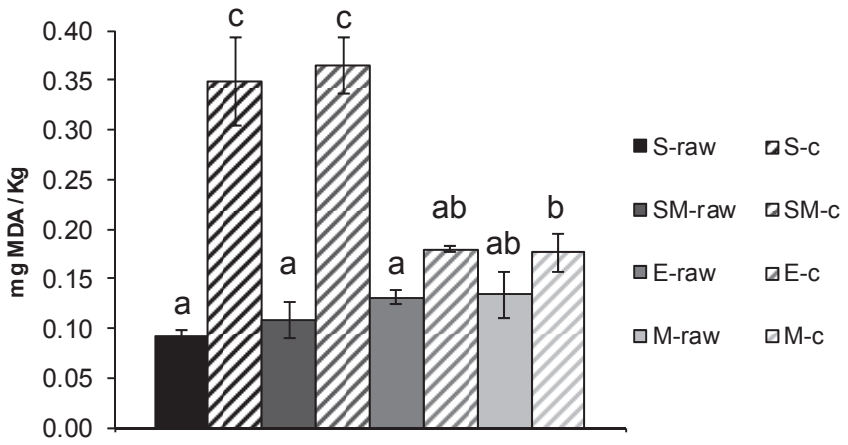

Figure 5. TBARS of beef patties (S, SM, E and EM). For each type of patty, the filled bar indicates raw sample and the striped bar indicates cooked sample. Different letters denote significant differences among samples ($p<0.05$).

Table 2. COPs concentration (µg / 100 g dry sample) in raw and cooked beef patties (S, SM, E and EM). Different letters denote significant differences among samples ($p<0.05$).

	S-raw	**S-c**	**SM-raw**	**SM-c**	**E-raw**	**E-c**	**EM-raw**	**EM-c**
7α-HC	76.26 a	155.21 b	72.84 a	168.06 b	55.24 a	69.26 a	52.66 a	62.21 a
7β-HC	113.81 a	234.76 b	106.17 a	242.25 b	80.83 a	113.25 a	83.96 a	105.89 a
5,6β-EC	286.57 ab	529.20 c	260.49 a	533.72 c	144.91 a	370.90 a	240.51 a	351.49 bc
5,6α-EC	55.21 ab	57.69 a	56.79 a	62.60 a	20.53 c	64.57 b	37.30 b	64.59 a
CT	22.55 a	21.76 a	29.63 ab	13.43 a	33.73 b	20.63 a	19.61 a	24.84 a
25-HC	11.38 a	13.01 b	11.73 a	9.94 a	15.54 c	12.84 b	12.84 b	14.11c
7-KC	347.79 ab	761.47 d	335.80 ab	700.05 d	187.43 a	378.54 a	270.87 a	349.25 bc
Total COPs	913.58 ab	1773.10 d	873.46 ab	1716.46 d	538.22 a	1030.01 c	717.75 a	972.39 bc

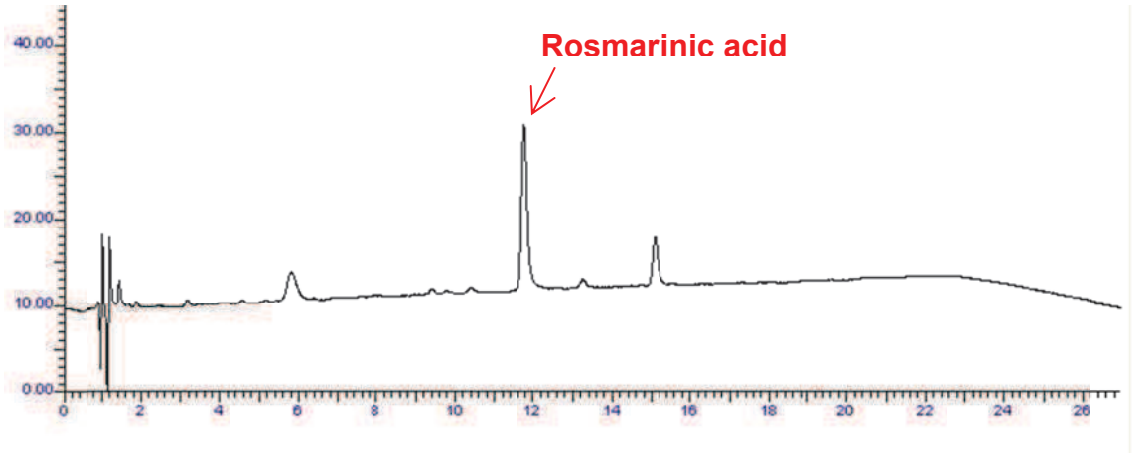

Figure 1 (SupMat). HPLC chromatogram of *Melissa officinalis* extract.

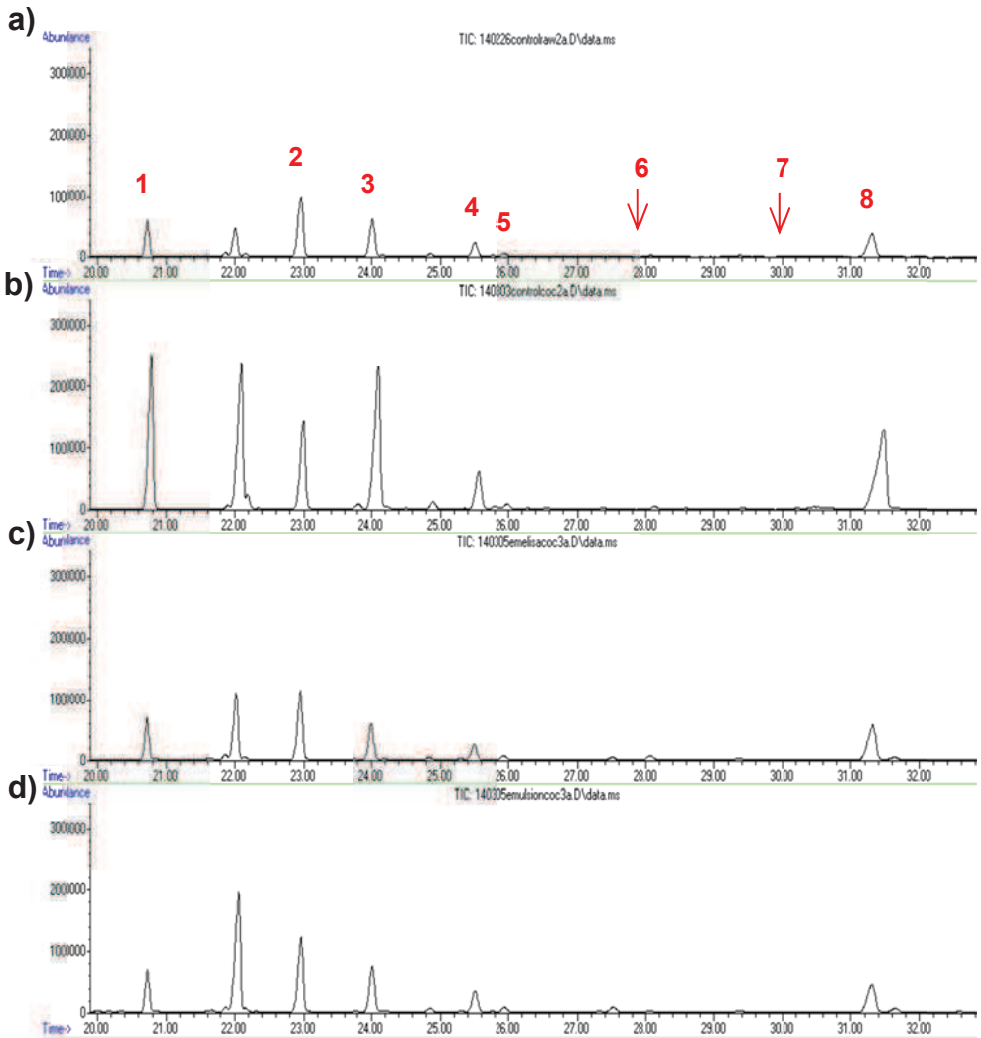

Figure 2 (SupMat). GC chromatograms of beef patties. a) S-raw b) S-cooked c) EM-cooked and d) E-cooked. Numbers indicate the identified COPs; 1: 7α-HC, 2: 19-hydroxycholesterol, 3: 7β-HC, 4: 5,6β-EC, 5: 5,6α-EC, 6: CT, 7: 25-HC, 8: 7-KC.

Table 1 (SupMat). Moisture (%), lipid (%) and cholesterol (mg/100 g) content of the beef patties.

	S-raw	S-c	SM-raw	SM-c	E-raw	E-c	EM-raw	EM-c
moisture	73.16 ± 0.83	66.05 ± 0.42	73.36 ± 0.95	65.88 ± 0.61	71.58 ± 0.06	65.83 ± 0.39	71.53 ± 0.05	66.32 ± 0.21
lipid	2.47 ± 0.16	3.12 ± 0.14	2.61 ± 0.25	3.30 ± 0.14	4.53 ± 0.22	6.09 ± 0.12	4.70 ± 0.21	5.58 ± 0.13
cholesterol	50.30 ± 1.34	54.08 ± 2.04	51.05 ± 1.60	54.22 ± 1.95	47.63 ± 0.66	54.70 ± 1.27	45.04 ± 1.32	54.32 ± 1.65

Results VI

Paper 5

Solanum sessiliflorum (mana-cubiu) antioxidant protective effect towards cholesterol oxidation: influence of docosahexaenoic acid

Plant Foods for Human Nutrition (under revision)

Solanum sessiliflorum (mana-cubiu) antioxidant protective effect towards cholesterol oxidation: influence of docosahexaenoic acid

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Abstract

Harmful health effects have been attributed to cholesterol oxidation products (COPs). Factors that modulate their formation in foods are light, oxygen, heat, and food matrix (such as antioxidants content or unsaturation degree of lipids), among others. The objective of this work was to asses the effectiveness of an extract obtained from *Solanum sessiliflorum* (mana-cubiu) (MCE) as a potential inhibitor of cholesterol oxidation under heating conditions. The influence of free DHA presence in the system was also evaluated. Results showed that MCE inhibited cholesterol degradation (44 % *vs* 18 % without and with MCE, respectively) and reduced 9-fold COPs formation in the absence of DHA. However, when DHA was present, the MCE was not effective towards cholesterol oxidation. In this case, MCE showed its antioxidant effect protecting DHA from degradation (89 % *vs* 64 %). Antioxidant properties of this solvent free natural extract make MCE a potential good ingredient in food products containing highly polyunsaturated lipids.

Keywords: *oxysterols, docosahexaenoic acid, oxidation, natural antioxidants*

Highlights:

1. Mana-cubiu aqueous extract inhibited cholesterol degradation and reduced COPs formation in a model system.
2. Mana-cubiu aqueous extract was not effective towards cholesterol oxidation in the presence of DHA.
3. Presence of DHA inhibited cholesterol degradation.
4. Refrigeration storage up to 72 h did not show any effect on lipid oxidation.

1. Introduction

Cholesterol chemical structure makes it an easy-to-oxidize molecule, leading to the formation of oxysterols. These oxidation products, usually named as Cholesterol Oxidation Products (COPs), have been related to several diseases (atherosclerosis, neurodegenerative diseases, mutagenic and carcinogenic effects, etc) (Otaegui et al., 2010). They have been found in a variety of animal food samples (Otaegui et al., 2010), and some studies have pointed out their potential absorption through the diet (Meunier et al., 2003; Baumgartner et al., 2013). Therefore, minimizing their formation during food manufacturing, processing and / or cooking is of great interest to reduce health risk. Use of additives or oxygen-restriction methods is commonly applied for that purpose.

In this sense, a growing interest in natural antioxidants found in plants is noticed, not only because of technological reasons, but also due to their potential ability to suppress oxidative stress and related diseases. Regarding their usefulness in food systems, successful cases in controlling cholesterol oxidation have been reported in several types of matrices (Mariutti et al., 2011; Sampaio et al., 2012; Karkwowska et al., 2014). Mana-cubiu (*Solanum sessiliflorum*) is a fruit native to Amazonia, which possess antioxidant properties attributed to the presence of carotenoids and phenolic compounds in its composition (Rodrigues et al., 2013).

On the other hand, the interest in highly unsaturated fatty acids has recently increased due to their health-related properties. Particularly, long-chain omega-3 polyunsaturated fatty acids have demonstrated cardiovascular disease lowering effects (Mozaffarian et al. 2011). Nevertheless, inadequate manufacturing and cooking conditions can lead to some loss in the content of these interesting compounds. A number of studies have dealt with the prevention of fatty acid degradation through antioxidant addition, after different cooking and storage conditions (Sampaio et al., 2012; Bhale et al., 2007; Sancho et al., 2011). A possible interaction between cholesterol oxidation and the surrounding fatty acids has been proposed by several authors as a factor that modulates cholesterol oxidation susceptibility, although no consensus on the subject has been found (Soupas et al., 2004; Xu et al., 2009; Ansorena et al., 2013a).

Foods are usually complex matrices where interferences among several components may hamper a clear view about the mechanisms of cholesterol oxidation. Therefore, model systems are a very useful tool to evaluate separately the factors that exert an influence in this process. A variety of antioxidants (Xu et al., 2012; Chien et al., 2006; Kmieck et al., 2011), and lipid matrices (Ansorena et al., 2013a; Lehtonen et al., 2012) have been tested in model systems.

Considering the exposed above, the aim of this study was to evaluate the antioxidant protective effect of a *Solanum sessiliflorum* lyophilized aqueous extract against cholesterol degradation and cholesterol oxidation products formation in a model system, with and without the presence of docosahexaenoic acid (DHA). Furthermore, the effect of refrigeration storage was also evaluated.

2. Material and methods

2.1 Material and reagents

Mana-cubiu fruits (~ 21 Kg) were acquired at CEAGESP (São Paulo General Warehousing and Centers Company, São Paulo, Brazil). Cholesterol, 22R-hydroxycholesterol, 22S-hydroxycholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, 5,6β-epoycholesterol, 5,6α-epoxycholesterol, 7-ketocholesterol and DHA standards were purchased from Sigma-Aldrich. 7α-hydroxycholesterol and 7β-hydroxycholesterol were purchased from Steraloids. The purity of the standards was at least 95% as determined by HPLC or GC analyses. Chloroform and methanol were purchased from Synth. Chromatographic grade hexane (minimum 63% n-hexane) and 2-propanol were purchased from Panreac.

2.2 Mana-cubiu extract preparation and characterization

Mana-cubiu fruits were lyophilized before extraction (Rodrigues et al., 2013). Fifty grams of lyophilized mana-cubiu fruit were homogenized with ultrapure water in a vortex for 5 min and centrifuged at 20000 g at 10 °C. The aqueous layer was lyophilized during 120 h at -92 °C below 40 μHg (Liobras, São Paulo, Brazil). The identification and quantification of the phenolic compounds of the mana-cubiu extract (MCE) was carried out according to Rodrigues et al. (2013).

2.3 Sample preparation and heating

Stock solutions of cholesterol (1 mg/mL in chloroform), DHA (1 mg/mL in chloroform) and MCE (2.5 mg/mL in methanol) were prepared. Four types of samples were prepared: cholesterol alone, cholesterol with MCE, cholesterol with DHA, cholesterol with DHA and MCE. Aliquots of cholesterol solution (1 mL) were poured in test tubes. For DHA and MCE containing samples, 1 mL and 0.2 mL of the corresponding stock solution was added, respectively. Solvent was evaporated under a stream of N_2 and uncapped tubes were placed in a dry block (Marconi, Brazil) at 180 °C. After 7 min heating, tubes were taken out and introduced into an ice water bath for 4 min and capped. Then, they were kept in the fridge (4 °C) for 72 h or in the freezer (-30 °C) until analysis. The experiment was carried out in triplicate.

2.4 Cholesterol and COPs determination

Each sample was dissolved with 1 mL hexane:2-propanol (97:3, v:v), analysed by HPLC-UV-RI, following the same procedure as in Mariutti et al., (2008). Identification of the compounds was confirmed by HPLC-APCI-MS/MS, as in and Zardetto et al., (2014). Chromatographic and MS and MS/MS data are shown in Table 1.

2.5 DHA determination

Docosahexaenoic acid was converted into its methyl ester according to Joseph & Ackman (1992) and analyzed with a gas chromatograph (GC 2010 model, Shimadzu, Kyoto, Japan) equipped with a fused silica CP-SIL 88 capillary column 100 m x 0.25 mm i.d., 0.20 um film thickness (Chrompack, Middelburg, The Netherlands) and flame ionization detector. Chromatographic conditions were described in detail by Sancho et al. (2011).

2.6 Statistics

The data obtained were analyzed by means of the software Stata 12 (SataCorp LP, Texas, U.S.A.). For the evaluation of the significant differences among the amounts of cholesterol and COPs of different samples, one factor ANOVA with Bonferroni post hoc multiple comparisons ($p < 0.05$) was applied. For the evaluation of the significant differences between the amounts of cholesterol, COPs and DHA after 0 and 72 h of storage under refrigeration, Student-t test was applied.

3. Results and discussion

3.1 MCE properties

The profile of phenolic compounds (Figure 1) in the aqueous mana-cubiu extract showed that 5-caffeoylquinic acid (5-CQA) was the main one, representing 2.48 mg/g extract. This is a slightly lower value than that obtained in a methanol-water extract of the same fruit (4.49 mg/g extract) (Rodrigues et al., 2013), where two other compounds were also found: bis- and tris-dihydrocaffeoylspermidine. In the current extract, a small amount of bis-dihydrocaffeoylspermidine was detected, but it was below the limit of quantification. Despite its lower phenolic content, the aqueous extract was interesting, since 5-CQA has demonstrated high antioxidant capacity. This compound is usually found in high amounts in coffee, especially green coffee, or coffee extracts, which have been applied both in model and food systems to protect them from oxidation or to increase their antioxidant capacity (Budryn et al., 2014; Lin et al., 2015). On the other hand, this extract was safe, environmentally friendly and potentially

applicable in foodstuffs, since it was free from organic solvents. Hence, the aqueous MCE was selected for the experiments carried out in this work.

3.2 Effect of MCE on cholesterol and DHA degradation

The initial amount of cholesterol (before heating) was 1.05 mg (Table 2) in all samples. All samples showed a significant decrease in the cholesterol content after heating (presenting values below 0.82 mg in all cases). As it can be observed in Figure 2, higher amounts of remaining cholesterol were found when MCE was added to the sample (80 %), compared to the remaining amount present when cholesterol was heated alone (55 %). This reduction in cholesterol degradation was attributed to the high content in 5-CQA of MCE and its antioxidant capacity. Similar reductions in cholesterol degradation have also been noticed in other model systems using phenolic compounds such as green tea catechins and quercetin (Xu et al., 2009; Chien et al., 2006).

On the other hand, similar values of remaining cholesterol (around 20%) were found for the two types of samples that included DHA in the mixture, regardless the presence of MCE. As compared to cholesterol alone, the presence of free DHA enhanced cholesterol degradation, and MCE could not counteract this effect. Therefore, MCE seemed to protect cholesterol from oxidation in the absence of DHA, but not in the presence of this fatty acid.

The initial amount of DHA (before heating) was 1.00 mg (Table 2). DHA remaining content after the thermal treatment was also analyzed (Fig 3). Both MCE lacking and containing samples showed a significant decrease in the DHA content after heating (0.11 and 0.36 mg remaining, respectively). Heating of DHA alone (without cholesterol nor MCE) resulted in 11.53 ± 3.05 % of remaining compound, so cholesterol had no effect on DHA thermal degradation given that the presence of cholesterol in the mixture yielded the same remaining amount (11 %). Results showed that DHA content was much higher in the presence of MCE (36 %), compared to the previously mentioned remaining amount found in the absence of the extract (11 %). Similar protective effects of natural extracts against DHA degradation have been also reported in studies dealing with fish meatballs and fish oil (Bhale et al., 2007; Sancho et al., 2011). Hence, it could be assumed that MCE antioxidant properties were devoted to protect DHA from degradation, lessening the protective effect towards cholesterol degradation.

3.3 Effect of MCE on COPs formation

COPs content was much higher in cholesterol-alone sample (227 μg COP / mg cholesterol) than in the presence of MCE (25 μg COP / mg cholesterol), as it can be observed in Table 3. On the other hand, similar values (around 87 μg COP / mg cholesterol) were found for both MCE containing and MCE lacking samples when DHA was present in the medium. So again, as it occurred with cholesterol degradation, MCE seemed to prevent from COPs formation in the absence of DHA, but not in the presence of this compound. COPs formation has been previously reported to be depleted in the presence of phenolic compounds (Xu et al., 2009; Barriuso et al., 2015).

Nine COPs were analyzed and only five were found in the samples. From these, 7-KC was the main one in most cases, followed by β-EC and 7β-HC (Table 3). A number of studies dealing with cholesterol oxidation in model systems have reported this profile of COPs (Xu et al., 2009; Barriuso et al., 2015; Rodríguez-Estrada et al., 2014; Derewiaka et al., 2015). The dominance of β-isomer was supported by the steric hindrance at C3 position. Interestingly, when comparing cholesterol and cholesterol+MCE samples, whereas a 90 % reduction in 7-KC was noticed; only a 40% reduction was reported in 7α-HC, becoming the main compound. So the antioxidant extract seemed to show differential behavior towards individual COPs formation. In this sense, reaction rate might be slowed down in the presence of MCE, remaining as 7α-HC for longer time before starting the conversion into 7-KC. Similarly, Kmiecik and co-workers (2009) found differences among sterol oxides distribution depending on the antioxidant applied. This selective inhibition towards individual derivatives could be related to the differences in chemical structure, that could hamper certain positions to be attacked and, hence, certain oxidation derivatives to be formed.

3.4 Effect of DHA on cholesterol degradation and COPs formation

Cholesterol degradation was higher when heated within DHA than when heated alone, as it can be observed in figure 3. The presence of a lipid unsaturated surrounding has been reported to protect cholesterol from oxidation (Ansorena et al., 2013a; Barriuso et al., in press). This discordance could be related to the higher ratio cholesterol:lipid matrix used in the current study (1:2) compared to those ones (1:100). Higher amounts of cholesterol could have hampered the physical protection and favoured cholesterol interaction with highly oxidated DHA. This way, Lehtonen and co-workers (2012), using cholesteryl esters (stechiometry 1:1) found higher levels of oxidation in cholesteryl linoleate than in free cholesterol (0.17 % and 0.084 %), which was attributed to the linoleate double bonds likelihood to radical formation.

Additionally, using free DHA as compared to triglycerides (main constituents of the matrix in Ansorena et al. (2013a) and Barriuso et al. (in press)) makes also an important difference regarding physical protection, chemical group interaction and viscosity, which are key factors in the process (Rodríguez-Estrada et al., 2014).

On the other hand, even though cholesterol degradation was enhanced by DHA, COPs formation was lower than in the absence of DHA, denoting that the routes of cholesterol oxidation were different. Consequently, the oxidation products formed were different, probably oligomers or volatile compounds (Derewiaka et al., 2015; Sosinska et al., 2014). It was also possible that reaction rates for COP degradation were higher than for COP formation in the presence of DHA, giving rise to the aforementioned compounds. Previous studies have shown no correlation between the sterols degradation and the oxides formed (Derewiaka et al., 2015; Oehrl et al., 2001).

3.5 Effect of refrigerated storage

Storage under refrigeration conditions (4 °C, 72 h) modified neither cholesterol levels nor COPs concentration in most cases, except for two samples. Chol+DHA sample slightly decreased its content in COPs, possibly due to degradation of the compounds (Derewiaka et al., 2015). DHA levels suffered no changes along the time either. This behaviour was attributed to the lack of water or any other solvents in the samples, what retarded the oxidation processes.

In conclusion, MCE protected against cholesterol degradation and COPs formation when there was no other lipid compound in the system, but not in the presence of DHA. On the other hand, DHA was effectively protected from oxidation by MCE addition. Considering that it implies a solvent-free extraction process, this mana-cubiu extract could be a potential good ingredient in food products containing highly polyunsaturated lipids.

4. Acknowledgements

We are grateful to the PIUNA (Plan de Investigación de la Universidad de Navarra) and Ministerio de Economía y Competitividad (AGL2014-52636-P) for their contribution to the financial support of this work. B. Barriuso acknowledges Banco Santander and Asociación de Amigos de la Universidad de Navarra for the grants received. We are grateful to “Red de Excelencia Consolider” PROCARSE (AGL2014-51742-REDC). N. Bragagnolo thanks FAPESP (grant # 2013/06489-1) and CNPq for financial support.

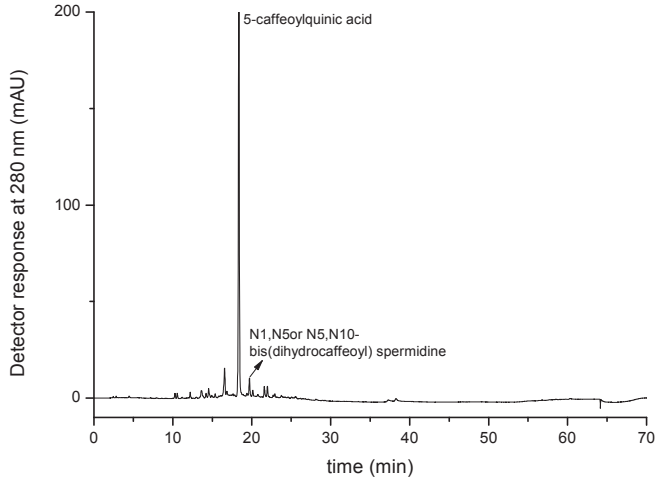

Figure 1. Chromatogram obtained by HPLC-DAD of the phenolic compounds from the aqueous mana-cubiu extract.

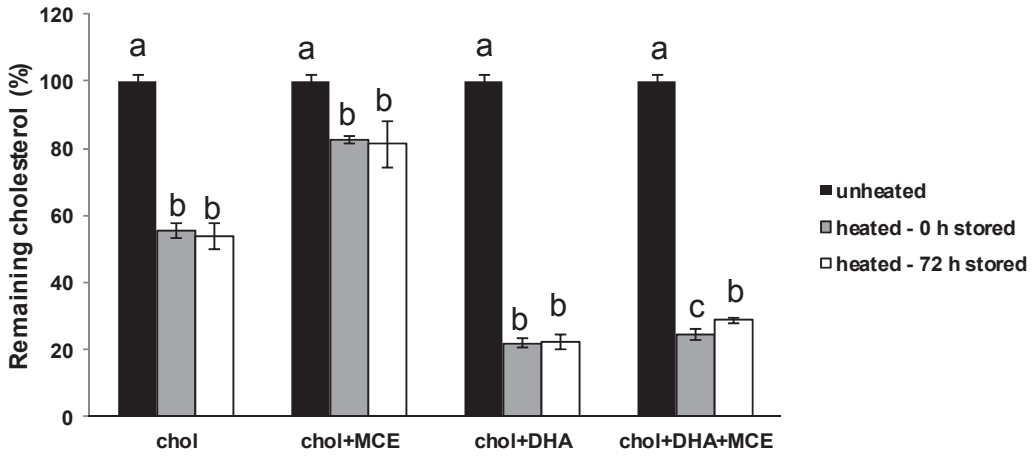

Figure 2. Remaining percentage of cholesterol of the unheated sample and the four heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples.

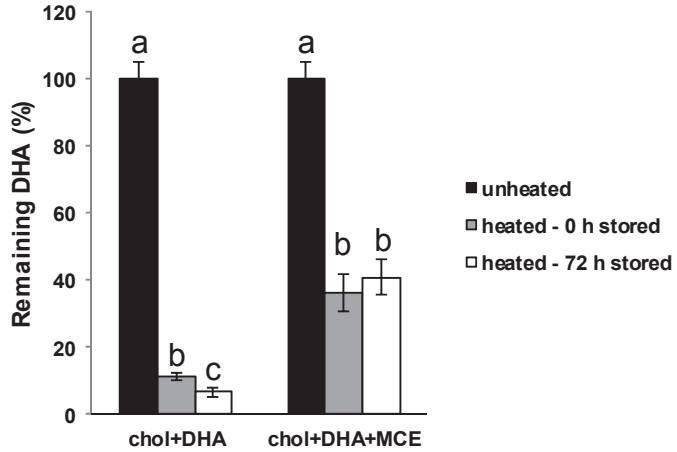

Figure 3. Remaining percentage of DHA of the unheated sample and the two heated samples after 0 and 72 h storage. Different letters for each sample denote statistical differences among the unheated, the 0 h stored and the 72 h stored samples.

Table 1. Chromatographic and mass spectrometry characteristics of cholesterol oxides obtained by HPLC-MS/MS.

Cholesterol oxide	t_r (min)	$[M+H]^+$ *(m/z)*	Fragment ions *(m/z)*
22R-hydroxycholesterol	3.7	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
22S-hydroxycholesterol	4.2	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
20α-hydroxycholesterol	4.4	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
25-hydroxycholesterol	4.7	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
7α-hydroxycholesterol	5.5	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
7-ketocholesterol	5.7	401	383 $[M+H-18]^+$, 365 $[M+H-18-18]^+$
7β-hydroxycholesterol	5.8	nd	385^* $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6β-epoxycholesterol	7.0	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$
5,6α-epoxycholesterol	7.6	403	385 $[M+H-18]^+$, 367 $[M+H-18-18]^+$

nd: Not detected. * In source fragmentation.

Table 2. Cholesterol and DHA content (mg) of unheated cholesterol and DHA, and the four heated samples during storage at 4 °C for 0 and 72 h.

	unheated	chol		chol+MCE		chol+DHA		chol+DHA+MCE	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
cholesterol	1.05 aA	0.56 c	0.54 C ^{ns}	0.82 b	0.81 B ^{ns}	0.22 d	0.22 D ^{ns}	0.25 d	0.29 D *
DHA	1.00 aA	-	-	-	-	0.11 c	0.07 C *	0.36 b	0.41 B ^{ns}

Different lower case letters denote statistical differences among the unheated sample and the heated samples after 0 h. Different capital letters denote statistical differences among the unheated sample and the heated samples after 72 h.
ns: non-significantly different content between 0 and 72 h within each type of sample.
*: significantly different content between 0 and 72 h within each type of sample.

Table 3. Cholesterol oxidation products (μg/mg cholesterol) content of the unheated sample and the four samples during storage at 4 °C for 0 and 72 h.

	unheated	chol		chol+MCE		chol+DHA		chol+DHA+MCE	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
7α-HC	nd	28.90 c	27.78 C ^{ns}	17.07 b	9.55 B *	6.47 a	0.18 A *	10.42 ab	0.18 A *
7β-HC	nd	48.25 c	47.17 C ^{ns}	1.84 a	4.74 A *	13.01 b	11.06 B ^{ns}	13.25 b	15.04 B ^{ns}
5,6β-EC	nd	61.06 c	63.12 C ^{ns}	1.21 a	1.11 A ^{ns}	20.78 b	15.29 B *	20.11 b	18.49 B ^{ns}
5,6α-EC	nd	29.22 c	31.81 C ^{ns}	0.65 a	0.59 A ^{ns}	15.33 b	10.78 B *	12.58 b	10.88 B ^{ns}
7-KC	nq	59.65 c	68.37 C ^{ns}	3.26 a	7.97 A *	31.10 b	25.71 B *	30.78 b	30.64 B ^{ns}
Total COPs	-	227.07 c	238.24 C ^{ns}	24.03 a	23.96 A ^{ns}	86.69 b	63.02 B *	87.14 b	75.22 B ^{ns}

Different lower case letters denote statistical differences among heated samples after 0 h. Different capital letters denote statistical differences among heated samples after 72h.
nd: not detected (detection limit: 7α-HC = 0.98 μg/mg, 7β-HC = 0.46 μg/mg, β-EC = 4.99 μg/mg, α-EC = 3.67 μg/mg)
nq: not quantitated (quantification limit: 7-KC = 1.01 μg/mg)
ns: non-significantly different content between 0 and 72 h within each type of sample.
*: significantly different content between 0 and 72 h within each type of sample

Results VII

Poster 2

Protective effect of a *Solanum sessiliflorum* (mana-cubiu) extract in tuna patties

Protective effect of a *Solanum sessiliflorum* (mana-cubiu) extract in tuna patties

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VIII Jornadas de Investigación en Ciencias Experimentales y de la Salud

INTRODUCTION

Lipid oxidation products exhibit undesirable sensorial attributes and toxic effects. Among them, Cholesterol Oxidation Products (COPs) have been associated with atherosclerosis and other pathologies. An aqueous extract of Mana-Cubiu (MCE) has been reported to exhibit antioxidant properties due to its high content in phenolic compounds.

OBJECTIVE

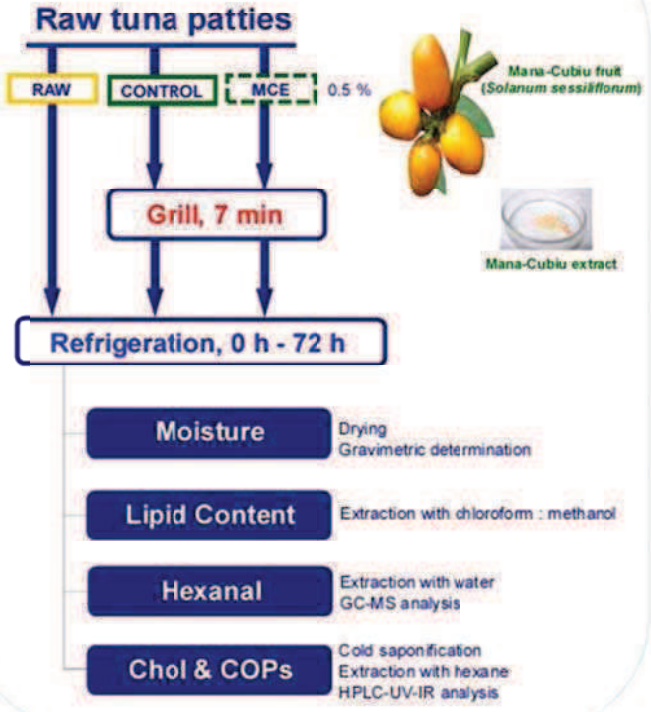
To study the evolution of tunna patties enriched with MCE after grilling and refrigeration storage, assessing:

i) The effect of cooking on general composition and oxidation (hexanal and COPs).

ii) The effect of MCE addition on oxidation (hexanal and COPs).

iii) The effect of storage.

EXPERIMENTAL DESIGN

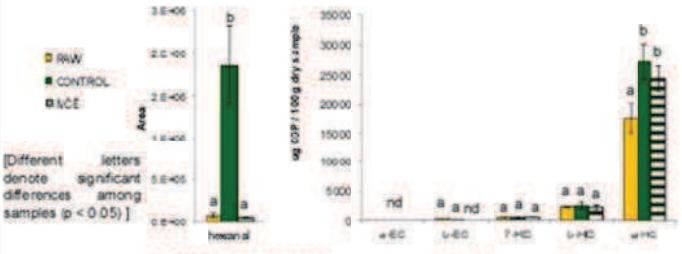


RESULTS

General composition

[Different letters denote significant differences among samples ($p < 0.05$)]	RAW	CONTROL	MCE
Moisture (g/100 g)	75.63 ± 0.46 a	71.01 ± 0.11 b	70.33 ± 0.38 b
Lipid (g/100 g)	1.05 ± 0.03 a	1.23 ± 0.03 b	1.30 ± 0.06 b
Cholesterol (mg/100g dry sample)	151 ± 11 a	145 ± 13 a	145 ± 8 a

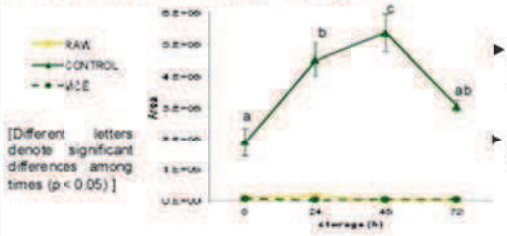
COPs and hexanal content after 0 h storage



▶ Cooking produced hexanal formation

▶ Cooking produced COPs formation

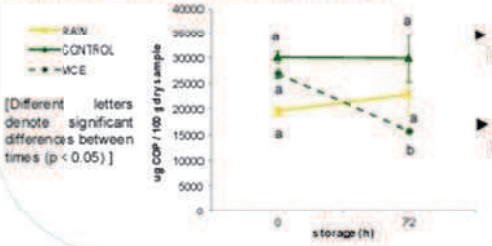
Hexanal content during storage



▶ MCE reduced hexanal formation

▶ Storage affected hexanal content

Total COPs content during storage



▶ MCE did not reduce COPs formation

▶ Storage did not affect COPs content

CONCLUSIONS

1) Cooking produced drying and formation of hexanal and COPs

2) MCE protected against overall lipid oxidation but not COPs formation

3) Storage produced changes in hexanal but not in COPs

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Results VIII

Paper 6

Cholesterol and stigmasterol within a sunflower oil matrix: Thermal degradation and oxysterol formation

Steroids (2015) DOI: 10.1016/j.steroids.2015.02.009

Cholesterol and stigmasterol within a sunflower oil matrix: thermal degradation and oxysterols formation

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Abstract

The characteristics of the lipid matrix surrounding sterols exert a great influence in their thermal oxidation process. The objective of this work was to assess the oxidation susceptibility of equal amounts of cholesterol and stigmasterol within a sunflower oil lipid matrix (ratio 1:1:200) during heating (180 °C, 0-180 min). Remaining percentage of sterols was determined and seven sterol oxidation products (SOPs) were analysed for each type of sterol along the heating treatment. Evolution of the fatty acid profile and vitamin E content of the oil was also studied. Overall oxidation status of the model system was assessed by means of Peroxides Value (PV) and TBARS. PV remained constant from 30 min onwards and TBARS continued increasing along the whole heating treatment. Degradation of both cholesterol and stigmasterol fitted a first order curve (R^2= 0.937 and 0.883, respectively), with very similar degradation constants (0.004 min^{-1} and 0.005 min^{-1}, respectively). However, higher concentrations of oxidation products were found from cholesterol (79 μg/mg) than from stigmasterol (53 μg/mg) at the end of the heating treatment. Profile of individual oxidation products was similar for both sterols, except for the fact that no 25-hydroxystigmasterol was detected. 7α-hydroxy and 7-keto-derivatives were the most abundant SOPs at the end of the treatment. PUFA and vitamin E suffered a significant degradation along the process, which was correlated to sterols oxidation.

Keywords: sterols, vitamin E, polyunsaturated fatty acids

Highlights

1. Cholesterol and stigmasterol fitted a first order degradation curve, with similar kinetic constants.

2. Higher concentrations of oxidation products were found from cholesterol than from stigmasterol.

3. Profile of individual oxidation products was similar for both sterols

4. PUFA and vitamin E degradation correlated to sterols oxidation.

1. Introduction

Sterol oxidation products (SOPs) have been extensively reported to be involved in a variety of pathologies and diseases (Vejux et a., 2008; Bjorkem et al., 2009; Vanmierlo et al., 2013; O'Callaghan et al., 2014). Their formation occurs endogenously both by enzymatic or non-enzymatic pathways, from sterols present in plasma and tissues (Iuliano, 2011). Furthermore, sterol oxidation can also take place in foods before consumption (Otaegui-Arrazola et al., 2010). In this issue, both cholesterol, as the main animal sterol, and plant sterols, which are used to enrich foods due to their hypocholesterolemic capacity, are susceptible to be oxidized in certain conditions, generating oxysterols. The incorporation of these exogenously formed oxysterols into the organism through the diet has been widely discussed. Although the intestinal absorption of these compounds (both from cholesterol and plant sterols) has been demonstrated (Meynier et al., 2005; Staprans et al., 2005; Leonarduzzi et al., 2002), the relevance of oxyphytosterol absorption *vs* endogenous formation of these compounds is still under debate, and some scientific evidence has been achieved (Bang et al., 2008; Baumgartner et al., 2013). So, assessment and control of factors affecting sterol oxidation in foodstuffs is a matter of interest for food safety purposes.

Among these influencing factors, processing, cooking and storage conditions clearly affect the oxysterols formation (Boselli et al., 2010; Lira et al., 2014; Hernández-Becerra et al., 2014). Furthermore, the presence of the surrounding lipids and their unsaturation degree exert some kind of effect, affecting the pattern of resulting oxidation products and also modulating the intensity and rate of oxidative reactions (Oehrl et al., 2001; Lehtonen et al., 2012; Xu et al., 2011; Ansorena et al., 2013a). Additionally, both synthetic and natural antioxidants have presented promising results against sterol oxidation, being phenolic compounds and tocopherols some of the most studied compounds (Kmiecik et al., 2011; Polak et al., 2011). To study in detail all these influencing factors, model systems have been frequently used: from net modelized studies where only chemical standards are used for the experiments (Lehtonen et al., 2012; Xu et al., 2011), often using stigmasterol as the plant sterol, to intermediate model systems, where chemical standards are mixed within foods (Oehrl et al., 2001; Polak et al., 2011; Tabee et al., 2008). This strategy allows isolating factors to be assessed, avoiding interferences among them. Thus, a deeper understanding of the underlying mechanisms is allowed and kinetic curves can be determined easily. Previous studies have been made in this regard, obtaining some mathematical models for sterols' degradation under thermal treatment, when they were heated alone and in the presence of pure triacylglycerols of increasing unsaturation degree (Ansorena et al., 2013a; Barriuso et al., 2012).

Considering all this, and taking into account that a combination of both animal and plant sterols can be found in foods intended for cholesterol-lowering purposes, the aim of the current study was to assess the thermal stability and oxidation susceptibility of a mixture of cholesterol and stigmasterol within a highly unsaturated oil (sunflower oil). The influence of both the saponifiable and unsaponifiable fractions of the oil on these processes was analysed.

2. Experimental

2.1 Reagents and materials

Cholesterol, stigmasterol, 5α-cholestane, heptadecanoic acid, α-tocopherol, tocopherol acetate, ammonium thyocianate and thiobarbituric acid 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, cyclohexanone, trichloroacetic acid, potassium chloride, potassium hydroxide, ammonium iron (II) sulphate, barium chloride, anhydrous sodium sulphate and sodium phosphate 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). Sunflower oil was purchased in a major local distributor.

2.2 Heating process

Cholesterol (30 mg), stigmasterol (30 mg) and sunflower oil (6 g) were dissolved in 25 mL of chloroform. Then, aliquots (1 mL) were placed in tubes and the solvent was evaporated under a gentle stream of N_2. Tubes were then placed in a Tembloc (P Selecta, Spain) and heated at 180 °C for 0, 5, 10, 20, 30, 60, 120 and 180 min. After each corresponding time, tubes were rapidly cooled down and each sample was dissolved in 1 mL chloroform, shaken vigorously to ensure homogenous and accurate aliquoting, added a stream of N_2 gas and sealed. Samples were kept under -20 °C up to one week until analysis (except for Peroxides Value, that was determined immediately after heating). The experiment was performed in triplicate.

2.3 Peroxides value

Peroxides Value (PV) was analysed following the method of Shanta and 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 dissolved 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 (n=6).

2.4 TBARS

TBARS values were determined according to the method described by Poyato et al. (2013). Briefly, an aliquot (1 mL) of sample was transferred to a tube and chloroform was evaporated under a stream of N_2. Distilled water (0.5 mL), BHT (20 µL, 1%) and the TBARS reagent (2 mL) were added to the sample and vortexed, placed in a boiling water bath for 15 min and then cooled down in an ice bath to room temperature. Cyclohexanone (4 mL) and ammonium sulphate (1 mL, 4M) were added to the mixture and vortexed. The mixture was centrifuged at 1300 g for 10 minutes. The absorbance was measured at 532 nm in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). Results were expressed in mg of malondialdehyde (MDA) / Kg sample.

2.5 Sterols determination

An aliquot (50 µL) of sample was transferred to a tube and chloroform was evaporated under a stream of N_2. Cholesterol and stigmasterol determination was performed as described in Barriuso et al. (2012), with slight modifications that only affected the chromatographic conditions applied. In this case, the equipment used was an Agilent 6890N-5975, a Column Varian VF-5ms CP8947 (50 m x 250 µm x 0.25 µm) with the oven conditions as follows: temperature started at 85 °C, increased to 290 °C at a rate of 50 °C /min, increased to 291 °C at a rate of 0.05 °C /min.

2.6 Sterol oxidation products determination

An aliquot (800 µL) of sample was transferred to a tube and chloroform was evaporated under a stream of N_2. 19-hydroxycholesterol (1 mL of 20 µg/mL in hexane:2-propanol, 3:2) was added as internal standard. Solid phase extraction (SPE) was performed for purification of SOPs as described in Ansorena et al. (2013a). The sample solutions of cholesterol and stigmasterol oxidation products were derivatized to trimethylsilyl (TMS) ethers. Their chromatographic analysis, identification and quantification were performed according to the validated method of Menéndez-Carreño et al. (2008b). Calibration curves of COPs were also used to quantify StOPs, 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 and Ulberth, 2004). Seven different SOPs from each sterol were determined: 7α-hydroxy (7α-

H), 7β-hydroxy (7β-H), 5,6β-epoxy (β-E), 5,6α-epoxy (α-CE), 3,5,6-triol (T), 25-hydroxy (25-H), 7-keto (7-K).

2.7 Fatty acids determination

An aliquot (800 μL) of sample was transferred to a round bottom flask and chloroform was evaporated under a stream of N_2. Fatty acid methyl esters (FAME) were prepared by derivatization with Boron trifluoride / Methanol, and their identification and quantification was done by means of Gas Chromatography-FID, as described in Ansorena et al. (2013b).

2.8 Vitamin E determination

The α-tocopherol (α-TOH) content was determined by HPLC-UV analysis according to the method described by Berasategi et al. (2012). Briefly, an aliquot (800 μL) of sample was transferred to a volumetric flask and chloroform was evaporated under a stream of N_2. α-tocopherol acetate (0.1 mL, 10 mg/mL solved in methanol) was added as internal standard and the flask was filled up to 10 mL with previously warmed (30 °C) supergradient HPLC grade methanol. Dilution was vortexed for 30 sec and filtered with 0.20 μm filter (Syringe-driven Filter Unit, Millex®). The sample (10 μL) was injected into the HPLC system and a isocratic elution with methanol/water (97:3) at 1.5 mL/min flow was performed. UV spectra were recorded at 295 nm on a Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA, using an analytical precolumn (3.8 mm x 8 mm with 4 mm x 3 mm of C18 cartridges*, Phenomenex, California, USA*) and a LC18 column (150 mm x 3.9 mm, 4 μm particle size; Waters). Identification of α-tocopherol was done using the retention time of the pure standard compound and its characteristic UV spectra. The quantification was performed using a calibration curve previously plotted with tocopherol acetate.

2.9 Statistics

For the statistical analysis of the data, Stata 12 program was used. Mean and standard deviation of data obtained from each replicate were calculated. Kruskal-Wallis test was applied to evaluate the significant differences on PV, TBARS, sterols, SOPs, vitamin E and fatty acids among different heating times. U de Mann-Whitney test was applied to evaluate the significant differences between cholesterol and stigmasterol data. Correlations were assessed by Spearman´s correlation test.

3. Results and discussion

Overall oxidation status of samples at every point of analysis was assessed by means of both primary and secondary oxidation products (Figure 1). These methods inform about the presence of oxidation compounds originated both from the lipid matrix and the added sterols. Peroxides Value (PV) sharply increased, reaching a maximum at 30 min and maintaining a constant formation-degradation rate afterwards. The maximum value found (16 meq O_2 / Kg) was significantly lower than that reported in a previous study (Ansorena et al., 2013a) in which sterols were heated within unsaturated matrices lacking antioxidants (up to 40 meq O_2 / Kg). On the other hand, TBARS values increased progressively along the whole heating process. Evolution of both parameters was the expected for oxidation processes.

3.1 Sterol-structure effect

Figure 2a showed the evolution of the remaining amounts of cholesterol and stigmasterol during heating. They both followed a very similar degradation pattern, fitting a first order curve (R^2 = 0.937 and 0.883) with similar kinetic constants: [cholesterol] = $93.34e^{-0.004\ t}$; [stigmasterol] = $89.19e^{-0.005\ t}$. This pattern was also observed by Xu et al. (2009), where cholesterol and sitosterol degraded equally under heating treatment. Despite this similar degradation rate during heating, some differences were noticed between the two sterols for the formation of their respective polar oxysterols (Figure 2b).

A small amount of oxidation products were noticed before the heating treatment started, being mainly StOPs, which mostly originated from the sterols standards used (over 95%). Once the heating process was started, higher concentrations of oxidation products were found from cholesterol than from stigmasterol already after 60 min heating. After 180 min treatment, a similar percentage of cholesterol and stigmasterol were still remaining (around 40%) and a difference of 30 μg/mg for oxysterols was detected. This is in accordance to previous works in model and food systems, where similar degradation rates had been noticed for different sterols, but higher amounts of COPs than of StOPs were found within the same experiment (Barriuso et al., 2012; Menéndez-Carreño et al., 2008a). Additionally, Xu and coworkers (2009) reported slightly higher amounts of oxysterols from cholesterol than those from sitosterol after heating samples in several oil matrices.

Cholesterol and stigmasterol are structurally very similar, only differing in their side alkyl chain. Therefore, similar susceptibility to oxidation is expected for their 5, 6 and 7 ring-positions (which were the major oxidation sites found in this work, as it will be explained later). Lengyel et al. (2012) calculated the enthalpy for C-H bond breakage in position 7 and obtained exactly

the same value for both sterols: 328 KJ/mol. Nevertheless, a variety of other factors different than thermodynamics might be involved in the differential oxysterol formation from cholesterol compared to stigmasterol. Different kinetics, distinct dimers formation rate and steric hindrance of the molecule could be taken into account to explain these frequently found results.

3.2 Oxysterols distribution

Despite the different total amount of COPs and StOPs, the distribution and evolution patterns of individual oxidation products were similar for both sterols (Table 1). All oxysterols except for epoxides, increased their presence during heating, being 7-keto derivative the major one during the first stages of the treatment. After 30 min a sharp increase of oxysterols was found, finding a maximum at 120 min heating. At this point, percentage of 43%, 40% and 15% for cholesterol derived compounds and 45%, 38% and 17 % for stigmasterol derived ones were achieved for 7-hydroxydes, epoxides, and 7-ketones, respectively. Although this is not an unusual distribution (Hernández-Becerra et al., 2014; Julien-David et al., 2014), 7-keto derivatives are more frequently found as the major ones, mainly in advanced stages of oxidation. This point was also critical for epoxides behaviour, which started to decrease afterwards, with beta-isomers being formed in higher amounts than alpha ones, as the steric hindrance of hydroxyl group in position 3 determines (Lampi et al., 2002). Oxysterols degradation after long-term high thermal treatments is commonly found. This behaviour has been related to the formation of dimers and oligomers of varying polarity (Struijs et al., 2010; Sosinska et al., 2014). Regarding oxidation that affects the side chain, whereas a small content on 25-hydroxycholesterol could be noticed, no 25-hydroxystigmasterol was detected in the sample. Although the theoretically calculated enthalpies for C25 would indicate a more labile bond for stigmasterol than for other sterols (and the subsequent higher formation of 25-hydroxycholesterol) (Lengyel et al., 2012), no formation of this compound under heating conditions of stigmasterol solved in vegetable oils has been previously reported (Oehrl et al., 2001).

Oxysterols did not counterbalance sterol loss, but they account for around 15% of total sterol degradation since other oxidation products apart from the analysed ones were supposed to be formed (Derewiaka et al., 2015).

3.3 Surrounding matrix effect

The effect of the lipid matrix on sterols oxidation is dependent on temperature and experimental conditions (Soupas et al., 2004). So, to study the effect of the sunflower oil

towards cholesterol and stigmasterol oxidation, data of a previous study performed in the same experimental conditions but without any lipidic surroundings were considered (Barriuso et al., 2012). Sterols heated alone suffered a more intense degradation than within the sunflower oil matrix. After 5 min treatment, the percentages of remaining cholesterol were 60% and 90 % in absence and presence of sunflower oil, respectively. Considering stigmasterol results, 50% and 90 % of the initial sterol content remained in same conditions. On the other hand, oxysterols were formed much faster when sterols were heated alone, reaching after 5 min heating similar values than after 180 min in the presence of sunflower oil. The amount of oxysterols formed was, after 10 min, 79 *vs* 4 µg COPs / mg cholesterol and 51 *vs* 7 µg StOPs / mg stigmasterol for sample alone *vs* sample within sunflower oil.

The protective effect of the lipidic surrounding matrix to sterol oxidation was also described by Ansorena et al. (2013a), using pure triacylglycerols, and by Yen and co-workers (2010;2011), who reported that cholesterol was oxidised more slowly within lard than alone. This behaviour could be related to dilution of the sample, physical protection or competition for oxygen of the surrounding lipids (Rodríguez-Estrada et al., 2014). Table 2 reported the evolution of fatty acids along treatment. Linoleic, the most abundant one in sunflower oil, decreased significantly from the first 10 min of treatment, and a 36% loss was noticed at the end of the heating process. Modification of the rest of fatty acids was less noticeable from the quantitative standpoint.

Furthermore, vitamin E content of the sunflower oil could also be behind this protective effect against the sterol oxidation. In this sense, Xu et al. (2009) attributed to tocopherol the slower oxidation rate of both cholesterol and β-sitosterol in the presence of corn and olive oil. In our work, vitamin E was rapidly affected by heating, giving that a 67% of the initial amount was degraded after 30 min, and a slower but continuous drop was observed thereafter, reaching 80 % loss after 180 min (Table 2). This drastic loss of vitamin E up to 30 min was simultaneous to the maximum in PV and the sharp increase of oxysterols formation, which have been previously mentioned. It seemed therefore that the presence of vitamin E protected from secondary oxidation processes.

To better illustrate this behaviour, Figure 3 plotted the evolution during heating of both sterols (Fig 3a) and their oxides (Fig 3b) along with the evolution of the vitamin E and the main lipid fractions [total polyunsaturated fatty acids (PUFA), total monounsaturated fatty acids (MUFA) and total saturated fatty acids (SFA)]. When correlation coefficients among these curves were calculated, high values for cholesterol and stigmasterol were obtained for PUFA and vitamin E (Spearman $R \geq 0.983$). MUFA behaviour did not correlate with sterol degradation as these fatty

acids only suffered a small degradation from 10 min, and neither SFA correlated as they remained practically stable along time. The same behaviour was observed for total COPs and StOPs: their formation was highly correlated to PUFA and vitamin E degradation (|Spearman R| ≥ 0.993).

In conclusion, when heated within sunflower oil at 180 °C, cholesterol and stigmasterol presented a very similar degradation pattern. Although a similar distribution of individual oxides was noticed, higher values of total oxysterols from cholesterol than from stigmasterol were observed at the end of the heating treatment. The oil matrix protected sterols from oxidation, with vitamin E and PUFA content playing a relevant role in this issue.

4. Acknowledgements

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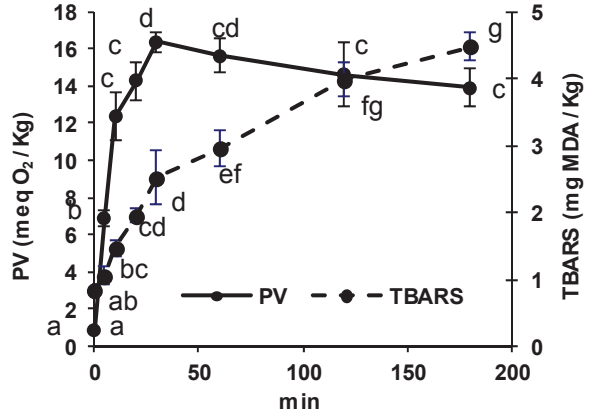

Figure 1. Peroxides Value meq O_2 / Kg and TBARS (mg MDA / Kg) along the heating process. Different letters for each parameter denote statistical differences ($p < 0.05$) along time.

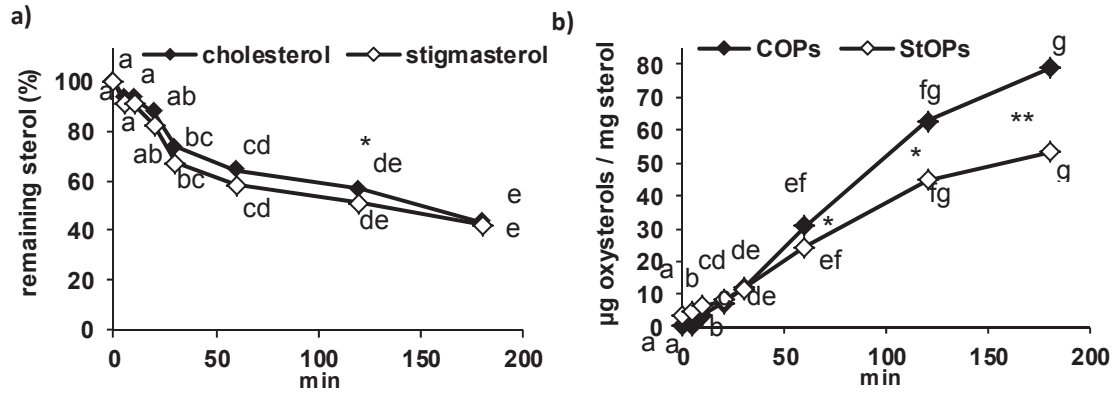

Figure 2. Remaining sterols (a) and content on total oxysterols (b) along the heating process. Different letters denote statistical differences ($p<0.05$) along time. The Mann-Whitney U test compared stigmasterol and cholesterol (and their oxysterols) at every time of analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

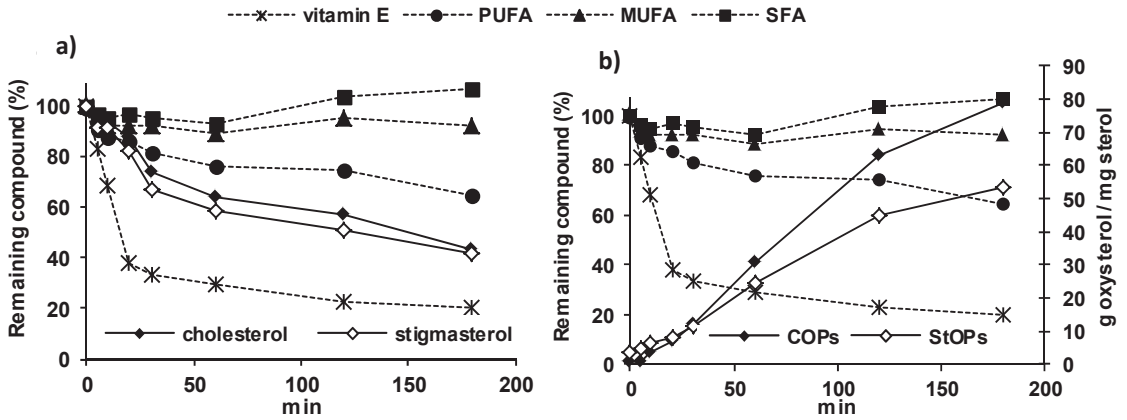

Figure 3. Vitamin E, PUFA (polyunsaturated fatty acids), MUFA (monounsaturated fatty acids) and SFA (saturated fatty acids) evolution as compared to sterols degradation (Figure 3a) or oxysterols formation (Figure 3b) in the sample along the heating process. COPs: total cholesterol oxidation products; StOPs: total stigmasterol oxidation products.

Table 1.
Individual oxysterols from cholesterol and stigmasterol along the heating process (µg oxysterol / mg initial sterol)

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
COPs (Cholesterol oxidation products)								
7α-HC	0.02 ± 0.01	0.07 ± 0.00	0.64 ± 0.12	1.32 ± 0.13	2.31 ± 0.06	6.44 ± 0.42	13.98 ± 0.69	20.24 ± 0.94
7β-HC	0.00 ± 0.00	0.06 ± 0.01	0.51 ± 0.10	1.14 ± 0.13	2.05 ± 0.07	5.99 ± 0.55	12.46 ± 0.65	17.58 ± 0.63
β-EC	0.05 ± 0.02	0.15 ± 0.01	0.95 ± 0.14	1.59 ± 0.09	2.80 ± 0.13	7.33 ± 0.56	14.84 ± 1.20	12.44 ± 0.41
α-EC	0.13 ± 0.02	0.18 ± 0.00	0.91 ± 0.10	1.54 ± 0.12	2.51 ± 0.21	6.09 ± 0.41	10.48 ± 0.90	6.84 ± 0.37
CT	0.00 ± 0.00	0.02 ± 0.00	0.04 ± 0.01	0.10 ± 0.02	0.11 ± 0.00	0.25 ± 0.05	0.45 ± 0.00	0.62 ± 0.08
25-HC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.00	0.16 ± 0.01	0.35 ± 0.03	1.29 ± 0.18	1.79 ± 0.23
7-KC	0.11 ± 0.06	0.33 ± 0.02	1.07 ± 0.17	1.45 ± 0.18	2.03 ± 0.14	4.38 ± 0.45	9.28 ± 1.00	19.51 ± 4.66
StOPs (Stigmasterol oxidation products)								
7α-HS	0.39 ± 0.02	0.51 ± 0.00	0.95 ± 0.08	1.44 ± 0.08	2.17 ± 0.04	5.02 ± 0.36	9.94 ± 0.45	14.08 ± 0.78
7β-HS	0.36 ± 0.08	0.60 ± 0.00	1.02 ± 0.07	1.55 ± 0.10	2.28 ± 0.06	5.34 ± 0.39	10.03 ± 0.52	13.62 ± 0.88
β-ES	0.40 ± 0.15	0.57 ± 0.03	1.12 ± 0.11	1.52 ± 0.08	2.37 ± 0.10	5.59 ± 0.44	10.19 ± 0.86	7.47 ± 0.60
α-ES	0.39 ± 0.14	0.46 ± 0.00	0.95 ± 0.09	1.25 ± 0.15	1.88 ± 0.14	3.93 ± 0.13	6.53 ± 0.84	3.25 ± 0.42
ST	0.10 ± 0.03	0.14 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	0.19 ± 0.01	0.27 ± 0.01	0.43 ± 0.04	0.49 ± 0.09
25-HS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7-KS	1.88 ± 0.39	2.32 ± 0.04	2.37 ± 0.15	2.39 ± 0.06	2.70 ± 0.05	4.33 ± 0.29	7.69 ± 0.73	14.43 ± 2.25

Table 2.
Content on vitamin E (mg / 100 g) and fatty acids (g / 100 g) along the heating process. Different letters denote statistical differences ($p < 0.05$) along time.

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
Vitamin E	71.09 ± 0.83 a	59.21 ± 8.71 b	48.67 ± 2.55 b	26.79 ± 4.89 c	23.79 ± 4.40 cd	20.81 ± 4.71 cd	16.10 ± 1.11 cd	14.24 ± 1.62 d
Palmitic	6.30 ± 0.72 a	6.04 ± 0.09 a	6.00 ± 0.14 a	6.15 ± 0.20 a	6.05 ± 0.28 a	6.01 ± 0.11 a	6.48 ± 0.23 a	6.96 ± 0.01a
t-Palmitoleic	0.02 ± 0.01 a	0.03 ± 0.00 a	0.03 ± 0.00 a	0.03 ± 0.00 a	0.05 ± 0.03 a	0.03 ± 0.00 a	0.03 ± 0.00 a	0.04 ± 0.00 a
Palmitoleic	0.11 ± 0.03 a	0.04 ± 0.00 b	0.05 ± 0.00 b	0.05 ± 0.01 b	0.04 ± 0.00 b	0.05 ± 0.01 b	0.05 ± 0.00 b	0.06 ± 0.00 b
Stearic	4.08 ± 0.26 a	3.61± 0.04 ab	3.55 ± 0.11 b	3.62 ± 0.13 ab	3.64 ± 0.15 ab	3.56 ± 0.13 b	3.97 ± 0.05 a	4.15 ± 0.04 a
Elaidic	0.27 ± 0.13 a	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.03 ± 0.00 b
Oleic	23.71 ± 0.93 a	22.45 ± 0.08 ab	21.91 ± 0.35 b	21.83 ± 0.64 b	21.83 ± 0.56 b	21.10 ± 0.48 b	22.50 ± 0.46 b	21.91 ± 0.38 b
Vaccenic	0.74 ± 0.11 a	0.73 ± 0.00 a	0.66 ±0.08 a	0.68 ± 0.10 a	0.58 ± 0.08 a	0.30 ± 0.03 b	0.70 ± 0.01 a	0.34 ± 0.02 b
t-Linoleic	0.12 ± 0.03 ab	0.05 ± 0.00 b	0.20 ± 0.01 a	0.13 ± 0.02 ab	0.11 ± 0.07 ab	0.09 ± 0.01 ab	0.12 ± 0.02 ab	0.19 ± 0.04 a
c-t linoleic	0.21 ± 0.02 a	0.20 ± 0.00 a	0.22 ± 0.03 a	0.21 ± 0.01 a	0.20 ± 0.03 a	0.20 ± 0.02 a	0.20 ± 0.01 a	0.22 ± 0.01 a
t-c linoleic	0.20 ± 0.06 a	0.21 ± 0.00 ab	0.22 ± 0.01 ab	0.22 ± 0.01 ab	0.21 ± 0.01 ab	0.22 ± 0.02 ab	0.22 ± 0.01 ab	0.25 ± 0.02 b
Linoleic	63.45 ± 3.38 a	57.50 ± 0.40 ab	55.41 ± 1.12 bc	54.34 ± 1.21 bc	51.35 ± 3.80 cd	47.85 ± 0.75 d	46.93 ± 0.98 d	40.50 ± 0.88 e
γ-linolenic	0.05 ± 0.03 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.02 ± 0.00 a
Eicosenoic	0.09 ± 0.02 a	0.01 ± 0.00 b	0.08 ± 0.00 a	0.09 ± 0.00 a	0.09 ± 0.00 a	0.09 ± 0.01 a	0.09 ± 0.01 a	0.08 ± 0.02 a
α-linolenic	0.07 ± 0.01 a	0.06 ± 0.02 a	0.06 ± 0.00 a	0.06 ± 0.00 a	0.06 ± 0.01 a	0.05 ± 0.01 ab	0.04 ± 0.00 b	0.03 ± 0.00 b

Results IX

Paper 7

Unsaturated lipid matrices protect plant sterols from degradation during heating treatment

Food Chemistry (under revision)

Unsaturated lipid matrices protect plant sterols from degradation during heating treatment

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Abstract

In recent years, there has been growing interest in plant sterols enriched foods due to their proven beneficial effects in the human population. In this study, we evaluated the effect of the unsaturation degree of different fatty acids methyl esters (FAME) on a mixture of three plant sterols (PS). Our results indicated that sterols presented a lower degradation rate 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 and 120 min in neat PS and lipid mixtures, respectively. These concentration values were lower in all cases when the unsaturation degree was increased. 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 optimizing the sterol-enriched products formulation, for the maintenance of their healthy properties during cooking or processing.

Keywords: phytosterols, sitosterol, oxidation, oxysterols, SOPs, POPs

Highlights:

1- The presence of FAME slowed down plant sterols degradation at 180 °C.

2- The presence and unsaturation degree of the lipid matrix delayed the POPs formation.

3- Unsaturated FAME reduced POPs formation.

4- Both sterols and FAME degradation fit a first order kinetic model.

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) was prepared in chloroform. Samples (240 mg) 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), 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. Birefly, 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 (Suplemmentary 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).

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), 5,6β-epoxy (β-E), 5,6α-epoxy (α-CE), 3,5,6-triol (T) 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 S. However, mixtures with O, L or 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 control, S, O, L and Ln samples, respectively. At the end of the heating process, the lowest remaining values were for control and S samples (2-10%), followed by 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 control, and 0.0042 to 0.0049 min^{-1} in L and Ln. In a recent publication from our group, similar k values were obtained when cholesterol and stigmasterol were heated in sunflower oil (0.004-0.005

min^{-1}) (Barriuso, Ansorena, Poyato, & Astiasarán, 2015) and also when cholesterol was heated in TAG (0.051-0.004 min^{-1}) (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 S, O, L and 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 PS and FAME. Formation of peroxides in 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 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 FAMES, 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 control samples than in the FAME-containing mixtures, except for the case of P+S samples. Besides, among the FAME-containing samples, the one that yielded the highest POPs content was S, followed by O, whereas L and 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 control sample from 60 min onwards. These results could indicate a slow POPs degradation in this kind of matrix. In the control 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 O, L and Ln samples, they were lower in C and 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).

The amount of total POPs (expressed as μg/mg total initial sterols), showed that those derived from sitosterol, referred to the control sample without FAME, were the most abundant (82 μg/mg in control), followed by derivatives from campesterol (63 μg/mg in control) and finally by those from stigmasterol (16 μg/mg in control), as it could be expected from the relative initial amounts of plant sterols (54 % sitosterol, 30 % campesterol and 15 % stigmasterol).

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 C, and 120 min in FAME-containing samples, values ranged from 71 to 286 μg/mg sterol, 43 to 130 μg/mg sterol and 50 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 FAMEs 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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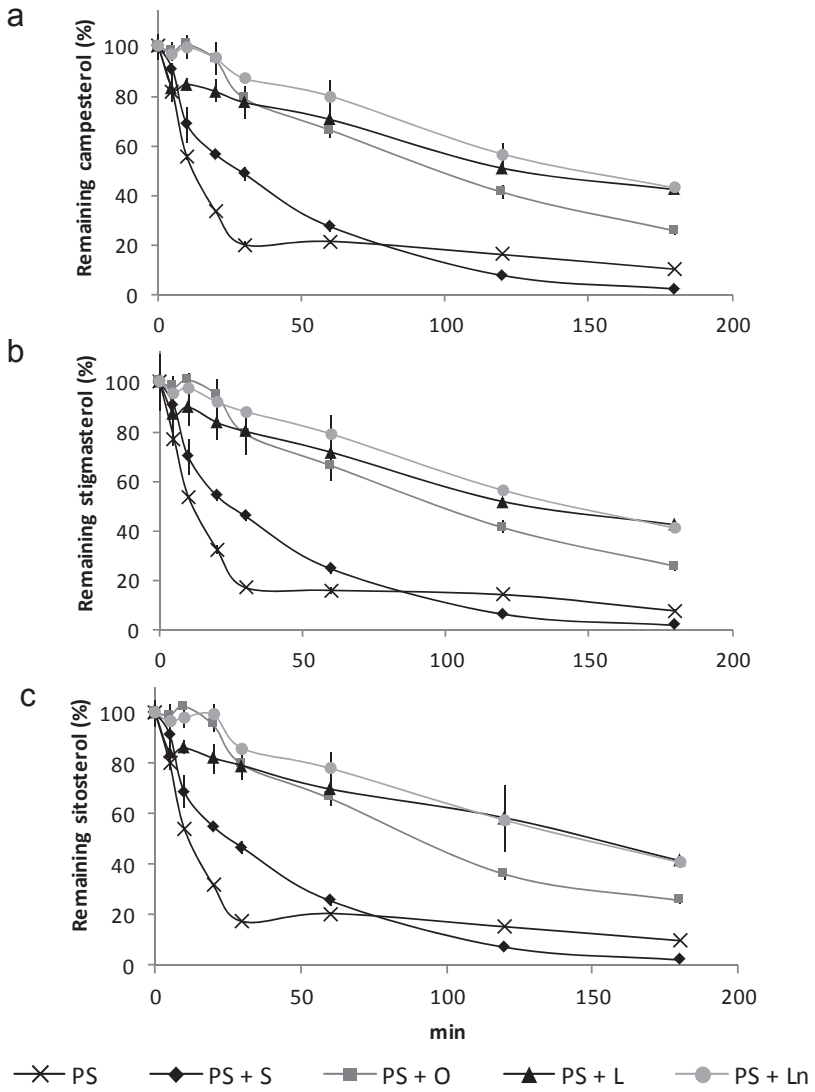

Figure 1. Remaining percentage of plant sterols throughout heating time for plant sterols (PS) and the different FAME-containing mixtures (S, O, L and Ln).

Table 1. Kinetic parameters for remaining FAME and plant sterols throughout the heating process, in plant sterols (PS) and FAME-containing mixtures (S, O, L and Ln).

	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
Plant Sterol	-	-	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 $ln\frac{FAME}{FAME_0} = -k.t$

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

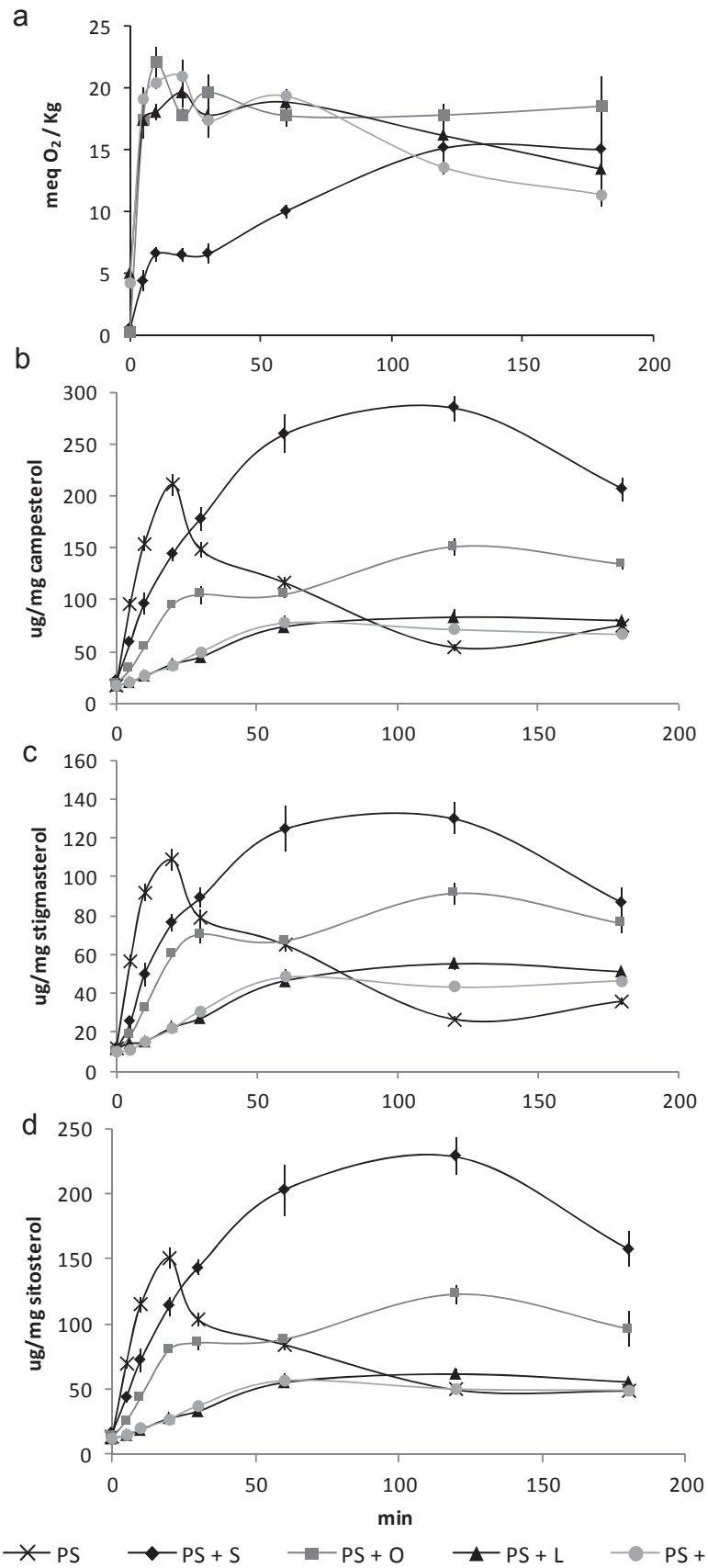

Figure 2. a) Peroxides Value (meq O_2 / Kg) and total plant sterol oxidation products from b) campesterol, c) stigmasterol and d) sitosterol throughout time for PS and the different FAME-containing mixtures (S, O, L and Ln). For each sterol, results are expressed in µg of total POPs per mg of their corresponding sterol.

Table 2. Concentration of campesterol oxidation products (μg/mg campesterol) in plant sterols sample and plant sterols + FAME mixtures, during heating at 180°C.

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
Plant sterols								
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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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 and plant sterols + FAME mixtures, during heating at 180°C.

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
Plant sterols								
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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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 and plant sterols + FAME mixtures, during heating at 180°C.

	time (min)							
	0	**5**	**10**	**20**	**30**	**60**	**120**	**180**
Plant sterols								
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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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
5,6β-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
5,6α-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.

Table S1. Retention times and characteristic ions of sterols within chromatography.

Compound	t_R (min)	Characteristic ions (m/z)
5α-cholestane	13.0	**217***, 357, 372
campesterol	20.5	**343**, 367, 382
stigmasterol	21.1	355, 394, **484**
β-sitosterol	23.2	357, 381, 396, 486

*Ions in bold denote the ion used for integration

Table S2. Retention times and characteristic ions of POPs within chromatography.

Compound	t_R (min)	Characteristic ions (m/z)
19-hydroxycholesterol	25.61	**353*** 366
7α-hydroxycampesterol	25.73	470 **471** 472
7α-hydroxystigmasterol	26.10	482 **483** 484
7α-hydroxysitosterol	27.94	484 **485** 486
7β-hydroxycampesterol	29.74	470 471 **472**
7β-hydroxystigmasterol	29.88	482 483 **484**
5β,6β-epoxycampesterol	31.74	370 383 398 **488**
5α,6α-epoxycampesterol	32.28	398 **380** 488
7β-hydroxysitosterol	32.54	484 485 **486**
5β,6β-epoxystigmasterol	32.59	253 382 410 **500**
5α,6α-epoxystigmasterol	33.13	253 **392** 410 500
campestanetriol	34.84	**417** 418 470 560
5β,6β-epoxysitosterol	35.14	384 394 412 **502**
stigmastanetriol	35.62	**429** 253 482 572
5α,6α-epoxysitosterol	35.78	**394** 397 412 502
7-ketocampesterol	38.74	**486** 381 487 396
sitostanetriol	38.75	**431** 432 484 574
7-ketostigmasterol	39.96	357 359 **498** 347
7-ketositosterol	43.70	395 **500** 510 410

*Ions in bold denote the ion used for integration

General discussion

Lipid and sterol oxidation analysis

Lipid compounds play a major role in foods, since they contribute with particular organoleptic notes to the flavour of products, and also because of their unquestionable nutritional function. From the nutritional perspective, there are essential lipids such as omega-3 fatty acids and hydrophobic vitamins that should be incorporated in the diet to meet recommended intakes. Some of them exert relevant physiological functions, and some others, such as plant sterols, exhibit particular beneficial effects at certain doses. However, the oxidation process affecting lipid compounds is one of the main problems for food industry due to sensory and toxicological consequences.

In this context, the adequate evaluation of the oxidative status of a lipid sample becomes a major need, for both industries and research laboratories. Nowadays, there is a wide variety of analytical methodologies to give response to this need, to satisfy time, cost, scientific reliability and simplicity requirements. Thus, a review of the technics which are currently being used for lipid oxidation analysis in foods was carried out. Most common methods and classical procedures were reviewed (PV, TBARS, SOPs and volatiles, among others). Besides, the increasing and promising use of some alternative methodologies (chemiluminescence, fluorescence emission, Raman spectroscopy, infrared spectroscopy and magnetic resonance) was also commented. To choose among all these possible methodologies, several issues should be considered. The first step is to decide the oxidation compound which is going to be assessed, taking into account the sample's characteristics and the oxidation conditions to which it has been exposed. Then, for selection of the particular method, factors such as analytical reliability and suitability, complexity of the procedure and cost-time efficiency should be taken into consideration.

The interest on sterols, especially on their oxidation derivatives, has risen during the last years. A high intake of cholesterol is related to CVD and its oxidation products have been closely linked to toxicological effects. In the case of phytosterols, their beneficial effects related to reductions in LDL plasma levels have promoted their incorporation into a variety of foodstuffs as functional foods. But their presence in this kind of products could be threatened by oxidation reactions. The analytical process needed for the evaluation of sterol oxidation products (SOPs) is among the most laborious and expensive methods for assessing lipid oxidation, so the optimization of the methodology used is a main goal. Time and cost-efficiency are factors to be considered, without disregarding the achievement of reliable results. Nowadays, there is a great diversity of protocols assessing SOPs among the different research groups. The complex laboratory procedure involves several critical steps, usually

including lipid extraction, saponification, unsaponifiables extraction, SPE purification, derivatization to trimethylsilyl ethers and chromatographic analysis. The analytical conditions play a crucial role in artefact generation, sensitivity thresholds achieved, accuracy, recovery, reproducibility, among other items.

In this work, a comparison between two different SPE purification procedures for SOPs determination was made: 1) purification with silica cartridges and hexane/diethylether mixtures or 2) purification with aminopropyl cartridges and hexane/ethyl acetate mixtures. The procedure using aminopropyl SPE cartridges was demonstrated to be the best in terms of cost, time and analyte recovered. Besides, the stability of the derivatized samples could be guaranteed for up to 13 h before GC-MS analysis.

Moreover, an inter-laboratory harmonization study of the methodologies used for sterols and SOPs determination among different European and non-European research groups was performed. The analysis were carried out in serum samples. Although the complete version of the discussion and conclusions of the whole work is still in preparation, preliminary results suggest a great dispersion of the data: slightly different concentrations are obtained depending on the laboratory. This was most probably due to the diversity of the applied methods. Diverse factors could have affected the results: from sample preparation and preservation, to chromatographic conditions. Consequently, the establishment of a standarized procedure to try to unify as much as possible the analytical protocols would be highly recommended. Therefore, a long-time collaborating-work should be promoted among the oxysterols researchers.

Effect of the heating treatment

Heating is a well-known lipid oxidation inducer, since the activation energy for the hydrogen abstraction is reduced and free radical reaction is enhanced under thermal treatments. Thus, heat intensity and time are key factors affecting sterol oxidation. Different experimental designs based on model systems including different sterols were carried out in this work in order to elucidate factors governing thermal degradation of sterols and formation of their oxides. All the model systems used a temperature at 180 °C during times ranging from 0 to 360 min. We selected 180 °C as the temperature for the experimental procedure since it is characteristic of cooking conditions. High sterol degradation and SOPs formation were observed in all the model systems analyzed. Table 1 summarizes the results obtained of the model systems containing the sterols, as pure standards, without any other matrix in the system.

Table 1. For each sterol, first column represents the percentage of sterol degradation after 10 min of heating and the second column represents the time of heating when the maximum SOPs concentration was achieved.

	cholesterol		**campesterol**		**stigmasterol**		**sitosterol**	
	% 10 m	**max**	***% 10 m***	**max**	***% 10 m***	**max**	***% 10 m***	**max**
Standards (Paper 3)	56	10	72	10	62	5	75	5
Standards (Paper 4)	36	30	-	-	-	-	-	-
Standards (Paper 5)	44 (7min)	-	-	-	-	-	-	-
Standards (Paper 7)	-	-	55	20	54	20	54	20

All the experiments pointed out that the sterols degradation was drastic from the beginning of the heating treatment, exceeding 50% degradation after 10 min. Cholesterol degradation was below this value in the experimental set where higher initial amounts of cholesterol were placed in the tubes (20 mg compared to around 1-2 mg in the other experimental sets). A higher amount of sample hampers the heating transfer and reduces the area-to-volume ratio, slowing down the oxidation process (Lampi et al., 2002).

One of the most changing experimental conditions in sterol degradation research is heating temperature. A diversity of results has arisen, even when comparing results from the same research group (Chien et al., 1998; Chien et al., 2006; Yen et al., 2010). Several works performed at 150 °C using cholesterol samples, have obtained around 50% degradation only after 30-60 min heating, most likely due to the low temperature applied (Chien et al., 2006; Derewiaka et al., 2015). When higher temperature was applied (200°C), up to 89% degradation was achieved in the first 10 minutes (Xu et al., 2005). At that same temperature (200°C), Thanh et al. (2006) obtained, slower phytosterol degradations than those noticed in our work. These authors applied a progressive heating slope instead of the sudden temperature rise used in our study, what could have contributed to obtain these different results.

Regarding experiments attained using similar to our thermo–oxidation treatment, Xu et al. (2005) found 61% of cholesterol degradation after 10 min heating at 175 °C. On the contrary, percentage of around 50% degradation of cholesterol, β-sitosterol and stigmasterol were only achieved after 60 min heating in some works (Xu et al., 2009; Menéndez-Carreño et al., 2010). It seems that the heat treatments applied in the current work have been more destructive than those applied in other studies, since the degradation percentages of sterol are, in general, higher. The capping or decapping disposal of the tubes could have influenced the results, as oxygen presence during heating clearly enhances oxidation susceptibility.

SOPs started to be formed ready at the beginning of the heating process, reached maximum values and started to decrease from 5 to 20 min onwards in most cases, as a consequence of the formation of complex derivatives, such as dimers or trimers, among others. When 20 mg of cholesterol were initially placed in the tube for heating, the maximum COPs value was delayed up to 30 min heating, which again could be attributed to the higher amount of sample compared to the other experimental sets.

Seckin and Metin (2005) and Derewiaka et al. (2015) concluded that changing processing temperatures had an important statistically significant effect on oxycholesterols content. Yen et al. (2010), Zhang et al. (2005) and Chien et al. (2006) did not find any decrease in oxysterol levels even after 60 min heating, noticing constant levels or even increasing trends up to that heating time. In those works, the temperature was 150 °C. However, at 200 °C, Zhang et al. (2005) noted the drop much earlier (20 min). In this context, it has been hypothesized that only drastic heating treatments may induce oxysterol degradation (Thanh et al 2006), and 150 °C could be not enough to achieve it. In this sense, Soupas et al. (2004) proposed 140 °C as the key temperature in the process..

At 175-180 °C, whereas Xu et al. (2005) found the maximum amount of COPs at similar times as our experiment, some other works (Kemmo et al., 2005; Menéndez-Carreño et al., 2010; Xu et al., 2011) obtained SOPs decreases only after 40, 60 and 90 min, respectively. This suggests that other experimental conditions besides temperature and time are behind the differential behavior of oxysterols (higher sterol initial amount, different evaporation procedures, application/lack of filtration methods or exposure to light/oxygen, to name a few). In reference to the incidence of light exposure it should be highlighted that our tubes 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 over-oxidation observed.

The apex in oxysterols levels was followed by a decrease in most of our experiments. The disappearance of oxysterols after a certain moment of the heating process, is related to the formation further oxidized compounds. They can 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 (Lampi et al., 2009; Struijs et al., 2010; Sosinska et al., 2014). They can also consist on steradienes and steratrienes, compounds formed from sterols, 7-ketosterols or 7-hydroxysterols (Bortolomeazzi et al., 2000). After 180 min at 180 °C Menéndez-Carreño et al. (2010) reported that dimeric and polymeric products contributed to 30% of stigmasterol degradation and found strong relationship between non-polar/mid polar and the other

indicators of oxidation reactions; Lampi et al. (2009) found at the same conditions similar percentages of this kind of compounds. Evidence was also provided for the possible formation of oligomeric species of campesterol, sitosterol and cholesterol during heating (Rudzinska et al., 2010; Derewiaka et al., 2015).

Considering the drastic loss in sterol content, alongside the sharp increase in SOPs yielded as a consequence of the heating at 180 °C, this temperature was applied throughout the whole experimental work, when combining the other influencing factors (lipid matrices and antioxidants).

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, FAME or without any surroundings was carried out.

Sterols seemed to degrade similarly regardless of their structure. The four sterols studied showed similar degradation patterns among them. This similar degradation pattern accounts for their similar dissociation entalpies in the sterol ring (Lengyel et al., 2012). Regarding the kinetic models, the parameters for an exponential adjustment of the results are compiled in Table 2.

Table 2. For each sterol, first column shows the kinetic constant and the second column shows the level of adjustment.

	cholesterol		**campesterol**		**stigmasterol**		**sitosterol**	
	k **(min^{-1})**	**R^2**	***k*** **(min^{-1})**	**R^2**	***k*** **(min^{-1})**	**R^2**	***k*** **(min^{-1})**	**R^2**
Standards (Paper 7)	-	-	0.0500	0.906	0.0553	0.943	0.0543	0.917
Sunflower (Paper 6)	0.0044	0.927	-	-	0.0050	0.878	-	-
Stearate (Paper 7)	-	-	0.0237	0.980	0.0261	0.983	0.0253	0.980
Oleate (Paper 7)	-	-	0.0076	0.980	0.0078	0.980	0.0081	0.961
Linoleate (Paper 7)	-	-	0.0046	0.918	0.0048	0.885	0.0042	0.865
Linolenate (Paper 7)	-	-	0.0047	0.952	0.0049	0.947	0.0049	0.952

First order kinetic model corresponding to $\ln(\text{sterol}/\text{sterol}_0) = -k.t$

Very similar kinetic constants were found among sterols during their thermo degradation within the same study and between different studies. Thus, mathematical modelling appears to be very useful for sterol degradation studies, since it enables easy comparison of data from different experimental sets. Several authors have performed kinetic studies concerning sterols (Medina-Meza and Barnaba, 2013), most of them using very elegant approaches and complex mathematical calculations. Chien et al. (1998) 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 here. 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 (Rudzinska et al., 2009; Lampi et al., 2009; Xu et al., 2011; Derewiaka et al., 2015). The evaluation of 7-9 points, as made in our experimental conditions, enables obtaining useful information to build reliable regression curves.

On the other hand, SOPs presented some differences in their formation rate depending on their original sterol. Results from the different experimental sets are compiled in Table 3.

Table 3. Oxidation rate (μg SOPs/mg initial sterol) of the four sterols within the different experimental sets, in decreasing order.

	Oxidation rate (μg SOPs/mg initial sterol)
Standards (Paper 3)	campesterol > sitosterol > cholesterol > stigmasterol
Standards (Paper 7)	campesterol > sitosterol > stigmasterol
Sunflower (Paper 6)	cholesterol > stigmasterol
Stearate (Paper 7)	campesterol > sitosterol > stigmasterol
Oleate (Paper 7)	campesterol > sitosterol > stigmasterol
Linoleate (Paper 7)	campesterol > sitosterol > stigmasterol
Linolenate (Paper 7)	campesterol > sitosterol > stigmasterol

As it can be observed, the behavior is uniform among the different experimental sets. Campesterol presents the higher oxidation rate, expressed as μg SOP/mg initial sterol, regardless of the initial ratio within the mixture of sterols. The following are sitosterol and cholesterol, being stigmasterol the least prone to oxidation.

Campesterol has been previously reported to yield more SOPs than other sterols (Bothelo et al., 2014; Kmiecik et al., 2015, González-Larena et al., 2015). González-Larena et al. (2015), postulated that the different surface activity of sterols could be behind this behavior, based on the results of Cercaci et al. (2007). Thus, 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.

On the contrary, 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 (Menendez-Carreño et al., 2008). This could be attributed to lower efficiency in the

oxidation reaction or slower kinetics. Besides, other oxides instead of the ones we analyzed could have been formed, such as 6-hydroxy, 20-hydroxy, 22-hydroxy, dienes, trienes… (Bortolomeazzi et al., 2003; Kemmo et al., 2005; Derewiaka et al., 2015). Moreover, even if the formation of the current SOPs would be favoured in cholesterol and stigmasterol (over that of campesterol and sitosterol) by their high surface activity, these SOPs could have decomposed to form oligomers, polymers and other compounds characteristic of advanced stages of oxidation (Sosinska et al., 2014; Derewiaka et al., 2015). Hence, the overall balance of SOPs would have been reduced in cholesterol and stigmasterol.

Furthermore, we used different relative initial amounts of sterols within each experimental set, which could also explain the differential oxidation rate, as thermo-oxidation is significantly dependent on the sample area-to-volume ratio (Lampi et al., 2002). In this sense, sterols in low amounts would be overexposed to oxygen since particles present a high area-to volume-ratio, so they show a greater exhibition to pro-oxidation agents. Nevertheless, according to Cercaci et al. (2007), higher sterol concentration would favour their presence in the interfaces and promote the oxidation, contrary as what occurs in the current work.

The double bond in C22 of stigmasterol could lead to the idea of a greater oxidation of this compound but, actually, the side chain double bonds do not affect the reactivity on the side chain (Kemmo et al., 2008; Lengyel et al., 2012). Therefore, it is not surprising that in this work stigmasterol was the less oxidized.

On the other hand, some studies have reported no difference in oxides production among these sterols (Kemmo et al., 2008; Xu et al., 2011).

As it has been reported, absorption of campesterol is higher than that of sitosterol (Ostlund et al., 2002). Considering the higher campesterol oxidation rate compared to sitosterol, potential higher toxicity of foods enriched with campesterol could be hypothesized. The European legislation establishes lower limits for campesterol (<40% of the sterols addition) than for sitosterol (<80% of the sterols addition) addition to functional foods.

Distribution of particular oxysterols was similar among the different kind of sterols. Detailed discussion on this particular distribution is provided below.

Effect of the unsaturation degree

The role that surrounding lipids play in sterol oxidation has been under debate during the last decade. Actually, the scientific discussion still remains alive, since opposing results have been reported by several research groups. Whereas some authors support the hypothesis of a protective effect of a surrounding lipid matrix over the susceptibility of sterols to oxidation, some others state that this condition promotes sterol oxidation. Both trends are supported by strong scientific evidence and have strong arguments behind them. On the one hand, lipids can compete for oxygen with sterols and reduce sterol oxidation by oxidizing themselves. 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. Depending on the experimental conditions, the balance can be displaced to one or another side. In this context, we performed several experiments trying to lighten the matter. Table 4 summarizes the results.

Table 4. For each sterol, first column represents the effect of the lipid matrix on sterol degradation rate and the second column represents the effect of the lipid matrix on oxysterol formation.

	cholesterol		**campesterol**		**stigmasterol**		**sitosterol**	
	Deg	SOPs	*Deg*	SOPs	*Deg*	SOPs	*Deg*	SOPs
Sunflower (Paper 6)	inh	inh	-	-	inh	inh	-	-
Stearate (Paper 7)	-	-	inh	inh	inh	inh	inh	inh
Oleate (Paper 7)	-	-	inh	inh	inh	inh	inh	inh
Linoleate (Paper 7)	-	-	inh	inh	inh	inh	inh	inh
Linolenate (Paper 7)	-	-	inh	inh	inh	inh	inh	inh
DHA (Paper 5)	pro	pro	-	-	-	-	-	-

Deg: effect on sterol degradation rate; SOPs: effect on oxysterols formation;
inh: inhibition; pro: promotion

In our study, the presence of a surrounding lipid was, in general, protective against sterols oxidation. Only in the case of cholesterol+DHA model system there was a promoting effect. The other lipid matrices applied (18:0, 18:1, 18:2 and 18:3, both as FAME and within natural TAG from vegetable oils) inhibited both sterol degradation and SOPs formation.

This study also tried to explain the behavior of sterol degradation during thermal treatment from a statistical point of view. Regression curves were built for every case, obtaining kinetic models, which k values can give an idea of the intensity and rate of sterol degradation. Regarding kinetic models (Table 2), higher k values are found for isolated sterols than within any lipid medium. And almost identical values are observed for stigmasterol within linoleate

and within sunflower oil (containing more than 60 % linoleic acid). Among the different lipid matrices, the more unsaturated ones exhibited a further protective effect, except for DHA, again. K values also decreased with increasing the unsaturation degree of the lipid matrix.

There are several possible explanations for the differential behavior of DHA. First, the high degree of unsaturation favors the formation of many radicals and their pro-oxidant effect is noted. Thus, the pro-oxidant effect of these radicals is noted over the competitive intervention of the unsaturations.

It is also possible that DHA is degraded to such a high extent that it cannot protect anymore the sterols. Actually, only 11% of the initial DHA concentration remained unaltered after the heating process.

As it has been previously commented, a diversity of results has been reported in this issue, not all of them according to the present data. In an attempt to understand the causes of those differences, several hypothesis 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 the surrounding lipids, the oxidation is promoted since the generated radicals are in close contact with the sterol oxidation sites. Hence, the more unsaturated the matrix is, the more sterol oxidation achieved (Lehtonen et al., 2011; 2012).

On the other hand, when sterols are free (not linked to the lipid matrix), the interaction with the lipid matrix is weaker. In these cases, the effect of competition for oxygen of the lipids would be the predominant and increasing the unsaturation degree would imply a reduction in sterol oxidation (Hu and Chen, 2002; Chien et al., 2003; Xu et al., 2011; Ansorena et al., 2013). However, when double bonds are in high numbers, lipid susceptibility to oxidation is extremely high and the large amount of generated pro-oxidant species promotes sterols oxidation. This would be the case of the current work, where free sterols are protected from oxidation in the presence of moderately unsaturated lipids (stearic, 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 results. In this sense, some of the works consider photo-oxidation at room temperature during several days, and some others oxidation induced by heat application. Among the thermos-oxidative experiments, different temperatures have been used, and this factor has been shown to be critical in the protective/promoting effects of the lipids towards sterol oxidation (Soupas et al., 2004). So close attention should be paid to all questions raised.

Effect of the presence of antioxidants

Plant foods are rich in compounds with antioxidant properties. Among them, the most outstanding are vegetable oils, fresh fruits and beverages from fruit and seeds, 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 (Xu et al., 2009; Chen et al., 2010; Mariutti et al., 2011; Hernández-Becerra et al., 2014). Particular antioxidant compounds have been associated with these properties, mainly tocopherols, carotenoids and phenolic compounds.

In this context, both α-tocopherol and phenolic compounds naturally present in sunflower and olive oil, respectively, were tested against cholesterol and stigmasterol oxidation in a model system and in beef patties. These compounds have been previously reported to exert protective effects towards sterols both in model systems and in foods (Polak et al., 2011; Kmiecik et al., 2015). In the current research, the significant content on α-tocopherol (71 mg/100 g) and phenolic compounds (14 mg/100 g) of the vegetable oils were responsible for the inhibition of oxysterols production. Hence, both sunflower and olive oil are potentially interesting vehicles of sterols, to enhance their consumption by limiting their oxidation. The inclusion of vegetable oils as ingredients in foodstuffs can be implemented directly or by means of an emulsified system, depending on the food characteristics (mainly texture and sensory attributes). This approach has been widely used in meat products in order to reduce their SFA content. But, to our knowledge, vegetable oils have not been included in formulations with the aim of protecting sterols, yet. It would be a promising market strategy, considering the increasing public interest towards natural ingredients.

The consumer's interest towards natural products has also promoted the obtention of plant extracts for their addition into foodstuffs (Devatkal et al., 2010; Berasategi et al., 2011; Figueiredo et al., 2014). We selected two promising species, considering their antioxidant properties, for their potential use against cholesterol oxidation: *Melissa officinalis* and *Solanum sessiliflorum*. The results obtained in the several experiments are summarized below:

Table 5. Effect of the presence of melisa and mana-cubiu aqueous extracts on cholesterol degradation and COPs formation.

	Dose	Cholesterol degradation	COPs
Melisa in model system (Paper 4)	2 g/100 g chol	82% inh (10 min)	94% inh (10 min)
Melisa in beef patty (Paper 4)	150 μg/g	No effect	No effect
Mana-cubiu in model system (Paper 5)	1 g/2 g chol	59% inh (7 min)	89% inh (7 min)
Mana-cubiu in tuna patty (Poster 2)	5000 μg/g	No effect	No effect

Their aqueous extracts were strongly effective in isolated model systems containing only cholesterol: they drastically reduced cholesterol degradation and COPs formation. Melisa and mana-cubiu were rich in phenolic acids: rosmarinic (123 mg/g) and 5α-caffeoylquinic acid (2.48 mg/g), respectively. So the antioxidant capacity was attributed to the presence of these compounds in the aqueous extracts. In this sense, Kmiecik et al. (2015) recently hypothesized about the stronger efficiency of phenolic acids compared to other phenolic compounds.

However, they were not effective within beef or tuna patties. In beef patties, the low doses of melisa applied (65 μg/g in non-emulsion containing patties and 150 μg/g in emulsion-containing ones) due to the sensory limitation, were most likely behind the lack of effectiveness. 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 standard of DHA 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 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.

When aiming to reduce COPs formation, the addition of antioxidant extracts to meat or fish patties should always take into account sensory aspects, since their off-flavors commonly impair the applicable dose (Valencia et al., 2008; Berasategi et al., 2011; Karwowska et al., 2014). This is mostly important in foodstuffs with few or none spices in their formulation, such as beef patties. If the extract flavour is not limiting 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, and it is 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 (Valencia et al., 2008; Sancho et al., 2011). Besides, when aiming to reduce COPs formation, antioxidant extracts have been most usually applied to moderately unsaturated foods, rather than highly unsaturated ones (Mariutti et al., 2011; Rodríguez-Carpena et al., 2012b).

Distribution of particular oxysterols

Sterol auto-oxidation yields primarily hydroperoxides, and afterwards alcohols, ketones and epoxides. In the present work, six different oxysterols derived from any of the four sterols were analyzed in the different experiments: 7α-hydroxysterols; 7β-hydroxysterols; 5,6α-epoxysterols; 5,6β-epoxysterols; 3,5,6-steroltriols; 25-hydroxysterols and 7-ketosterols. Table 6 summarizes the profile of oxysterols distribution (excluding 25-hydroxysterol) at the moment of the maximum oxysterol concentration for each experiment, as well as the prevalence alpha/beta epimer of 7-hydroxy and 5,6-epoxy derivatives.

Table 6. Distribution of the oxysterols found at the moment of the maximum oxysterol concentration for each experiment and prevalence of alpha/beta epimer of 7-hydroxy and 5,6-epoxy derivatives.

	distribution	major 7-H epimer	major 5,6-E epimer
Standards (Paper 3)	7-K > 7-H > 5,6-E > triol	alpha	alpha
Standards (Paper 4)	7-K > 5,6-E > 7-H > triol	beta	beta
Standards (Paper 5)	7-K > 5,6-E > 7-H	beta	beta
Standards (Paper 7)	7-K > 5,6-E > 7-H > triol	beta	alpha
Sunflower (Paper 6)	7-H > 5,6-E > 7-K > triol	≈	beta
Stearate (Paper 7)	7-K > 5,6-E > 7-H > triol	beta	alpha
Oleate (Paper 7)	7-K > 5,6-E > 7-H > triol	alpha	beta
Linoleate (Paper 7)	7-K > 5,6-E > 7-H > triol	≈	beta
Linolenate (Paper 7)	7-K > 7-H > 5,6-E > triol	≈	beta
Beef (Paper 4)	7-K > 5,6-E > 7-H > triol	beta	beta
Tuna (poster 2)	7-H > 7-K > 5,6-E	alpha	beta

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, except for sunflower and tuna matrices. 7-ketosterols are commonly found as the main oxysterols both in model and food systems (Xu et al., 2011; Derewiaka et al., 2015). These compounds are moderately stable, being formed either directly from hydroperoxides or through 7-hydroxysterols dehydration (Iuliano, 2011), and are frequently the end of the oxidation route. Hence, they have been extensively reported to be good markers of sterols oxidation (Rodríguez-Estrada et al., 2014).

Nevertheless, in sunflower and tuna matrices 7-hydroxy and 5,6-epoxy were the major compounds. Given the different formation routes possible for these compounds, several hypothesis 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 mechanism, due to the complex environmental feature of the sunflower oil, yielding higher 5,6-epoxy amounts. Photo-oxidation processes could also have influenced the results, by enhancing 7-

hydroxysterols formation. Furthermore, the higher levels of 7-hydroxysterols could indicate that first stages of oxidation are still going on, and low amounts of 7-keto have been formed from them, yet. Although it is not the most common behavior, some works have previously reported this type of distribution profile for oxysterols (Kmiecik et al., 2015; González-Larena et al., 2015).

Triol and 25-hydroxy derivatives were, by far, the less abundant. Triols formation from epoxides is favoured in aqueous acidic mediums, so the low levels found are not unexpected (Menéndez-Carreño et al., 2010; Iuliano et al., 2011). 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 samples analyzed. They were not found among plant sterols' oxides, neither it has been reported by other authors (Oerhl et al., 2001).

Between the pairs of epimers, most of the experiments yielded higher amounts of beta than alpha isomers, except for a few of them. Beta isomers are usually expected to be the predominant because of the steric hindrance at C3 (Iuliano et al., 2011), but a number of studies have reported on the opposing trend (Soupas et al., 2005; Kmiecik et al., 2011; Hernández-Becerra et al., 2014; González-Larena et al., 2015).

Concluding remarks

Sterols' thermo-oxidation is a multifactorial process which strongly depends on time-temperature combination, producing a high sterol oxidation from the beginning of the process. The presence and unsaturation degree of the lipid matrix, as well as the presence of phenolics and tocopherols significantly protected sterols from oxidation in model systems. The inclusion of plant extracts in foodstuffs to achieve this same goal, appeared to be promising if sensory aspects and characteristics of the sample are taken into account. The monitoring of sterol oxidation through the measurement of the oxides generated is a complex issue (as for general lipid oxidation assessment), hence a scientific consensus to achieve a standardized methodology is still needed.

Conclusions

The current work has led to the following conclusions:

1. Purification of oxysterols constitutes a key step during their analysis. The use of aminopropyl cartridges washed with hexane/ethyl acetate resulted in a better strategy than the use of silica cartridges in terms of cost and time of analysis. Considering the complexity of oxysterols analysis and data dispersion obtained in interlaboratory trials, a scientific consensus to achieve a standardized methodology is essential.
2. Heating cholesterol and plant sterols at 180 °C produced high sterol degradation already from the beginning of the treatment, leading to oxysterols formation. Maximum amounts of oxysterols were observed after 10-20 min in the absence of a surrounding matrix, whereas this maximum was delayed up to 120 min in the presence of lipids or of an antioxidant aqueous melisa extract.
3. Under heating treatments at 180 °C for up to 360 min, cholesterol, campesterol, stigmasterol and sitosterol showed similar degradation rates. However, greater amounts of oxidation products derived from campesterol were observed, compared to those derived from the other three sterols.
4. The unsaturation degree of the surrounding lipids protected against sterol oxidation. In general, the higher the unsaturation degree, the higher the protective effect.
5. *Melissa officinalis (melisa)* and *Solanum sessiliflorum* (mana-cubiu) aqueous extracts protected cholesterol from oxidation in model systems, reducing approximately 90% the formation of oxycholesterols. However, both extracts showed difficulties in their application into foodstuffs. Particularly, when added to beef patties, the melisa extract was not effective since its off-flavor limited the applicable dose to 150 μg/g. When added to tuna patties, the mana-cubiu extract was not effective because the highly unsaturated fatty acids of this food competed with cholesterol for the antioxidant effect.

El presente trabajo ha permitido concluir que:

1. La purificación de esteroles constituye un paso clave durante su análisis. El uso de cartuchos de aminopropil lavados con hexano/acetato de etilo resultó una mejor estrategia que el uso de cartuchos de sílice, en cuanto a costes y tiempo de análisis. Teniendo en cuenta la complejidad del análisis de esteroles y la dispersión de datos obtenida en el estudio interlaboratorio, es esencial un consenso científico para conseguir una metodología estandarizada.
2. El calentamiento de colesterol y esteroles vegetales a 180 °C produjo una alta degradación de esteroles ya desde el inicio del tratamiento, llevando a la formación de esteroles. Las cantidades máximas de oxiesteroles se observaron tras 10-20 min en ausencia de matriz circundante, mientras que este máximo se retrasó hasta los 120 min en presencia de lípidos o de un extracto acuoso antioxidante de melisa.
3. El tratamiento térmico a 180 °C durante un máximo de 360 min produjo una degradación similar de colesterol, campesterol, estigmasterol y sitosterol. Sin embargo, se observaron mayores niveles de productos de oxidación derivados del campesterol que de los demás esteroles.
4. El grado de insaturación de la matriz lipídica en la que se encuentran protegió a los esteroles de la oxidación. En general, a mayor grado de insaturación, se observó un mayor grado de protección.
5. Los extractos acuosos de *Melissa officinalis (melisa)* y *Solanum sessiliflorum* (mana-cubiu) protegieron al colesterol de la oxidación en sistemas modelo, reduciendo aproximadamente un 90% la formación de oxiesteroles. Sin embargo, ambos extractos mostraron dificultades en su aplicación en alimentos. Concretamente, cuando se incorporó en hamburguesas de ternera, el extracto de melisa no fue efectivo puesto que su sabor desagradable limitó la dosis aplicable a 150 µg/g. Cuando se incorporó en hamburguesas de atún, el extracto de mana-cubiu no fue efectivo porque los ácidos grasos altamente insaturados de este alimento compitieron con el colesterol por el efecto antioxidante.

List of abbreviations

LIST OF ABBREVIATIONS

ABC	ATP-binding casette
ACAT	acyl-CoA acyltransferase
ANOVA	Analysis Of the Variance
AOAC	Association of Official Analytical Chemists
BHT	Butylated Hydroxytoluene
CL	Chemiluminescence
COPs	Cholesterol Oxidation Products
CVD	Cardiovascular Diseases
DHA	Docosahexaenoic acid
DHS	Dynamic Headspace
DNPH	2,4-dinitrophenylhydrazine
EFSA	European Food and Dafety Association
EPR	Electron Paramagnetic Resonance
ESI	Electrospray *Ionization*
FAME	Fatty Acids Methyl Esters
FID	Flame Ionization Detector
FTIR	Fourier Transform Infrared
GC	Gas Chromatograph
HS	Headspace
HDL	High Hensity Lipoproteins
HPLC	High Performance Liquid Chromatography
HPSEC	High Performance Size Exclusion Chromatography
IR	Infrared
LDI-TOF	Laser Desorption/Ionization-Time Of Flight
LDL	Low Density Lipoproteins
LXR	Liver X Receptor
MDA	Malondialdehyde
MSD	Mass Spectrometer Detector
MUFA	Monounsaturated Fatty Acids
NMR	Nuclear Magnetic Resonance
ORAC	Oxygen Radical Absorbance Capacity
PAV	Para-Anisidine Value
POPs	Phytosterol Oxidation Products

PUFA	Polyunsaturated Fatty Acids
PV	Peroxides Value
RPDE	Reduced Pressure Distillation Extraction
SDE	Simultaneous Distillation Extraction
SERS	S*urface Enhanced RamanSspectroscopy*
SHS	Static Headspace
SFA	Saturated Fatty Acids
SIM	Single Ion Monitoring
SOPs	Sterol Oxidation Products
SPME	Solid Phase Micro-Extraction
SREBPs	Sterol Regulatory Element Binding Proteins
StOPs	Stigmasterol Oxidation Products
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TEP	1,1,3,3-tetraethoxypropane
TG, TAG	triglycerides, triacylglycerides
TMP	1,1,3,3-tetramethoxypropane
TMS	Trimethylsilyl
TPC	Total Phenolic Compounds
UV-Vis	Ultraviolet-Visible
VLDL	Very Low Density Lipoproteins

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Dissemination of results

ARTICLES IN SCIENTIFIC JOURNALS

B. **Barriuso**, A. Otaegui-Arrazola, M. Menéndez-Carreño, I. Astiasarán, D. Ansorena. (2012) "*Sterols heating: Degradation and formation of their ring-structure polar oxidation products*". Food Chemistry, 135, 706–712 Q-1 (Food Science and Technology)

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B. **Barriuso**, I. Astiasarán, D. Ansorena "*Cholesterol and stigmasterol thermoxidation within a sunflower oil matrix*". 4th ENOR Symposium, Coimbra (Portugal), 2014

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B. **Barriuso**, I. Astiasarán, D. Ansorena. "*Kinetic behaviour of sterols and sterol oxidation products during heating" V Jornadas de Investigación en Ciencias Experimentales y de la Salud*, Pamplona (Spain), 2012

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B. **Barriuso**, L.R.B. Mariutti, D. Ansorena, I. Astiasarán, N. Bragagnolo. "*Protective effect of a Solanum Sessiliflorum (Mana-cubiu) extract in tuna patties" VIII Jornadas de Investigación en Ciencias Experimentales y de la Salud*, Pamplona (Spain), 2015