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STUDY OF THE EFFECT OF MINOR COMPOUNDS ON THE OXIDATIVE STABILITY AND PHYSICAL PROPERTIES OF BULK OIL, OIL-IN-WATER EMULSION, AND FOOD EMULSIONS

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1. Abstract

1. Abstract

The present thesis was focused on the study of the effect of minor compounds on the oxidative stability and physical properties of bulk oil, oil-in-water emulsions, and food emulsions. For this purpose, various classes of lipophilic minor components (plant sterols and α-tocopherol) were selected. The choice of these types of molecules was based on the fact that they are widely used in the food industry due to their biological and antioxidant properties. However, it should be considered and understood how these minor components could impact lipid oxidation in food as related to matrix nature/composition and heat treatment and how their physical location could affect the chemical and physical stability of the food product.

Plant sterols/stanols (PS) have demonstrated a cholesterol-lowering effect, so they are used to enrich food products (1-3 g on a daily basis consumption). In general, the percentage of sterols in PS-enriched food products greatly vary up to 8.0%, depending on the type of food product, the consumption market area and the daily consumption volumes. Analytical methods for the determination of PS have been developed for the evaluation of edible oils or food vegetable extracts with ≤1% of sterols as naturally occurring minor components. Conventional GC-FID is usually performed for quali-quantitative chromatographic analysis of PS in enriched and non-enriched products, while GC–MS is often employed for peak identification. Considering the explosion of PS-enriched products in the market, there is a need for developing new and fast analytical methods able to quantify PS in such enriched food. A Fast gas chromatography-mass spectrometry method for plant sterols/stanols analysis in functional dairy products (milk and yogurt) was developed, using a short capillary gas chromatography column (10 m x 0.1 mm internal diameter x 0.1 μm film thickness) coated with 5% diphenyl-polysiloxane. A silylated mixture of the main plant sterols/stanols standards (β-sitosterol, campesterol, stigmasterol, campestanol, sitostanol) was well separated in 1.5 min, with a good peak resolution (>1.4,

determined on a critical chromatographic peak pair (β-sitosterol and sitostanol)), repeatability (<13%) and sensitivity (<0.017 ng/mL). The suitability of this Fast chromatography method was tested on plant sterols/stanols-enriched dairy products (yogurt and milk). The analytical performance and significant reduction of the analysis time and consumables, demonstrated that Fast gas chromatography-mass spectrometry could be also employed for the plant sterols/stanols analysis in functional dairy products.

On the other hand, it is also necessary to determine how enrichment with minor components can impact lipid oxidation in foods, especially as related to matrices and thermal treatment. Lipids and lipid-containing food are particularly sensitive to microwave heating as the specific heat of lipids is low and thus they are quickly warmed up. This cooking method can differently impact lipid oxidation depending on the treatment conditions used (power, temperature and time), as well as on food composition. Due to their chemical structure, PS can undergo oxidation with analogous mechanisms to those observed for cholesterol, giving rise to phytosterol oxidation products (POPs) with different characteristic functional groups (7-hydroxy, 5,6-epoxy, triol and 7-keto derivatives) of known adverse biological effects. The oxidative stability of PS during microwave heating was evaluated. Two different model systems (a solid film made with a phytosterol mixture (PSF) and a liquid mixture of phytosterols and triolein (1:100, PS+TAG)) were heated for 1.5, 3, 6, 12, 20 and 30 min at 1000 W. PS degraded faster when they were microwaved alone than in presence of TAG, following a first order kinetic model. Up to 6 min, no POPs were generated in both systems. At 12 min of heating, POPs content reached a higher level in PSF (90.96 µg/mg PS) than in PS+TAG (22.66 µg/mg PS), but after 30-min treatment, the opposite trend was observed. 7-keto derivates were the most abundant POPs in both systems. The extent of PS degradation depends on both heating time and the surrounding medium, which can impact the quality and safety of the food product destined to microwave heating/cooking.

Many minor lipid components are included in emulsion systems and can affect the rate of lipid oxidation because, due to their surface active character, they partition at the droplet interface, where oxidation primarily takes place. Since PS have prove to be surface active molecules, they could be particularly prone to oxidation when they are incorporated in the lipid droplet. The oxidative stability of an oil-in-water emulsions containing plant sterol esters (PSE), ω-3 fatty acids and phenolic compounds, were evaluated at the beginning, after heating (90°C for 45 min) and after a 14-day storage at room temperature. Emulsions added with *Echium* oil (as omega 3 (ω-3 FA) source) and PSE were prepared without or with phenolic compounds extracted from red propolis (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans,trans*-farnesol, rutin, gallic acid or sinapic acid). *Tert*-butylhydroquinone (TBHQ) and a mixture containing ascorbic acid and $FeSO_4$, were applied as negative and positive controls of the oxidation, respectively. Phytosterol oxidation products (POPs) were evaluated as oxidative markers of plant sterols. Among the 16 POPs identified in the samples, 7-Keto and triol derivatives were the most abundant. The samples containing water-soluble compounds such as ascorbic acid and iron, showed the highest content of total POPs amount and a significant increase in 7-keto derivatives.

Finally, some minor lipophilic components may increase oxidative stability of food systems due to their antioxidant activity. However, a series of critical factors (physical location, partitioning, and distribution of minor compounds between oil, water and interfacial regions) are to be considered when dealing with antioxidant strategies. In this research, the α-tocopherol partitioning and antioxidant activity was determined in the presence of excess sodium dodecyl sulfate (SDS) in stripped soybean oil-in-water emulsions. A 70% increase in displacement of α-tocopherol to the aqueous phase was observed when SDS concentrations were increased from 5 mM to 7 mM. The surfactant-antioxidant co-micelles also increased the oxidation lag phase by 5

days, as compared to the control. Considering these results, surfactant micelles could play a key role as an antioxidant carrier, by potentially increasing the accessibility of hydrophobic antioxidant to the emulsion droplet interface.

***Keywords**:* Fast GC-MS, functional food, microwave, phenolic compounds, phytosterol oxidation products, plant sterol/stanol, oil-in-water emulsion, triolein, α-tocopherol, ω-3 fatty acids.

2. Introduction

2. Introduction

2.1 Minor compounds

Food and processed food products contain different lipid classes, such as fatty acids, sterols, phospholipids and glycerides, whose degradation is closely related to the food shelf-life. One of the main deterioration mechanisms of natural or processed foods is lipid oxidation. Different food systems, such as mayonnaise, salad dressings, creams, ice cream, and butter, have emulsion-based structures in which the lipids are important ingredients. Therefore, the physical structure of the lipid system (*e.g.* bulk oil or an emulsion) and minor lipid components could impact the oxidation process extent, thus contributing to food shelf-life.

Minor components represent a wide range of food molecules that are naturally present in vegetable and animal lipids. These lipophilic compounds represent only 1-2% of the total lipid composition and are separated in two classes, saponifiable and unsaponifiable matter. Those in the latter category include hydrocarbons, carotenes, tocopherols, tocotrienols, linear fatty alcohols, triterpenic alcohols, methyl sterols, sterols and pigments. Minor components are of particular interest because of their antioxidant and biological properties. They may be natural food ingredients (*e.g.* oils, eggs, milk, vegetables, etc.), or they may be added as specific functional ingredients (*e.g.* ω-3 fatty acid, carotenoids and phytosterols (PS)). The critical factor determining whether a food lipid is bioactive is its potential to promote human health beyond simply being an energy source (Chen et al., 2013). Epidemiological studies suggest that plant-based diets, which contain high amounts of these minor compounds, have protective effects against cardiovascular disease (CVD), various cancers, or other chronic disease (Kris-Etherton et al., 2002). To evaluate this phenomenon, many bioactive compounds such as phenolics, phytoestrogens, and plant sterols (PS) have been studied in depth to evaluate their health effects (Gylling et al., 2014; Yao et al., 2004). Espley et al. (2014) used genetically engineered apples

with increased flavonoids, non-transformed apples, and a control diet (without apple) to evaluate the effects of dietary flavonoids on inflammation and gut microbiota in mice. In particular, the study showed that high-flavonoid apple was associated with a decrease in some inflammation markers and changes in gut microbiota when fed to healthy mice. Kuhnt et al. (2014) studied the effect of long chain ω–3 polyunsaturated fatty acid (PUFA) from *Echium* oil dietary supplementation in plasma and cellular blood lipids, and its efficacy was determined in humans. The daily intake of *Echium* oil (15-20 g) increased eicosapentaenoic acid and docosapentaenoic acid in blood, but had no influence on docosahexaenoic acid. Also, *Echium* oil lowered cardiovascular risk markers, such as serum triacylglycerides, which is particularly relevant for individuals with metabolic syndrome. Hence, it could be advantageous to fortify food or beverages with these bioactive minor compounds. An example of this application is the production of PS enriched foods that have become popular for their ability to decrease total and low-density lipoprotein "bad" cholesterol (McClements et al., 2007). While it is important to understand the health implications of lipid minor components, such as PS, it is also necessary to determine how their supplementation impacts lipid oxidation in foods, especially in relation to matrices and thermal treatment. It is important to understand how minor components impact lipid oxidation, because the latter results in decreased sensory quality, due to the production of secondary oxidation products, and also the destruction of essential nutrients in foods (Chen et al., 2013). For example, PS contain an unsaturated ring structure and therefore are susceptible to oxidation during processing and storage in bulk oil, oil-in-water emulsions, and infant foods (Cercaci et al., 2007). Thus, their degradation lead to POPs, volatile flavor components and oligomers which could impact the sensory profile as well as nutritional quality of the food system. Volatile compounds are important from a sensory standpoint and their formation can be a source of off-flavor formation in thermally treated oil (Rudzinska et al., 2009). However, the information about this issue are neglected. The chemical structure of minor compounds will

influence their prooxidant or antioxidant action, as well as their stability that is largely dependent on the context of the food system. From a physical point of view, some minor compounds are amphiphilic molecules with a surface activity that can form physical structures in presence of small quantity of water in bulk oil. These physical structures, known as association colloids, can serve as reaction centers to promote lipid oxidation and destabilize the food system (Chen et al., 2011). Minor compounds may also be prooxidative by altering the physical properties of emulsion droplets as a result of their surface activity (Chen et al., 2011). However, some minor components may increase oxidative stability of the food system, which is especially important in the prevention of lipid oxidation in enriched functional foods. Several compounds, such as tocopherols, phenols, and carotenoids, can limit oxidation by their ability to scavenge free radicals, chelate metal ions, and inhibit the decomposition of hydroperoxides (Decker et al., 2010). It is of outmost importance the physical location, partitioning, and distribution of minor compounds are critical factors to take in consideration when evaluating antioxidant strategies. These factors help to determine whether the minor compound will behave as a prooxidant or as an antioxidant and protect bioactive lipids. Thus, the effect of minor compounds in real foods is difficult to discern because the matrices are extremely complex and their stability can be affected by several extrinsic factors (*e.g.* light, temperature abuse, oxygen). Given the paradoxical oxidative effect of minor lipids compounds, more research is needed to specifically determine the context under which health-promoting minor compounds may positively or negatively impact the stability of foods.

2.2 Food emulsions

Many common food products exist as emulsions, such as beverages, salad dressings, sauces, soups and deserts (McClements, 2005). Generally, an emulsion consists of at least two immiscible liquids (usually oil and water), with one of the liquids being dispersed as small

droplets in the other (Dickinson et al., 1982; Dickinson, 1992). In oil-in-water (O/W) emulsions, lipids are important ingredients that are dispersed as miniscule droplets within an aqueous continuous phase. On the contrary, lipids can be the continuous phase with water droplets dispersed as in water-in-oil (W/O) emulsions (McClements et al., 2000). Emulsions are thermodynamically unfavorable systems that tend to break down over time, due to a variety of physicochemical mechanisms that include gravitational separation, flocculation, coalescence, and Ostwald ripening (McClements, 2005). The use of emulsifiers can stabilize emulsions to be kinetically stable and prevent the aggregation of the droplets. Emulsifiers are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, thus forming a protective layer that prevents droplet aggregation. Besides emulsifiers, other surface active materials, such as antioxidants, minor lipid components (*e.g.*. sterols and diacylglycerols) and mineral ions, are included in interface composition (Waraho et al., 2012; Waraho et al., 2011). All these compounds can affect the chemical properties of the interfacial membrane, such as electrical charge, and impact the rate of lipid oxidation (Cercaci et al., 2007; Waraho et al., 2011).

Furthermore, O/W emulsions are technologically important as delivery systems for a wide range of minor bioactive lipids that can be directly incorporated in lipid-based food (*e.g.* shortenings, butters, margarines, and spreads) (Chen et al., 2013). Through emulsion technology, milk, yogurt, salad dressing, fitness bars, ice cream, and meat patties have been enriched with bioactive compounds, such as omega-3 fatty acids, arachidonic acid, conjugated linoleic acid, lycopene, astaxanthin, lutein, and plant sterols (Boon et al., 2008; Cercaci et al., 2007; Chee et al., 2007; Let et al., 2007). Therefore, these physical constituents are also important for food industry, because they can protect the bioactive lipids from chemical deterioration during the processing and storage of foods, as well as to increase their absorption and bioavailability (Chen et al., 2013; McClements et al., 2007).

2.3 Phytosterols: an overview

Among minor components, sterols are the majority of the unsaponifiable fraction of edible fats and oil. Depending on their origin, sterols can be classified as animal sterols or plant sterols. Cholesterol is a sterol unique to vertebrate animals and its consumption has been widely associated with atherosclerosis (Otaegui-Arrazola et al., 2010). On the other hand, plant sterols dietary intake, or phytosterols, have a beneficial effect on reduction and low-density lipoprotein "bad" cholesterol. PS are constituted by more than 200 different types of molecules found in the higher plants. Additionally, ergosterol is a sterol found in the lipid fraction of yeast and fungi.

Sterol molecules function as structural components of plant membranes, and also as communicating molecules (e.g. steroidal hormones) in cell signaling. Specifically, the free form stabilizes phospholipid bilayers in plant cell membranes, leading to membrane rigidification.

In terms of structure, plant sterols and cholesterol share the steroid nucleus (tetracyclic cyclopentana[a]phenanthrene ring), including a 3β-hydroxy group on the ring A and a 5,6 double bond on the ring B.

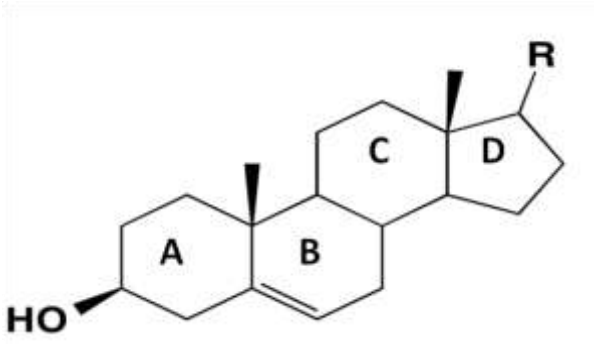

Figure 1. Sterol structure

Plant sterol's molecular structure differs from cholesterol due to presence of an extra methyl or ethyl group, and a double bond in the side chain attached to the C-17 of the ring D (not shown). Most PS side chains contain 9-10 carbon atoms, instead of 8 found in cholesterol.

It is possible to further divide the sterols based on the number of methyl groups at the C-4 position, distinguishing the 4-desmethyl sterols (*i.e.* with no substituent on carbon-4), 4α-methyl sterols and 4,4'-dimethyl sterols (also known as linear and triterpenic alcohols). The last two compounds represent the intermediates in the plant sterols biosynthesis and exist at lower levels than the terminal product 4-desmethyl sterols. In general, the position of a double bond in the steroid nucleus of 4-desmethyl sterols is between C-5 and C-6, but in some plant sterols it can be between C-7 and C-8. This is the reason why the 4-desmethyl sterols are categorized in two groups: Δ5-sterols and Δ7-sterol. Phytostanols are another subgroup of naturally occurring fully saturated plant sterols, but are only present in trace amounts.

Depending on the substitution at position C-3, plant sterols can be found in nature as free (3β-hydroxy group) or as conjugated ones, The C-3 position can be esterified (fatty acid or a hydroxycinnamic acid (steryl ester)), glycosylated (carbohydrate moiety, like glucose (steryl glycoside)) or steryl glycosides acylated.

The difference in structural sterol characteristics are related to their biological functions. In particular, they can affect the cell membrane stability according to the type of molecule; for instance, stigmasterol can have a disordering effect on membranes and the molar ratio of stigmasterol to other PS in the plasma membrane increases during senescence (Moreau et al., 2002). In addition to these important roles, plant sterols are also precursors of a group of plant growth factors like brassinosteroids, which are steroidal plant hormones that are involved in the growth and development of plants (Saoussem, 2012). In particular, plant stanols, such as

campestanol and stigmastanol, are precursors of C-28 and C-29 brassinosteroid, respectively (Piironen et al., 2000; Saoussem, 2012).

The biosynthetic route of PS occurs in the plant cells cytoplasm. The major plant sterols biosynthesis end products are sitosterol (stigmasta-5-en-3β-ol), stigmasterol (stigmasta-5,22-dien-3β-ol), campesterol (campest-5-en-3β-ol), brassicasterol (ergosta-5,22-dien-3β-ol) and avenasterol (ethylidenecholest-5,24-en-3β-ol). The difference between campesterol and sitosterol is an additional methyl and ethyl group at C-24, which allows the distinction between 28-carbon and 29-carbon PS. The 29-carbon stigmasterol contains an ethyl group at C-24 and an ethylenic bond at C-22 (Moreau et al., 2002). Phytostanols (fully saturated) are sitostanol (also called stigmastanol (stigmastan-3β-ol)) and campestanol (24-methylcholestan-3β-ol).

In the PS biosynthesis, it has been reported that the isoprene units, fundamental building blocks in the biosynthesis of sterols, are supplied exclusively from the mevalonate pathway (Piironen et al., 2000; Thimmappa et al., 2014). In this pathway, the cycloartenol (present in higher plant and algae) and lanosterol (present in fungi and animals) are the first products formed by cyclization of squalene oxide via chair-boat-chair conformation. Subsequently, the conversion of cycloartenol to other plant sterols involve the C-24 alkylation of the side chain, demethylation of the C-4 and C-14 methyl groups and double bond formation (Thimmappa et al., 2014). On the other hand, saturated PS can be produced by hydrogenation of plant sterols in which all double bonds are reduced. This chemical reaction produces sitostanol from sitosterol or stigmasterol and campestanol from campesterol or brassicasterol. The conversion of campesterol into campestanol in the C-6 oxidation pathway involves a four-step process, which proceeds from campesterol to 24-methylcholest-4-en-3β-ol, to 24-methylcholest-4-en-3-one, to 24-methylcholest-5α-cholestan-3-one and finally to campestanol (Kim et al., 2005). A study on transgenic cotton seeds found a

direct correlation between 3-hydroxysteroid oxidase levels and phytostanol levels, suggesting the enzyme's role in the formation of stanols (Venkatramesh et al., 2003).

Concerning the natural source of PS in the human diet, fruits and vegetables only make up a small proportion, typically less than 0.05% wt/wt (wet weight basis) (García-Llatas et al., 2011). However, nuts and vegetable oils contain more than 1% of PS. Cereal products and vegetable oils represent the best natural source of PS in the diet, accounting for up to 40% of daily intake of plant sterols (Wasowicz et al., 2010). However, it is important to note that oil and fat refining processes greatly impact the final concentrations of PS. Refining can lead to a 10% to 70% loss in PS (mainly free ones), depending on the applied conditions (García-Llatas et al., 2011). The initial content of the plant sterols in the majority of crude vegetable oils vary from 1 to 5 g/kg of oil. Corn oil along with rapeseed, rice bran and wheat germ oils contain the highest amount of sterols. The total amount of PS reported for corn oil range from 8.09 to 15.57 g/kg of oil, but fully refined corn oil resulted in a sterol loss of 36% (Piironen et al., 2000). In vegetable oils, sterols are present in both their free form and as fatty acids esters, with their proportions varying amongst oils. For example, canola, avocado and corn oils are very rich in steryl fatty acid esters (56-60% of total sterol content) as compared to other oils (soybean, olive and sunflower oils) where free sterols are predominant (57-82%) (Piironen et al., 2000; Saoussem, 2012). Among free sterols, it was found more stigmasterol and less campesterol in the peanut oil, whereas the free fraction of corn oil contained more campesterol and less Δ^5-avenasterol than the ester fraction. In all oils, sitosterol (as steryl ester and free sterol fractions) represents 50% or more of total sterols (Worthington et al., 1984).

Regardless of their form, phytosterols can be recovered from vegetable oil refining and be reapplied in health, pharmaceutical and food applications (Fernandes et al., 2007). Among all vegetable oils, corn oil, corn fiber oil, wheat germ oil and soybean oil are the most commonly

used in this practice. Phytosterol extraction, mostly in free form, takes place during the deodorization step of crude vegetable oil refining (García-Llatas et al., 2011). In addition, the PS extraction can also come from tall oil, a by-product of wood pulp industry. The commercially grown coniferous trees (*Pinus sp.*) are the predominant source of wood for this application (Fernandes et al., 2007). Furthermore, the tall oil is also rich in naturally-occurring phytostanols even though hydrogenated PS are usually employed for food-grade ingredient purposes.

Depending on the eating habits, country, and traditional cuisine, the average daily intake of PS varies from 150 to 440 mg in different populations (García-Llatas et al., 2011; Lagarda et al., 2006). This average can increase up to more than 1 g/day in the case of a vegetarian diet (Piironen et al., 2000).

2.4 Role of bioactive lipids in health

Plant foods contain hundreds of non-nutrient microconstituents with significant biological activity, generally called bioactive compounds or phytochemicals, which seem to play a role in the maintenance of human health (Saura-Calixto et al., 2009). Several bioactive lipids that exert this function are fat-soluble vitamins, PS, carotenoids, and polyunsaturated fatty acids. On the other hand, an exceeding amount of some lipids can negatively impact human health. In particular, overconsumption of saturated fatty acids (FAs), *trans*-FAs, and possibly cholesterol are believed to be detrimental to human health (Chen et al., 2013). Thus, overconsumption of lipids has been correlated to various metabolic and neuroendocrine diseases.

Recently, health problems such as diabetes and obesity have been linked with a widespread increase in premature atherosclerosis and cardiovascular diseases. The increase in disease rates has largely been attributed to a change in dietary habits. Atherosclerosis is considered a complex disorder in which both innate and adaptative immune-inflammatory mechanisms play a major

role (Alemany et al., 2014). Atherosclerosis is a chronic, progressive disease typically initiated during the first three decades of life, which results in the hardening and narrowing of blood arteries (Gylling et al., 2014). Data obtained with studies in genetically modified mouse- and cell-based models, suggest that an early decrease of dietary low density lipoprotein-cholesterol (LDL-C) may substantially delay, or even prevent, the onset of atherosclerosis, particularly in coronary circulation (Gylling et al., 2014). While these results are promising, it is important to note that the experiment's short duration and high pharmacological dosage make it uncertain if these results are to be matched in a human diet.

Inflammation plays a significant role in the etiology of atherosclerosis, from development of fatty streaks to plaque rupture and thrombosis. While dietary habits are known to contribute to the inflammation process, food minor components have also been shown to reduce its occurrence. For instance, plant sterols have been shown to reduce the plasma levels of inflammatory cytokines CRP, IL-6, TNF-a, phospholipase A1, and fibrinogen. This effect may be the result of beneficial alterations in the membrane composition, membrane fluidity, sensitivity, and signaling pathways. All of these changes may subsequently alter the immune response by influencing the synthesis and secretion of eicosanoids, leukotrienes, and prostaglandins (Othman et al., 2011). Further observations in animal models have pointed out the anti-carcinogenic (especially upon the inhibition of colon cancer), anti-atherosclerotic, anti-inflammatory and antioxidant effects of PS (Lagarda et al., 2006). In recent years, some studies have been conducted on PS supplementation because of their cholesterol-lowering effects and the potential contribution to lowering the risk of cardiovascular diseases (Alemany et al., 2014; García-Llatas et al., 2011). PS seem to attenuate intestinal cholesterol absorption through a different path (Gylling et al., 2014).

Given the effect of PS on cholesterol, a potential strategy is to use PS to reduce the cholesterol content of chylomicrons and very low density lipoprotein (VLDL) remnants and to

lower LDL-C levels, in order to diminish cardiovascular disease (Gylling et al., 2014; Marangoni et al., 2010). The hypocholesterolemic effects of PS have been known since 1950's. A reduction of plasma cholesterol levels by 27% was observed in 26 healthy subjects to whom 5-10 g/day of PS were administered for 2 weeks (Pollak, 1953). In a subsequent study, a reduction of up to 20% of blood cholesterol levels was observed in subjects supplemented with high doses (>10 g/day) for 3-5 weeks (Marangoni et al., 2010). The mechanism by which PS induce the reduction of cholesterol absorption has not been completely elucidated, but several possible mechanisms have been proposed. In brief, the disruption of the intraluminar solubilization step occurs when phytosterols compete with cholesterol molecules for incorporation in mixed micelle formation, due to their greater physico-chemical affinity, in the intestinal tract. Micelles serve as vehicles for liposoluble components (phytosterol, cholesterol) due to their excessive hydrophobicity. Co-crystallization with cholesterol, yielding an increased fecal excretion of cholesterol and its metabolites, also takes place; however, several authors report that this mechanism has a low impact on plasma cholesterol levels (García-Llatas et al., 2011; Gylling et al., 2014; Marangoni et al., 2010). Moreover, PS may also affect cholesterol levels through the up-regulation of ATP-binding cassette transporters ABCG-5 and ABCG-8, located on the apical surface of enterocytes. These transporters regulate the secretion of cholesterol and sterols from intestinal enterocytes into the gut lumen and from hepatocytes into the biliary space. Therefore, up-regulation of ABCG5 and ABCG8 promotes biliary cholesterol secretion and decrease cholesterol absorption, which leads to an increase in hepatic cholesterol synthesis (Godhia et al., 2015). This mechanism has been confirmed in a cohort study, implicating ABCG5 and ABCG8 polymorphisms in variation of circulating plant sterols levels (Gylling et al., 2014). Furthermore, PS may also reduce plasma cholesterol by stimulating cholesterol excretion via a non-biliary route. This route, called trans-intestinal cholesterol excretion, may involve stimulation of intestinal cells,

where the efflux of excess cholesterol goes from peripheral tissues towards the liver, followed by biliary secretion and subsequent disposal via the feces (Lottenberg et al., 2012).

The importance of PS as cholesterol-lowering effect has been recognized in several studies, but conflicting reports exist from cell line, animal, and human studies, thus questioning the usefulness of PS in preventing coronary heart disease (Lottenberg et al., 2012). Surprisingly, an increased risk of cardiovascular events has been associated with high PS concentrations; in particular, a high sitosterol concentration (>5.25 μmol/L) was significantly associated with a CHD risk found in plasma and specific tissues (Assmann et al., 2006). It has also been demonstrated that PS may induce inflammation and reduce cholesterol efflux from macrophages, conditions that have been directly implicated in the development of atherosclerosis (Sabeva et al., 2011). These contradictory results, combined with the lack of studies that follow long-term intake of high-dose plant sterols, have caused PS supplementation in nutritional intervention strategies to remain the subject of debate (Alemany et al., 2014).

2.5 Food formulation

Due to their mentioned properties, in the last decade the market has launched a wide number of PS-enriched products. The latter are complemented by new food habits in which the animal fat has been substituted with vegetable oils, thus contributing to a steady increase in PS intake (Garcia-Llatas et al., 2013). There is some EC regulations (No 1997/258 and No 2004/608 of the European Parliament and the Council on "Novel Foods") that deal with PS use and their esters ingredients in foodstuff, the. According to these regulations, if new phytosterol- and stanol-enriched products are to be marketed within the EU, they should be considered as a novel food or novel food ingredient. However, the complicated regulatory application process can be simplified if a newly launched product is substantially equivalent to an existing food application. The Scientific Committee on Food (SCF) and the European Food Safety Authority (EFSA)

through its "Scientific Panel on Dietetic Products, Nutrition and Allergies", has recommended a precautionary intake limit of 3 g PS/day by multiple dietary sources (http://www.ifst.org/knowledge-centre/information-statements/phytosterol-esters; García-Llatas et al., 2011). Scientific studies indicate that PS consumption (1-3 g/day) reduces low-density lipoprotein cholesterol (LDLc) blood levels by about 5-15% (European Commission Regulations 1997/258; Normén et al., 2003). Recently, the EC authorized health claims made on PS-enriched foods that refer to the beneficial effect of PS and stanols in helping to maintain normal blood cholesterol levels. These claims declare that "plant sterols and plant stanol esters have been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary heart disease" (European Commission Regulation 2010/384). Several types of PS-enriched food products have been marketed, such as yellow fat spreads, salad dressings, dairy products, fermented dairy products, soya drinks, cheese products, and yoghurts (EEC No. 2004/333 and 2004/335)**.** Among these products, milk-based beverages are a convenient way for consumers to obtain the daily recommended amount of PS (1.5–3 g). In general, the phytosterol/phytostanol contents vary greatly (0.3-8.0%), depending on the type of food product and market area (Barnsteiner et al., 2012), as well as on the daily consumption volumes and on food amounts to be ingested. Several companies have adopted a one-daily dose approach (Laakso, 2005). However, it must be pointed out that the consumption of high doses of PS can also have side effects, namely a significant reduction in the blood levels of carotenoids without having an additional effect on cholesterol (García-Llatas et al., 2011).

The importance of cholesterol lowering ability of PS was emphasized specifically when plant stanol esters were used to make functional margarines introduced to the Finnish market in 1995 (García-Llatas et al., 2011; MacKay et al., 2011). To warrant that PS work effectively, it is important that the formulation of the PS and its delivery vehicle allow the PS incorporation into the mixed micelles of the intestinal chyme. Their high melting point and tendency to form

insoluble crystals, when extracted from vegetable oils, prevent PS solubilization in food formulation (MacKay et al., 2011; McClements et al., 2007). To overcome this problem, PS have been added to high-fat foods and/or they have been esterified with fatty acids, which facilitate their dispersion and increase sterol solubility by a factor of ten. Although this kind of PS product formulation was innovative, this delivery system may not be ideal for individuals following low fat diets. Therefore, lower fat or fat free functional foods could be prepared by introducing PS into aqueous-based system (McClements et al., 2007). Functional foods have since been developed, in which free PS have been incorporated directly into foods, through homogenization and emulsion techniques often using stabilizing agents such as lecithin (MacKay et al., 2011). The free PS ingredients comprises PS, food-grade fats or oil and water, and its physical state is mainly microcrystalline (Soupas et al., 2006). The most common approaches employed for delivery of functional lipophilic components into food are emulsion-based technologies and spray drying (Sanguansri et al., 2010). In addition to the latter, different nanotechnology methods (such as microemulsion by solvent displacement or rapid expansion of supercritical solution into aqueous solution) have been developed to incorporate PS in functional food. At the same time, the encapsulation technologies have a protective effect on PS ingredients from adverse processing and storage conditions, which are a critical point for PS stability and POPs formation. Their manufacturing, physical state (power, liquid, microencapsulated, emulsified) and chemical form (free or esterified) can affect the PS oxidation rate and POPs profiles (Alemany et al., 2014; González-Larena et al., 2011).

2.6 Phytosterol oxidation

Bioactive lipids must maintain their potential bioactivity within the food product during its manufacture, storage, transport, and utilization. In addition, the desirable physicochemical and sensory attributes of food product must remain also unaltered during processing and storage.

Depending on the components and the treatment and storage conditions, some bioactive lipids could be chemically labile or physically unstable. Chemical degradation is a major challenge for a number of important minor lipids, because they are susceptible to oxidation in complex food matrices. The deterioration of bioactive lipids is often accelerated by several factors, such as light, heat, enzymes, metals, metalloproteins, and microorganisms, leading to autoxidation, photoxidation, thermoxidation, or enzymatic oxidation (Chen et al., 2013; Shahidi et al., 2011).

PS contain an unsaturated ring structure and therefore are susceptible to oxidation during processing and storage in bulk oil, O/W emulsions, and infant foods (Cercaci et al., 2007). PS can undergo oxidation via analogous mechanisms to those observed for cholesterol, giving rise to phytosterol oxidation products (POPs) with different characteristic functional groups (7-hydroxy, 5,6-epoxy, triol and 7-keto derivatives) (García-Llatas et al., 2011).

The enzymatic and nonenzymatic pathways involved in POPs formation, mostly react with the side chain and sterol ring of sterol structure, respectively. Like other bioactive lipids, several environmental and chemical factors can trigger phytosterol autoxidation. In free radical chain reactions, PS can be oxidized by many different species of oxygen, such as oxygen in its ground state ($^3O_2$), ozone (O_3), singlet oxygen ($^1O_2$), hydroperoxides (H_2O_2), dioxygen cation (O_2^+) and hydroxyl radical (García-Llatas et al., 2011). The susceptibility to autoxidation or thermoxidation stems from the presence of C=C double bonds, which easily undergo free radical attack, followed by hydrogen abstraction on the carbon atoms in α-positions with respect to the double bonds (Choe et al., 2009; Lengyel et al., 2012).

The initial step in free radical-mediated reactions involves the formation of C-7 carbon centered radical adjacent to the double bond in the ring B of Δ5-phytosterol (C5-C6). The formation of a free radical allows triplet oxygen ($^3O_2$), the primary oxygen species involved, to overcome its electronic spin restriction and form one of the two unstable 7-hydroperoxide

epimers (7α-OOH or 7β-OOH). These hydoperoxides subsequently decompose by a dismutation reaction to the corresponding hydroxyl (7α-OH or 7β-OH) and 7-keto derivatives. Epoxides and triols are other major oxidation products that can be formed from oxidation of the ring structure. Furthermore, interaction of hydroperoxides radicals with another sterol molecule can lead to the formation of both epimers of the epoxy derivatives (Rudzinska et al., 2010; García-Llatas et al., 2011). In an acidic environment, the hydration reactions could occur and epoxides form triols, through epoxy ring opening. The 7α- and 7β-OOH are subjected to redox turnover, making them reactive intermediates, whereas the other more stable POPs are usually stable end products. Further oxidation of POPs end products (7-hydroxy (α and β), 7-keto, 5,6-epoxy (α and β) derivatives) implicate the dehydration of the hydroxyl group on C3, which generates conjugated dienes and trienes (Dutta, 2003; Lercker et al., 2002).

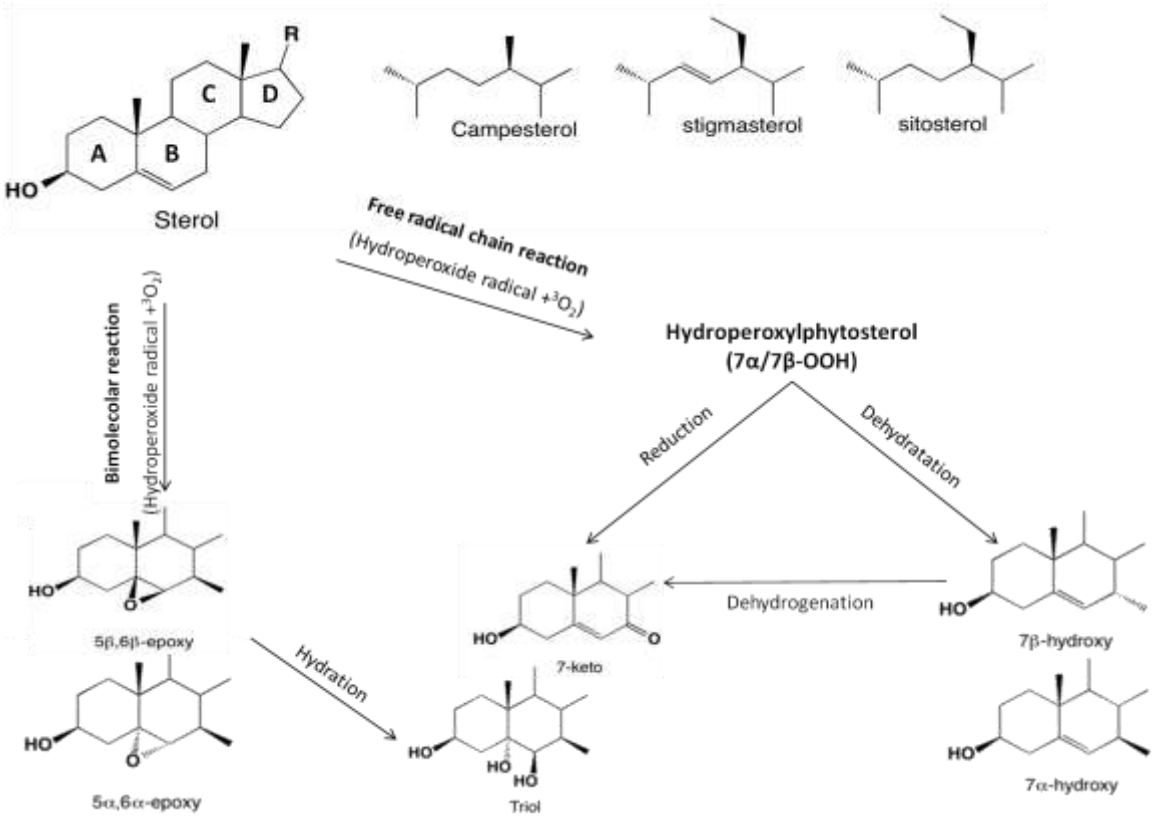

Figure 2. Autoxidation of sterols

Autoxidation of the phytosterol side-chain takes place in a similar manner to the ring moiety, but is much less pronounced than that of the ring structure (Smith, 1992). The major attack sites for the formation of hydroperoxides are C20, C24 and C25. The additional methyl or ethyl group at C24 allows sitosterol, campesterol and stigmasterol to form a wider range of oxidation products than cholesterol (García-Llatas et al., 2011; Kemmo et al., 2005). Despite the possibility to form oxidation products in the side-chain, research in this area has been limited (Dutta, 2004). The phytostanols, the saturated molecule, is considered to be more stable than its counterpart (Dutta, 2003). The main oxidation products from sterols are polar compounds that include hydroxy, keto, epoxy, and triol derivatives. Some authors have pointed out that polymers and other high molecular weight products can be formed during sterol oxidation at high temperatures (García-Llatas et al., 2011; Rudzinska et al., 2010; Wasowicz et al., 2010). Further thermoxidation of sterols and polar oxidation products form non-polar and medium polar oxidation products. Recently, studies on stigmasterol thermoxidation model systems have reported the formation of dimeric, polymeric products, and non-polar monomers (Lampi et al., 2009; Menéndez-Carreño et al., 2010).

2.7 An overview on thermoxidation of phytosterols: model systems and food products

2.7.1 Sterol degradation

Given that food processing and cooking can affect lipid oxidative stability, it is important to know their impact on sterol degradation and POPs content in food products. The rate of lipid oxidation is largely affected by the thermal treatment that food undergoes during its preparation. High temperatures will accelerate propagation reactions, as well as lipid hydroperoxide decomposition, which results in more free radicals that promote lipid auto-oxidation (Soupas et al., 2004). These reactions and the resulting oxidative destabilization of food, may help to

explain the changes in phytosterol levels during sample heating (Kmiecik et al., 2011; Kmiecik et al., 2015). In order to better understand this issue, model systems are used to identify which factor (alone or in combination) influence this process. In the study by Xu et al. (2009), about 75% of β-sitosterol was thermally degraded when it was oxidized alone at 180°C for 2 h in the oven. Barriuso et al. (2012) investigated the behavior of β-sitosterol, stigmasterol, and campesterol at 180°C when heated for 6 h. In the first five heating points (0-30 min), the authors observed significant differences in PS content at each time, meaning that a drastic drop occurs in the first 30 min. Thereafter, small differences were found. After 30 min heating, about 88% of the initial PS was degraded, of which 41.80% during the first 5 min of treatment. The sterols showed a different susceptibility to degradation, according to the following decreasing order: campesterol ~ β-sitosterol ≥ stigmasterol. The same authors also tried to predict the loss of PS using a non-linear regression model, which fit with a logarithmic model for all sterols.

Although high temperature is among factors that could impact PS losses, it is also necessary to consider the nature of components sorrounding phytosterol, in particular lipids and lipophilic compounds. Different researches have focused on phytosterol oxidation when heated with fatty acids or triacylglycerides at temperature/time conditions generally used in food processing (Kmiecik et al., 2015; Kmiecik et al., 2011; Xu et al., 2011; Xu et al., 2009). Kmiecik et al. (2011) evaluated the PS content after 4 h at 180°C in a mixture of PS, triacylglycerols and antioxidants. The authors observed a decrease in total PS content up to a maximum of 9.5% compared to an unheated sample. This decrease varied and depended on the antioxidant used. It is important to note that some individual PS may be particularly susceptible to oxidation. For instance, Kmiecik et al. (2011) found higher losses of campesterol (10.5%) in the sample with natural tocopherol or phenolic compounds as compared with the unheated samples. Based on β-sitosterol changes, the highest losses (15.6%) was observed in the sample heated without added antioxidant.

β-sitosterol has proven to be susceptible to thermoxidation, as it can result in an 85% decrease of its initial level after 1.5/2 h oven heating at 180°C in several model systems containing fatty acids at different ratios (Xu et al., 2011). During the first hour of heating, a faster β-sitosterol degradation was noticed in presence of fatty acids than in their absence (control). At 1.5 h, the percentage of β-sitosterol degradation was similar in all model systems; however, after 2 h of heating, the oxidation of β-sitosterol was greater in the control as compared to the fatty acid-containing systems, showing a protective effect of fatty acids towards sterol oxidation. Similarly, after 1.5 h treatment at 180°C, β-sitosterol oxidized faster when heated alone than in presence of corn oil, lard, and olive oil. After that time, no differences were observed among the single-sterol system and those combined with oils and fats (Xu et al., 2009).

These researches highlighted that the initial phase of oxidation (the induction) is a critical point, especially in the fatty acid-containing systems where fatty acids accelerated PS oxidation in the first hour and thereafter their oxidation-promoting effect decreased, thus exhibiting a noticeable protective effect towards sterol if compared with the control sample. Although the chemical mechanisms behind these behaviors are still unknown, two hypotheses have been proposed to try to explain this fact. The first hypothesis is that both pure fatty acids and triacylglycerides could increase the solubility of β-sitosterol, to evenly disperse it, and thus increase the contact with oxygen and penetration of the latter into the medium to accelerate sterol oxidation. The second hypothesis is that fatty acids could undergo oxidation and produce fatty acid oxidation products and free radicals, which in turn accelerate the degradation of β-sitosterol (Xu et al., 2011). However, the latter hypothesis was not completely supported by the results obtained in the study by Xu et al. (2011), where the prooxidative effect of fatty acids was not related to their degree of unsaturation.

Among commercially available PS enriched-products, milk is the one that is typically subjected to heat treatment like microwave or electrical heating. These applications result in a

60% decrease of total PS after drastic treatment at 90°C for 15 min with electrical heating or after 2 min of microwaving (Menéndez-Carreño et al., 2008). A time reduction to 1.5 min of microwaving led to a half decrease of total PS (30%), as compared to 2 min. In this work, β-sitosterol and campesterol were the most affected compounds by the oxidation process, decreasing 65 and 61%, respectively.

2.7.2 POPs formation

The oxidative behavior of phytosterols is similar to that of cholesterol in terms of oxidative rate and oxidation products pattern, because they are structurally similar except for the side chain (Barriuso et al., 2012; Xu et al., 2009). After thermal treatment of PS, 7-keto derivatives are normally the most abundant POPs, but 7-hydroxy, 5,6-epoxy and triol derivatives can also be found (Barriuso et al., 2012; Xu et al., 2011; Xu et al., 2009). Xu et al. (2009) observed that 20% of total POPs of β-sitosterol were generated considering the initial amount of β-sitosterol, when heated to 180°C for 2 h. Concerning the individual POPs trends, the authors found an increasing tendency from 30 to 90 min of treatment, followed by a decline after that time. Barriuso et al. (2012) investigated not only the behavior of individual phytosterols, but also the formation and degradation pattern of the oxidation products during heating at 180°C up to 6 h. The maximum level of POPs was observed between 5-10 min of heating, depending on individual PS, with the highest concentration of total campesterol and β-sitosterol oxides. In another study, maximum POPs levels were found at 1 h or longer times when PS (β-sitosterol and stigmasterol) were heated at 180°C (Menéndez-Carreño et al., 2010; Xu et al., 2011). Since processing temperatures have an important effect on the formation of oxysterols, the oxidation mechanism of stigmasterol at 100°C (from 0 to 7 days) and 180°C (1 h) in an oven was investigated and showed different oxidative behavior depending on the conditions (Kemmo et al., 2005). The stigmasterol oxidation products reached the maximum levels after only 10 min at 180°C, showing a sharp rate

of oxidation at this temperature, which is in agreement with previous research (Soupas et al., 2004). At 100°C, a moderate increase of stigmasterol oxidation products was observed over the entire 7-day oxidation period. At both temperatures, the same POPs are formed, but with different ratios and percentage distribution. 7-ketostigmasterol, the most abundant POP in both treatments, was the dominant POPs after 40 min in the sample heated at 180°C and throughout the oxidation period in those heated at 100°C (Kemmo et al., 2005).

The aforementioned research primarily focused on the effect of heating temperature (typically 180°C) on POPs formation, but another important aspect to be considered on sterol oxidative stability is the nature and composition of the sorrounding lipids or oil, as well as their interactions. The same oxidation products pattern was also found in the fat-containing systems. When β-sitosterol, in presence of various fatty acids, was heated at 180°C for 2 h, 7-ketositosterol, 7α-hydroxysitosterol, 7β-hydroxysitosterol and 5,6-epoxysitosterol were the most abundant POPs (Xu et al., 2011). Less β-sitosterol oxides were found in the fatty acid-containing system when heated at 120, 150, and 180°C for 60 min as compared to the amount formed during oxidation of phytosterol alone (Xu et al., 2009). The date reported in this research work highlighted the increasing oxidative stability due to the "protective" and/or competing effect of the matrices components. The unsaturated fatty acids in corn oil, lard and olive oil compete for oxygen with phytosterols, thus contributing to a decreased formation rate of POPs during heating.

In other investigations (Xu et al., 2011; Xu et al., 2009), the formation of oxidation products was strongly promoted during the initiation phase of oxidation in the system containing fatty acids as compared to the system with PS alone. After the initiation phase, the production of individual POPs demonstrated a drastic drop (Kemmo et al., 2005; Menéndez-Carreño et al., 2010; Xu et al., 2011); this trend was also noted in the study by Barriuso et al. (2012), but it occurred earlier. These authors (Barriuso et al., 2012) assayed the best degradation model for all

POPs, finding that the inverse and the logarithmic models fit 7-hydroxy and 5,6β-epoxy derivatives, respectively; the remaining POPs (7-keto, α-epoxy and the total oxyphytosterols deriving from each individual sterol) exhibited an exponential fitting trend.

POPs decreasing trends observed with increasing heating times, could be ascribed to further oxidation of POPs into volatile compounds (Rudzinska et al., 2009; Soupas et al., 2004; Zhang et al., 2005; Xu et al., 2009; Xu et al., 2011) or to their conversion into high-molecular weight oxidation products (oligomers and/or polymers) (Lampi et al., 2009; Menéndez-Carreño et al., 2010; Rudzinska et al., 2010).

Esterification with fatty acids can also contribute to phytosterol oxidation by an intramolecular oxidation mechanism. At 100°C, unsaturated fatty acids moieties in phytosteryl ester molecules increased the formation of phytosterol oxides as compared to free phytosterols in tripalmitin matrices, while at 180°C the latter were slightly more reactive (Soupas et al., 2005). The distribution and the proportion of individual POPs seemed to be associated with the phase of oxidation. 7-ketosterols accumulated when oxidation had not yet reached the propagation phase, as observed for free β-sitosterol when heated at 100°C for 0–48 h and for sitosteryl ester when heated at 100°C for 0–24 h. Once oxidation reached a propagation phase, the major products were 5,6-epoxysterols and 7-hydroxysterols, as can be seen for free and esterified sitosterols heated at 180°C for 1–3 h; this is probably due to the high heating temperature in which the lower oxygen availability could be a limiting factor (Soupas et al., 2005).

The oxidation of phytosterol can occur not only in oils/fat used in the cooking process, but also in other food matrices. A few studies have discussed the effect of heat treatments (different type, time and temperature conditions) on the phytosterol oxidation and POPs formation in food products where phytosterols occur naturally or were added to enrich the product. Menéndez-Carreño et al. (2008) quantified the 7-hydroxy, 7-keto, epoxy, and triol derivatives of β-sitosterol and campesterol, as well as 7-hydroxystigmasterol in heated PS-enriched milk using a GC–MS

method in selected ion monitoring (SIM) mode. The POPs amount found in the PS-enriched milk heated in drastic conditions (microwaved (900 W, 2 min) or cooked on an electrical heating plate for 15 min) was lower than expected in microwave-treated samples for 1.5 min, probably due to the POPs conversion into non-detected high-molecular weight oxidation products (oligomers and/or polymers). In this work, the oxidation percentage showed that β-sitosterol and stigmasterol were the least affected compounds by the oxidation process. Usual heating conditions (1.5 min of microwaving) maintained phytosterol content on physiologically active values (301 mg/100 g of milk), with phytosterol oxidation percentages around 0.12–0.40%. The authors concluded that commercial PS-enriched milk constitutes an adequate phytosterol source even when it is heated for consumption. However, when the heating conditions are too drastic, there is a significant decrease in sterol content without a corresponding increase of sterol oxidation products, which could not be ascribed to oxidation products breakdown only (Menéndez-Carreño et al., 2008), but also to their interaction with other milk components, such as Maillard reaction products and proteins (García-Llatas et al., 2011).

Overall, the stability of sterols is an issue to examine in depth, considering all factors involved in their degradation, in their breakdown as volatile compounds, in their oxidation as POPs and/or further conversion into oligomers and/or polymers. Thus, it is important to take into account the technological or cooking processes, temperature, exposure time, and the nature/composition of the matrix for product formulation.

2.8 Phytosterol stability in enriched food

2.8.1 Sterol degradation

Product stability over time is an essential measure to ensure food quality. Phytosterol-enriched foods, in particular, deserve attention considering their daily intake. During long-term storage, phytosterols are susceptible to oxidation, generating POPs that could potentially trigger

the stability and safety of the product, as well as lower the initial PS content of the food. Commercial spreadable fats, margarines, milk, and yoghurts that are formulated with phytosterols are currently available on the market. Over the past few years, several researches have been conducted on the evaluation of the oxidative stability of sterols in PS-enriched foods during their formulation, production and storage. González-Larena et al. (2012) tested the stability of three PS-enriched milk-based fruit juices, fruit juices, and milk beverages stored at 4, 24, and 37°C during 6-month storage. For these types of products, the organoleptic properties were preserved during the whole storage time, which corresponded to the turnover period in the grocery. The sample were stable under all treatments, since no losses in total PS content were observed. In another work (González-Larena et al., 2015), the authors found different oxidation susceptibility among the aforementioned samples during storage. In the fruit-based samples, the PS oxidation rate (from 0 to 6^{th} month) was lower (<0.08%) compared to those found in the milk beverage (<0.12%), probably due to the natural antioxidant compounds in the raw material used to formulate the enriched product. Also, the individual PS showed different susceptibility to the oxidation (campesterol > β-sitosterol), which could be explained considering their diverse surface activity behavior. The β-sitosterol oxidation rate found by Alemany et al. (2013) was <0.02% in all PS-enriched beverages manufactured at industrial scale, which was lower than those previously reported for similar beverages (0.07%) prepared at laboratory scale. Menéndez-Carreño et al. (2008) studied the oxidative stability of PS-enriched milk subjected to Schaal oven conditions (equivalent to 1 month of storage at room temperature) and observed just a 4% decrease of total phytosterol content. The PS stability seems warranted by the antioxidants included in the formulation. An elevated oxidative stability was also found in PS-enriched chocolate bars during 5 storage months, being only 0.1% of the initial plant sterols oxidized (Botelho et al., 2014). Rudzińska et al. (2014) used GC and GC/MS to quantify the phytosterol content of PS-enriched margarines; they found that the phytosterol/phytostanol content dropped

about 20% and 31% during 4 months storage at 4 and 20°C, respectively. The authors highlighted the importance of temperature as storage condition on the stability of PS-enriched margarines.

In general, despite the different conditions (time, temperature, matrices) of all these studies, all these PS-enriched products generally demonstrated a good oxidative stability, regardless of the treatments used in their formulation and long-term storage.

2.8.2 POPs formation

Considering the wide range of functional products developed to exploit the health claims, it is thus important to evaluate the oxidation reactions as related to the interactions among ingredients, matrices, fatty acid composition, processing and storage conditions. Regarding POP contents in PS-enriched dairy matrices, several studies have been carried out (Alemany et al., 2012; Alemany et al., 2013; Conchillo et al., 2005; González-Larena et al., 2015; Menéndez-Carreño et al., 2008; Soupas et al., 2006). Soupas et al. (2006) evaluated the POPs level in PS-enriched milk powder (7% phytosterols) and heat-treated milk (0.4% free phytosterols, 0.5% phytosterol esters, 0.5% phytostanol esters), during processing and long-term storage. PS-enriched milk powder, stored at 38°C for 12 months, exhibited the largest increase in the β-sitosterol oxide content as comapred to the one kept at room temperature. The main POPs quantified throughout the storage period at both temperatures were 7α- and 7β-hydroxysitosterol. Free-fat cow's milks enriched with free or esterified phytosterols, or with phytostanol esters, contained low levels of phytosterol/stanol oxides; in these products, no significant changes in the amounts of sitosterol/stanol oxides were detected during the 6-month storage at room temperature or at 4°C. β-sitosterol was slightly more stable in its free form rather than in its esterified one, probably due to the presence of an unsaturated fatty acid as steryl moitie of the esterified sterol molecule. The major sitosterol oxides found in these milk samples were the

two7-hydroxysitosterol epimers, as well as 7-ketositosterol. González-Larena et al. (2015) observed an increase in the total POPs content in all PS-enriched samples analyzed, which varied according to storage time rather than to temperature (4, 24, 37°C). The milk beverage showed higher POPs content than the fruit-containing beverages. The β-sitosterol and campesterol oxides found were 7α- and 7β-hydroxy, α- and β-epoxy, 7-keto and triol derivatives. The beverages showed low PS oxidation levels (<0.17%), and the authors were able to use this information to establish predictive models of POP content versus storage time. According to the mathematical predictive models applied to PS oxidation in PS enriched-beverages as a function of storage time, total POPs content and individual POPs content showed a R^2 (percentage of variability explained by the model) over 75% and 50%, respectively. Among all individual oxides, the authors confirmed that 7-ketositosterol and 7-ketocampesterol could be used as markers of PS oxidation level in beverages as they exhibited the highest R^2 value. POPs contents were also evaluated in fruit beverages enriched with PS from tall oil and with esterified PS from vegetable oils, manufactured at both laboratory and industrial scales (Alemany et al., 2013; Alemany-Costa et al., 2012). In these studies, only β-sitosterol oxides (7-hydroxy, epoxy, 7-keto and triol derivatives) were detected, since β sitosterol was the most abundant PS. The total POPs content in beverages prepared at industrial scale was 10-times lower than those manufactured at laboratory scale, indicating that the beverages had a higher oxidative stability at industrial scale. A recent assessment of PS-enriched dark chocolate over a 5-month storage period used sensory analysis and GC/MS to compare formulations that contained palm oil and phytosterol esters with samples that had phytosterol esters plus added antioxidants. The peak of hydroperoxides content, indicator of primary oxidation of unsaturated fatty acids, was observed at 30 days (30°C) and at 60 days (20°C). The most commonly identified POPs after 5 months of storage at 30°C were 7-hydroxy, 7-keto, epoxides and triols derivatives of campesterol, stigmasterol and β-sitosterol. Campesterol was the most susceptible phytosterol to oxidation in the PS-enriched chocolate samples, followed by

β-sitosterol and stigmasterol. Sitostanetriol, 6-ketositosterol and 6β-hydroxycampesterol were the major quantified POPs (Botelho et al., 2014). The same authors suggested that the dark chocolate bars could represent an option as functional food, since only 0.10% of the initial plant sterols were oxidized and this level did not change after 5 months of storage. From a sensory standpoint, the physical changes observed after 90 days of storage did not reduce the overall acceptability of the enriched product. Rudzińska et al. (2014) noticed an increase in POPs levels when PS-enriched margarines were stored for 18 weeks at 4 and 20°C, being two times higher at 20°C than at 4°C. Among the oxidized sterols, the major oxidation products in all PS were the 7-hydroxy derivatives, followed by the 7-keto and the 5,6-epoxide ones. The epoxy derivatives exhibited a maximum after 6 weeks of storage at 20°C. There is a clear trend for sterol/stanol loss and POP formation when stored at higher temperatures. Since PS oxidation usually follow the free radical mechanism, analogous to monounsaturated fatty acids, radicals deriving from the latter and the temperature conditions could initiate and propagate oxidation (Dutta, 2004; Rudzińska et al., 2014). Cercaci et al. (2007) suggested that the oxidation in emulsions (*e.g.* margarine) occurs at the droplet interface, so the surface active character of PS could particularly promote PS oxidation when they are incorporated in the lipid droplet.

Overall, previous researches (Conchillo et al., 2005; Grandgirard et al., 2004; Johnsson and Dutta, 2006; Moreau et al., 2002; Soupas et al., 2004) and the aforementioned studies underline that the POPs profile and their formation in PS-enriched food products is the result of the combined effect of added enriching ingredients, the level and type of PS enrichment, the chemical form of PS (free or esterified), matrices types, processing and storage conditions (time/temperature).

2.9 Effect of antioxidants on PS stability

The addition of antioxidants to PS-enriched food could be a useful tool to modulate POPs formation during their processing and storage. Antioxidants may be synthetized (butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ)) or obtained from natural vegetable sources (oils, plant tissues, vegetables and herbs (rosemary, sage, tea leaves)) (Kmiecik et al., 2015). However, food industry trends to remove synthetic additives have place pressure on food manufacturers to rely on antioxidants of natural origin. When TAGs were heated in the presence of phytosterols at 180°C for 4 h, Kmiecik et al. (2011) reported that the addition of tocopherols decreased total POP content from 347 to 137 mg/kg in the model system as compared to a control without antioxidant. Tocopherols have proved to be able to decrease POPs formation by quenching free radicals in frying oils. It is also important to note that the abundance of individual sterol oxides can change depending on the antioxidant added. Among the dominant group of oxyphytosterols, the hydroxy and epoxy derivatives of β-sitosterol and campesterol were found in heated samples of triacylglycerols/phytosterols which had been added with rosemary extract, phenolic compounds, synthetic tocopherols or acid sinapic (Kmiecik et al., 2011). In a recent study carried out by the same authors, the epoxy derivatives of β-sitosterol and campesterol were the most abundant POPs in heated samples of triacylglycerols/phytosterols which had been added with green tea extract, rosemary extract or a mix of synthetic tocopherols (Kmiecik et al., 2015). The antioxidant occurrence (like tocopherols) in vegetable oils/fats can slow down POP formation during heating at high temperature as 180°C (Xu et al., 2009). Furthermore, the same authors found that green tea catechin, α-tocopherol and quercitin were more effective antioxidants than butylhydroxytoluene in preventing oxidation when β-sitosterol was heated at 180°C alone rather than in the presence of corn oil or olive oil (Xu et al., 2009). As a result of their protection against POPs formation, antioxidants are routinely employed to stabilize PS-enriched food products, such as milk, to prevent their oxidation during storage (Menéndez-Carreño et al., 2008).

The phytosterol stability and antioxidant parameters (total carotenoids, total polyphenols, and total antioxidant capacity) in PS-enriched functional beverages were assessed during 6 months of storage at 4, 24 and 37°C (González-Larena et al., 2012). No PS losses were detected under the storage time and temperatures studied, so the authors hypothesized that the increase in total antioxidant capacity might have helped PS maintenance throughout storage. Nevertheless, authors did not found a correlation between PS contents and TAC values.

2.10 Minor compounds: antioxidant activity

The human diet contains several compounds with antioxidant activity that stabilize food quality and extend its shelf-life. Antioxidants may be defined as any substances which are capable of delaying, retarding, or preventing the development of oxidation rancidity or other flavor deterioration in food (Gülçin, 2012; Halliwell, 1990). These components are widely distributed in plant materials, animal tissue and microorganism, providing natural health protection to humans (Shahidi et al., 2010). The most well-known dietary antioxidants are vitamin C, tocopherols, carotenoids, and flavonoids. These minor components can be extracted from natural sources (*e.g.* typically plant-based) and used for different applications, such as food preservation, nutritional supplementation, medical and cosmetic purposes. Fruit, vegetables, cereals, grains, oils and seeds are important sources of plant-derived antioxidants. Tocopherols are particularly important plant-derived antioxidants, which are commonly added to lipid-containing foods to extend their quality. Besides that, they are also of interest because their biological activity as vitamin (Vitamin E). Tocopherols and tocotrienols occur as four isomers in nature (α, β, γ, δ) (Choe et al., 2009). The antioxidant property and the vitamin activity vary between the four isoforms in opposite manner, being the vitamin E activity almost ascribed to α-tocopherol, followed by β, γ and δ (α>β>γ>δ) (Evans et al., 2002). Despite the antioxidant activities decrease from δ to α-tocopherol, the optimum antioxidant activities of tocopherols

depend on the their concentration in the systems. From this point of view, α-tocopherol is the most important antioxidant among the isomers, given that its better antioxidant activity was shown at lower concentration compared to δ-tocopherol, but at higher concentration the activities are inverted (Evans et al., 2002; Huang et al., 1996; Seppanen et al., 2010). Thus less α-tocopherol is necessary to get the maximum antioxidant protection, even if it is necessary consider the fat system involved. Tocopherols are a group of compounds that are highly lipophilic and are located in the cell membranes or lipoproteins of biological systems. Vegetable oils are a major source of liposoluble vitamin E, followed by nuts and seeds. When the oil seeds are crushed, tocopherols are extracted along with the oil, but tocopherol concentrations tend to decrease during the oil refining process (Chen et al., 2011). In general, tocopherols act as antioxidants by donating the hydrogen of the hydroxyl group to the lipid peroxyl radical to act as a chain-breaking antioxidant (Gülçin, 2012). The tocopherol radical is then stabilized through delocalization of the solitary electron over the aromatic ring structure. The antioxidant activity of tocopherols in fats, oils and lipoproteins is largely attributed to their low electron reduction potential, which indicates that tocopherols are capable of donating a hydrogen to a free radical with a higher electron reduction potential (*e.g.* allylic, peroxyl, and alkoxyl radicals) (Decker et al., 2010). Related to their bond dissociation energies, α-tocopherol (79.1 kcal/mol) is the most reactive and less stable form of tocopherol, hence during the lipid oxidation it is oxidized faster than β- (80.2 kcal/mol), γ- (80.1 kcal/mol), and δ-tocopherols (82.2 kcal/mol) (Decker et al., 2010). The hydrogen transfer from tocopherols is more thermodynamically facilitated for those molecules with the lower bond dissociation energy, thus α-tocopherol is more susceptible as compared to the other isoforms (α > β > γ > δ) (Timmermann, 1990; Wright et al., 2001). The ability to scavenge lipid peroxyl radicals is an important function of tocopherols that is directly linked to inhibition of lipid peroxidation. This reaction leads to the formation of lipid hydroperoxides and a tocopheroxyl radical. The latter can form non-radical products, including

stable peroxides, which can be reduced to tocoquinones and tocopherol dimers (Choe et al., 2009; Gülçin, 2012). In addition to its chain breaking effects, α-tocopherol has also been associated with the delay of hydroperoxide decomposition. Due to the powerful antioxidant effect of tocopherols, it is important to evaluate how the structure and composition of food matrices can impact the antioxidant efficacy of tocopherols. Since foods are complex chemical combinations of diverse components, there are a wide range of factors that can affect the depletion of tocopherols therein, such as oxygen pressure, light, transition metals, location within the food systems, the unsaturation degree of coexisting lipids and storage temperature (Chen et al., 2013).

2.11 Role and interactions of antioxidants in emulsions

A major concern of the food industry is how to retard, or inhibit, food oxidation to improve food quality. This challenge is becoming increasingly difficult since consumers' demand for all natural foods has limited the use of effective synthetic food-grade additives. The addition of free radical scavengers is one of the main methods employed by food scientists to prevent the propagation step of lipid oxidation (Panya et al., 2012). The effectiveness of antioxidant is affected by different factors, such as molecular structure, polarity (hydrophilic, lipophilic, amphiphilic), concentration, temperature, substrate composition, physical state of the system (*e.g.* bulk oil or emulsion), the presence of antioxidant synergists and proxidants, and their synergic effects (Shahidi et al., 2010).

Many common foods (*e.g.*, milk, salad dressing, sauces, soups) exist as emulsions of lipids dispersed in an aqueous phase and stabilized by emulsifiers that can provide the lipid physical protection. In emulsion systems, lipid oxidation proceeds differently from bulk oils because each phase of the system contains both prooxidants and antioxidants molecules; in addition, the oil-water interface impacts the interaction between the oil droplet and water components (Frankel et

al., 1994; Waraho et al., 2011). Within this complex system, the polarity of an antioxidant determines its location and environment (e.g. water, oil, or interface), which in turn affect its ability to scavenge free radicals to retard lipid oxidation (McClements, 2014). To this end, the antioxidant "polar paradox" has been a way to describe how its physical location is important to explicate its activity (Frankel, 1998; Porter. et al., 1989;). Regarding antioxidant effectiveness, the polar paradox theory states that hydrophobic antioxidants are more effective in O/W emulsions than their hydrophilic homologs (Porter et al., 1994). Several studies have demonstrated that non-polar antioxidants (*e.g.*, α-tocopherol, ascorbyl palmitate, carnosol) are more effective than their polar counterparts (Trolox, ascorbic acid, carnosic acid and rosmarinic acid) in emulsion systems containing stripped oil (Chaiyasit et al., 2005; Frankel et al., 1994; Frankel et al., 1996; Hopia et al., 1996; Huang et al., 1996). The higher activity of non-polar antioxidants in oil-in-water (O/W) emulsions has been proposed to be dependent on antioxidant retention in the emulsion droplet or partitioning at the droplet interface, where oxidation primarily takes place (Cercaci et al., 2007; Frankel et al., 1994; Schwarz et al., 2000). Hence, antioxidant surface active antioxidants, such as δ-tocopherol, α-tocopherol, *tert*-butylhydroquinone, and propyl gallate, may be located at the oil-in-water interface (Chaiyasit et al., 2007) or associated with surfactant micelles (Heins et al., 2007). At the interface, lipophilic antioxidants scavenge free radicals and prevent their crossing through the droplet membrane and come in contact with lipids (Shahidi et al., 2011). Gunaseelan et al. (2006) found that 73% of α-tocopherol is located in the interfacial region of a Brij 30-stabilized octane-in-water emulsion. The nature of tocopherol as surfactant-like character, with a polar phenolic head group attached to a hydrocarbon tail, explains why the large percentage of tocopherol is located at the interface where the lipophilic tail is oriented into the oil droplet (Gunaseelan et al., 2006). However, the prediction of the activity of an antioxidant to inhibit lipid oxidation in O/W emulsions cannot be explained considering just the polarity. Real foods are complex systems in which the antioxidant

activity can be increased or decreased depending upon other compounds present (Alamed et al., 2009). Indeed, recent publications highlighted that hypothetical expectations of the polar paradox do not always accurately predict antioxidants' behavior (Bakır et al., 2013; Laguerre et al., 2015). These studies were carried out changing the antioxidant polarity of rosmarinate acid by esterification with diverse fatty acids and showed that the activity-polarity relationship is affected also by molecular size of the side chain in both positive and negative manners (Laguerre et al. 2015; Shahidi et al., 2011). For instance, in O/W emulsions, it was observed that increasing antioxidant hydrophobicity by adding fatty acid chains to an antioxidant rises its activity up to a certain point after which further increasing hydrophobicity actually decreased antioxidant activity (Laguerre et al., 2015; Panya et al., 2010). Laguerre et al. (2009) stated that the antioxidant activity of cholorogenic acid in a stripped tung O/W emulsion stabilized with Brij 35 increase when esterified with methyl, butyl, octyl, and dodecyl hydrocarbons. However, further increments in the alkyl chain length (C20) led to a collapse in the antioxidant effectiveness, indicating a threshold for the dodecyl chain. This observation was termed ‘‘the cut-off effect’’ by these authors and was also highlighted with lipophilic antioxidants in which the lipophilized rosmarinates esterified with octyl hydrocarbons showed the maximal antioxidant capacity (Laguerre et al., 2010). More evidence was reported by Panya et al. (2012) where the influence of esterification of rosmarinic acid was determined on its ability to inhibit lipid oxidation in Tween 20-stabilized stripped soybean O/W emulsions during storage. Esterification with eicosyl hydrocarbons were less effective at inhibiting lipid oxidation than esters with shorter fatty acyl chains (C4, C8, C12). The authors suggested that the increased hydrocarbon length could lead to an internalization of the antioxidant away from the interface where it could scavenge free radicals into lipid droplet where it cannot explain the function. Other hypotheses were that reduced mobility of the antioxidant, as well as self-aggregation inside the emulsion droplet, may also play a role in reduced antioxidant activity. In the "reduced mobility hypothesis", the

mobility of the lipophilic antioxidant decreases as its alkyl chain is lengthened, consequently decreasing its ability to move towards the numerous oxidation sites. The hydrophobic interaction between the antioxidant and lipophilic compounds may also lower the diffusion of long chain antioxidants to the reaction centers. The most recent hypothesis of "self-aggregation" suggests that micellization of antioxidant in water phase can explain the collapse of its activity (Laguerre et al., 2015). Furthermore, it is important to consider the effect of the food matrices on the antioxidant activity. Alemán et al. (2015) determined how fatty alcohols chain length affects antioxidant activity in two different fish oil enriched food products (mayonnaise and milk). The authors evaluated the activity of caffeic acid and caffeates C1–C18 and caffeic acid and caffeates C1–C20 in mayonnaise and milk, respectively. The authors showed that there is an optimal chain length to protect against the oxidation depending on food matrices. In particular, the esterification with medium alkyl chain produced a better protection against oxidation in mayonnaise, whereas in fish oil enriched milk emulsions the most effective caffeates were those with shorter alkyl chains.

Besides polarity, other phenomena should be taken into account to explain the antioxidant activity of a molecule. The antioxidant polar paradox hypothesis states that non polar antioxidants are more effective in O/W emulsions presumably due to the greater retention of the non-polar antioxidants at the interface (Frankel et al., 1994). Factors like antioxidant concentration (Bakır et al., 2013), chemical composition of the emulsion, emulsifier type, and concentration also play a key role on the antioxidant partition in the medium (Shahidi et al., 2011). Therefore, its effectiveness could be mainly impacted by displacing the antioxidant to a different region of the emulsion, which in turn is affected by the emulsifier. The emulsifier can provide physical protection to the oil droplet by saturating the interface, and thus excluding other surface active molecules. In other words, emulsifier competes with antioxidants and prooxidants for localization at the interface, where the oxidation reactions prevalently occur.

Once that the emulsifier saturated the interface adsorbing onto the oil droplet, its molecules in excess are dispersed in the continuous phase as a monomeric molecules. This takes place when the emulsifier concentration is below to the critical point known as critical micelle concentration (CMC). If the concentration of the emulsifiers in continuous phase is above this point, the CMC, then surfactant micelles form and can solubilize small lipophilic or amphiphilic compounds, thus altering the partitioning behavior between the oil, water and interfacial regions (Kiralan et al., 2014; Laguerre et al., 2015; Nuchi et al., 2002; Panya et al., 2012; Richards et al., 2002). Given this, the antioxidants could be trapped in these self-assembled structures, contributing to antioxidants mass transfer among the emulsions compartments (Waraho et al., 2011). The exchange between the micelles and interface is also affected by the emulsifier type (ionic or non-ionic), because the antioxidant displacement could be hindered due to electrostatic repulsion between the ionic interface and surfactant micelles. Antioxidant displacement could compromise its activity and therefore the oxidative stability of the emulsion system. Several research projects have found that increasing the concentration of hydrophobic antioxidants in the aqueous phase of O/W emulsions, via the formation of surfactant micelles, increases the overall antioxidant activity (Kiralan et al., 2014; Laguerre et al., 2009; Panya et al., 2012). In a recent research, Kiralan et al. (2014) hypothesized that a reservoir of tocopherols in Tween 20–tocopherol co-micelles could replace oxidized tocopherol in emulsion droplets thus an rapid exchange of lipid components between micelles and emulsion droples to maintain an optimum level of antioxidants.

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3. Objectives

3. Objectives

The purpose of the whole research was focused on the study of the effect of minor compounds, such as phytosterols and α-tocopherol, on the oxidative stability and physical properties of bulk oil, oil-in-water emulsions, and food emulsions. The choice of these types of molecules was based on the fact that they are widely used in the food industry, due to their biological and antioxidant properties. In particular, the cholesterol-lowering effect of PS has resulted in the development of a wide range of phytosterol-enriched foods, which represent one of the most important growing areas of the functional food market. Therefore, these minor components are introduced into aqueous-based foods, such as milk, but they need to be either suspended or emulsified. The analysis of plant sterols/stanols present in enriched foodstuff has been usually carried out by conventional gas chromatography (GC) coupled to a flame ionization detector (FID), while GC–MS is often employed for peak identification. Considering that most methods have been set up to analyze the PS amount in edible oils or vegetable extracts, which are far below the concentration range of PS-enriched products, there is a need for developing new and fast analytical methods able to quantify PS in enriched food. Therefore, the objective of the first part of PhD research was to develop a sensitive Fast GC-MS method for analysis of the main phytosterol in sterol-enriched dairy products in a single run and short time. The method was first set-up with phytosterol standards and its suitability was later tested in various phytosterol-enriched dairy products.

However, sterols can oxidize and generate a series of oxidation products (such as phytosterol oxidation products (POPs)) with adverse biological effects, so it would be important to understand how cooking techniques can impact phytosterol oxidation in model systems, oils and food matrices. In particular, microwave ovens are widely used for fast cooking and reheating of food, so it would be interesting to highlight the effects of microwave heating on edible oils and

lipid-containing food. In fact, there are no available data on phytosterol oxidation during microwaving. The aim of this second part was to evaluate the oxidative stability of PS during microwave heating, using two different model systems (a solid film made with a phytosterol mixture and a liquid mixture of PS and triolein).

Since previous research showed that the rate of PS oxidation in O/W emulsions is higher than in bulk oil, other aim of this Ph.D. thesis was to evaluate the oxidative stability of an O/W emulsion containing both plant sterol esters and *Echium* oil as ω-3 fatty acids source in the presence of phenolic compounds extracted by red propolis. This study was carried out in three steps. Firstly, it was evaluated the effect of some known artificial and natural antioxidants and pro-oxidants on emulsions containing *Echium* oil and plant sterol esters (PSE), aiming to establish positive and negative controls of reactions to monitor the lipid oxidation. Samples were analyzed at the beginning, after heating and also after 30 days of storage at room temperature. In a second step, 11 phenolic compounds were added to the emulsion and the oxidative stability was evaluated after emulsion preparation, after heating, and 14 days of storage at room temperature. Phenolic compounds that showed better performance in the second step, were also evaluated in terms of quantification of hexanal, malondialdehyde (MDA), POPs and fatty acids composition.

Given the paradoxical oxidative effect of minor lipids compounds, more research is needed to specifically determine the context under which health-promoting minor compounds impact the stability of foods. The last part of the Ph.D. thesis was devoted to investigate the α-tocopherol partitioning in excess of anionic surfactant in stripped soybean oil-in-water emulsion. To this purpose, different concentrations of SDS were used to monitored the displacement of α-tocopherol in the O/W emulsion system. Subsequently, an oxidation study was carried out to

understand how the micelles affected the antioxidant activity of α-tocopherol in the O/W emulsion.

4. Results

4. Results

4.1 The effects of microwave heating in edible oils and lipid-containing food

4.2 Analysis of phytosterols and phytostanols in enriched dairy products by Fast gas chromatography with mass spectrometry

4.3 Effect of microwave heating on phytosterol oxidation

4.4 Antioxidant activity of phenolic compounds added to a functional emulsion containing omega-3 fatty acids and plant sterol esters

4.5 Increasing antioxidant activity by partitioning α-tocopherol into SDS-micelles in oil-in-water emulsions

4.1THE EFFECTS OF MICROWAVE HEATING IN EDIBLE OILS AND LIPID-CONTAINING FOOD

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The effects of microwave heating in edible oils and lipid-containing food

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Abstract

Lipids and lipid-containing food are particularly sensitive to microwave heating as the specific heat of lipids is low and thus they are quickly warmed up. Microwave heating mainly promotes lipid oxidation, but it can also cause lipolysis and polymerization. This cooking method can differently impact lipid oxidation depending on the treatment conditions used (power, temperature and time), as well as on food composition. This review provides a picture of the main degradation effects of microwave heating on vegetable oils and lipid-containing food, making emphasis on both fatty acids and cholesterol oxidation.

1. Introduction

Microwave (MW) heating is a common and fast procedure for food preparation and manufacturing. Microwaves are electromagnetic waves with a frequency range from 300 MHz to 30 GHz. To avoid their interaction with telecommunication devices, only 915 and 2450 MHz are approved for food processing at industrial level and home MW ovens, respectively.

Food MW heating results from two type of interactions between food components with MW: the *ionic conduction* and the *dipolar rotation*. In *ionic conduction*, the positively charged ions will rotate in the direction of the electric field of the MW oven, while negatively charged ions will move in the opposite direction. The electric field will change its direction depending on the frequency, causing thus a modification of the ions motion direction as well. Moving particles will collide with the adjacent particles, and the temperature of the particle will consequently increase (Malheiro et al., 2011). In the presence of polar molecules, heat generation in MW oven takes place by the mechanism of *dipolar rotation*. When polar molecules interact with the electric field, they try to orient themselves in the direction of the field and they collide with the other molecules. The change of the field direction results in further collision as they try to line up with the reversed directions, leading to agitation and heating. Therefore, the food dielectric properties (dielectric constant and dielectric loss factor), which are related to food composition, will greatly influence the cooking/heating time. However, it might be pointed out that the food temperature reached during microwaving is related to geometry/dimension and quality level of the MW oven.

Among food macro components, lipids are also particularly sensitive to this treatment as the specific heat for lipids is low and they are heated quickly. In particular, MW heating of vegetable oils, which are commonly used as heat transfer medium in complex food, accelerates their oxidation, causing polymerization and thermal-oxidative decomposition. MW treatment,

similarly to other cooking methods, impacts the lipid quality, nutritional value and safety; however, the domestic application does not require high exposure times and temperatures, except for MW frying.

This article will give an overview on the effects of MW heating in edible oils and lipid-containing food.

2. Microwave heating effects on edible oils

The effects of MW cooking on edible oils results in a degradation pattern involving oxidation, hydrolysis, and polymerization. Generally, vegetable oils subjected to MW treatments show the formation of reactive free radicals that quickly generate hydroperoxides and secondary oxidation products. Oxygen availability promotes this degradation pathway by reacting with unsaturated fatty acids. The unsaturated fatty acids content and composition, as well as the presence of antioxidants (tocopherols, chlorophylls, carotenoids, phenolic compounds), influence the extent of the first oxidative stage known as induction period. MW heating can also cause a drop in antioxidant content, which may modify the protection capacity from oxidation initiators. Hydroperoxides are well known to be unstable under microwaving, which promotes their rapid conversion into secondary oxidation products, in particularly aldehydes, alcohols, ketones, dimers, trimers, polymers and cyclic compounds. The rate of hydroperoxides transformation could be related to MW conditions and to the initial oxidative status of edible oils. Extreme heating conditions (temperature and time) favor polymer formation in vegetable oils with a high level of unsaturated fatty acids, leading to an increase of density and viscosity which can affect the rate of fatty acid breakdown and promote hydrolysis. Such heating conditions, however, are unusual for MW domestic procedures.

In a recent study, the effects of MW heating on the oxidation of fatty acid methyl esters (FAME) that are characteristic of edible oils (methyl oleate, linoleate and linolenate), were evaluated (Cardenia et al., 2012). The authors reported that the oxidation lag phase decreased as the FAME unsaturation degree increased. Methyl linoleate displayed a rise on conjugated dienes, whereas the latter and conjugated trienes were both found in methyl linolenate. Main volatile compounds (aldehydes, short-chain methyl esters, epoxy derivates, ketones, alcohols and hydrocarbons) formed faster in FAME with a higher unsaturation degree. However, no *trans* isomerization and dimer formation were detected. These oxidative and composition changes must be considered for the formulation of ready-to-eat food to be MW cooked as this can affect their nutritional and sensory profile, especially if highly unsaturated vegetable oils are used therein.

Another important change that occurs during MW heating of vegetable oils is lipolysis, which can lead to an acidity increase depending on treatment conditions (temperature, heat, oil-water interface, water and steam presence). Final acidity level will be, in any case, influenced by the initial free fatty acid (FFA) content, which varies among vegetable oils according to the extraction technology and refining conditions. In fact, virgin olive oils have a higher acidity value than commercial vegetables oils that undergo refining.

Chiavaro et al. (2010) evaluated the modifications on the differential scanning calorimetry (DSC) profiles and thermal properties (upon cooling and heating) of refined peanut, high-oleic sunflower and canola oils at different times of MW heating and compared the results with those obtained for standard chemical indices (free acidity, peroxide value, *p*-anisidine, oxidative stability index (OSI time)). Oils were exposed at a frequency of 2450 Hz at medium power (720 W) for 1.5, 3, 6, 9, 12 and 15 min. Considering all oxidative indices, canola oil was more markedly oxidized by MW heating than the other two oils, which might be related to its higher unsaturation degree. FFA formation was also remarkable at prolonged times of MW heating,

especially in peanut and canola oils (5 and 6 times higher than in the untreated oils, respectively). MW heating also altered both cooling and heating profiles of the vegetable oils tested (mainly in peanut and canola oils), shifting phase transitions towards lower temperature and enlarging temperature range. DSC thermal properties could be correlated to the formation of primary lipid oxidation products only for those oils that exhibited extensive lipid oxidation after prolonged MW heating, such as canola oil.

The same research groups (Chiavaro et al., 2010) evaluated the influence of MW heating on some chemical indices (oxidation parameters, free acidity, phenolic compound degradation, water content), and DSC thermal properties (upon cooling and heating) of three commercial categories of olive oil for direct human consumption (extra virgin olive oil (EVOO), pomace olive oil and olive oil) at different times of treatment. The authors applied the same MW conditions as those described in the aforementioned work (Chiavaro et al., 2010). Water content of unheated EVOO was higher with respect to the other two oils and it was found to decrease with increasing treatment time in all oils, particularly in EVOO (after 3 and 12 min of heating). Lipolysis was also noticeable in the latter only at highest treatment times (12 and 15 min). Peroxides greatly decreased (76-85%) in all oils up to 6 min of heating and remained approximately constant until the end of the heating treatment, while secondary oxidation products showed a sinusoidal trend in EVOO, and olive oil with a maximum at 6 min and 15 min, respectively, while they gradually increased in pomace olive oil. About 30% of phenols initially found in EVOO were lost after 6-min heating; nevertheless, among the different classes of phenolic compounds, *o*-diphenols and lignans showed the highest MW heating resistance in the whole time range of MW heating, which is of noticeable importance from a nutritional point of view. Regarding the DSC thermal properties, the authors reported marked changes of DSC cooling profiles for EVOO and pomace olive oil after MW, with the major exotherm that shifted towards lower temperature and decreased height with increasing treatment time. Cooling thermal

properties changed in all samples: crystallization enthalpy significantly decreased and the phase transition developed over a larger temperature range, due to more heterogeneous chemical composition of all oils that resulted from hydrolysis and oxidation products. Heating profiles of EVOO and pomace oil were also affected by MW treatment, as the minor endotherm progressively disappeared, significantly shifting offset temperature of transition towards lower temperature. Olive oil did not show such changes of thermal properties and phase transition profiles as described for EVOO and pomace oil.

3. Microwave heating effects on lipid containing food

3.1 Fish

The fat content and fatty acid composition of fish display a large variability associated with different biotic (eg. species, reproductive cycle, muscle type) and abiotic (eg. temperature, pH, diet) factors. Among the main fatty acid classes, long-chain polyunsaturated fatty acids (PUFA) n-3 are the most representative fish lipids, in particular eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are of great nutritional importance, as they prevent human coronary artery diseases. However, they are particularly sensitive to oxidation due to their high unsaturation degree.

A recent study (Zhang et al., 2012) reported the effects of different cooking methods (boiling (98°C/12 min), steaming (12 min), microwaving (4 min at high power), grilling (250°C/12 min), pan-frying (180°C/8 min) and deep-frying (180°C/5 min)) on lipid oxidation and fatty acid composition of grass carp fillet. In general, frying and grilling led to the most extensive modifications in fish composition, but also MW treatment induced some changes on this food product. In fact, when microwaved, the moisture content of the carp fillet decreased significantly as compared with raw fish and samples obtained with “wet processing methods”

(boiling and steaming); fried fish also registered a drop in moisture content, with a simultaneous gain of fat from the frying oil. Such water loss led to a corresponding increase of fat and protein concentrations in microwaved, grilled and fried fish with respect to the other samples. Fried fish showed the highest content of polar lipids, followed by MW and grilled samples which had twice as much the level found in other treatments. All cooking methods led to a general decrease of FFA, which could be due to lipase inactivation and/or loss of volatile FFA; both MW-baked and grilled samples underwent a similar FFA decrease. Regarding oxidation, no significant effects of cooking methods were observed on the peroxide level, but all heating treatments promoted secondary lipid oxidation (including volatile compound generation), especially in grilled and MW samples. Despite lipid oxidation, MW heating did not change the n-3/n-6 ratio (0.97) with respect to the one found in raw fillets (0.95) (Zhang et al., 2012); only fried fish registered a decrease in such ratio, due to oil absorption from the frying medium.

3.2 Meat

Meat lipids can oxidize during heating/cooking and generate several oxidation products that are responsible for desirable and undesirable sensory attributes. The extent of such modifications will depend on time/temperature conditions and will also impact the meat nutritional profile. Oxidation will not only affect unsaturated fatty acids, but it will also involve other lipophilic, unsaturated molecules, such as cholesterol, which can generate neo-formation products (cholesterol oxidation products or COPs) that are known for their asserted toxicity and involvement in different chronic and degenerative diseases, such as atherosclerosis and cancer. Most common COPs are those formed in the ring structure, having different functional groups (hydroxy, epoxy and keto derivatives); in general, 7-ketocholesterol is the most abundant and it is often used as marker of cholesterol oxidation.

Broncano et al. (2009) studied the effects of several cooking methods on lipid oxidation of *Latissimus dorsi* muscle from Iberian pigs. In particular, meat samples were grilled (190°C/2 min on each side), fried (in refined olive oil at 170°C/2 min on each side), microwaved (80°C/90 s at 450 W) and roasted (150°C/20 min). No significant changes were observed in the total lipid and moisture contents of meat cooked with the different methods. However, some differences were found on the level of secondary oxidation products (hexanal and thiobarbituric reactive acid substances (TBARs)). MW meat had a lower TBARs level with respect to roasted and fried samples, but it was similar to the grilled one; on the contrary, hexanal was lower in grilled meat as compared to the other cooking treatments. It is possible that temperatures reached during the MW heating were not high enough to promote extensive decomposition of secondary oxidation products and/or reaction with other molecules (such as proteins), as it may have occurred during grilling. The formation of COPs was not differently affected by cooking methods; main COPs found in samples from all cooking methods were 7α-hydroxycholesterol and 7β-hydroxycholesterol, while only traces of 7-ketocholesterol were detected.

3.3 Milk

Fresh milk contains about 3-4% of fat, which is composed mainly by triglycerides with fatty acids having a wide range of hydrocarbon chain length (from short to long chain), unsaturation and ramification degree. The milk fat is organized as globules surrounded by a membrane, which is able to retain the globules in emulsion thanks to the presence of polar lipids, phospholipids and specific membrane proteins. Milk also has some minor components, such as cholesterol (about 0.012% in fat), which is prone to oxidation, as previously described. Although the risk of cholesterol oxidation in fresh milk or fresh dairy products is low due to fat organization into micelles and milk composition, the manufacturing processes used for dairy products (eg. high

pressures), as well as the cooking methods (high temperatures combined with heating time and heating sources like MW/conventional one), could favor cholesterol oxidation.

In a recent study, conventional and MW heating were applied to full-cream milk to evaluate COPs formation as related to temperature and time (Calderon-Santiago et al., 2012). In the MW treatment, full-cream was heated for 30, 60, and 90 s at 360 W, whereas an electrical heater plate was used for conventional heating for 5, 10, 20, 30, and 40 min. In both cooking methods, heating times were chosen as to avoid reaching milk boiling point, which could alter the stability of the lipid phase. In the first 5 min of heating, conventional cooking induced a rise of total COPs level, which decreased thereafter; this could be ascribed to further degradation of these oxidation products and/or interaction with other matrix components (such as proteins). In MW heating, maximum COPs amounts were reached after 60 sec, being similar to those found after 5 min in conventional cooking; the great effectiveness of MW heating in inducing COPs formation is also confirmed by other studies. 7β-hydroxycholesterol was the most abundant COP in both treatments, and its behavior during heating greatly influenced the overall COP trend. Although 7-ketocholesterol was detected in samples from both cooking methods, it displayed a different behavior as it did not increase during microwaving, but it showed a sinusoidal trend in conventional cooking and degraded mainly after 30 min.

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4.2 ANALYSIS OF PHYTOSTEROLS AND PHYTOSTANOLS IN ENRICHED DAIRY PRODUCTS BY FAST GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY

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Analysis of phytosterols and phytostanols in enriched dairy products by Fast gas chromatography-mass spectrometry

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Abstract

A Fast gas chromatography-mass spectrometry method for plant sterols/stanols analysis was developed, using a short capillary gas chromatography column (10 m x 0.1 mm internal diameter x 0.1 μm film thickness) coated with 5% diphenyl-polysiloxane. A silylated mixture of the main plant sterols/stanols standards (β-sitosterol, campesterol, stigmasterol, campestanol, sitostanol) was well separated in 1.5 min, with a good peak resolution (>1.4, determined on a critical chromatographic peak pair (β-sitosterol and sitostanol)), repeatability (<13%) and sensitivity (<0.017 ng/mL). The suitability of this Fast chromatography method was tested on plant sterols/stanols-enriched dairy products (yogurt and milk), which were subjected to lipid extraction, cold saponification and silylation prior to injection. The analytical performance (sensitivity <0.256 ng /mL and repeatability <10.36%) and significant reduction of the analysis time and consumables, demonstrates that Fast gas chromatography-mass spectrometry could be also employed for the plant sterols/stanols analysis in functional dairy products.

Keywords: Enriched products, Fast GC-MS, functional dairy products, phytosterols, phytostanols

1. Introduction

Plant sterols/stanols (PS) are a mixture of 4-desmethylsterols (mainly β-sitosterol, campesterol and stigmasterol), which have demonstrated a cholesterol-lowering effect (García-Llatas et al., 2011). In nature, PS can be found as free molecules or conjugated with a fatty acid or hydroxycinnamic acid (Lagarda et al., 2006). PS can be extracted from tall oil, a by-product of the wood pulp industry, and from deodorizer distillates of vegetable oil refining. Phytostanols are obtained through hydrogenation of unsaturated sterols (Kawamura, 2008). PS are widely used to formulate enriched-food products that meet the health claims associated with sterol fortification. Scientific studies indicate that PS consumption (1-3 g/day) reduces low-density lipoprotein cholesterol (LDLc) blood levels by about 5 to 15% (European Commission Regulations 1997/258; Normén et al., 2003). In 2008, the European Food Safety Authority (EFSA) concluded that PS dietary intakes that do not exceed 3 g per day could be considered as safe. Higher daily PS consumption should be avoided, as it can reduce carotenoid plasma levels without having an additional effect on cholesterol (EFSA, 2008).

Analytical methods for the determination of PS have been developed for the evaluation of edible oils or food vegetable extracts with ≤ 1% of sterols as naturally occurring minor components. In PS-enriched food products, the percentage of sterols varies up to 8%, highly over the concentration range of edible oils or food vegetable extracts (Duchateau et al., 2002; Laakso, 2005). Current analytical methods for PS determination are generally time-consuming, as they include lipid extraction, saponification, extraction of unsaponifiable matter, purification (by thin-layer chromatography (TLC) or solid-phase extraction (SPE)) and derivatization of sterols. The first step of sample preparation is to isolate the sterol fraction and to convert all conjugated or esterified PS into free molecules for gas chromatography (GC) analysis. To achieve this goal, it is important to choose the adequate extraction method, which is closely related to the sample matrix and the original form of the phytosterol in the sample

[http://lipidlibrary.aocs.org/topics/phytosterols/index.htm]. In some cases, such as blood serum, the determination of total sterols requires hydrolysis to convert bonded sterols (also present in the lipoproteins) into their free form. Mendiara et al. (2012) developed a fast sample treatment by using ultrasound assistance to reduce the reaction time during the alkaline hydrolysis and they also compared different SPE phases (two reversed-phase sorbents (DSC-18 and polymeric Oasis-HLB) and a novel zirconia-coated silica) for metabolite concentration. Esche et al. (2012) described a SPE method, which enables the effective isolation of free sterols/stanols, steryl/stanyl fatty acid esters and steryl/stanyl phenolic acid esters from corn, rye, wheat, and spelt grain oils. The same authors investigated a method for the analysis of intact phytosteryl/phytostanyl esters in enriched dairy products with substantial amounts of protein and fat; the method involves the combined used of acid digestion, prior to lipid extraction, and on-line liquid chromatography (LC)-GC (Esche et al., 2013).

However, if sterols are to be analyzed by capillary GC, oils or fat extracts are generally subjected to direct alkaline or acid hydrolysis followed by extraction of the unsaponifiable matter. The latter may contain other lipophilic compounds (such as 4-methylsterols, 4,4'-dimethylsterols and triterpenic alcohols), which may co-elute with 4-desmethylsterols on GC columns. To overcome this partial or total overlapping problem, pre-fractionation by thin-layer chromatography (TLC) is widely used to isolate PS, as requested by the official methods for the accurate capillary GC determination of sterols in olive oil and other seed oils [http://lipidlibrary.aocs.org/topics/phytosterols/index.htm; European Commission Regulation No.2568/91, NP EN ISO 12228, 199). Quali-quantitative chromatographic analysis of PS is usually performed by GC equipped with a flame ionization detector (FID) (Abidi, 2001; Laakso, 2005), while GC coupled to a mass spectrometer (MS) is often employed for peak identification (Kawamura, 2008). Considering all these facts, there is a need for developing new and fast analytical methods able to quantify PS in enriched-food. To optimize time and resources, Fast

GC coupled with an electron impact MS could be a suitable alternative for PS determination, as it can provide a highly sensitive, simultaneous identification and quantification of the compounds of interest (Abidi, 2001; Cardenia et al., 2012), thus preventing misrecognitions and inaccurate quantification arising from partial overlappings and/or matrix interference. Reduction of analysis time is achieved by using a micro-bore capillary column, high inlet pressure and fast temperature program rates; these devices allow to obtain a higher resolution power, to operate at higher speed and to preserve the same efficiency than a conventional GC instrument (Cardenia et al., 2012). To the best of our knowledge, no Fast GC-MS method has been developed for the analysis of all plant sterols/stanols present in sterol-enriched food (Duchateau et al., 2002; Laakso, 2005), even though several researchers (Alemany-Costa et al., 2012; González-Larena et al., 2011; Menéndez-Carreño et al., 2008) had focused on the study of PS stability in enriched food through the determination of their presence and the formation of phytosterol oxidation products (POPs). Alemany-Costa et al. (2012) identified and quantified campesterol, stigmasterol, β-sitosterol, campestanol and sitostanol in PS-enriched milk-based fruit beverage and fruit beverage by conventional GC-FID, whereas Menendez-Carreño et al. (2008) determined the sterol content in phytosterol-enriched milk by GC-FID to evaluate their stability under heating conditions. A recent work reported a Fast GC-MS method for determination of cholesterol oxidation products, which highlights the great potential of this technique for reduction of analysis time and consumables (Cardenia et al., 2012).

The aim of the present study was to develop a sensitive Fast GC-MS method for analysis of the main PS in sterol-enriched dairy products in a single run and short time. The method was first set-up with PS standards and its suitability was tested in various PS-enriched dairy products.

2. Materials and methods

2.1 Reagents and solvents

Chloroform, *n*-hexane, methanol, isopropanol, potassium chloride and diethyl ether were purchased from Merck (Darmstadt, Germany). Anhydrous sodium sulfate and potassium hydroxide were supplied by BDH (Poole, England) and Prolabo (Fontenay, France), respectively. N°1 filters (70 mm diameter) were used (Whatmann, Maidstone, England). 5α-cholestan-3β-ol (dihydrocholesterol, IS, purity: 95%), 3β-stigmast-5-en-3-ol (β-sitosterol, purity: 60%), 3β-ergost-5-en-3-ol (campesterol: 37.5%), 3β-hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol, purity: 98%), hexamethyldisilazane, trimethylchlorosilane and palladium (purity: 99.99%), were purchased from Sigma (St. Louis, MO, USA). Dried pyridine was used (Carlo Erba, Italy). The silylation mixture was prepared with dried pyridine, hexamethyldisilazane and trimethylchlorosilane, at a ratio of 5:2:1 by volume.

2.2 Samples of PS-enriched dairy products

PS-enriched yogurt samples (fermented cow's milk (MY) and fermented soya drink (SY)) and PS-enriched milk samples (skimmed cow's milk (MM) and soya milk (SM)), were purchased in different Italian (Bologna) and Spanish (Granada) supermarkets. The yogurt and milk samples were stored in refrigerator at 4°C and within one week were analyzed. Two packs of yoghurt of the same batch were used as two independent replicates. Table 1 shows the nutritional information reported in the products' labels.

Table 1. Nutritional information reported in the label of the PS-enriched products (for 100 mL), as provided by the suppliers**.**

	MY	**SY**	**MM**	**SM**
Energy (Kcal/KJ)	40/167	47/197	40/169	38/159
Protein (g)	3.3	1.4	3.7	3.0
Carbohydrate (g)	4.4	3.6	4.7	2.3
of which are sugar (g)	4.1	2.9	4.7	2.3
Fat (g) (excluding PS)	1.1	2.8	0.7	1.8
of which saturated(g)	0.1	0.3	0.3	0.3
of which monounsaturated (g)			0.2	
of which polyunsaturated (g)			0.2	
Fiber (g)	0.7	1.0	0	0.5
Salt (g)	0.04	Trace	0.06	0.04
Plant sterol (g)	1.6		0.3	0.37
Plant stanol (g)		3		
Vitamin B1 (mg)			0.22	
Vitamin B6 (mg)		0.85		
Folic Acid (µg)		75		
Calcium (mg)			130	120

2.3 Preparation of PS standard solution

2.3.1 Hydrogenation of sterol standards

Since stanol standards are not commercially available, a hydrogenation reaction of sterol was carried out. β-sitosterol standard (418 mg) was dissolved in 10 mL of isopropanol:diethyl ether mixture (3:2, v/v) in a reaction flask and then 10.6 mg of palladium were added. A hydrogen pressure of 3.04 bar was employed for the reaction. Once the latter was completed, the catalyst was removed by filtration and the solvent was evaporated by rotavapor at 40°C. Stanol crystals were placed in a vial, covered with an aluminum foil and stored at 4°C.

The performance and efficiency of the hydrogenation reaction were controlled by NMR spectra and mass spectrometry; the spectra obtained were compared with those reported in literature. As for sterol standards, purity of the synthesized stanols standards was determined by GC-FID (area percentage), which was 51% for sitostanol and 33% for campestanol; such data were also confirmed by NMR.

2.3.2 Standard solutions of sterols/stanols

Two standard stock mixtures of sterols (mix A) and stanols (mix B), respectively, were prepared in *n*-hexane:isopropanol (3:2, v/v). The mix A contained β-sitosterol, stigmasterol and campesterol at a concentration of 2.13 mg/mL, 1.10 mg/mL and 1.51 mg/mL, respectively. The mix B contained sitostanol and campestanol at a concentration of 7.62 mg/mL and 4.83 mg/mL, respectively.

Calibration curves were generated at six different concentration levels, ranging from 0.125 to 5 mg/mL of total sterols and stanols (in *n*-hexane:isopropanol (3:2, v/v)). In order to overcome and to correct eventual PS losses during preparation and purification sample steps, 0.500 mg of dihydrocholesterol (used as IS) was added to each level of concentration. Solvents were dried under nitrogen flow; the residues were silylated (Sweeley, 1963), dried under nitrogen flow at 40°C and dissolved in 5 mL of *n*-hexane. Each calibration point was made in triplicate.

The calibration curves in Fast GC-MS were obtained by plotting the ratio of an individual sterol/stanol area to the internal standard area as function of the ratio of individual sterol/stanol standard concentration to internal standard concentration.

2.4 Sample preparation of PS-enriched dairy products

Lipids from yogurt drink and milk were extracted according to a modified version (Boselli et al., 2001) of the method described by Folch et al. (1957). A 25 mg lipid subfraction of the Folch extract was added with 250 µL of a solution containing dihydrocholesterol (2 mg/mL in chloroform), used as internal standard. Subsequently, the sample was dried under nitrogen and 30 mL of 2 M KOH solution in methanol were added to perform a cold saponification for 18 h (Sander et al., 1989). The extraction of the unsaponifiable matter was carried out with 10 mL of water and 10 mL of diethyl ether; the samples were shaken and the diethyl ether fraction was collected. The extraction with 10 mL of diethyl ether was repeated twice. The three ethereal fractions were combined and added with 5 mL of a 0.5 N KOH solution,. The resulting ethereal extract was washed twice with 5 mL of a saturated sodium chloride solution. The ether solution was separated and finally evaporated by rotary evaporator. The unsaponifiable matter was then derivatized with 1 mL of silylation mixture and heated in thermoblock at 40°C for 20 min (Sweeley et al., 1963). Thereafter, the samples were dried under nitrogen stream, dissolved in 1 mL of *n*-hexane, stirred and centrifuged at 1800 rpm (600 RCF) for 3 min. The upper layer was collected, dried under nitrogen stream and dissolved in 10 mL of *n*-hexane. One µL of the silylated plant sterols was then analyzed by Fast GC-MS. Three independent replicates were run per sample.

2.5 Determination of phytosterol and phytostanol

Fast GC-MS analysis was performed using a GC Shimadzu QP 2010 Plus (Kyoto, Japan) equipped with a split-splitless injector and coupled to an electron impact (EI) mass spectrometric detector. A fused silica capillary column Restek RTX-5 (10 m x 0.1 mm i.d. x 0.1 µm film thickness; Bellafonte, PA, USA) coated with 95% dimethyl- and 5% diphenyl-polysiloxane, was used. A non-silanized standard split liner (95 mm x 5 mm O. D. x 3.4 mm I. D.) was utilized. The temperature was programmed from 220 to 325°C at 5°C/min. The injector and the ion

source temperatures were set at 325°C and 320°C, respectively. The injection was performed in the split mode (1:50) and helium was used as carrier gas at a linear velocity of 47.7 cm/s. The electron energy was 70 eV. A mass range from 50 to 650 *m/z* was scanned at a rate of 2500 amu/s, which corresponded to 0.3 event time. The acquisition and integration modes were Full Scan (TIC) and Single Ion Monitoring (SIM), respectively. PS were recognized and quantified by their corresponding characteristic ions: 343 *m/z* (campesterol), 83 *m/z* (stigmasterol), 396 *m/z* (β-sitosterol), 488 *m/z* (sitostanol), and 459 *m/z* (campestanol).

The results obtained by Fast GC-MS were compared with those achieved by conventional GC-FID, as the latter is the most used analytical method for the chromatographic determination of sterols. The GC-FID analysis was carried out by an HRGC 5300 Mega Series (Fisons, Rodano, Italy) equipped with a split injector and a flame ionization detector (FID). A fused-silica capillary column CP-Sil 5 CB Low Bleed/MS (Chrompack-Varian, Middelburg, The Netherlands) (30 m x 0.32 mm i.d. x 0.25 μm film thickness) coated with 100% dimethyl-polysiloxane was used. The oven temperature was programmed from 265 to 280°C at a rate of 0.5°C/min, then from 280 to 325°C at rate of 4°C/min and held for 30 min at 325°C. The injector and detector temperatures were both set at 325°C. Helium was used as carrier gas at a flow rate of 2.8 mL/min. The split ratio was 1:11. PS were identified by comparing their retention times with those of standards. Sterols were then quantified by comparing the peak areas of the internal standard (dihydrocholesterol) and its concentration with the peak areas of the individual sterols/stanols standard. In order to obtain a more accurate measurement and avoid overestimation, the GC response factor of PS was calculated according to the following expression:

$$K = (A_x \times C_{is})\,(A_{is} \times C_x)^{-1} \quad (1)$$

where A_x and A_{is} are peak areas, whereas C_x and C_{is} represent the concentration of individual sterols/stanols and IS, respectively.

2.6 Resolution, sensitivity and repeatability

The peak resolution was determined on a critical chromatographic peak pair (β-sitosterol and sitostanol) and in terms of average peak width at the peak base (McNaught et al., 1997), according to the following expression:

$$R= 2(t_{R2}-t_{R1}) (w_1+w_2)^{-1} \quad (2)$$

where t_R is the retention time of chromatographic peak and w is the peak width at its base level.

The sensitivity of the method was evaluated in both standards solutions and samples by Fast GC-MS and conventional GC-FID; limits of detection (LOD) and quantification (LOQ) for each sterol/stanol were defined. LOD and LOQ were calculated by examining the signal-to-noise ratios; LOD was expressed as a concentration (ng/mL) at a specified signal-to-noise ratio (3:1), whereas a signal-to-noise ratio of 6:1 was used to determine LOQ.

To evaluate the repeatability of the Fast GC-MS method with that of the conventional GC-FID, intraday and interday precision were calculated in standard solutions (at 0.125 mg/mL and 1 mg/mL level for Fast GC-MS and for the conventional methods, respectively) and samples. Silylated sterol/stanol standard solutions and samples were injected (n=3) on the same day (intraday precision) for 3 consecutive days (interday precision, n=9). All injections were performed manually and by different operators. In both methods, the coefficient of variation (% CV) of the individual sterol/stanol area to the internal standard area (SIM mode in Fast GC-MS method) ratio was determined.

3. Results and discussion

Since stanol standards were not commercially available when the analysis were carried out, the phytosterol standards (β-sitosterol:campesterol, 60:40) were hydrogenated. Figure 1 shows the efficiency of hydrogenation reaction, monitored by GC-FID and Fast GC-MS; in the latter, the spectra were compared with those reported literature (Dutta, 2002) or in library system (NIST 08s). As reported in Figure 1, the TMS derivatives of stanol eluted after their corresponding sterols (elution order: campesterol, campestanol, β-sitosterol and sitostanol).

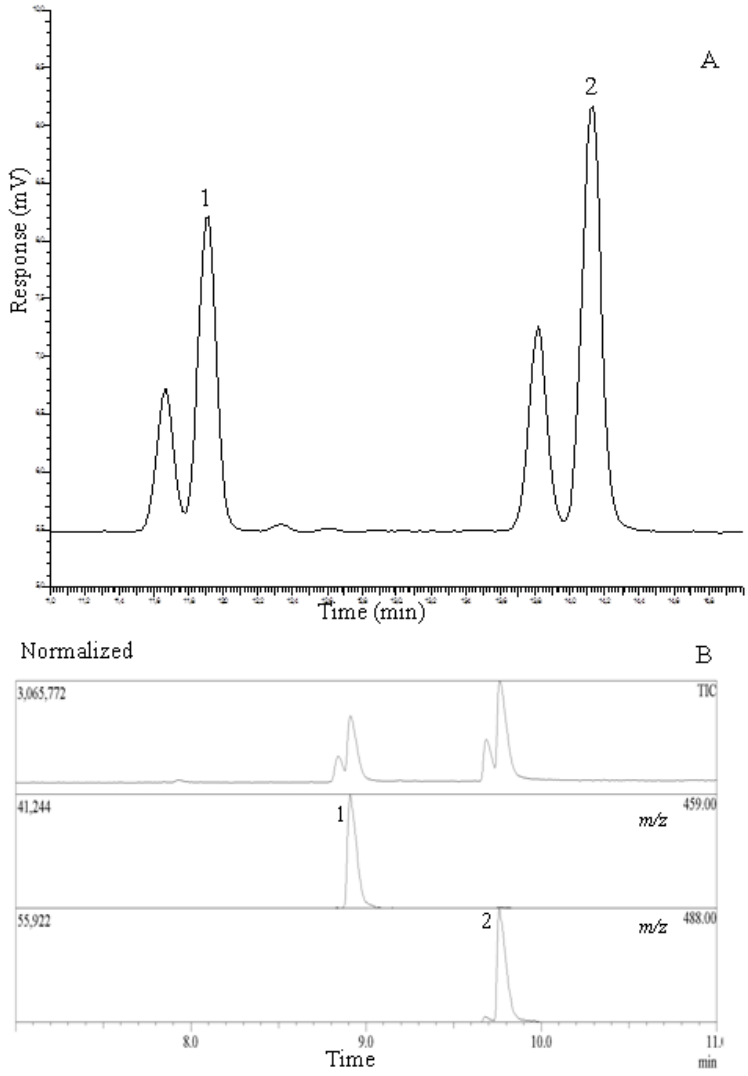


Figure 1. GC-FID (A) and Fast GC-MS chromatograms (B) (full scan (TIC) and single ion monitoring (SIM)) of TMS derivatives of hydrogenated sterol standard. The peak numbers in the chromatograms refer to the following plant sterols: 1, campestanol (*m/z* 459); 2, sitostanol (*m/z* 488).

3.1 Chromatographic separation of phytosterol and phytostanols

TMS derivatives of the PS standard mixture (β-sitosterol, campesterol, stigmasterol, sitostanol, campestanol) were analyzed by Fast GC-MS using the conditions reported in paragraph 2.5 of this paper. Figure 2 (A and B) displays the Fast GC-MS traces of the TMS

derivatives of the phytosterols and phytostanol standard mixture, using TIC and SIM acquisition modes, respectively.

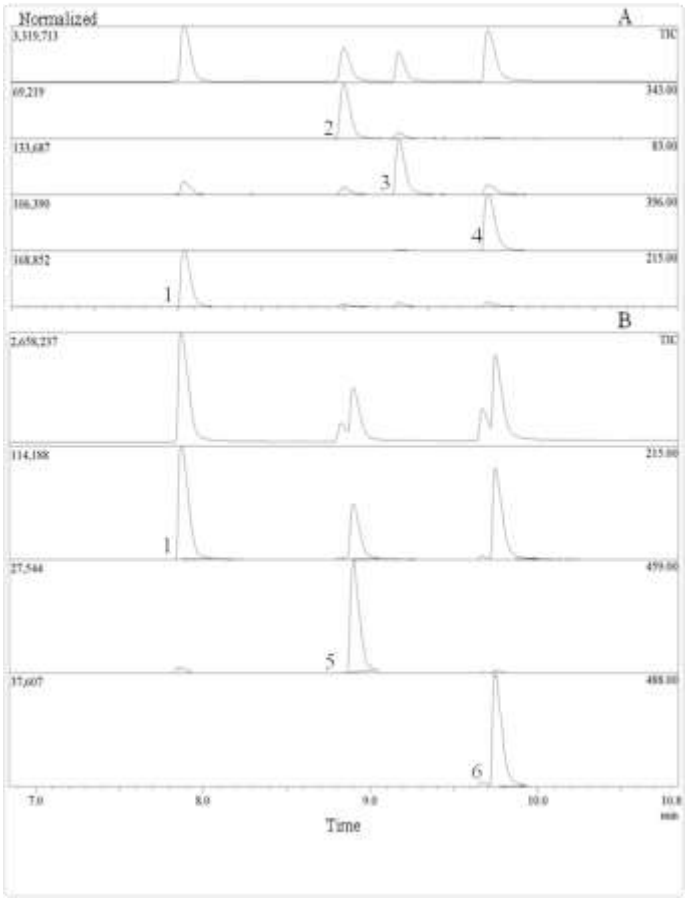
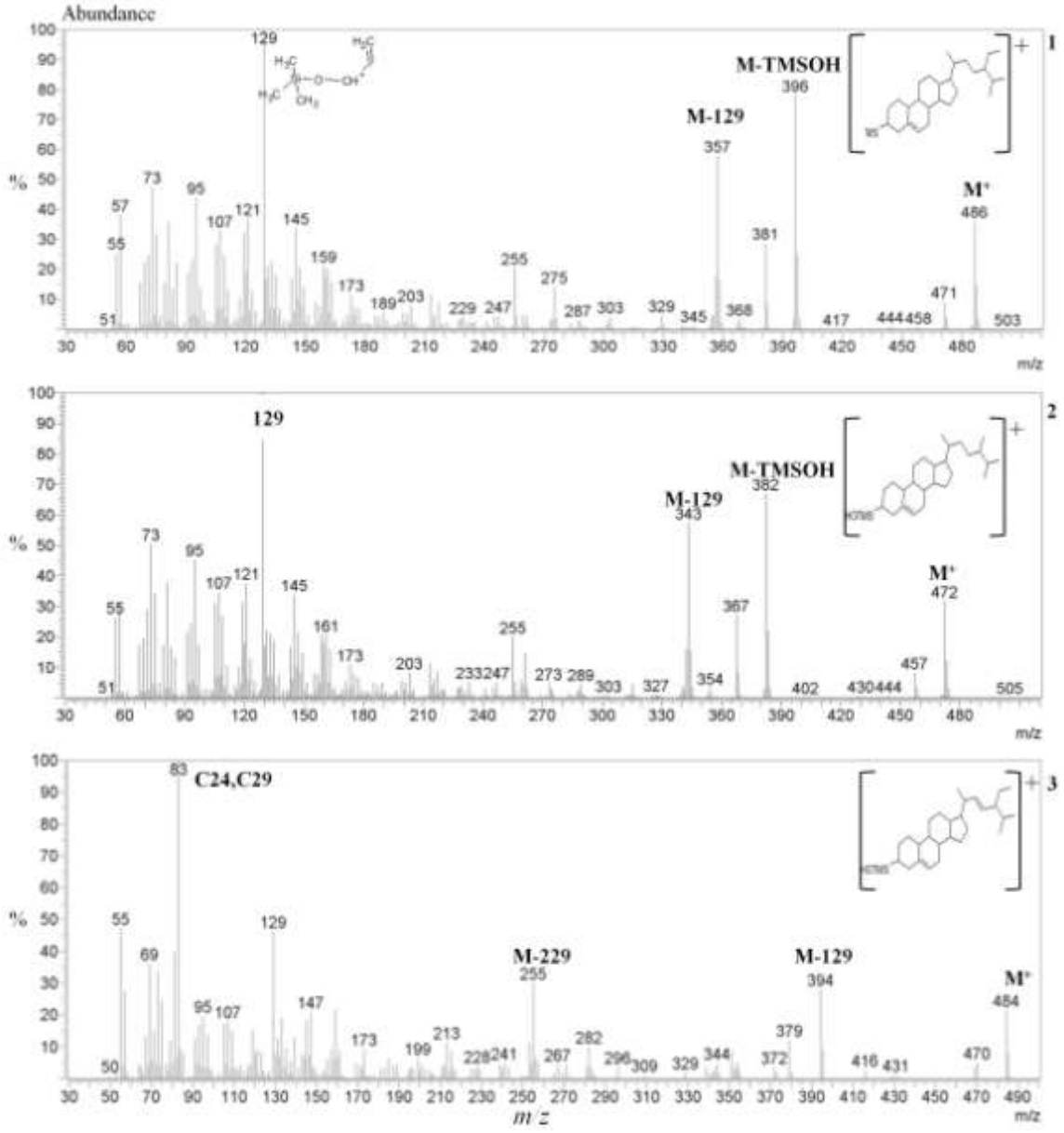


Figure 2. Fast GC-MS traces obtained with Full Scan (TIC) and Single Ion Monitoring (SIM) modes of TMS derivatives of phytosterols (A) and phytostanols (B) standard mixtures. Peak identification: 1, IS; 2, campesterol; 3, stigmasterol; 4, β-sitosterol; 5, campestanol; 6, β-sitostanol.

Each PS was recognized using its characteristic mass fragmentation pattern produced by electron impact (Figure 3). Two specific ions allowed to distinguish between TMS derivatives of unsaturated and saturated PS, in particular the ion fragmentation at *m/z* 129 (C1, C2, C3/TMSOH) for sterols and the ion fragmentation at *m/z* 215 (M^+ - side chain - 42 – CH-TMSOH) for stanols. The ions *m/z* 396, 343, 83, 488 and 459, which correspond to [M-

TMSOH], [M-129], [C24,C29], [M^+], [M-15] fragments, respectively, were selected to quantify β-sitosterol, campesterol, stigmasterol, sitostanol and campestanol (Figure 3).



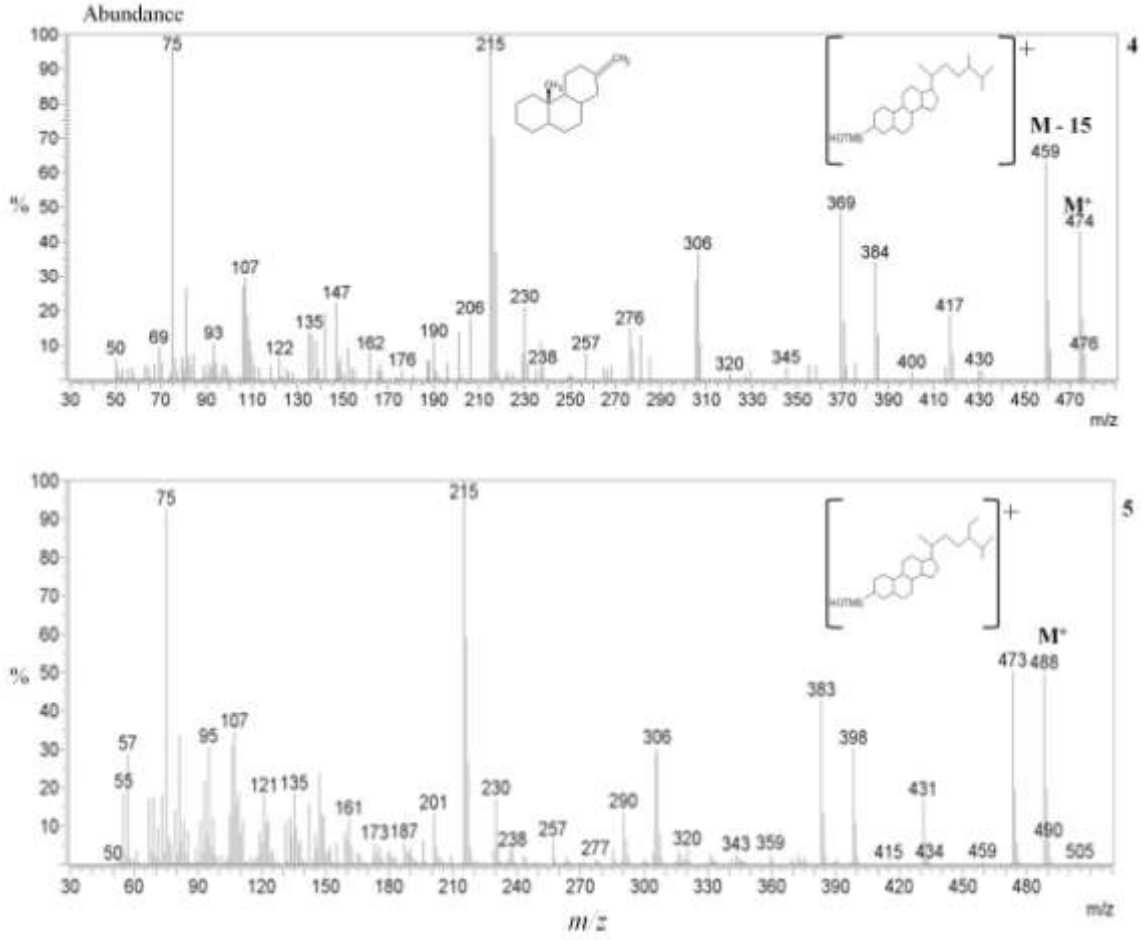


Figure 3. Characteristic mass fragmentation pattern of plant sterol: 1, β-sitosterol; 2, campesterol; 3, stigmasterol; 4, campestanol; 5, sitostanol.

The separation efficiency of the Fast GC-MS method was compared with that obtained by GC-FID, by injecting the standard solutions and PS-enriched dairy samples under the instrumental conditions reported in section 2.5. Peak resolution in the Fast GC-MS method (R>1.35) was greater than the one found in conventional GC-FID (R>1.03). Regarding the PS retention time (Figure 4), the Fast GC-MS method allowed a good PS separation in the first 10 min (1.3 min time frame), while the chromatographic separation in the conventional GC-FID was achieved in 20 min.

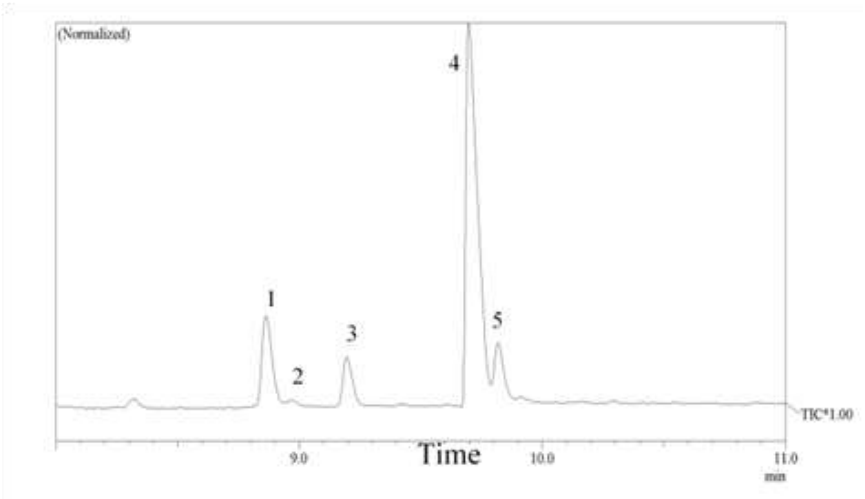


Figure 4. Fast GC-MS chromatogram of phytosterols and phytostanols in PS-enriched dairy products: 1, campesterol; 2, campestanol; 3, stigmasterol; 4, β-sitosterol; 5, sitostanol.

3.2 Linearity, sensitivity, and repeatability

Although the sensitivity of the GC-MS and GC-FID instruments is known to be different, the results achieved with the Fast GC-MS method were compared with those obtained with GC-FID, as the latter is widely used for determination of food sterol composition (Barnsteiner et al., 2012; Borkovcová et al., 2009; Conchillo et al., 2005; Contarini et al., 2002). In addition, conventional GC-MS has proven to give similar analytical parameters to those achieved by Fast GC-MS, as described by Cardenia et al. (2012); in fact, the authors reported that the Fast GC-MS method allowed a significant reduction in the analysis time and consumables as compared with conventional capillary GC.

The linearity of the Fast GC-MS method was evaluated by analyzing the standard solutions of phytosterol and phytostanol at 6 different levels, which ranged from 0.125 to 5 mg/mL of total sterols. Three replicates of each concentration level were analyzed. For each individual sterol/stanol, calibration curves were generated. As shown in Table 2, these curves

displayed a linear behavior within the concentration ranges tested, having determination coefficients (R^2) that varied from 0.9924 to 0.9950 for individual sterols and stanols.

Table 2 Response parameters of phytosterol and phytostanol standard mixture (concentration range= 0.125-5 mg/mL) in the Fast GC-MS method.

Sterol and Stanol	**Calibration curve[a]**	**R^{2b}**
β-sitosterol	y=1.1272x - 0.1862	0.9928
Campesterol	y=0.8727x - 0.0849	0.9950
Stigmasterol	y=2.395x - 0.1712	0.9939
Campestanol	y=0.4915x - 0.0617	0.9925
Sitostanol	y=0.4307x - 0.062	0.9924

a: y=sterol area/IS area; x= mg/mL
b: R2= determination coefficients

The sensitivity of the Fast GC-MS method was evaluated in both standard solutions and PS-enriched dairy products. Fast GC-MS LOD and LOQ (Table 3) varied from 0.002 to 0.128 ng/mL and from 0.005 to 0.256 ng/mL, respectively.

As reported in Table 3, the Fast GC-MS method displayed a higher sensitivity than the conventional GC-FID one. The sensitivity of Fast GC-MS method was about 10-1000 times higher, in particular for stigmasterol in PS-enriched food as it was below the LOD in the conventional GC-FID. The sensitivity data reported in Table 3 confirmed the suitability of Fast GC-MS for phytosterol and phytostanol analysis in PS-enriched sample.

Table 3. Limits of detection (LOD, ng/mL) and limits of quantification (LOQ, ng/mL) of PS by Fast GC-MS and conventional GC-FID methods, determined in PS standard mixtures (0.125 mg/mL for Fast GC-MS and 1 mg/mL for GC-FID) and PS-enriched dairy products (soya milk for sterols, SM; soya drink for stanols, SY).

	Fast GC-MS method				**Conventional GC-FID method**			
	PS standard mixture		PS-enriched		PS standard mixture		PS-enriched	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
Campesterol	0.005	0.010	0.128	0.256	0.198	0.396	9.986	19.973
Stigmasterol	0.002	0.005	0.030	0.061	0.189	0.377	nd	nd
β-sitosterol	0.002	0.005	0.004	0.008	0.211	0.422	10.798	21.596
Campestanol	0.006	0.011	0.011	0.022	0.318	0.636	22.320	44.640
Sitostanol	0.009	0.017	0.027	0.053	0.339	0.677	22.706	45.412

Intraday and interday precision of both Fast GC-MS and GC-FID methods were calculated by manually injecting the silylated sterol and stanol standard solutions (n=3) in the same day (intra-day precision) for 3 consecutive days (inter-day precision, n=9). The injections were performed by different operators for testing the method precision. Different levels of standard solutions (0.125 and 1 mg/mL for Fast GC-MS and GC-FID, respectively) were used, since the detectors showed different sensitivity. Similarly, intraday and interday precision of the enriched milk and yogurt were calculated.

The coefficient of variation (%CV) was determined by the ratio of the individual sterol/stanol peak area to the IS peak area. As reported in Table 4, the results for repeatability of standard solutions showed an intra-day precision that ranged from 4.66 to 11.85% and 1.55 to 10.69% in Fast GC-MS and conventional GC-FID analysis, respectively. The inter-day precision

was from 8.12 to 12.48% for Fast GC-MS and from 3.05 to 7.89% for conventional GC-FID analysis. In both Fast GC-MS and GC-FID methods, the inter-day and intra-day values observed for PS-enriched dairy products were similar to those found for solution standards.

Table 4. Intra-day and inter-day precision of the Fast GC-MS and conventional GC-FID methods in PS standard mixtures (0.125 and 1 mg/mL for Fast GC-MS and GC-FID, respectively) and PS-enriched dairy products (soya milk for sterols, SM; soya drink for stanols, SY).

	Fast GC-MS method				**Conventional GC-FID method**			
	PS standard mixture		PS-enriched		PS standard mixture		PS-enriched	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Campesterol	6.73	11.30	3.97	4.47	2.99	6.05	4.20	2.99
Stigmasterol	11.85	8.46	5.19	5.35	1.55	3.05	5.53	3.80
β-sitosterol	10.45	8.12	7.04	6.40	6.51	4.90	2.43	1.50
Campestanol	8.18	12.48	8.02	7.32	4.57	4.46	7.78	3.85
Sitostanol	4.66	12.47	10.33	10.36	10.69	7.89	5.90	3.05

The PS-enriched milks and yoghurt drinks were selected to demonstrate the suitability of the Fast GC-MS method for determination of phytosterols and phytostanols in PS-enriched dairy products. As already mentioned, phytosterols and phytostanols (1-3 g on a daily basis consumption) are added to foods, to reduce human blood cholesterol levels. In general, the phytosterol/phytostanol contents vary greatly (from 0.3 to 8.0%), depending on the type of food product and the consumption market area (Barnsteiner et al., 2012). The concentrations depend on the daily consumption volumes or the food amounts to be ingested. Several companies have also adopted a one-daily dose approach (Laakso, 2005).

Preliminary tests of pre-fractionation of the unsaponifiable matter by TLC (visualized with 2',7'-dichlorofluorescein) showed that other lipophilic compounds (such as 4-methylsterols,

4,4'-dimethylsterols and triterpenic alcohols) were found at trace levels in the PS-enriched dairy products. Therefore, it was not necessary to carry out a TLC clean-up step prior to Fast GC-MS analysis. Moreover, the TIC and SIM acquisition and quantization modes further warranted the correct quantification of sterol peaks by using their characteristic ions, thus removing eventual interferences.

Phytosterol and phytostanol in PS-enriched dairy food products were quantified by Fast GC-MS and conventional GC-FID method under the same analytical conditions used for the standard solutions. All individual phytosterol and phytostanol identifications in GC-FID were confirmed by comparing their retention times with those of the corresponding standards, while in Fast GC-MS both retention times and mass spectra were used to confirm the PS identifications (Figure 4).

Table 5 shows the phytosterol/phytostanol content (mg phytosterol and phytostanol/100 mL of sample) of enriched food analyzed by both methods. In general, a difference of 5-13% in the phytosterol/phytostanol content was found between the two methods here applied. The higher PS contents found in enriched food analyzed by Fast GC-MS method, might be due to the higher sensitivity of the mass spectrometry detector. In the phytosterol-enriched sample, the relative abundance was β-sitosterol > campesterol > stigmasterol, whereas in the phytostanol-enriched one the order was sitostanol > campestanol, which are in agreement with those reported by European Commission Decision (European Commission Decision 2004/335).

Table 5. Content of PS (mg PS/100 mL of food product) in PS-enriched milk and yogurts, determined by both Fast GC-MS and conventional GC-FID.

	MY		**SY**		**MM**	
	Fast GC-MS	GC-FID	Fast GC-MS	GC-FID	Fast GC-MS	GC-FID
Campesterol	125.77 ± 5.34	143.09 ± 0.65	nd	nd	23.40 ± 1.67	34.71 ± 1.83
Campestanol	Nd	nd	780.54 ± 97.29	838.22 ± 36.27	nd	nd
Stigmasterol	10.49 ± 0.10	nd	nd	nd	1.48 ± 0.23	nd
β-sitosterol	1436.75 ± 64.75	1224.43 ± 170.87	nd	nd	141.97 ± 17.68	157.62 ± 14.96
Sitostanol	229.55 ± 16.99	275.33 ± 14.42	4388.69 ± 582.97	3480.06 ± 184.04	18.84 ± 2.09	nd

Abbreviations: nd, not detected; MY, fermented milk; SY, soya drink; MM, skimmed milk

4. Concluding remarks

The analytical performance of the Fast GC-MS method for the determination of plant sterols (phytosterols and phytostanols), together with the consequent significant reduction of the analysis time and consumables, demonstrates that Fast GC-MS represents a good alternative to conventional GC-FID analysis and evinces its great potential for the analysis of PS-enriched food.

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4.3 EFFECT OF MICROWAVE HEATING ON PHYTOSTEROL OXIDATION

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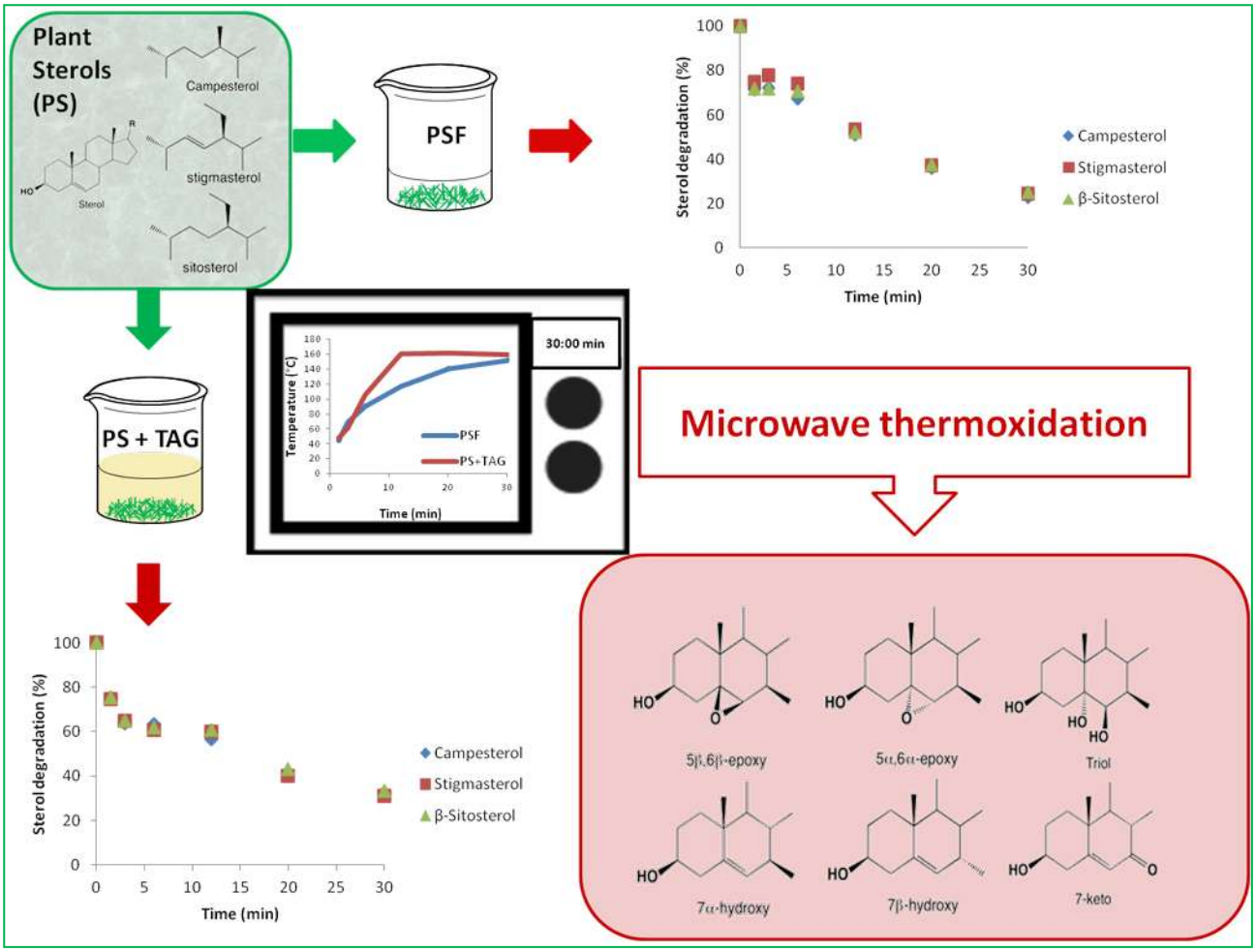

Effect of microwave heating on phytosterol oxidation

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Abstract

The oxidative stability of phytosterols during microwave heating was evaluated. Two different model systems (a solid film made with a phytosterol mixture (PSF) and a liquid mixture of phytosterols and triolein (1:100, PS+TAG)) were heated for 1.5, 3, 6, 12, 20 and 30 min at 1000 W. PS degraded faster when they were microwaved alone than in presence of TAG, following a first order kinetic model. Up to 6 min, no phytosterol oxidation products (POPs) were generated in both systems. At 12 min of heating, POPs content reached a higher level in PSF (90.96 µg/mg phytosterols) than in PS+TAG (22.66 µg/mg phytosterols), but after 30-min treatment, the opposite trend was observed. 7-keto derivates were the most abundant POPs in both systems. The extent of phytosterol degradation depends on both heating time and the surrounding medium, which can impact the quality and safety of the food product destined to microwave heating/cooking.

Keywords: Phytosterols oxidation product, oxidation, microwave, heating, β-sitosterol, triolein, GC-MS

1. Introduction

Phytosterols (PS) are synthesized by plants and introduced into human body through the diet. The major PS are β-sitosterol, campesterol and stigmasterol (Figure 1), β-sitosterol being the most abundant one.

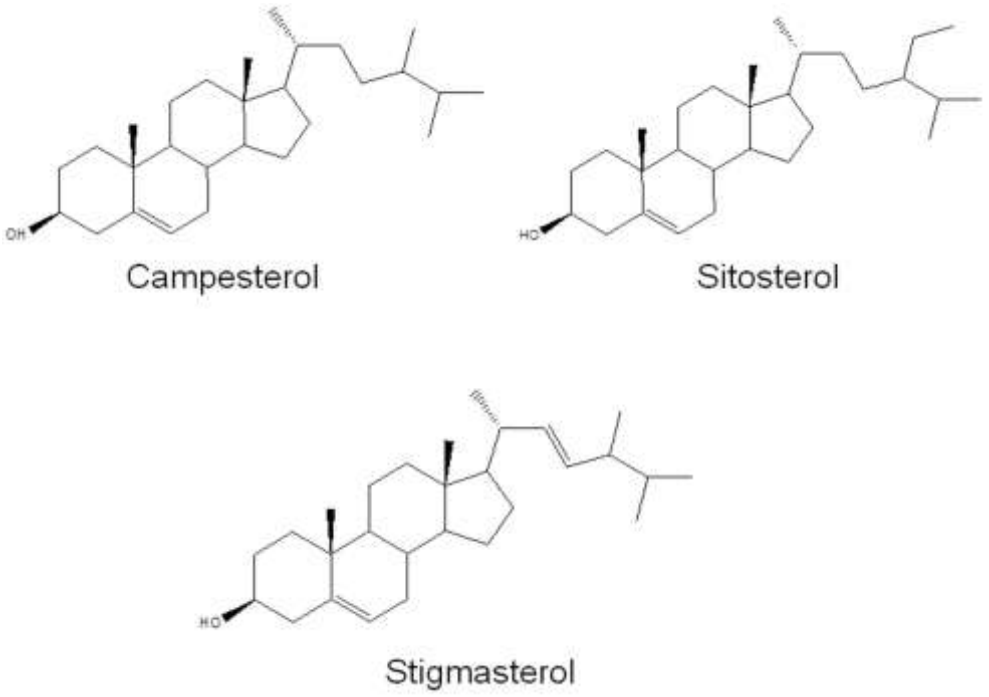


Figure 1. Main structures of phytosterol.

The main sources of PS are oils, fats, spreads, nuts, seeds, and, to a lesser extent, cereals, bakery products, vegetables and fruits (García-Llatas et al., 2011; Marangoni et al., 2010). PS dietary intake ranges from 150 to 440 mg/day, and can reach 1 g/day in vegans (Escurriol et al., 2010; García-Llatas et al., 2011). PS consumption (2 g/day) results in a cholesterol reduction of approximately 9% and exerts beneficial effects upon other lipid variables (Hernández-Mijares et al., 2010); in addition, PS have been described as anti-inflammatory and anti-cancer compounds (Alemany-Costa et al., 2012; García-Llatas et al., 2011; Marangoni et al., 2010). Despite their structural similarity with cholesterol, PS have a much lower intestinal absorption rate in humans.

For these reasons, many PS-enriched foods have been commercialized since 1995, with a noticeable increasing trend in the market (García-Llatas et al., 2011).

However, PS can undergo oxidation with analogous mechanisms to those observed for cholesterol, giving rise to phytosterol oxidation products (POPs) with different characteristic functional groups (7-hydroxy, 5,6-epoxy, triol and 7-keto derivatives) (García-Llatas et al., 2011). PS can be oxidized through a free radical chain reaction, by many different species of oxygen, such as the oxygen in the ground state ($^3O_2$), ozone (O_3), singlet oxygen ($^1O_2$), hydroperoxides (H_2O_2), dioxygen cation (O_2+) and hydroxyl radical (HO•) (García-Llatas et al., 2011). The susceptibility to autoxidation or thermoxidation results from the presence of double bonds, which easily undergo free radical attack followed by hydrogen abstraction on the carbon atoms in α-positions to the double bonds (Choe et al., 2009; Lengyel et al., 2012). While toxicological aspects of cholesterol oxidation products (COPs) have been well documented for their wide range of adverse biological effects and possible role in the induction of atherosclerosis (Otaegui-Arrazola et al., 2010; Rodriguez-Estrada et al., 2014), the evidence on the biological effects of POPs is still limited (García-Llatas et al., 2011). Some investigations have highlighted the potential atherogenicity and the role in the inflammation as well as cytotoxicity effects of POPs (Alemany et al., 2014). High level of oxidative stress, glutathione depletion, mitochondrial dysfunction and elevated caspase activity have also been linked to POPs (Gylling et al., 2014; O'Callaghan et al., 2014). Studies in humans have shown that POPs may be absorbed from the diet, but that they could also form *in vivo*, as found in the serum of healthy subjects (García-Llatas et al., 2011).

Sterols oxidize when subjected to heating treatments and/or high temperatures. The effects of culinary processes (such as frying and oven cooking) have been imitated by choosing 180ºC as heating temperature for most investigations (Barriuso et al., 2012). After 1-h heating in a conventional oven at this temperature, Menéndez-Carreño et al. (2010) found a progressive decrease in stigmasterol content, as well as formation and subsequent degradation of POPs. (Xu et

al., 2009) investigated the oxidative stability of β-sitosterol in a mix with edible oils heated at 180ºC in an oven; after 120-min heating, a 75% degradation of β-sitosterol was observed. The same research group Xu et al. (2011) used a mix of β-sitosterol and triacylglycerols with different fatty acid profiles, observing that heating at 180°C for 120 min led to 85% degradation of β-sitosterol; it seems that the surrounding fatty acids affected sterol oxidation in a time-dependent manner and their effect was unlikely related to their degree of unsaturation.

Among the different cooking techniques, the use microwave (MW) has widely spread in the last two decades at both domestic and catering levels for either cooking, reheating or thawing food, as it is fast and easy technique to be employed for the consumption of processed and frozen foods. MW heating of food results from two types of interactions between food components with microwaves: the ionic conduction and the dipolar rotation (Malheiro et al., 2011). The MW cooking/heating time will be thus greatly influenced by food dielectric properties (dielectric constant and dielectric loss factor), which are related to food composition (Inchingolo et al., 2013). Despite the large utilization of MW, to the best of our knowledge there are no available data on PS oxidation during microwaving; in fact, only the influence of frying, boiling, and oven heating on sterol oxidation in model systems (Ansorena et al., 2013; Barriuso et al., 2012; Kmiecik et al., 2015; Soupas et al., 2004) and food (Derewiaka et al., 2012; Dutta, 1997; Menéndez-Carreño et al., 2008) has been studied. For a better understanding of how MW could impact PS stability, it would be therefore important to first assess PS behavior in a model system during microwaving.

The aim of this study was to evaluate the oxidative stability of PS during MW heating, using two different model systems (a solid film made with a phytosterol mixture (PSF) and a liquid mixture of phytosterols and triolein (PS+TAG, 1:100, wt/wt).

2. Materials and Methods

2.1. Reagents and solvents

All solvents used were analytical grade. Reagents were supplied by Carlo Erba Reagenti (Rodano, Italy) and Merck (Darmstadt, Germany). β-sitosterol was purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany) (purity: 41% β-sitosterol, 23% campesterol, 23% stigmasterol). Triolein was supplied by Nu-Check (Elysian, MN, USA). 5α-cholestan-3β-ol (dihydrocholesterol, IS, purity: 95%), cholest-5-en-3β,19-diol (19-hydroxycholesterol, 19-HC) (purity: 99%), cholest-5-en-3β,7α-diol (7α-hydroxycholesterol, 7α-HC) (purity: 99%), cholest-5-en-3β,7β-diol (7β-hydro-xycholesterol, 7β-HC) (purity: 90%), 5α,6α-epoxy-cholestane-3β-ol (α-epoxycholesterol, α-CE) (purity: 87%), 5β,6β-epoxy-cholestan-3β-ol (β-epoxycholesterol, β-CE) (purity: 80%), cholestane-3β,5α,6 β-triol (cholestanetriol, triol) (purity: 99%), cholest-5-en-3β-ol-7-one (7-KC) (purity: 99%), 5-cholestene-3β,25-diol (25-HC) (purity: 98%), were purchased from Sigma (St. Louis, MO, USA). Glass thin-layer chromatography (TLC) silica plates (20 cm x 20 cm x 0.25 mm film thickness) were supplied by Merck (Darmstadt, Germany). Silica solid-phase extraction (SPE) cartridges (500 mg/3 mL Strata cartridges) from Phenomenex (Torrence, CA, USA), were utilized for POPs purification. The silylation mixture was prepared with dried pyridine, hexamethyldisilazane and trimethylchlorosilane at a ratio of 5:2:1 by volume.

2.2. Experimental design

Two different model systems were used:

a) a phytosterol film (PSF) was prepared with a solution of PS in chloroform (1 mg/mL). An aliquot of 1 mL was transferred to a previously weighed vial (5 mL, 22.00 x 38.25 mm) and taken to dryness under nitrogen flow.

b) a mixture of phytosterols and triolein (PS+TAG) was prepared with a solution of PS in chloroform (1 mg/mL). An aliquot of 1 mL was transferred to a previously weighed vial (5 mL, 22.00 x 38.25 mm), taken to dryness under nitrogen flow, weighed again, added with about 100 mg of triolein, and thoroughly mixed by vortexing.

A domestic multimode MW oven was used for sample heating (Panasonic NN-CT756, Domestic MW, Bracknell, UK). Samples in the opened vials (n=4 for each model system, of which 3 for sampling and 1 for temperature control) were placed on the rotatory turntable plate of the oven at equal distance and exposed at a power of 1000 W. Samples were independently heated for 1, 1.5, 3, 6, 12, 20 and 30 min. All heated samples were allowed to cool at room temperature (23 ± 1°C) after thermal treatment. For each run time, a vial, with triolen or film, was used to measure the temperature of the system. Each tube was added with 1 mL of chloroform, shaken vigorously for 40 sec and kept under -20°C until analysis. The MW heating experiment was run in triplicate for each heating time and model system.

Temperature of samples was measured immediately after MW exposure, by inserting a thermocouple (K-type; Ni/Al-Ni/Cr) connected to an acquisition system (HI 98804, Hanna Instrument, Villafranca Padovana-PD, Italy) at approximately the geometrical centre of the control sample.

2.3. Qualitative thin-layer chromatography (TLC) of oxidized model systems

A qualitative silica TLC was carried out to verify the formation of polymerization products in the PS +TAG system during oxidation in MW. PS+TAG (about 100 mg) was placed in an open vial and heated in MW for 30 min. The sample was then resuspended in 1 mL chloroform and an aliquot (0.2 mg/20 µL) was spotted on a 10 cm x 20 cm silica TLC plate. A triolein standard was also loaded as a reference spot (0.2 mg). The mobile phase was a mixture of diethyl ether:*n*-hexane (35:65, v/v); the TLC was developed at 4°C for 20 min. The TLC bands were visualized by spraying a molybdatephosphoric acid solution (20 wt % in ethanol) and then were charred in an oven at 180°C for 20 min.

2.4. Purification and quantification of PS

PS purification was achieved by using the silica SPE method described by Bortolomeazzi et al. (1990). A known amount of 5α-cholestan-3β-ol (internal standard; 500 µL from a 2 mg/mL solution in *n*-hexane:isopropanol (3:2, v/v)), was added to 1/10 of sample for PS quantification. Briefly, apolar lipids were removed by 5 mL of *n*-hexane:diethyl ether (8:2, v/v), while the polar fraction that contained PS was eluted with a *n*-hexane:diethyl ether solution (1:1, v/v) and methanol. The eluted fraction was taken to dryness under nitrogen flow and PS were silylated at 40°C for 30 min (Sweeley et al., 1963). The silylated samples were dried under nitrogen flow at 40°C and dissolved in 1 mL of *n*-hexane.

The GC determination of sterols was performed with a GC HRGC 5300 (Carlo Erba, Milan, Italy) instrument, equipped with a split–splitless injector, a flame ionization detector (FID) and a computerized data acquisition system (Turbochrom Navigator software Ver.6.1.1.0.0: K20, Perkin Elmer, Norwalk, CT, USA). The separation of the silylated sterols was performed with a CP-SIL 5CB Low Bleeds/MS column (30 m x 0.32 mm i.d. x 0.25 µm film thickness) (Varian Chrompack, Middelburg, The Netherlands), coated with 100% dimethyl-polysiloxane. The oven temperature was programmed from 265°C to 280°C at 0.5°C/min and then from 280°C to 325°C at 4°C/min; the injector and detector temperatures were both set at 325°C. Helium was used as carrier gas at a flow of 2.9 mL/min; the split ratio was 1:15. The analysis was run in triplicate.

Sterols were identified by comparing their retention time with those of a solution of silylated sterol standards and further confirmed by injection into Fast gas chromatography-mass spectrometry (Fast GC-MS; same conditions used for POPs analysis and reported in section 2.5). Sterols were quantified with the internal standard method, using 5α-cholestan-3β-ol. Their response factors were evaluated with respect to the internal standard.

2.5 Purification and analysis of POPs

Regarding the determination of POPs, the remaining 9/10 of the initial sample solution were purified by silica SPE (Supelclean LC-Si, 500 mg/3 mL) (Supelco, Bellefonte, PA, USA), according to the method suggested by Guardiola et al. (1995). Twenty µL of internal standard 19-hydroxycholesterol (0.5 mg/mL of *n*-hexane: isopropanol (4:1, v/v)) were added to the sample, dried under nitrogen flow, and redissolved in *n*-hexane:diethyl ether (95:5, v/v) before sample purification. The SPE cartridge was previously activated with 5 mL of *n*-hexane before sample loading and then washed with *n*-hexane (5 mL) and different solvent mixtures of *n*-hexane:diethyl ether (10 mL, 30 mL and 10 mL of 95:5, 90:10 and 80:20, v/v, respectively). The POPs fraction was finally eluted with 10 mL of acetone, subjected to silylation (see paragraph 2.3) and dissolved in 0.5 mL of *n*-hexane.

POPs were determined and quantified by Fast GC-MS. A GC Shimadzu QP 2010 Plus (Kyoto, Japan) equipped with a split–splitless injector and coupled to an electron impact (EI) mass spectrometric detector, was used. The separation was carried out by a fused-silica capillary column Restek RTX-5 (10 m x 0.1 mm i.d. x 0.1 µm film thickness; Bellafonte, PA, USA), coated with 95% dimethyl- and 5% diphenyl-polysiloxane. A non silanized standard split liner (95 mm x 5 mm o.d. x 3.4 mm i.d.) was utilized. The temperature was programmed from 210 to 325°C at 15°C/min and kept for 5 min to 325°C. The injector, the ion source and the transfer line temperatures were set at 340, 200 and 340°C, respectively. The injection was performed in the split mode (1:50). Helium was used as carrier gas at a linear velocity of 47.7 cm/sec. The electron energy was 70 eV. A mass range from 40 to 650 *m/z* was scanned at a rate of 2500 amu/s. The acquisition and integration modes were full-scan total ion current (TIC) and single ion monitoring (SIM), respectively. Identification of POPs was performed by comparing their retention time and mass spectra with those reported in previous studies (Alemany et al., 2013; Toschi et al., 2014). POPs were recognized and quantified by their corresponding characteristic ions with a high abundance obtained

in the SIM mode (*m/z*): IS (19-hydroxycholesterol) (353), 7α-hydroxycampesterol (470), 7α-hydroxystigmasterol (482), 7α-hydroxysitosterol (484), 7β-hydroxycampesterol (470), 7β-hydroxystigmasterol (482), 7β-hydroxysitosterol (484), 6β-hydroxycampesterol (417), 6β-hydroxystigmasterol (429), α-epoxysitosterol (412), β-epoxysitosterol (412), α-epoxystigmasterol (253), β-epoxystigmasterol (253), 7-ketocampesterol (381), 7-ketostigmasterol (386), 6-ketositosterol (473), 7-ketositosterol (500). Considering that most POP standards are not commercially available and assuming that POPs fragmentation is similar to that of cholesterol oxidation products (COPs), POPs quantification was performed by using the calibration curves obtained for cholesterol oxides in the SIM mode (Cardenia et al., 2012). Three independent replicates were run per sample.

2.6 Statistical analysis

Mean and standard deviation of each sample data were calculated. One way ANOVA, with Tukey's post hoc multiple comparisons ($p<0.05$), was applied to evaluate the significant differences on PS and POPs amounts over MW heating time and between PSF and PS+TAG models throughout the MW heating process. Pearson correlation coefficients ($\alpha = 0.05$) were used to examine possible relationships between each system, PSF or PS+TAG, and the temperature. SPSS 15.0 program (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis of the data.

For the kinetic evaluation of PS degradation, a first order regression analysis of data was performed using Excel 2008 and the fitting goodness was evaluated on the basis of the regression coefficient (R^2) and the associated *p*-value.

3. Results and discussion

Table 1 reports the temperatures reached at each heating time during the two MW experiments, showing that they varied according to the type of model system. The temperature recorded for PFS was lower than that of PS+TAG .

Table 1. Temperature reached by the model systems during heating in a multimodal microwave oven

	Temperature (°C)	
Time (min)	**PSF**	**PS+TAG**
1.5	43.9	47.5
3	68.8	61.6
6	90.5	105.9
12	117.05	160.4
20	140.9	161.9
30	151.8	159.5

This could be mostly attributed to their different composition and physical status (PSF was a solid film, while PS+TAG was liquid), which could have affected their dielectric properties and thus their heating capacity. Another factor that might have partly contributed is the technology of the multimodal domestic MW oven, since the electromagnetic waves enter the cavity, move and bounce off the walls, generating pockets (called modes) of high and low energy that create a non-uniform microwave field. Moreover, this type of MW oven is unable to deal with power variations and to maintain a constant temperature as unimodal microwave ovens do.

3.1. Unreacted PS trends during MW heating

The presence of crystals was observed in the PS+TAG model after 20 and 30 min of heating, so a silica TLC was performed to verify the occurrence of dimers in the mixture, as they could

modify the stationary phase of the GC column and affect its peak resolution capacity. Figure 2 shows the TLC of the PS+TAG model after 30 min of heating, which qualitatively evidenced a higher degree of polymerization with increasing heating time Therefore, a silica SPE was run for all samples before GC analysis of PS, to remove apolar lipids (including dimers).

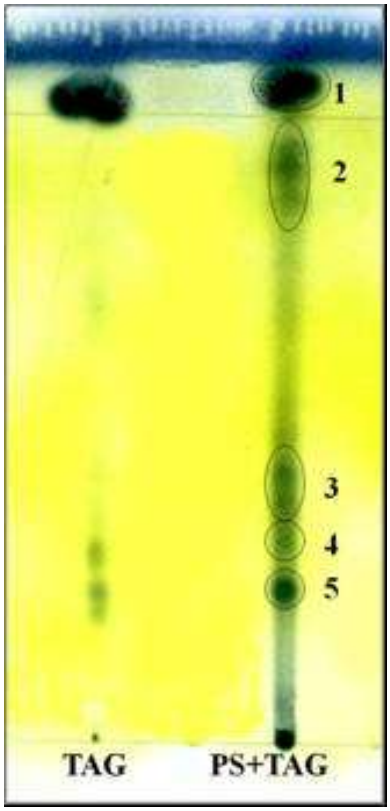
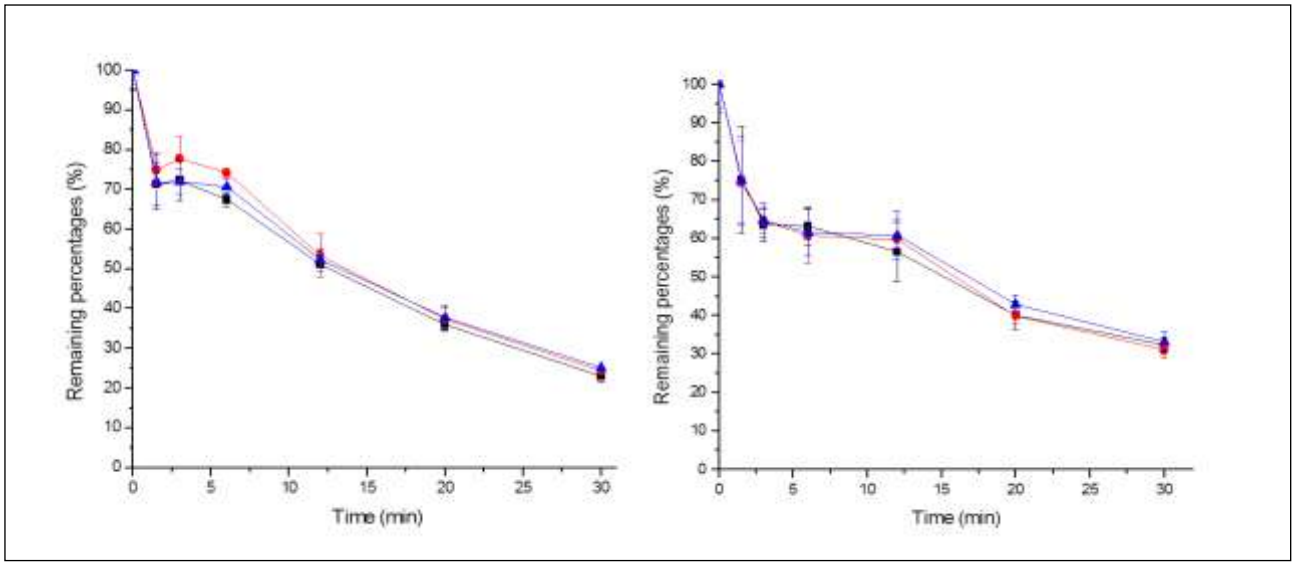


Figure 2. TLC plate of a mixture PS+TAG model after 30 min of MW heating. Peak identification: 1, triolein; 2, polymerization products (three bands); 3, free fatty acid; 4, diacylglycerols; 5, phytosterols.

Figure 3 and supplemental table, reports the amounts of remaining PS in both model systems after MW heating (0-30 min). In the PSF system, a marked PS degradation (~ 30%) was noted in the first 1.5 min of microwaving, which remained almost constant up to 6 min of heating; thereafter, PS showed a continuous, fast drop rate and, at the end of 30-min heating, only about 24% of the initial PS content was observed. The single PS displayed a similar degradation trend, being slightly more pronounced for campesterol than for the other two PS.

Time (min)	PSF			PS+TAG		
	Sitosterol	**Campesterol**	**Stigmasterol**	**Sitosterol**	**Campesterol**	**Stigmasterol**
0	100.00±4.57e	100.00±5.22d	100.00±3.53d	100.00±6.32c	100.00±7.37d	100.00±6.36c
1.5	71.86±6.97d	71.33±5.48c	74.88±4.47c	75.19±11.25b	75.22±13.89c	74.42±10.87b
3	72.01±3.20d	72.27±4.91c	77.67±5.51c	64.62±4.44b	63.56±4.44c	64.56±3.11b
6	70.61±1.93d	67.41±1.73c	74.18±1.02c	61.55±5.88b	63.13±4.91c	60.70±7.05b
12	52.30±2.68c	51.16±0.04b	53.37±5.37b	60.75±6.31b	56.49±7.73bc	59.72±5.20b
20	37.79±3.12b	35.96±1.16a	37.31±2.99ab	43.02±2.31a	40.07±3.67ab	39.83±1.86a
30	25.09±0.33a	22.94±1.51a	24.40±1.05a	33.18±2.57a	32.35±1.92a	31.02±2.20a

*Each value represents the mean ± standard deviation of three replicates. Mean values in the same column followed by different letters are significantly different ($p < 0.05$).



Supplemental Table and Figure 3. Remaining percentages (%) of phytosterols during microwave heating of PSF and PS+TAG.

These results are similar to those obtained by Barriuso et al. (2012), who found the following sterol susceptibility to degradation: campesterol ~ β-sitosterol ≥ stigmasterol. The authors reported that the experimental heating conditions (temperature/exposure time, modality (e.g. MW, convection, conduction), initial PS amount, sample area/container volume ratio, open/closed container during heating) and PS composition (single PS or mixtures with diverse relative percentages) can differently affect the extent of PS degradation. In PSF, the extent of PS degradation was lower (~ 76%) than the one observed (~ 88%) at 180°C after 30 min of heating in a

thermoblock (Barriuso et al., 2012); such differences could be mainly ascribed to both the diverse temperature and heating modality applied (MW vs. conduction), as they led to different real sample temperatures. Xu et al. (2009) found ~ 50% degradation of initial β-sitosterol when heated at 180°C for 30 min in an oven, while Menéndez-Carreño et al. (2010) observed ~ 38% degradation of initial stigmasterol at 180°C after 1 h in an oven.

Regarding PS+TAG system, PS also greatly degraded (about 25%) in the first 1.5 min of heating, remaining almost constant up to 12 min. Thereafter, as for PS+TAG system, a gradual PS drop was noted and about 32% of the initial PS content was detected after 30 min of heating. In this case, all PS exhibited an analogous degradation behavior. However, the extent of PS degradation was much higher (~ 68%) than the one found (~ 21%) when cholesterol+triolein was heated at 180°C for 30 min in an oven (Ansorena et al., 2013); this distinctive behavior might be attributed to the different heating modality (MW vs. convection), whose influence is more evident in a liquid system than in a solid one.

When comparing the two model systems, PS degraded faster when they were microwaved alone than in the presence of TAG. Table 2 reports the sterol loss rate throughout the MW heating process in terms of the observed first order rate constants. All data satisfactory fit the kinetic model with very high determination coefficients. All sterols in the PS+TAG model exhibited similar degradation rates (k values of 0.03), which were lower than those of the PSF model (0.04), thus confirming that sterol degradation within the same models was similar; furthermore, their degradation was slower in the PS+TAG system.

Table 2. First-order kinetic parameters (rate constant (k) and corresponding coefficient of determination (R^2)) for remaining sterols in both PSF and PS+TAG models throughout the MW heating process.

	PSF			PS+TAG		
	Sitosterol	Campesterol	Stigmasterol	Sitosterol	Campesterol	Stigmasterol
k	0.042	0.044	0.044	0.031	0.033	0.033
R^2	0.968	0.973	0.979	0.899	0.911	0.910
p	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001

p **=** probability value

The behavior described in the previous paragraph could be attributed to the physical/steric protection of TAG towards PS, combined with the dilution effect, which led to a lower air contact thus hampering oxidation (Yen et al., 2011). Moreover, triolein used as TAG in the model system may have also competed with PS for oxidation. Similar results were found when the influence of the degree of unsaturation of different TAG on cholesterol oxidation was evaluated at 180°C in an oven (Ansorena et al., 2013); in this study, both the presence of TAG and their unsaturation degree inhibited cholesterol thermooxidation. When Xu et al. (2011) evaluated the interaction of fatty acids with β-sitosterol oxidation, they noted ~ 15% degradation of initial β-sitosterol in presence of oleic acid if heated at 180°C for 30 min in an oven. Four fatty acids (with 18 C atoms and different degrees of unsaturation) accelerated the degradation of β-sitosterol for the first 60 min at 180°C and thereafter their oxidation-promoting effect became weaker or diminished; the authors concluded that the surrounding fatty acids affected PS oxidation in a time-dependent manner and their effect was unlikely related to their level of unsaturation.

Another aspect to consider is that, at elevated temperatures (> 150°C), polymerization, dehydration and formation of mid-polar and polar compounds can occur (http://lipidlibrary.aocs.org; Lampi et al., 2009; Lercker et al., 2000; Menéndez-Carreño et al., 2010; Rudzinska et al., 2009; Rudzinska et al., 2010). In the present study, PS+TAG reached 161.9

and 159.5°C after 20 and 30 min of MW heating, respectively, so dimers could form, as evinced by TLC fractionation (Figure 2). The proportion of the different dimers depends on the conditions used; Dobarganes (http://lipidlibrary.aocs.org) reported that only dehydrodimers have been found in significant amounts at 150°C, while mono-, bi- and tricyclic dimers are mainly generated above 250°C. Some authors have reported that the average polymerization temperature of polar sterol oxides is approximately 180°C (Menéndez-Carreño et al., 2010; Soupas et al., 2006; Rudzinska et al., 2010).

3.2 POPs formation

The TMS derivatives of POPs were analyzed by Fast GC-MS using the method reported in Section 2.4. The characteristic mass fragmentation pattern produced by EI (Table 3) was used to identify the main POPs, based on those reported in previous studies (Alemany et al., 2013; Toschi et al., 2014; Dutta, 2002). As reported in Table 3, sixteen POPs were identified and a specific ion was selected for each POP, based on abundance and specificity. In particular, 7-hydroxysterols (α and β), 6β-hydroxysterols, epoxysterols (α and β) and 7-ketosterols originated from oxidation of the main phytosterol (β-sitosterol, stigmasterol and campesterol) were formed in both systems.

Table 3. Characteristic Mass Fragmentation Pattern of the Main Phytosterol Oxidation Products (POPs) and the Selected Ions for their Quantification.

Phytosterol Oxidation Products	***m/z*[a]**	**Characteristic fragmentation**
7*α*–hydroxycampesterol	**470**	129, 233, 455, 470, 471, 472, 560
7*α*-hydroxystigmasterol	**482**	208, 233, 343, 482, 483, 484, 467, 572
6*β*-hydroxycampesterol	**417**	417, 455, 470, 545
7*α*-hydroxysitosterol	**484**	233, 253, 484, 485, 486, 574
6*β*-hydroxystigmasterol	**429**	429, 467, 482, 543, 557, 572
7*β*-hydroxycampesterol	**470**	129, 209, 233, 381, 455, 470, 471, 472, 486, 560
7*β*-hydroxystigmasterol	**482**	209, 233, 482, 483, 484, 572
α-epoxystigmasterol	**253**	83, 253, 349, 410, 500
β-epoxystigmasterol	**253**	83, 129, 157, 211, 253, 349, 410, 442, 471, 500
7*β*-hydroxysitosterol	**484**	233, 484, 485, 486, 574
α-epoxysitosterol	**412**	253, 384, 394, 412, 431, 502, 503, 575
β-epoxysitosterol	**412**	253, 384, 394, 412, 474, 502, 503
7-ketocampesterol	**381**	129, 381, 396, 469, 486
7-ketostigmasterol	**386**	129, 269, 357, 359, 386, 455, 498
6-ketositosterol	**473**	473, 474, 487
7-ketositosterol	**500**	129, 161, 395, 410, 483, 500, 501, 502

[a] *m/z* column reports the ions selected for POPs quantification purposes.

All PS examined in this study (campesterol, stigmasterol, and β-sitosterol) produced detectable and quantitative amounts of hydroxy-, epoxy-, and keto- derivatives in the ring structure. The PS oxides found are analogous to the major cholesterol oxidation products obtained by auto- and thermo-oxidation, following similar reaction pathways. The major site of POPs formation is in the C-7 on the second-ring, where hydroperoxides, hydroxy and keto sterols are formed. Other major oxidation products that can be produced in the ring structure are epoxides and triols. The formation

of epoxides by auto-oxidation occurs by a bimolecular mechanism that involves one hydroperoxy radical and one unoxidized sterol molecule (García-Llatas et al., 2011).

Table 4 reports the single and total POPs contents (expressed as µg/mg phytosterols) of the PSF and PS+TAG models heated in a multimodal MW oven. The evolution of 16 different POPs was followed in this study. POPs formation in both model systems was observed just after 12 min of MW heating; prior to that time, no detectable amounts of POPs were found. This result is the opposite to the one reported by Menéndez-Carreño et al. (2008), since they found that the highest POPs level in phytosterol-enriched milk were formed in the first 1.3 min of heating in microwave oven at 900 W and 69°C. However, a significant decrease of POPs content was already noticed after 2 min of treatment (304.71 vs. 265 µg/mg of fat after 1.5 and 2 min of treatment, respectively).

In our study, an increase of the total amount of POPs was observed at 12 min of treatment, following different trends depending on the model system. In the PSF, the POPs content did not significantly change from 12 min until 30 min of heating, remaining around 100 µg/mg phytosterols. The PS+TAG system displayed a sinusoidal behavior in which a small amount of POPs (22.66 µg/mg phytosterols) was observed at 12 min of microwaving, and thereafter an exponential increase was noticed from 20 min of MW heating onwards. At 12 min of heating, POPs level in PSF was higher than in PS+TAG (90.96 vs. 22.66 µg/mg phytosterols), but at 30 min the POPs content showed the opposite trend; in fact, the maximum POPs levels reached at the end of heating were 107.00 and 159.00 µg/mg phytosterols for PSF and PS+TAG, respectively. To our knowledge, other studies reported the time course changes of POPs content deriving from thermo-oxidation of phytosterols. Xu et al. (2011) reported that the fatty acids accelerated β-sitosterol oxidation for the first 60 min of treatment as compared to the control; indeed a change in total β-sitosterol oxidation products >10 % and <5 % was reported for the system with fatty acids and just β-sitosterol; starting at 90 min and thereafter, their levels were slightly higher in the system with β-

sitosterol alone than the sample with fatty acids. This study emphasizes that pure fatty acids might promote the formation of β-sitosterol oxides in an initial phase of oxidation. Another research paper demonstrated that frying oil/fat decreased β-sitosterol oxides up to 50% when heated for 60 min at 180°C, probably due to antioxidants naturally occurring in the oil (Xu et al., 2009). Barriuso et al. (2012) reported that the content of sterol oxidation products increase during the first 5–10 min of heating at 180°C, and subsequently their degradation rate was higher than their formation rate.

Table 4. Single and total POPs contents (μg/mg phytosterols) of the PSF and PS+TAG models heated in a multimodal MW oven.

		Time[a] (min)			
		From 0 to 6	12	20	30
			PSF		
Campesterol	7*α*-OH-	nd	2.37±0.50a	1.73±0.49a	1.26±0.80a
	6*β*-OH-	nd	0.22±0.07a	0.22±0.13a	0.28±0.09a
	7*β*-OH-	nd	5.91±0.44a	5.21±0.60a	3.54±2.18a
	7-keto-	nd	9.75±1.25c	12.42±0.24b	14.92±0.90a
	Σ Total	nd	18.25±1.95aA	19.58±1.36aA	20.01±3.96aB
Stigmasterol	7*α*-OH-	nd	1.94±0.26a	1.54±0.47a	1.04±0.67a
	6*β*-OH-	nd	0.19±0.04a	0.23±0.03a	0.25±0.08a
	7*β*-OH-	nd	5.23±0.48a	4.85±0.54a	3.25±1.78a
	5*α*,6*α*-epoxy	nd	2.46±0.47a	2.28±0.28a	2.80±0.52a
	5*β*,6*β*-epoxy	nd	2.53±0.16a	3.34±0.69a	4.44±1.68a
	7-keto	nd	4.92±1.05a	6.61±0.28a	7.83±0.41a
	Σ Total	nd	17.27±1.68aA	18.85±1.79aB	19.61±4.52aB
***β*-sitosterol**	7*α*-OH-	nd	3.77±0.53a	2.90±1.02a	1.91±1.29a
	7*β*-OH-	nd	10.68±1.25a	10.02±1.32a	6.97±4.24a
	5*β*,6*β*- epoxy	nd	4.55±1.19a	5.84±1.30a	4.36±2.54a
	5*α*,6*α*- epoxy	nd	5.78±1.56b	7.75±0.55a	7.97±2.07a
	6-keto	nd	1.20±0.27a	1.87±0.21a	2.50±0.96a
	7-keto	nd	29.46±5.637b	35.76±2.12ab	43.67±1.56a
	Σ Total	nd	50.89±7.80aA	58.29±2.88aA	63.02±6.54aB
TOTAL POPs		nd	90.96±12.46aA	102.56±6.62aA	107.00±17.45aB
			PS+TAG		
Campesterol	7*α*-OH-	nd	1.53±0.45c	7.53±0.41b	9.36±0.20a
	6*β*-OH-	nd	nd	0.20±0.05a	0.25±0.08a
	7*β*-OH-	nd	1.75±0.45c	7.67±0.18b	8.73±0.21a
	7-keto-	nd	1.47±0.18c	8.27±0.67b	10.62±0.81a
	Σ Total	nd	4.75±1.08cB	23.67±1.11bA	28.96±0.87aA

	7*α*-OH-	nd	1.23±0.29c	6.21±0.25b	8.01±0.06a
	6*β*-OH-	nd	0.09±0.01b	0.18±0.02a	0.21±0.03a
	7*β*-OH-	nd	1.49±0.43b	6.51±0.27a	7.23±0.04a
Stigmasterol	5*α*,6*α*-epoxy	nd	0.36±0.19b	4.97±1.54a	5.80±2.44a
	5*β*,6*β*-epoxy	nd	1.98±0.38c	10.72±0.91b	13.61±0.79a
	7-keto	nd	0.77±0.18c	3.91±0.59b	5.59±0.55a
	Σ Total	nd	5.92±1.39cB	32.49±3.06bA	40.44±1.77aA
	7*α*-OH-	nd	2.22±0.61c	12.27±0.64b	14.44±0.89a
	7*β*-OH-	nd	2.80±0.71c	12.70±0.51b	14.19±0.34a
	5*β*,6*β*- epoxy	nd	2.72±0.59c	17.28±0.97b	21.55±1.27a
***β*-sitosterol**	5*α*,6*α*- epoxy	nd	0.25±0.11c	5.54±1.54b	11.34±1.11a
	6-keto	nd	nd	0.94±0.10a	1.09±0.32a
	7-keto	nd	4.47±0.69c	20.32±1.11b	27.00±2.73a
	Σ Total	nd	11.99±2.70cB	69.05±4.63bA	89.60±5.44aA
TOTAL POPs		nd	22.66±5.16cB	125.21±8.51bA	159.00±7.14aA

[a] Each value represents the mean ± standard deviation of three replicates. Different small letters within the same row denote significant differences ($p < 0.05$) among different heating times. For Σ total of individual oxidized sterols (β-sitosterol, stigmasterol and campesterol) and Total POPs, different capital letters within the same column denote significant differences ($p < 0.05$) for each time among different system.

As stated before, the MW heating of edible oils, which are commonly used as heat transfer medium in complex foods, accelerates their oxidation, causing polymerization and thermal oxidative decomposition of sterols in foods (Inchingolo et al., 2013). The higher content of POPs in PS+TAG observed between 20 and 30 min of MW treatment could have been affected by the TAG used as lipid medium. At this temperature (~ 160°C), fatty acids could have undergone oxidation and produced hydroperoxide radicals, which in turn could have accelerated the degradation of phytosterol. It is well-known that MW produces fatty acid hydroperoxides at 2–3 times higher rates than the conventional heating (Kreps et al., 2014). On the other hand, TAG might have exerted a protective effect against phytosterol oxidation for the first 20 min. Ansorena et al. (2013) found that cholesterol alone degraded faster than TAG+cholesterol at 180°C, concluding that TAG seemed to provide physical protection to sterol against oxidation. In the present study, it appears that TAG diluted PS during heating transfer and exerted physical protection, thus slowing down their oxidation as compared to the PSF system where PS were directly exposed to MW. This behavior can be observed in PS degradation (Figure 3), where there is no change on POPs levels in the

PS+TAG system between 3 and 12 min. In the PSF system, the stable content of total POPs observed from 12 to 30 min could depend on the equilibrium reached between their thermo-formation and further degradation into volatile compounds and further evaporation of oxides formed (Zhang et al., 2005) and/or conversion into high-molecular weight oxidation compounds (oligomers and/or polymers) (Rudzinska et al., 2010). Other factors may impact their formation and differences in total POPs contents, such as temperatures, physical state and sterol composition.

As for the generation of oxyphytosterols from the different PS, β-sitosterol oxides were the most abundant, followed by those of campesterol and stigmasterol. This is mainly caused by the relative PS concentrations in the original mixture (41% β-sitosterol, 23% campesterol, and 23% stigmasterol). Regarding the total POPs content of each sterol, there were no significant modifications during heating of PSF, while a significant difference was observed in PS+TAG between 12 and 20 min of heating. Comparing the time course of total POPs from each sterol between the two systems, the major POPs formation observed at 12 min was in the PSF. From 20 to 30 min of heating, contrasting data trend were found, as singe PS from PS+TAG system had the highest oxides content. In fact, the behavior of the POPs originated by the single sterols reflects that observed for the total POPs in each model system.

It is generally expected that the main oxidation products of phytosterols and cholesterol are the 7-hydroxy and 7-keto (González-Larena et al., 2015), at least during the early stages of oxidation. In the PSF system (Table 4), POPs from all phytosterols contained in the mix were found, being the 7-keto oxide the predominant one (42% of total POPs at 30 heating time), followed by 7-hydroxy (7% of total POPs) derivatives.

The content of total POPs generated from β-sitosterol oxidation, showed a non-significant increasing trend with heating time (50.89, 58.29 and 63.02 μg/mg of phytosterol for 12, 20 and 30 min, respectively). The 7-keto derivative of this sterol was the predominant POP formed during microwaving, which showed a significant rise directly correlated with the treatment time (29.46,

35.76 and 43.67 µg/mg of phytosterols, for 12, 20 and 30 min, respectively). As it was illustrated by Rodriguez-Estrada et al. (2014), 7-ketocholesterol is easily formed and its content could be >30% of total cholesterol oxidation products, hence it is the most representative oxysterol. However, the trend of this oxidation marker could change depending on time/temperature conditions, interactions with the system (model or food), or with other oxidation compounds (González-Larena et al., 2015; Rodriguez-Estrada et al., 2014). According to mathematical predictive models applied to PS oxidation in PS enriched-beverages as a function of storage time, 7-ketositosterol and 7-ketocampesterol could be used as markers of PS oxidation level as they showed the highest R^2 value (percentage of variability explained by the model) (González-Larena et al., 2015). Other β-sitosterol POPs, like 7α-OH and 7β-OH, did not significantly change (p>0.05) during heating; between these two epimers, 7β-OH was predominant (about 10 µg/mg of phytosterols). Regarding campesterol and stigmasterol, total POPs formed after 12 min of microwaving, without showing significant differences during the rest of the heat treatment. The total amounts of POPs originated from each sterol were about 20 µg/mg of phytosterols. The individual POPs generated from these phytosterols exhibited the same trend of β-sitosterol's POPs but with different ratios, reflecting the PS concentrations of the original mixture. No epoxy derivatives of campesterol were detected, though. Barriuso et al. (2012) obtained similar results after 5 and 10 min of heating at 180ºC in a conventional oven.

In the PS+TAG model (Table 4), different amounts of 7-hydroxy and 7-keto were found, which accounted for less than 10% and 20% of total POPs at 30 min heating time, respectively. The content of total POPs generated from degradation of β-sitosterol displayed an increasing tendency with increasing heating time. A significant, rapid change was observed between 12 min and 20/30 min of microwaving; this behavior was also observed for single and total campesterol and stigmasterol oxides (Table 4). The total amount of stigmasterol oxides generated after 30 min of heating were higher as compared to campesterol ones; this could be partly ascribable to the

formation of both epoxy derivatives from stigmasterol, which were not found among campesterol's POPs. The epoxidation of the 5,6-double bond of the sterol comes from a bimolecular reaction mechanism between a hydroperoxide radical and an unoxidized sterol. The additional double bond on the side chain of stigmasterol as compared to the other sterols, represents a further attack point for free radicals, which can promote hydroperoxide formation at 100°C and 180°C (Kemmo et al., 2005) and may thus affect the rate of oxidation of this sterol (Barriuso et al., 2012). Ansorena et al. (2013) found that the concentration of COPs in a cholesterol-TAG system model heated in a conventional oven at 180ºC, displayed an increasing tendency at different heating times depending on the TAG unsaturation degree.

It must be noted that, in any case, the most abundant POPs were similar in either model system and that the main differences were due to the relative oxide concentrations. In the PSF system, the concentration of individual POPs were greatest for 7-keto, almost twice as much as in the other model system after 30 min of microwaving (43.67 vs. 27.00 µg/mg phytosterols). In PS+TAG, the 7-hydroxy (α and β) were present in the same proportion and their contents were higher than in the PSF system, where 7β-hydroxy derivatives were predominant. In both systems, the epoxy derivatives (α and β) from sitosterol and stigmasterol were formed; however, in the presence of TAG, β-epoxy was more abundant than the corresponding α epimer.

The correlation analysis mainly emphasized the differences between the two systems, PSF and PS+TAG, as related to the temperature reached during microwaving. In the PSF system, the temperature was directly correlated with POPs formation (r= 0.999, p=0.026), while it was inversely correlated with the sterol content (r= - 0.933, p=0.007). Although *β*-sitosterol was the most abundant in the phytosterol mixture, total POPs were only positively correlated with the total oxidation products of stigmasterol (r= - 0.999, p=0.033) and campesterol (r= 0.999, p=0.022). In addition, 7-ketostigmasterol formation was inversely correlated with the sterol content (r= - 0.999, p=0.029). In the PS+TAG system, POPs formation was not related to the temperature (r= - 0.097,

p=0.938), which further confirms the important "protective/dilution" role of TAG towards PS oxidation, despite the prooxidizing effect of temperature actually detected in PSF.

In conclusion, phytosterols displayed a different oxidative stability during MW heating which varied according to the model system. Up to 6 min of microwaving, no POPs were detected in both systems; at 12 min of heating, POPs content reached a higher level in PSF (90.96 µg/mg phytosterols) than in PS+TAG (22.66 µg/mg phytosterols). At the end of the MW treatment (30 min), the POPs content were highest in PS+TAG (159 µg/mg phytosterols). In this case, the presence of TAG greatly impacted PS stability in the first heating period, exerting a protective effect through different possible mechanisms (such as dilution, physical protection, competition for oxidation) (Ansorena et al., 2013). Although household MW heating and cooking does not usually require high exposure times and temperatures, these results prove that MW can differently affect the oxidative stability of sterols according to the medium composition. This aspect should not be disregarded for food product formulation, especially if intended to be cooked in MW for long time periods (> 12 min at the power and temperature settings we tested), as it can potentially impact the quality, nutritional value and safety of the food product.

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4.4 ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS ADDED TO A FUNCTIONAL EMULSION CONTAINING OMEGA-3 FATTY ACIDS AND PLANT STEROL ESTERS

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Antioxidant activity of phenolic compounds added to a functional emulsion containing omega-3 fatty acids and plant sterol esters

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Short running title: Antioxidant activity of phenolic compounds added to an emulsion

Abstract

The aim of this study was to compare the effect of eleven compounds extracted from red propolis on the oxidative stability of a functional emulsion. Emulsions containing 1.63 g/100 mL of α-linolenic acid (ALA), 0.73 g/100 mL of stearidonic acid (SDA) and 0.65 g/100 mL of plant sterol esters (PSE) were prepared without or with phenolic compounds (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans,trans*-farnesol, rutin, gallic acid or sinapic acid). *Tert*-butylhydroquinone and a mixture containing ascorbic acid and $FeSO_4$ were applied as negative and positive controls of the oxidation. Hydroperoxide, thiobarbituric acid reactive substances (TBARs), malondialdehyde, hexanal and phytosterol oxidation products (POPs) were evaluated as oxidative markers. Based on hydroperoxide and TBARs analysis, sinapic acid and rutin (200 ppm) showed the same antioxidant activity than TBHQ, representing a potential alternative as natural antioxidant to be applied in a functional emulsion containing ω-3 FA and PSE.

Keywords: oil-in-water emulsion, omega-3 fatty acids, phenolic compounds, plant sterol esters, antioxidant activity, oxidation, *Echium*, propolis.

1. Introduction

Atherosclerosis is an inflammatory condition associated with the genesis of several cardiovascular diseases (CVDs), including stroke and myocardial infarction (Libby et al., 2011), which constitute the primary cause of mortality in many countries. Although CVD manifests mostly in the adult and elderly population, the atherosclerotic process begins in childhood (Mendis et al., 2005). For this reason, strategies that target the initial prevention are important to reduce further disease progression. Among the factors that can be manipulated, dietary lipids are relevant, due to the influence of low-density lipoprotein cholesterol, triacylglycerols and saturated fat on CVD development (Waqar et al., 2010). Some bioactive lipid compounds, such as omega 3 fatty acids (ω-3 FA) and plant sterol esters (PSE), have been highlighted in the scientific literature as the most effective at improving cardiovascular protection (Garcia-Llatas et al., 2011; Harris et al., 2008).

There are different sources of ω-3 FA that can be added to food formulations. These compounds can be of animal or vegetal origin. Animal oils can be obtained from fish or algae and are rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, whereas vegetable oils such as *Echium* oil is rich in α-linolenic (ALA) and stearidonic acid (SDA) (Whelan, 2009). Biological activity of ALA and SDA most likely relates to their conversion to EPA (Calder, 2012; Whelan, 2009). The action of these specific fatty acids in animal metabolism is still under discussion, but the general mechanism involves a hypolipideamic effect based on the downregulation of liver X receptor (LXRα), with a subsequent inhibition of fatty acids synthesis, associated with the upregulation of peroxisome proliferator-activated receptors (PPARα) which promotes fatty acids β-oxidation (Adkins et al., 2010; Calder, 2012). Besides reducing very low density lipoprotein and triacylglycerol (TAG), ω-3 FA show a relevant anti-inflammatory effect, due to the replacement of arachidonic acid as substrate for enzymatic oxidation mediated by lipoxygenase and cyclooxygenase, resulting in eicosanoids with milder inflammatory action (Calder, 2012). Plant sterols are compounds with a molecular structure similar to that of cholesterol, found in seeds,

vegetable oils and cereals. These molecules are able to displace cholesterol during micelle formation in the intestine due to their higher hydrophobicity, reducing cholesterol absorption. Additionally, plant sterols act to increase the expression of ABCG 5 and ABCG 8, carriers involved in the reverse transport of cholesterol from enterocyte to intestinal lumen. PSE also reduce the activity of acetyl-coenzyme A acetyltransferase (ACAT), an enzyme that re-esterifies cholesterol, which is a necessary step for its incorporation into chylomicrons (Garcia-Llatas et al., 2011).

Therefore, the combination of the anti-inflammatory and hypotriglyceroleamic effects promoted by ω-3 FA with the hypocholesteroleamic effect of PSE in a food emulsion, could be an interesting strategy to reduce the risk of CVD. In addition, as ω-3 FA can increase total cholesterol, their combination with PSE could also contribute to reduce this effect (Castro et al., 2005). However, the susceptibility to oxidation increases according to the fatty acids unsaturation degree (Decker et al., 2010), becoming the ω-3 highly polyunsaturated fatty acids (such as EPA, DHA and SDA) prone to oxidise when added to an emulsion. Although *Echium* oil has been reported as a potential source of ω-3 FA to be used in functional foods, information about its oxidative stability as bulk oil or as part of an emulsion is scarce. Oxidized *Echium* oil presents a strong fishy odour that makes completely unfeasible its application in food systems. Gray et al. (2010) observed that *Echium* bulk oil oxidizes relatively fast, forming thiobarbituric acid reactive substances (TBARs) and 2,4-heptadienal after 2 days of storage at 40^{o}C, while lipid hydroperoxides increased since from the first day. Plant sterols are also susceptible to oxidation after heat treatment, contact with oxygen or exposure to sunlight, forming phytosterol oxidation products (POPs) (Garcia-Llatas et al., 2011; Otaegui-Arrazola et al., 2010). For this reason, it is necessary the use of antioxidant compounds able to delay lipid oxidation. The most efficient antioxidants are synthetic and controlled compounds that have been shown to be toxic and mutagenic at high dosages (Giraldo et al., 2007). Thus, the application of these artificial antioxidants in a functional food is contrary to the concept of healthy and should be discouraged. In this case, natural antioxidants could be applied instead of

artificial ones. Several natural compounds obtained from vegetal sources or from their processing by-products or waste products may represent interesting alternatives to replace artificial antioxidants in food emulsions (Capitani et al., 2009). Among these compounds, the cinnamic and benzoic acid derivatives, as well as flavonoids, are well known for their antioxidant properties (Natella et al., 1999). Phenolic compounds act as antioxidants due to their capacity of transferring single-electron and/or hydrogen-atom to free radicals, and also due to their ability to bind potentially pro-oxidant metal ions, resulting in a stable phenoxyl radical (Craft et al., 2012). However, besides the bond dissociation energy (BDE) and ionization potential (IP), the action of these natural polyphenols as antioxidants in an oil-in-water (O/W) emulsion depends on several factors, including their concentration, physical location, chemical structure, steric issues, nature of the lipid system, interaction with other compounds and relative polarity to the type of lipids present in the emulsion (Sørensen et al., 2011; Shahidi et al., 2011; Jayasinghe et al., 2013). Considering the necessity of replacing artificial by natural antioxidants in functional foods formulation, the objective of this study was to evaluate the antioxidant action of eleven phenolic compounds extracted from red propolis on the oxidative stability of an O/W emulsion containing both ω-3 FA (from *Echium* oil) and PSE.

2. Materials and methods

2.1 Reagents and solvents

Echium oil (NEWmega™ Echium Oil, Ref.15200) was purchased from De Wit Speciality Oils (De Waal, Tescel, The Netherlands). It was a refined, bleached, deodorised and winterised oil obtained from *Echium* seeds (*Echium Plantagineum* species). The oil presented the following major fatty acids as measured by gas chromatography according to Shirai et al. (2005): C16:00 – 7%, C18:00 – 3%, C18:1 ω9 – 15%, C18:2 ω6 – 15%, C18:3 ω-3 – 32%, C18:3 ω6 – 11% and C18:4 ω-3 – 14%. Plant sterol esters (CardioAid-S WD ™) was a mixture containing 41% of total sterols (β-

sitosterol: 47.3%, campesterol: 25.3%, stigmasterol: 17.2%, β-sitostanol: 1.1%, campestanol: 0.6%, brassicasterol: 3.6% and other sterols: 4.9%) supplied by Archer Daniels Midland Company - ADM ® (Decatur, IL, USA), as previously reported by Botelho et al. (2014). Phenolic compounds were provided by the College of Agriculture "Luiz de Queiroz" of University of São Paulo (São Paulo, Brazil), which were obtained from an ethanolic extract of Brazilian red propolis, fractioned by liquid-liquid extraction with hexane and chloroform and purified by semi-preparative reverse-phase HPLC (Oldoni et al., 2011). Analytical and HPLC-grade solvents were purchased from Merck & Co. (Whitehouse Station, NJ, USA). Reagents and phenol standards were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Silica solid-phase extraction (SPE) cartridges (Strata, 70A, 500 mg/3 mL) from Phenomenex (Torrence, CA, USA) were utilised for POPs purification. Standards of sterols and cholesterol oxidation products were purchased from Steraloids (Newport, RI, USA).

2.2 Experimental design

This study was carried out in three steps. Firstly, it was evaluated the effect of some known artificial and natural antioxidants (tocopherol, Trolox, TBHQ and ascorbic acid) and pro-oxidants (iron and iron + ascorbic acid) on emulsions containing *Echium* oil and PSE, aiming to establish reference values for chemical markers applied to monitor the lipid oxidation. Samples were analysed at the beginning (T_0), after heating (T_H) and also after 30 days of storage at room temperature (T_{30}). From this first step the positive and negative controls of the reaction were selected. In a second step, 11 phenolic compounds (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans*,*trans*-farnesol, rutin hydrate, gallic acid and sinapic acid) were added to the emulsion and the oxidative stability was evaluated. Samples were analysed at the beginning (T_0), after heating (T_H) and also after 14 days of storage at room temperature (T_{14}). Phenolic compounds that showed better performance in

the second step, were also evaluated in terms of quantification of hexanal, malondialdehyde (MDA) by HPLC, POPs and fatty acids composition. Afterward, the *in vitro* antioxidant activity of the compounds extracted from red propolis was compared with those commercially synthesised, using ORAC methodology. All assays were performed in duplicate.

2.3 Emulsion preparation

Oil-in-water emulsions were prepared using a sodium acetate-imidazole buffered solution (10 mmol/L each, pH 7.0) containing 0.6% Tween 20. The emulsions were prepared by mixing the *Echium* oil (5.0 g/100 mL), CardioAid (1.6 g/100 mL) with water, using a high-pressure homogenizer (Homolab mod A-10, Alitec, São Paulo, Brazil) at a pressure of 500 bar. During each step of the emulsion preparation, the samples were covered as much as possible to reduce light exposure and were kept in an ice bath. At the first step seven emulsions were prepared. Emulsions were added with no antioxidants (CONT), with antioxidants: tocopherol (TOC), ascorbic acid (ASC), *Tert*-butylhydroquinone (TBHQ) and Trolox (TLX), or with pro-oxidants: ascorbic acid (5.35 mg/g oil) + $FeSO_4$.7 H_2O (4.42 mg/g oil) (ASCIR),or 4.96 mg $FeSO_4$.7 H_2O/g oil (IRON), heated at 84 ^oC/90 min and stored at room temperature for 30 days. The proportion between ascorbic acid and $FeSO_4$.7 H_2O used to prepare the pro-oxidant mixture was based on a previous study (Branco et al., 2011) . In the second step, emulsions containing the phenolic compounds (200 ppm) were evaluated. The heat treatment was carried out at 90^oC for 45 min and the emulsions were stored at room temperature for 14 days, covered with aluminium foil. Selected phenolics (quercetin, rutin and sinapic acid) were also evaluated at 500 and 1000 ppm. Sampling was performed from the individual sealed screw-cap vials, keeping the same headspace in the samples during all assay.

2.4 Fatty acid composition

Lipids from the *Echium* oil and emulsions were derivatised by direct esterification (Shirai et al., 2005) and its composition was determined by gas chromatography (GC) (Agilent 7890 A GC

System, Agilent Technologies Inc., Santa Clara, CA, USA). A polyimide coated fused silica capillary column (J&W DB-23 Agilent 122-236; 60 m x 250 mm inner diameter, 0.15 mm film thickness) was used. Oven temperature was programmed from 80 to 175°C at 5°C/min, and from 175 to 230°C at 3°C/min; the final temperature was kept for 5 min. The injector and detector temperatures were set at 250 and 280 ^{o}C, respectively. High-purity helium was used as carrier gas at a flow rate of 1 mL/min, with a split ratio of 1:50. The fatty acids were identified by comparing their retention times with those of four purified standard mixtures of fatty acid methyl esters: 4-7801; 47085-U; 49453-U and 47885-U (Sigma Chemical Co.; St. Louis, MO, USA). Results were expressed as g/100 g of total fatty acids. Two independent replicates were run per sample.

2.5 Lipid hydroperoxide and TBARs analysis

Lipid hydroperoxide concentrations were determined according to the procedures described by Shantha and Decker (1994). Emulsion samples (300 µL) were mixed with 1.5 mL of an isooctane/2-propanol solution (3:1, v/v), resulting in a final volume of 1.80 mL. The mixture was vortexed three times for 10 s, and 200 µL of the mixture was added to a 2.8 mL solution of methanol/1-butanol (2:1, v/v). A thiocyanate/ferrous solution was prepared by mixing 500 µL of 3.94 M thiocyanate solution with 500 µL of 0.072 M Fe^{+2} solution. The 0.072 M Fe^{+2} solution was obtained from the supernatant of a mixture of 1.5 mL of 0.144 M $FeSO_4$ and 1.5 mL of 0.132 M $BaCl_2$ in 0.4 M HCl. 30 µL of thiocyanate/ferrous solution were added to 3.0 mL of methanol/1-butanol mixture, vortexed and incubated at room temperature for 20 min. Following the incubation period, the samples' absorbance readings were measured at 510 nm using a UV–Vis mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). The hydroperoxide content was determined using a standard curve prepared with known concentrations of cumene hydroperoxide. Concentrations were expressed as meq/kg of oil. The amount of thiobarbituric-acid-reactive substances (TBARs) was determined according to the method proposed by McDonald et al. (1987). Measurements were

taken in duplicate, and the values were expressed as μmol/kg of oil. Two independent replicates were run per sample.

2.6 Oxygen radical absorbance capacity (ORAC)

The ORAC method is based on the inhibition by antioxidants of the peroxyl-radical-induced oxidation of fluorescein, initiated by thermally induced decomposition of the azo-compound 2,2‘-azobis-2-amidino-propane-dihydrochloride (AAPH) (Prior et al., 2003). Phenolic solutions (5 g/L in methanol) were diluted to 2 mg/L with 75 mM phosphate buffer (pH 7.1). The samples and blank (25 μL) were incubated with 40 nM fluorescein (150 μL) in a 96-well microplate at 37°C for 30 min. Thereafter, 153 mM AAPH (25 μL) was added to the reaction media and the fluorescence (485 nm excitation and 525 nm emission) was monitored for 1 h by a multi-detection microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). The difference between the integrals of the decay curve for samples and blank was calculated using a Gen 5 software (version 1.06.10, BioTek Instruments Inc., Winooski, VT, USA) and the concentration equivalent of Trolox was calculated by linear regression (6.25-100.00 μM). Results were expressed as μM of Trolox equivalent (μM TE)/mg of phenolic compound. Two independent replicates were run per sample.

2.7 Phytosterols oxidation products (POPs)

Phytosterols oxidation products (POPs) were determined according to a modified version of the methods described by Cercaci et al. (2007) and Alemany-Costa et al. (2012). Briefly, emulsions were subjected to a direct cold saponification, and the unsaponifiable matter was fractionated by SPE, to obtain the purified and enriched POPs fraction. The identification and quantification of POPs were carried out using fast gas chromatography-mass spectrometry (GC-MS) according to Botelho et al. (2014) with minor modifications. In particular, the oven temperature was set from 210°C to 325°C at the rate of 15°C/min. The injector, transfer line, ion source and interface temperatures were set at 325, 280, 200 and 330°C, respectively. The acquisition and integration

modes were total ion current (TIC) and single ion monitoring (SIM), respectively. Identification of POPs was performed by comparing the retention time and mass spectra with those reported in our previous works and literature. POPs were recognised and quantified by their corresponding characteristic ions that show a high abundance by SIM mode (*m/z*): IS (19-hydroxycholesterol) (353), 7α-hydroxycampesterol (470), 7α-hydroxystigmasterol (482), 7α-hydroxysitosterol (484), 7β-hydroxystigmasterol (482), 7β-hydroxysitosterol (484), α-epoxysitosterol (412), 7-ketocampesterol (486), 6β-hydroxycampesterol (417), stigmastentriol (429), sitostanetriol (431), 6-ketositostanol (473), 7-ketositosterol (500). Considering that POP standards are not commercially available and that POPs fragmentation is similar to that of cholesterol oxidation products (COPs), POPs quantification was performed using the calibration curves obtained for cholesterol oxides in the SIM mode (Cardenia et al., 2012). Two independent replicates were run per sample.

2.8 Malondialdehyde (MDA) analysis

MDA concentration was determined by reverse phase high-performance liquid chromatography (HPLC) (Hong et al., 2000). Emulsion (0.05 mL) was submitted to alkaline hydrolysis with 12.5 µL of 0.2% butylated hydroxytoluene in ethanol and 6.25 µL of a 10 M sodium hydroxide aqueous solution. This mixture was incubated at 60°C for 30 min and 750 µL of 7.2% TCA aqueous solution containing 1% KI were added. The samples were kept on ice for 10 min and centrifuged at 13,000 x g for 10 min. The supernatant (500 µL) was mixed with 250 µL of 0.6% TBA and heated at 95°C for 30 min. After cooling, the MDA was extracted from the solution with 750 µL of *n*-butanol, and 50 µL were analysed by HPLC (Agilent Technologies 1200 series; Santa Clara, CA, USA). The TBA-MDA conjugate derivative was injected into a Phenomenex reverse-phase C18 analytical column (250 mm x 4.6 mm; 5 µm, Phenomenex, Torrance, CA, USA) with a LC8-D8 pre-column (Phenomenex AJ0-1287) and was fluorometrically quantified at an excitation and emission wavelengths of 515 nm and of 553 nm, respectively. The analysis were run

under isocratic conditions, using a mobile phase of 60% phosphate buffered saline (PBS) (50 mmol/L, pH 7.1) + 40% methanol at a flow rate of 1.0 mL/min. A standard curve (0.5-20 μmol/L, r = 0.995) was prepared using 1,1,3,3-tetraethoxypropane. Four independent replicates were run per sample.

2.9 Hexanal by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC–MS)

Hexanal content of the samples was determined according to Garcia-Llatas et al. (2007) after some modifications. In this study, hexanal was analysed as an oxidative marker of ω-6 fatty acid (γ-linoleic acid) present in the *Echium* oil. Emulsions (990 μL) were added with 10 μL of internal standard (1 μL of MBIK/mL in methanol) and hermetically sealed in a 20 mL headspace glass vial with a polypropylene hole cap and PTFE/silicone septum (Supelco, Bellefonte, PA, USA). A Combi PAL autosampler was used for automated HS-SPME analysis. The vials were agitated (400 rpm) at 40^{o}C for 15 min. After this period, a conditioned (at 300^{o}C/2 h) fibre (carboxen/polydimethylsiloxane (CAR/PDMS); StableFlex fibres; 85 μm; Supelco 57295-U, Bellefonte, USA) was exposed to the headspace of the emulsions for 45 min under agitation at 40^{o}C. Then, the fibre was introduced into the GC-MS injector at 250^{o}C for 5 min under a split ratio of 1:10. During the GC analysis, the fibre was thermally desorbed at 250^{o}C for 3 min. Vials containing only water were inserted after each 5 samples to prevent residual peaks from the fibre. The analysis was carried out in an Agilent 7890 A GC-MS (Palo Alto, CA, USA). The stationary phase was a ZB-5 MS capillary column (5% polysilarylene/95% polydimethylsiloxane; 30 m × 0.32 mm; 1 μm film thickness; Phenomenex®; Torrance, USA). The ion source and quadrupole temperatures were set at 230 and 150°C, respectively. Ultra pure helium was the carrier gas operated at a constant flow of 3.0 mL/min. Oven temperature was kept at 40 ^{o}C for 5 min, increased until 100^{o}C at 4^{o}C/min, and then taken to 220^{o}C at 17 ^{o}C/min; the final temperature was kept for 10

min. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV, adopting a mass range of 35–300 *m/z*. TIC and SIM were used as data acquisition mode using the NIST library. The following retention times and quantification ions were used: IS 8.7 min (43, 58 and 100 *m/z*) and hexanal 11.7 min (44, 56 and 72 *m/z*). All quantification was based on the peak area ratio of the signal of the analyte and the IS signal. A standard curve (μg/mL hexanal = 39.112 hexanal/IS area ratio; r = 0.995) was prepared with five concentrations of hexanal (0-0.08 μg/mL of fresh emulsion), keeping the same amount of IS. Results were expressed as μg hexanal/mL of emulsion. Two independent replicates were run per sample.

2.10 Statistical analysis

Values were expressed as mean ± standard error of mean (SEM). Variance homogeneity and normality were previously evaluated for all variables. Differences among the samples of the experiment were evaluated by Repeated Measures ANOVA, followed by Tukey's HSD or Dunnett's test (vs CONT) in each time interval. Equivalent non-parametric tests were applied when the distribution normality was not verified. Significance was set at *p*-values less of 0.05. All analyses were performed with the software STATISTICA version 9.0 (StatSoft, Inc., Tulsa, Ok, USA).

3. Results

Figure 1 presents the LOOH and TBARs values of the control sample (CONT), the emulsions containing antioxidants (TOC, ASC, TBHQ and TLX) and the emulsions added with pro-oxidants (ASCIR and IRON), at the beginning (T_0), after heating (T_H) and after 30 days (T_{30}) of storage at room temperature. No significant differences in the hydroperoxide concentration were observed among samples at T_0 and T_H (Figure 1A). After 30 days of storage, emulsions containing tocopherol (TOC) and the mixture of ascorbic acid + Fe^{2+} (ASCIR) showed the highest LOOH values. No

significant differences in the TBARs concentration were detected among the samples during the assay (Figure 1B). Based on these results, TBHQ and ASCIR were selected as negative and positive controls, respectively, in this study.

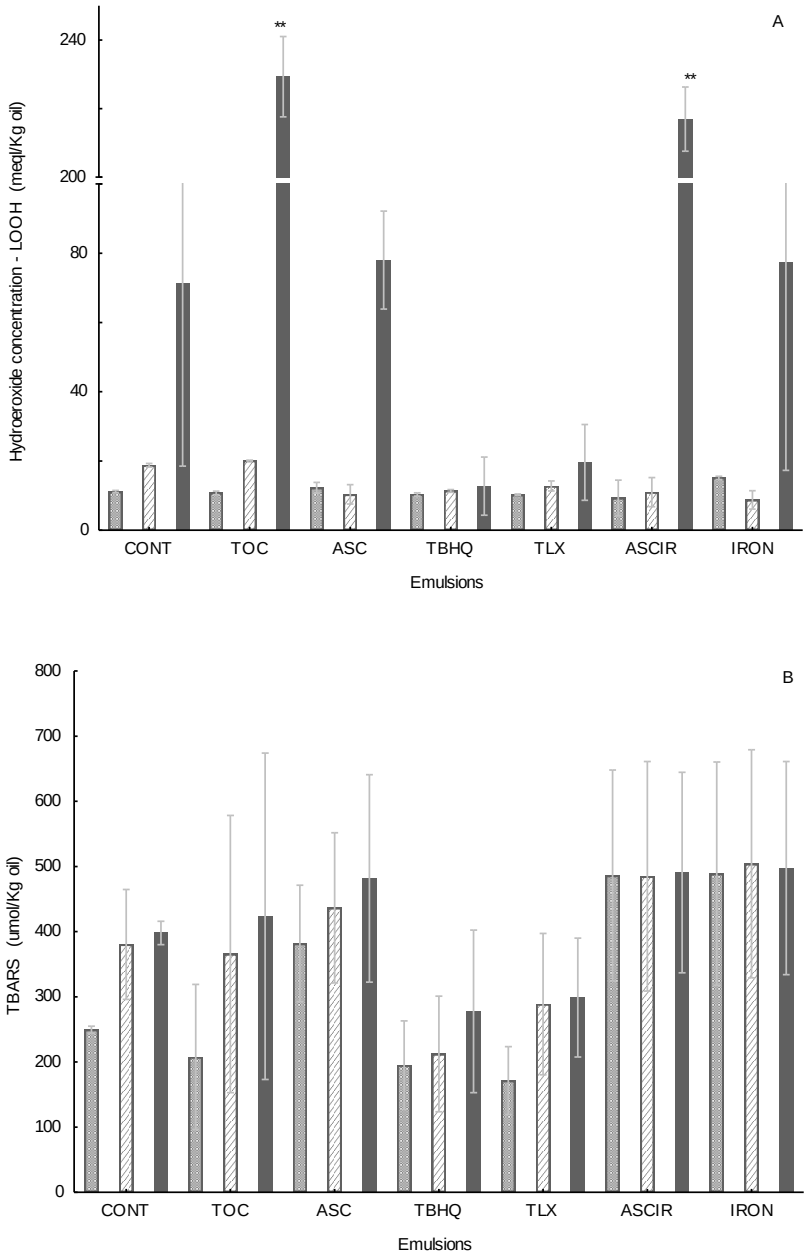
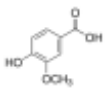
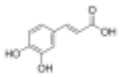
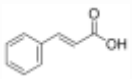
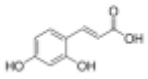
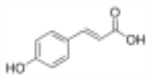
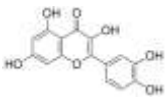
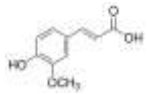
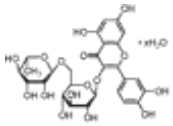
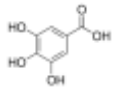
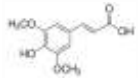
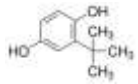


Figure 1. Hydroperoxides (meq/kg oil) and TBARs concentration (μmol/Kg oil) in functional O/W emulsions containing both ω-3 FA and PSE. Samples without antioxidants (CONT), with antioxidants (tocopherol (TOC), ascorbic acid (ASC), TBHQ (TBHQ) and Trolox (TLX) at 1.75 mg/g oil), and with prooxidants (ascorbic acid (5.26 mg/g oil) + $FeSO_4.7\ H_2O$ (4.38 mg/g oil) (ASCIR), and iron (48.8 mg $FeSO_4.7\ H_2O$/g oil) (IRON)), analyzed at the beginning (▪ T_0), after heating (⌜ T_H) and after 30 days (■ T_{30}) of storage at room temperature. Figure 1A: LOOH concentration, *p* (samples) = 0.008 and *p* (time) < 0.001. Figure 1B: TBARs concentration, *p* (samples) = 0.689 and *p* (time) < 0.001. Error bars are SEM of the values for each emulsion (n=2); ***P* < 0.01.

At the second step, emulsions prepared with 11 phenolic compounds at 200 ppm were compared with emulsions prepared with TBHQ and ASCIR, before and after heating, and after 14 days of storage at room temperature (Table 1). Regarding LOOH values, samples added with sinapic acid, rutin, quercetin, TBHQ and ASCIR showed lower LOOH values than CONT. The lower LOOH concentration observed in the ASCIR samples was due to the higher LOOH degradation, as evidenced by the highest concentration of TBARs in the ASCIR samples (Table 1).

Table 1. Hydroperoxides (LOOH) and TBARs concentration of the emulsions prepared with eleven natural compounds (200 ppm) compared with emulsions prepared with TBHQ, ASCIR and CONT, before (T0), after heating(TH) and after 14 days of storage (T14).

			LOOH (meq/kg oil)			TBARs (µmol/kg oil)		
Compound[a] and Molecular structure	**MW (g)[b]**	**µMol[c]**	**T_0**	**T_H**	**T_{14}**	**T_0**	**T_H**	**T_{14}**
Vanillic acid 	168.15	67.8	9.2±0.4	25.1±5.9	41.4±1.3	45.1±1.6	149.2±13.6	257.1±10.4
Caffeic acid 	180.16	63.3	8.1±0.3	23.6±4.9	30.7±0.8	41.3 ±0.6	117.8 ±8.9	170.1±11.2
trans-Cinnamic acid 	148.16	76.9	7.3±1.7	25.8±6.1	38.5±0.9	45.0 ± 6.5	135.6±27.3	259.1±30.9
2,4-Dihydroxycinnamic acid 	180.16	63.3	6.9±2.5	27.0±5.1	45.5±5.0	44.2±3.3	140.2±14.7	266.1±13.3
p-Coumaric acid 	164.16	69.4	9.0±1.3	27.1±8.1	40.5±0.6	50.4±5.1	153.9±22.5	273.1±19.1
Quercetin 	338.27	33.7	5.0±3.7	12.5±0.4	26.5±0.8*	40.0±0.0	101.8±6.9	176.0±6.1
trans-Ferulic acid	194.18	58.7	9.9 ± 0.5	18.3±0.6	39.6 ± 0.5	50.5 ± 9.5	142.3 ±20.8	231.3 ± 10.4

 Rutin hydrate	610.52	18.7	7.2±0.0	13.5±0.3	20.5±0.1*	45.0±12.7	99.4±15.3	134.2±3.4*
 Gallic acid	170.12	67.0	10.1±0.0	18.3±0.9	33.1±2.1	39.8±3.1	139.9±16.6	210.1±28.5
 Sinapic acid	224.21	50.8	8.6±0.1	13.4±0.9	14.6±0.7*	44.7±10.4	93.6±13.4	93.9±1.0*
 trans,trans-Farnesol	222.37	51.3	8.0±0.8	18.1±0.6	37.6±2.6	44.0±2.6	140.5±23.9	211.2±5.9
 TBHQ (CONT-)	166.22	68.6	8.5±0.6	11.5±0.4	11.2±0.3*	37.3±4.1	68.3±11.9	90.3±3.7*
CONT	-	-	7.1±2.3	22.9±3.7	38.5±3.3	43.0±3.3	139.6±19.4	230.9±32.1
ASCIR (CONT+)	-	-	4.3±1.1	7.4±2.6	16.2±2.4*	700.9±12.0*	1655.3±32.5*	1698.2±8.8*
P[d]	-	-	0.367	0.034	<0.001	<0.001	<0.001	<0.001

[a]Values are mean ± SEM (n= 2).
[b] Molecular weight (g).
[c]Concentration expressed as μMol/L emulsion equivalent to 200 ppm (based in oil content).
[d]Probability value obtained by ANOVA.*p<0.05) from CONT, as evaluated by Dunnet test (2-tailed).

Still regarding TBARs, samples containing sinapic acid, rutin and TBHQ showed lower values than CONT. Taking these data into account, sinapic acid, rutin and quercetin were chosen for the next step of the study. The selected phenolic compounds concentration effect on oxidative stability of the emulsions was also evaluated (Figure 2). The compounds concentration did not influence the antioxidant activity of the sinapic acid, rutin and TBHQ as measured by LOOH (Figure 2A), but quercetin at 200 ppm showed lower antioxidant activity than at 500 and 1000 ppm. No difference was verified in TBARs concentration by increasing the phenolics concentration from 200 to 1000 ppm (Figure 2B).

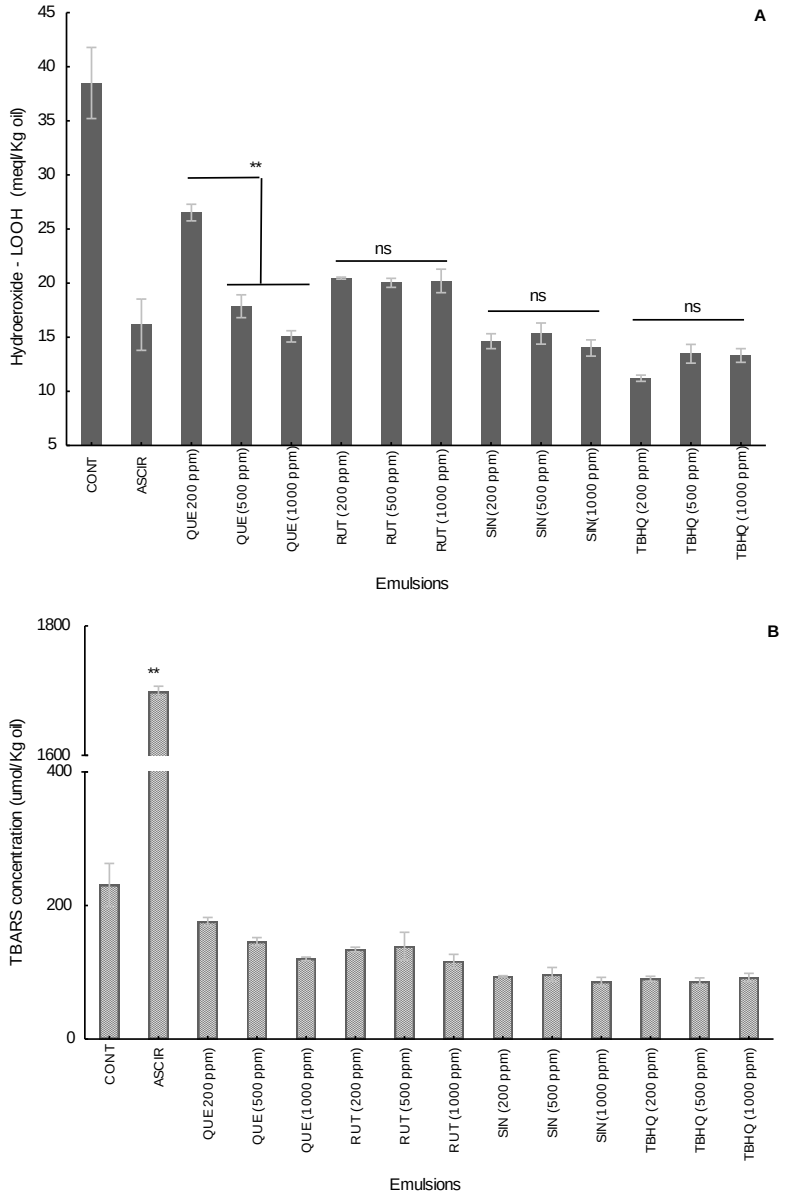


Figure 2. Hydroperoxides (LOOH) and TBARs concentration in the functional emulsions containing n-3 FA and PSE. Samples without antioxidants (CONT), with pro-oxidant ascorbic acid (5.26 mg/g oil)+ FeSO4.7 H2O (4.38 mg/g oil) (ASCIR), and with phenolic compounds: quercetin (QUE), rutin (RUT) sinapic acid (SIN) and TBHQ at 200, 500 and 1000 ppm, measured after 14 days of storage at room temperature. Error bars are SEM of the values for each emulsion (n=2); $^{**}p < 0.01$.

In the third step of this study, the oxidative stability of the functional oils (ω-3 FA and PSE) present in the emulsions added with sinapic acid (200 ppm), rutin (200 ppm) and quercetin (500 ppm) was evaluated after 14 days of storage at room temperature, and compared with ASCIR and TBHQ (200 ppm). The amount of the two ω-3 FA present in the *Echium* oil composition, α-linolenic (18:3 Δ9,12,15) and stearidonic (18:4 Δ6,9,12,15) acids, did not change among the samples. Secondary products of oxidation are shown in Figure 3. Hexanal content was measured by HS-SPME-GC-MS, and just the samples containing the pro-oxidant ASCIR showed higher values than CONT (Figure 3A). As the TBARs determination is a non-specific methodology for MDA quantification, a HPLC analysis of the latter was also carried out with these emulsions. Data present in Figure 3B also confirmed that only samples containing the pro-oxidant ASCIR showed higher values than CONT.

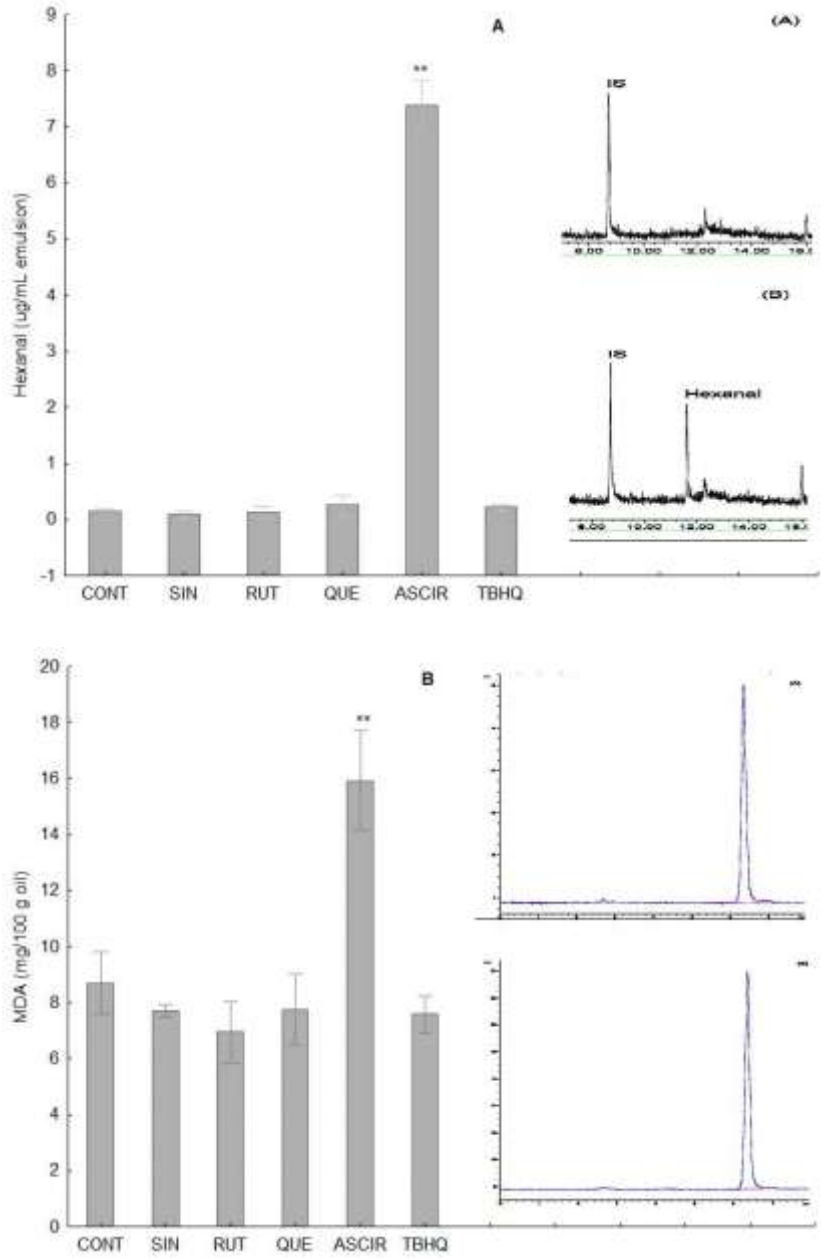


Figure 3. Secondary products of oxidation measured in O/W emulsions without antioxidants (CONT), with pro-oxidant (ASCIR), and with phenolic compounds (rutin (RUT), sinapic acid (SIN) and TBHQ at 200 ppm, and quercetin (QUE) at 500 ppm), after 14 days of storage at room temperature. **Figure 3A.** Hexanal (mg/mL emulsion). Error bars are SEM of the values for each emulsion (n=2); $^{**}p<0.01$. Chromatograms (A): TBHQ and (B): ASCIR. **Figure 3B.** Malonaldehyde (mg/100 g oil). Error bars are SEM of the values for each emulsion (n=4); $^{**}P < 0.01$. Chromatograms (A): TBHQ and (B): ASCIR.

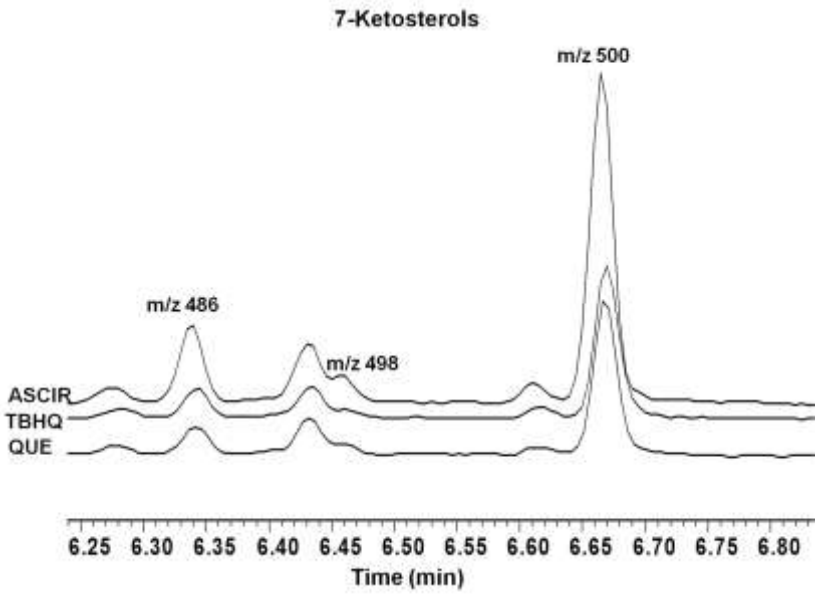
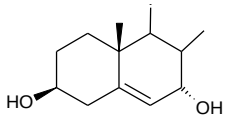
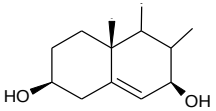
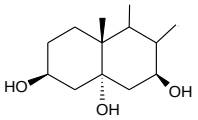
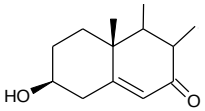
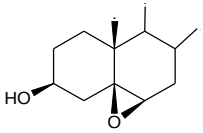
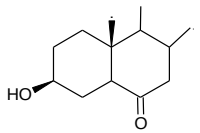


Figure 3C. Peaks relative to 7-Keto derivatives (7-Keto-campesterol, 7-Keto-stigmasterol and 7-Keto-sitosterol).

Finally, the major POPs were analysed in the emulsions after 14 days of storage (Table 2). Among the 16 POPs identified in the samples, 7-Keto and triol derivatives were the most abundant. No significant difference was found in triol-derivates among samples, while 7-Ketocampesterol ($p=0.011$) and 7-Ketositosterol differed (p=0.009) among the emulsions tested.

Table 2. POPs concentration of the emulsions without antioxidants (CONT), with pro-oxidant (ASCIR), and with phenolic compounds: rutin (RUT), sinapic acid (SIN) and TBHQ at 200 ppm, and quercetin (QUE) at 500 ppm, after 14 days of storage at room temperature.

	7-α- hydroxy			**7-β hydroxy**			**Tri**			**7-keto**			**Epoxi**[c]		**6-keto**
															
Sample	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**5α,6α-**	**5β,6β-**	**Sito-**
CONT	2.26	1.99	3.13	1.12	0.82	1.96	0.37	1.43	14.60	1.06	2.61	12.83	1.53	1.85	1.60
SIN	2.46	2.11	3.72	1.29	1.08	2.59	0.45	1.38	13.74	1.04	3.24	13.45	2.19	2.36	1.25
TBHQ	2.39	2.02	3.59	1.16	0.86	2.23	0.40	1.21	10.11	1.11	3.38	12.48	2.05	1.45	0.10
ASCIR	2.30	1.96	2.91	1.58	1.38	3.33	0.55	1.35	10.39	2.80*	6.09	28.27**	7.88	3.74	2.05
RUT	2.23	2.00	3.28	1.11	0.96	2.15	0.37	1.36	15.16	0.50	2.26	12.29	2.16	3.38	1.73
QUE	2.20	1.96	3.02	1.07	0.67	2.07	0.36	1.12	10.09	0.95	2.47	8.92	1.62	2.42	0.13
SEM[a]	0.05	0.04	0.15	0.08	0.09	0.24	0.02	0.04	1.04	0.23	0.50	2.00	0.81	0.31	0.35
p^b	0.834	0.939	0.643	0.576	0.247	0.710	0.109	0.357	0.583	0.011	0.229	0.009	0.132	0.204	0.514

[a]Pooled Standard Error of Mean

[b]Probability value obtianed by ANOVA.*p<0.05; ** p<0.001 as evaluated by Tukey post-hoc test.

[c]Structural configuration of 5α,6α

Likewise the results shown by other markers, ASCIR emulsions containing ascorbic acid and iron presented higher values of 7-ketosterols than the other emulsions. Regarding the 7-ketositosterol (Figure 3C), ASCIR emulsions showed higher values than the emulsions containing TBHQ and quercetin (p=0.031).

As the phenolic compounds applied in this study were obtained from red propolis, the antioxidant activity of these molecules was compared with the synthetic ones. No difference was observed to antioxidant activity between the molecules obtained by extraction. This result was found when the samples were analysed by ORAC methodology adapted for hydrophilic (Figure 4A) and lipophilic substrates (Figure 4B). In fact, a high correlation between the antioxidant activity of the hydrophilic and lipophilic fractions was noted (r= 0.839; p<0.001), while no correlation was found between the *in vitro* antioxidant capacity (ORAC) and chemical oxidation markers (LOOH and TBARs).

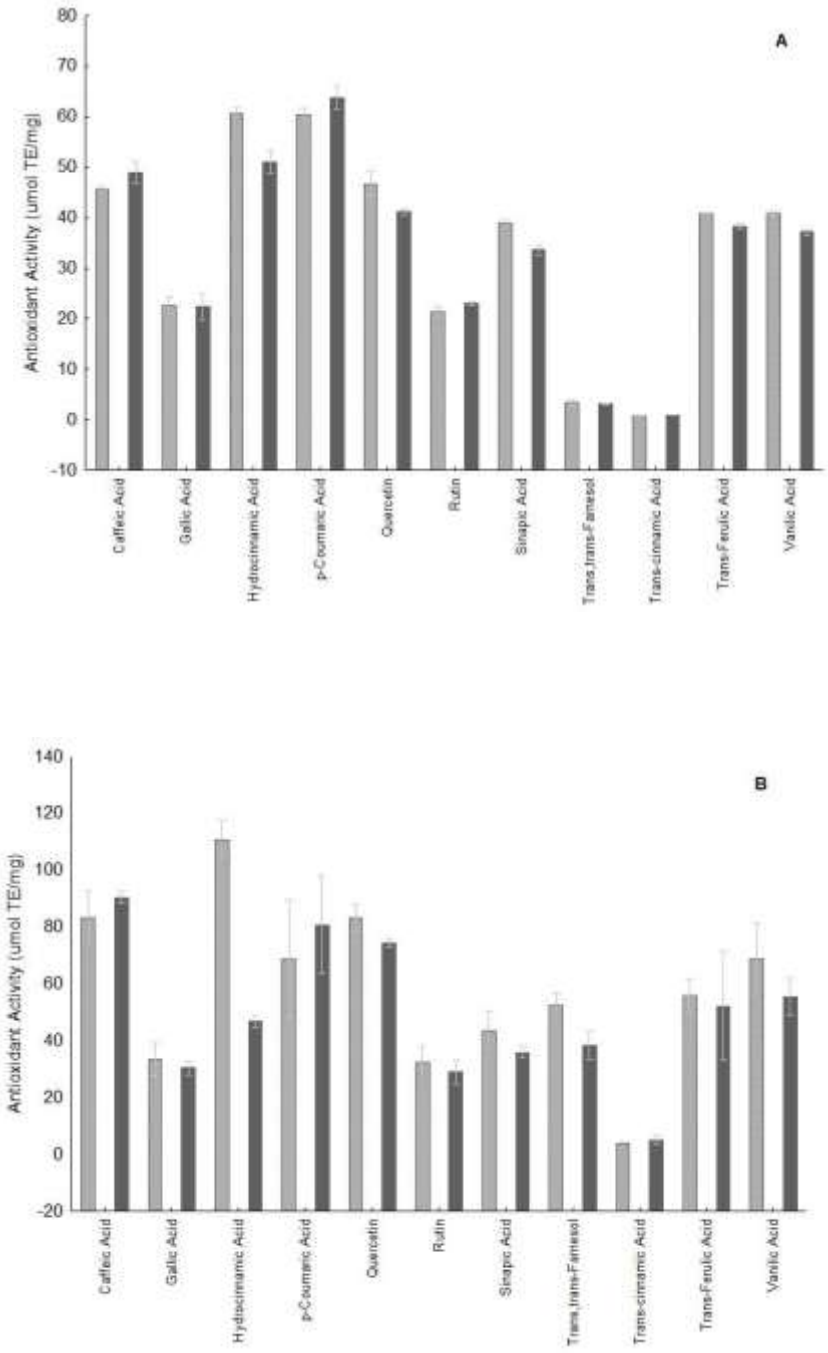


Figure 4. Antioxidant activity of the phenolic compounds extracted from red propolis and standards measured by ORAC for both hydrophilic (4A) and lipophilic (4B) fractions obtained by extraction or synthesis. Error bars are SEM of the values for each emulsion (n=2).

4. Discussion

The present data show that emulsions prepared with *Echium* oil and PSE are prone to oxidation after heat treatment, forming hydroperoxides and other secondary lipid oxidation products. The oxidative damage observed in the functional emulsion was higher when ascorbic acid and iron were present, regardless of the temperature. Even before the heat treatment, the energy generated during homogenization under high pressure was enough to initiate oxidation of samples containing the pro-oxidant (ASCIR). Pro-oxidant effect of ascorbic acid alone or combined with metals in emulsions is well known (Jayasinghe et al., 2013; Sørensen et al., 2011). In a previous study, Branco et al. (2011) showed that when pH increases from 3.0 to 7.0 and the ascorbic acid content was greater than 1.0 mmol/L, TBARs values increased from 3.0 to 12.0 mmol/L, regardless of the iron concentration. Similar results were also observed in ω-3 FA-enriched O/W emulsions added with ascorbic acid and kept at 35^{o}C/48h (Jayasinghe et al., 2013). In fact, as found in our model, Jayasinghe et al. (2013) observed oxidative instability in the initial stage of the storage period. Pro-oxidant effect of ascorbic acid in emulsions is due to its capacity to reduce Fe^{3+} to Fe^{2+}, thus accelerating lipid oxidation (Frankel et al., 2000). Cuvelier et al. (2000) applied a mixture of ascorbic acid with Fe^{2+} to induce oxidation in emulsions at 30^{o}C. In a study carried out by Sørensen et al. (2011), ascorbic acid (free or conjugated with fatty acids) acted as pro-oxidant at the end of the storage period in O/W emulsions containing ω-3 FA. Therefore, ascorbic acid should not be applied in ω-3 FA O/W emulsions neither as antioxidant nor as source of vitamin C, regardless of the processing treatment or temperature of storage.

Among the eleven phenolic compounds tested, sinapic acid (200 pmm), rutin (200 ppm) and quercetin (500 ppm) inhibited the oxidation of the functional emulsion at the same level as TBHQ (200 ppm). Although the effectiveness of an antioxidant strongly depends on its BDE and IP, other factors such as reaction environment, solubility, concentration and presence/influence of other

compounds contribute to its antioxidant activity (Lengyel et al., 2012; Leopoldini et al., 2011). DBE of the O-H bonds in natural phenols does not present a widely range of variation (Evgeny et al., 2011). Thus, it can be suggested that in emulsions, phenolics antioxidant activity depends on impact of their chemical structures on media characteristics (Evgeny et al., 2011). In the present study, TBHQ, sinapic acid, rutin and quercetin were able to react with part of the radicals and transition metals in the aqueous phase or in the oil-water interface, inhibiting fatty acids and plant sterols oxidation. Several factors can be involved in the higher antioxidant activity displayed by these four molecules, including solubility and chemical structure. Sinapic acid is a phenolic acid derived from the hydroxyl *trans*-cinnamic acid, quercetin and rutin are flavonols, and TBHQ is a hydroquinone. A number of studies have investigated the phenolics characteristics associated to their higher capacity of transferring a hydrogen atom (HAT) or a single electron (SET) to a radical molecule (Capitani et al., 2009; Craft et al., 2012). Zhiyong et al. (2003) suggested that the best phenolic antioxidants are compounds that contain an electron donor group directly attached to an aromatic ring. It has also been reported that the antioxidant activity rises with increasing number of phenolic rings, occurrence of multiple OH groups attached to the ring (preferentially in the ortho-dihydroxy configuration), planar structure and presence of additional functional C=C and C=O (Kim et al., 2004; Leopoldini et al., 2011). However, in the emulsion model used in our study, none of these statements was conjointly present and could justify the higher antioxidant activity observed in samples containing these four compounds.

Phenolic compounds are predominantly polar and expected to be located in the aqueous phase of an emulsion (Sørensen et al., 2008). Based on the "Polar Paradox" theory, non-polar antioxidants are more effective in media of relatively higher polarity (Porter, 1993). But our results showed that molecules with similar solubility presented opposite performance as antioxidants, reinforcing that other factors besides polarity are involved in the reaction (Shahidi et al., 2011). Regarding the SET mechanism, the IP is the most significant parameter; the lower the IP, the easier

the electron abstraction by the radical molecule (Leopoldini et al., 2011). At neutral pH, pKa of the phenolics could contribute to later their solubility, altering their distribution between oil, interface and aqueous media. Conversely, this condition does not explain the differences observed in our study, since pKa of the phenolic compounds are similar (Costa et al., 2013). Beside HAT or SET mechanisms, molecules containing *o*-diphenol groups are able to chelate metal ions such as iron (Sørensen et al., 2008). This fact explains the results observed in emulsions containing rutin and quercetin, as these molecules offer all possible chelating sites with $Fe^{(II)}$, thus preventing their involvement in the Fenton reaction (Leopoldini et al., 2011). However, this cannot justify the results observed in the emulsions formulated with sinapic acid or TBHQ. Therefore, there is not a common single aspect that justifies and explains the lower lipid oxidation in O/W emulsions prepared with these four phenolic compounds. It is possible that a combination of some of the aforementioned factors has improved their antioxidant activity when compared with the other compounds evaluated in this model.

Although sinapic acid, rutin, quercetin and TBHQ reduced LOOH and TBARs, no difference was observed among these samples and CONT when hexanal and MDA were separately analysed. The unsaturation degree is the major factor responsible for fatty acids susceptibility to oxidation. In *Echium* oil, 47% of the most unsaturated fatty acids (stearidonic and α-linolenic acids) are ω-3 FA, followed by 10% of ω-6 γ-linolenic. Secondary lipid oxidation products measured in our study are mainly formed from the oxidation of fatty acids with more than three double bonds (MDA) or from ω-6 FA (hexanal). This fact justifies the lack of significant differences among the treatments, except for ASCIR samples when MDA and hexanal were individually taken as markers. For instance, Gray et al. (2010) evaluated TBARs and 2,4-heptadienal (*t,t*) in *Echium* oil during storage and observed a higher alteration of 2,4-heptadienal (*t,t*) than TBARs, as 2,4-heptadienal (*t,t*) originates from linolenate hydroperoxide and is a volatile marker of ω-3 FA. Although a significant increase of both LOOH and TBARs had been observed in the emulsions containing the pro-oxidant

mixture (ASCIR), no alterations were found in the ω-3 FA content (ALA and SDA) after 14 days of storage, reinforcing that fatty acids profile cannot be used as marker to monitor earlier stages of lipid oxidation.

In our study, molecules extracted from red propolis showed similar antioxidant activity that their synthesised analogues, suggesting that further practical applications can be performed with either extracted or synthesised molecules. However, antioxidant activity measured by ORAC methodology did not correlate with the results observed in the emulsions. Similar conclusions were reported by Sørensen et al. (2013) when comparing the antioxidant activity of canola phenolic compounds by *in vitro* assays and emulsion markers. In fact, inconsistent results have been obtained for a number of recognised antioxidants depending on the methods used to test their antioxidant activity (Frankel et al., 2000). A weakness of the ORAC method is that the antioxidant protection of the fluorescent probe cannot be extrapolated to biological or food substrates. As already discussed, the antioxidant effectiveness is strongly dependent on the test system, including substrates, positioning, method employed and stages of the oxidation (Frankel et al., 2000). Thus, indirect methods, such as ORAC, should be applied just for initial screening on antioxidant activity of new or modified molecules.

The most commonly consumed plant sterols are Δ^5 ones, which have a double bond between C5 and C6 carbons in the sterol nucleus. During food processing and/or storage, this site is susceptible to oxidation by free radical attack, so the hydrogen abstraction represents the first step of the oxidation (Lengyel et al., 2012). In an emulsion system, Cercaci et al. (2007) suggested that oxidation occurs at the droplet interface. Since plant sterols are surface active compounds, they could be particularly prone to oxidation when they are incorporated in emulsions. This is particularly noticeable in the samples containing water-soluble compounds as acid ascorbic and iron, which showed the highest content of total POPs amount. Among the 16 POPs identified in our samples, only 7-keto derivatives (7-ketocampesterol, 7-ketostigmasterol and 7-ketositosterol)

showed a significant increase due to the oxidation induction. As for cholesterol (Rodriguez-Estrada, et al., 2014), 7-keto-phytosterols can be used as markers to monitor the plant sterols oxidation process in model systems, since they are easily formed and are the most representative ring POPs, as confirmed by the sterol thermoxidation study carried out by Barriuso et al. (2012). In the present work, the most abundant was 7-ketositosterol, followed by 7-keto-stigmasterol and 7-ketocampesterol. While the amount of 7-ketositosterol reflected the relative presence of β-sitosterol in the emulsion system (47.3%), the same cannot be stated for campesterol and stigmasterol. This trend is not in agreement with results reported by Barriuso et al. (2012), as they found that stigmasterol was less prone to oxidation with respect to the other two main plant sterols (campesterol ~ β-sitosterol>> stigmasterol) when heated at 180°C for 360 min, in presence of cholesterol. Such behaviour differences could arise from the diverse mixture sterol composition (with the consequent chemical equilibrium among the various chemical species), the different physico-chemical state of the model system (dried bulk sterols vs. sterol-enriched O/W emulsion), diverse heat transfer modalities and heating temperature/exposure time conditions (180 vs. 90°C) used in both studies.

5. Conclusions

Ascorbic acid combined with iron was able to oxide a functional emulsion containing 1.63 g/100 mL of α-linolenic acid (ALA), 0.73 g/100 mL of stearidonic acid (SDA) from Echium oil and 0.65 g/100 mL of plant sterols at room temperature, resulting in a significant increase of all oxidative markers evaluated in this study. Among 11 natural phenolic compounds extracted from red propolis, sinapic acid and rutin hydrate were the most efficient to delay lipid oxidation. There, these two compounds represent a potential alternative as natural antioxidant to be applied in a functional food emulsion containing ω-3 FA and plant sterols as bioactive lipids.

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4.5 INCREASING ANTIOXIDANT ACTIVITY BY PARTITIONING *α*-TOCOPHEROL INTO SDS-MICELLES IN OIL-IN-WATER EMULSIONS

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Increasing antioxidant activity by partitioning α-tocopherol into SDS-micelles in oil-in-water emulsions

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Abstract

The emulsifier in oil-in-water (O/W) emulsion functions as stabilizer and once it has saturated the droplet surface, the excess emulsifier tends to partition into the aqueous phase. Aqueous phase surfactants can increase the activity of antioxidants but it is not known if this is due to chemical or physical effects. A 70% increase in displacement of α-tocopherol to the aqueous phase was observed when SDS concentrations were increased from 5 mM to 7 mM. This increase in aqueous phase α-tocopherol occurred when SDS formed surfactant micelles and the increased aqueous phase α-tocopherol indicated that SDS and α-tocopherol where forming co-micelles. At 37 °C, the oxidative degradation of α-tocopherol in the control emulsion occurred faster than in the emulsion with SDS micelles. The surfactant-antioxidant co-micelles also increased the oxidation lag phase 5 days compared to the control. Considering these results, surfactant micelles could play a key role as an antioxidant carrier, potentially by increasing the accessibility of hydrophobic antioxidant to the emulsion droplet interface.

***Keywords*:** Oil-in-water emulsion; Tocopherol; Partitioning; Sodium dodecyl sulfate; Anionic surfactant; Lipid oxidation.

1. Introduction

Emulsion systems are important physical constituents of a wide variety of food products and are responsible for many of their physico-chemical properties (McClements, 2005). Lipids are important ingredients of these systems and they can found as oil-in-water (O/W) or water-in-oil (W/O) emulsions (McClements, 2014). In either formulation, lipids are susceptible to oxidation, which may occur at any stage of food processing and storage (Shahidi et al., 2011). Therefore, a major concern of the food industry is how to retard or inhibit this chemical reaction to improve food quality. The addition of antioxidants as free radical scavengers is one of main methods employed by food scientists to prevent the propagation step of oxidation (Panya et al., 2012).

Regarding antioxidant effectiveness, the polar paradox theory states that hydrophobic antioxidants are more effective in O/W emulsions than hydrophilic homologs (Porter et al., 1989; Frankel et al., 1994). This higher activity of non-polar antioxidants in O/W emulsions has been proposed to be dependent on antioxidant retention in the emulsion droplet or partitioning at the droplet interface where oxidation primarily takes place (Schwarz et al., 2000; Frankel et al., 1994; Cercaci et al., 2007). When the lipophilic antioxidants are at the interface, free radicals are scavenged before they can cross the droplet membrane and enter the lipid phase (Shahidi et al., 2011). However, recent publications highlighted that hypothetical expectations of the polar paradox do not always accurately predict an antioxidant's behavior (Laguerre et al., 2015; Bakır et al., 2013). For instance, in oil-in-water emulsions it was observed that increasing antioxidant hydrophobicity by adding fatty acid chains to an antioxidant increases activity only to a point after which further increasing hydrophobicity actually decreased antioxidant activity. This means that hydrophobicity could critically affect the antioxidant activity in both positive and negative manners (Laguerre et al., 2015). These authors suggested that these observations could be due to factors such as "reduced mobility", "internalization" and "self-aggregation of the antioxidants in the emulsion

droplet. When Alemán et al. (2015) evaluated the antioxidant effect of caffeic acid esterified with fatty alcohols of different chain length on two fish oil enriched food systems (mayonnaise and milk), the results showed that the optimal alkyl chain length of phenolipids for oxidation prevention greatly depends on the matrix studied. On the other hand, Bakır et al. (2013) highlighted that the polar paradox validity depends on a critical concentration of antioxidant employed. Furthermore, other factors, such as chemical composition of the emulsion, play a key role in antioxidant partitioning, thus affecting its activity.

The addition of emulsifier in the formulation helps to stabilize the emulsion system, but once it has saturated the emulsion droplet surface, the excess partitions to the aqueous phase. If aqueous phase surfactant are high enough, micelles form which could promote the solubilization of lipophilic antioxidant out of the emulsion droplet and into the aqueous phase. This was confirmed by Richards et al. (2002), when the solubilization of propyl gallate in the continuous phase was increased 2.3 fold after rising the Brij micelles from 0.3 to 2.8%. Our recent work also found that adding Tween 20 to an O/W emulsion increased partitioning of tocopherol homologues into aqueous phase with partitioning increasing with increasing number of methyl group on the tocopherol (e.g. δ-tocopherol > α-tocopherol) (Kiralan et al., 2014). This work also found that the antioxidant activity of tocopherols was enhanced when the surfactant partitioned the tocopherol into the aqueous phase. Unfortunately, in these emulsions with Brij, all the emulsions had surfactant micelles in the aqueous phase so it was not known whether this effect was due to surfactant solubilization or to the Brij chemically inhibiting oxidation or increasing the activity of tocopherols.

The aim of the study was to investigate the α-tocopherol partitioning in excess of anionic surfactant (sodium dodecyl sulfate, SDS) in stripped soybean oil-in-water emulsion. To this purpose different concentrations of SDS were used to monitored the displacement of α-tocopherol in the O/W emulsion system. SDS was chosen because it has a higher critical micelle concentration than Brij and thus emulsions could be prepared with aqueous phase surfactant in both monomer or

micelle forms. Subsequently, an oxidation study was carried out to understand how the micelles affected the antioxidant activity of α-tocopherol in the O/W emulsion.

2. Material and methods

2.1 Reagents and solvents

Refined soybean oil was purchased from a local retail store in Hadley (MA, USA). Iso-octane, 2-propanol, methanol, 1-butanol, *n*-hexane, hydrochloric acid and sodium phosphate dibasic were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Chempure Ultra (Houston, TX). Silicic acid (100–200 mesh), activated charcoal (100–400 mesh), sodium dodecyl sulfate (SDS), sodium phosphate monobasic, barium chloride dihydrate, ammonium thiocyanate, iron (II) sulfate heptahydrate, cumene hydroperoxide, hexanal, and (±)-α-tocopherol were supplied by Sigma-Aldrich (St. Louis, MO, USA). Solvents were HPLC grade, and all other chemicals were analytical grade. Double distilled and deionized water were used throughout the study. Glassware was incubated in 2 M HCl overnight to remove metals, followed by rinsing with double-distilled water before use.

2.2 Preparation of stripped soybean oil

Stripped soybean oil was prepared according to the method of Boon et al. (2008) and was used in all experiments. Briefly, silicic acid (100 g) was washed three times with a total of 3 L of distilled water and activated at 110°C for 20 h. The activated silicic acid (22.5 g) and activated charcoal (5.63 g) were suspended in 100 and 70 mL of *n*-hexane, respectively. A chromatographic column (3.0 cm internal diameter × 35 cm height) was then packed sequentially with silicic acid, followed by activated charcoal and then another layer of silicic acid. Thirty grams of soybean oil were dissolved in 30 mL of *n*-hexane and passed through the column by eluting with 270 mL of *n*-hexane. In order to retard lipid oxidation during stripping, the collected soybean oil was held in an ice bath and covered with aluminum foil. After complete elution, *n*-hexane was removed with a

vacuum rotary evaporator (Model RE 111, Buchi, Flawil, Switzerland) at 37°C and traces of the remaining solvent were evaporated under a nitrogen stream. The stripped soybean oil was flushed with nitrogen and stored at −80°C until use. Removal of tocopherols in stripped soybean oil was verified by HPLC.

2.3 Emulsion preparation

Stripped soybean oil (0.5 %, wt) and surfactant SDS (0.05%, wt; final emulsion concentration 1.73 mM) in 10 mM phosphate buffer solution (pH 7.0), were used for the formulation of the control O/W emulsion. To minimize oxidation during analysis of antioxidant partitioning, 200 μM of EDTA was included in the phosphate buffer solution. In the first step, a coarse emulsion was made by blending with a hand-held two-speed homogenizer (Model M133/1281-0, Biospec Products, Inc., Bartlesville, OK, USA) at the high speed setting for 2 min. After this time, three passes at a pressure of 9 Kbar were carried out using a microfluidizer (Model M-110L Microfluidics, Newton, MA, USA) to obtain O/W emulsions with very fine droplet size. During this process, ice was used to cover the homogenizer chamber and coil, in order to maintain the emulsion temperature at 25°C.

Subsequently, additional SDS was added in 20 g of control emulsion to obtain a final emulsifier concentration of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5 mM, followed by mixing with an electronic stirrer (Model 2008, Thermo Fisher Scientific Inc., Raleigh, NC, USA) at 500 rpm for 30 min at room temperature and in the dark. For the determination of antioxidant partitioning, a solution of α-tocopherol in methanol was put in all batches of O/W emulsions at final concentration of 100 μM. For the oxidative stability study, two O/W emulsions with additional SDS (2.5 and 6.0 mM) were chosen and a solution of α-tocopherol in methanol was added at a final concentration of 30 μM. Controls had no added α-tocopherol. Table 1 reports the

surfactant and α-tocopherol concentrations of the O/W samples and the corresponding abbreviations used in the Figures.

Table 1. Surfactant and α-tocopherol concentrations of the O/W emulsions and the corresponding abbreviations used in the Figures.

Emulsion	Surfactant concentration in the final emulsion (mM)	No α-tocopherol	With α-tocopherol (30 μM)
Control	1.73	C-	C+
Low	2.5	L-	L+
High	6.0	H-	H+

For the lipid oxidation study of the emulsions (tocopherol loss, hydroperoxide and hexanal formation in the emulsion system), 1 mL of the emulsion treatments were transferred into 10 mL GC vials, capped with aluminum caps with PTFE/Silicone (tetrafluoroethylene) septa and stored at 37°C in darkness during the whole storage study (13 days). The samples were taken out daily from a fix-temperature chamber to perform the analysis. For studies on the evaluation of the loss of α-tocopherol in the emulsion, 7 mL of the emulsion treatments were transferred in the same GC vials instead of 1 mL and subjected to the same storage conditions. Storage studies were carried out in duplicate and each sample was analyzed in triplicate. Particle size distributions of the final emulsions were measured using a dynamic light scattering instrument (Zetasizer Nano-ZS Malvern Instruments Ltd., Worcestershire, UK). Samples for particle size distribution measurements were diluted into 10 mM phosphate buffer solution (pH 7.0) at an emulsion:buffer ratio of 1:50, in order to prevent the multiple scattering effects. Each measurement was performed in triplicate at room temperature. The average emulsion droplet size was 174.14 ± 11 nm.

2.4 Determination of antioxidant partitioning

The content of α-tocopherol was determined in the aqueous phase of the O/W emulsion by high-performance liquid chromatography (HPLC), according the method described by Panya et al.

(2012). Briefly, the emulsions containing different concentrations of SDS (Section 2.3) plus α-tocopherol were centrifuged at 162102 *x g* (46,000 rpm) for 1 h at 4 °C using a PTI F65L-6x13.5 rotor with a Sorvall WX Ultra 80 high-speed centrifuge (Thermo Fisher Scientific Inc., Asheville, NC, USA). After centrifugation, α-tocopherol present in the aqueous phase (1 mL) was extracted with a 2 mL of iso-octane:2-propanol solution (3:1, v/v). The mixed solution was centrifuged at 4000 rpm for 5 min (CL10 centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1 mL of upper solvent phase was dried using a Speed Vac concentrator centrifuge at 300x for 15 min (Savant Instruments Inc.,Farmingdale, NY). The dried extract was dissolved in 200 µL of methanol, filtered with a PTFA membrane (Acrodisc CR 13 mm syringe filter with 0.45 µm PTFE Membrane, Pall life Science) and injected into an HPLC instrument (10A VP model, Shimadzu, USA), which was coupled to a C18 reversed phase column (150 mm x 4.6 mm i.d., particle size 5 µm, Beckman Coulter), a C18 security guard (ODS) (4 mm x 3 mm i.d., particle size 5 µm) (Phenomenex, USA) and a fluorescence detector (Scanning Fluorescence Detector, Waters 474, USA). The separation was carried out with a isocratic elution using methanol as mobile phase, at a flow rate of 1 mL/min. α-Tocopherol detection was performed using an excitation wavelength of 290 nm and emission wavelength of 330 nm. The concentration of α-tocopherol was calculated using an external standard calibration curve.

2.5 Evaluation of lipid oxidation

For the lipid oxidation studies, hydroperoxide formation was determined using a modified method described by Shantha et al. (1993). Hydroperoxide analysis was performed after headspace hexanal determination was complete. Emulsion samples (0.3 mL) were mixed with 1.5 mL of isooctane:2-propanol solution (3:1, v/v) and vortexed (10 s, three times). The mixed solution was centrifuged at 3,400 *x g* for 10 min (Centrific TM Centrifuge, Thermo Fisher Scientific Inc., Fairlawn, NJ, USA). The upper organic layer (0.2 mL) was mixed with 2.8 mL of a

methanol:butanol solution (2:1, v/v), followed by the addition of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of Fe^{2+} solution. The clear Fe^{2+} solution was prepared freshly from the upper phase of a mixture of equal amounts of 0.132 M $BaCl_2$ (in 0.4 M HCl) and 0.144 M $FeSO_4$. The solution was vortexed and, after 20 min of incubation at room temperature, the absorbance of the samples was measured at 510 nm using a UV−Vis spectrophotometer (Genesys 20, Thermo Fisher Scientific Inc., Waltham, MA, USA). Hydroperoxide concentrations were determined using a standard curve prepared from cumene hydroperoxide.

Headspace hexanal was measured according to the method described by Panya et al. (2010), using a gas chromatography instrument coupled to a flame ionization detector (GC-FID, Model GC-2014, Shimadzu Co., Tokyo, Japan) and equipped with an autosampler (Model AOC-5000, Shimadzu Co., Tokyo, Japan). Emulsions (1 mL) in 10 mL glass vials capped with aluminum caps with polytetrafluoroethylene (PTFE)/Silicone septa were heated at 55°C for 10 min in the autosampler heating block before measurements. After equilibration time, a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) stable flex solid phase microextraction (SPME) fiber (Supelco Co., Bellefonte, PA, USA) was inserted into the vial headspace for 2 min to adsorb volatile compounds. The desorption was carried out at 250°C for 3 min. The injection port of the GC-FID was operated in the split mode at a 1:7 ratio. Helium was used as carrier gas, at a constant flow of 1 mL/min. Volatile compounds were separated on a fused-silica capillary Equity-1 Supelco column (30 m x 0.32 mm i.d. x 1 μm), coated with 100% polydimethylsiloxane (PDMS) at 65°C for 10 min. The detector temperature was set at 250°C. Hexanal concentrations were determined from peak areas using a hexanal standard curve.

The loss tocopherol in emulsion was also carried out after hexanal determination. Briefly, 0.5 mL of O/W emulsion sample was mixed with 1 mL iso-octane:2-propanol solution (3:1, v/v). After extraction, the sample was dried using a vacuum centrifuge as described above and resuspended in

200 μL of methanol prior to HPLC analysis (see section 2.4). The loss of α-tocopherol in the aqueous phase was conducted as described in section 2.4.

2.6 Statistical analysis

All data shown represent the mean values ± standard deviation of triplicate measurements. The data obtained were analyzed by one-way analysis of variance (ANOVA), using SPSS version 21 (SPSS Inc., Chicago, IL, USA). The differences between mean values were compared using Tukey's HSD test with a level of significance of $p<0.05$.

3. Results and discussion

3.1 Physical characteristics of emulsions

The droplet size of all emulsions used in this study was measured immediately after their preparation. For the entire duration of the experiment, all samples remained visible stable and no changes in particle size or emulsion creaming was observed. The SDS-stabilized emulsion had a negative surface. The further addition of SDS in O/W emulsion increased the surface charge from ca. - 95.9 mV ± 1.6 in the control emulsion to - 102.4 ± 2.4 and 107.8 ± 1.8 in the O/W emulsion system with 2.5 and 6 mM of SDS, respectively.

3.2 Displacement of α-tocopherol by increasing anionic surfactant

İn this study, the percentage of α-tocopherol in aqueous phase of 0.5% stripped soybean oil-in-water emulsion was determined after addition of increasing concentrations of SDS. The final concentration of anionic surfactant in emulsion varied from 2.0 to 7.5 mM. In the range of concentrations used, the excess of SDS in aqueous phase could be present as monomers and/or micelles. The surfactants in aqueous phase are organized as individual molecules when their concentration is below a critical point, known as critical micelle concentration (CMC); above this point, the molecules spontaneously assemble into thermodynamically stable structures knows

micelles (McClements, 2005). The CMC of a surfactant depends on the chemical structure of molecules, as well as on the emulsion composition and environmental conditions (McClements, 2014).

As highlighted in Figure 1, the physical location of tocopherol in aqueous phase was highly dependent on the concentrations of SDS. When low concentrations of SDS (2-4 mM) were present, the percentage of α-tocopherol in aqueous phase did not exceed 8%. Thereafter, a noticeable inflection in the plot was observed, which corresponded to a 70% α-tocopherol increase in the aqueous phase at an SDS concentrations of 5.75 mM. α-Tocopherol is extemely low water solubility which explains why less than 5% is in the aqueous in the absence of added SDS. Further increasing SDS concentration resulted in a dramatic increase in aqueous phase α-tocopherol suggesting that the SDS had formed micelles and these micelles were able to solubilize α-tocopherol out of the emulsion droplets and into the aqueous phase.

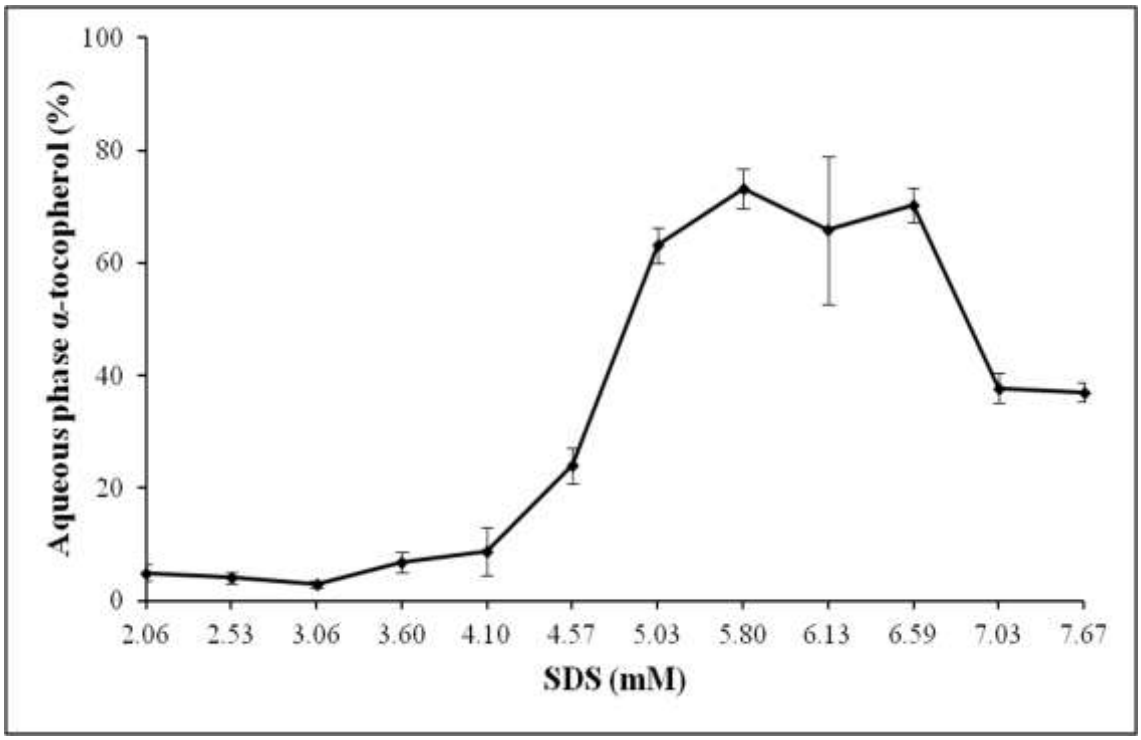
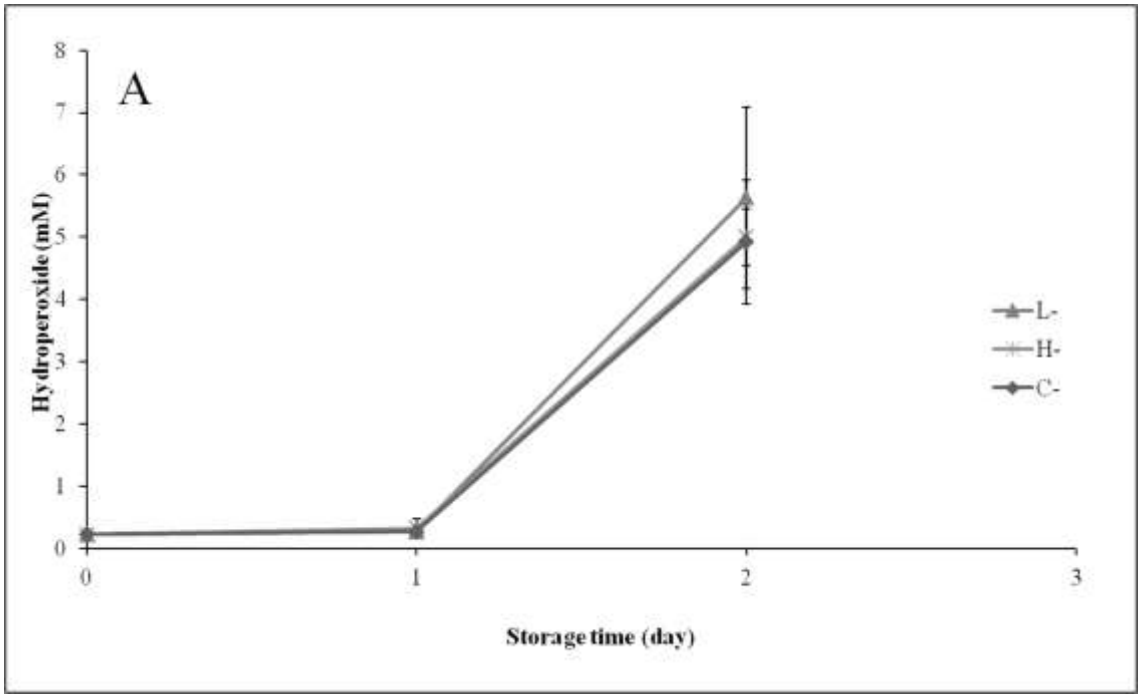


Figura 1 Effect of SDS addition (from 2 to 7.5 mM) on the displacement of α-tocopherol (100 µM) into the aqueous phase of 0.5% stripped soybean oil-in-water emulsions. Data points and error bars represent means (n = 3) ± standard deviations.

Panya and coworkers (2012) investigated the physical location of the rosmarinic acid esters in the presence of varying concentrations of Tween 20 in an O/W emulsion. Increasing Tween 20 concentrations to 0.1% led to an increase in the aqueous phase concentration of eicosyl rosmarinate over 7.5-fold which was suggested to be due to surfactant micelle solubilization. This increase in aqueous phase eicosyl rosmarinate increased at a much lower concentration than SDS which is likely due to the lower CMC of Tween 20. Berton-Carabin et al. (2013) also found that the lipophilic spin probe, 4-phenyl-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl nitroxide in emulsion system stabilized with anionic surfactant (SDS) migrated from the lipid droplet core to the micellar pseudophase when 35 mM and 70 mM of SDS was added in excess.

3.3 Evaluation of lipid oxidation in O/W emulsions

The O/W emulsions with different added SDS, below (2.5 mM) and above (6.0 mM) the CMC were chosen to understand if the different location of antioxidant, in surfactant micelles or emulsion droplets, could impact the oxidative stability of the emulsion during storage at 37°C in the dark. The study was carried out in emulsions with and without 30 μM of α-tocopherol. The formation of both primary and secondary lipid oxidation products (hydroperoxides and headspace hexanal, respectively) were monitored. İn absence of α-tocopherol, the control emulsion (1.75 mM) and emulsions with additional SDS (2.5 and 6.0 mM) had the same behavior, displaying similar lag phases. The length of lag phase for lipid hydroperoxide and hexanal formations in the control was 2 days and in the presence of added SDS decreased to 1 days (Figure 2).



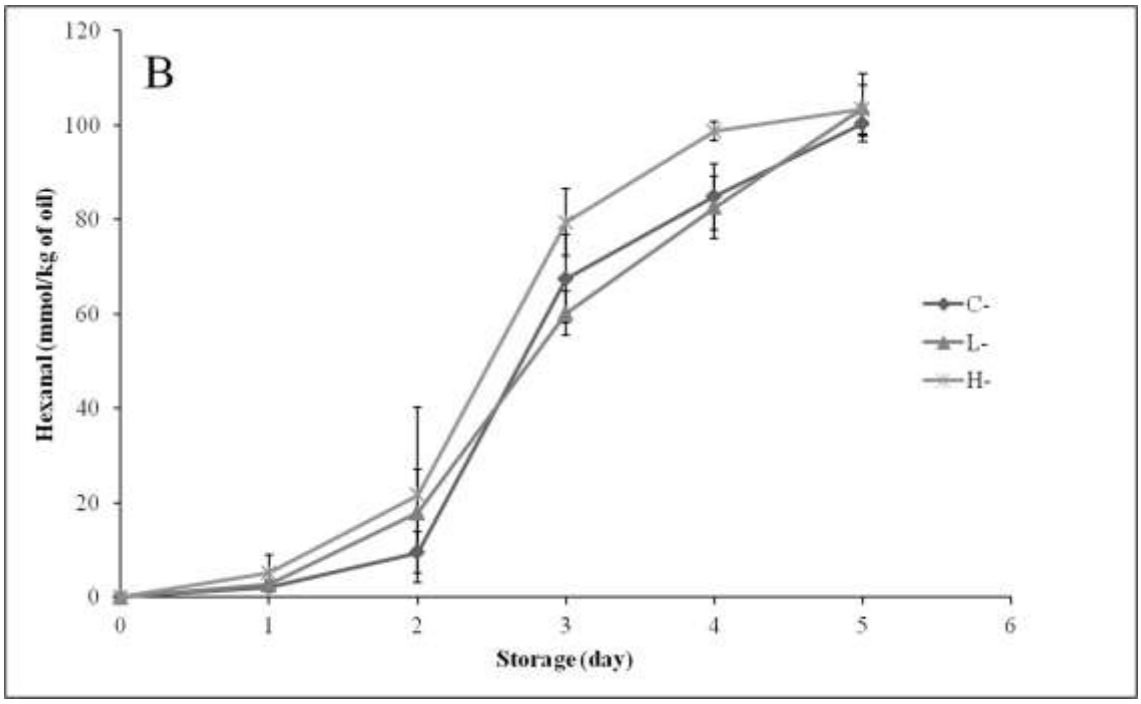


Figure 2. Lipid hydroperoxide (A) and hexanal (B) formation in 0.5% stripped soybean oil-in-water emulsions without α-tocopherol during storage at 37°C. Different concentration of SDS were added: control (C-, 1.75 mM), low concentration (L-, 2.5 mM) and high concentration (H-, 6.0).

Previous research has shown that aqueous phase SDS can increase oxidation rates presumably by binding iron and increasing its activity (Mei et al., 1998). The reduction of lipid hydroperoxide and hexanal formation by increasing aqueous phase anionic surfactants was also noted in water-in-walnut oil emulsions stabilized by polyglycerol polyricinoleate (Yi et al., 2014). Addition of α-tocopherol (30 μM) to the aqueous phase inhibited lipid oxidation to different extents depending on the emulsifier concentration. The lag phases for both lipid hydroperoxides and headspace hexanal increased with increasing SDS concentration (Figure 3). Differences in the lag phase from the control and 2.5 mM SDS only differed by 2 days. However, increasing SDS to 6.0 mM increased the hydroperoxide and hexanal lag phases 6 and 4 days beyond the control and 2.5 mM SDS emulsions, respectively.

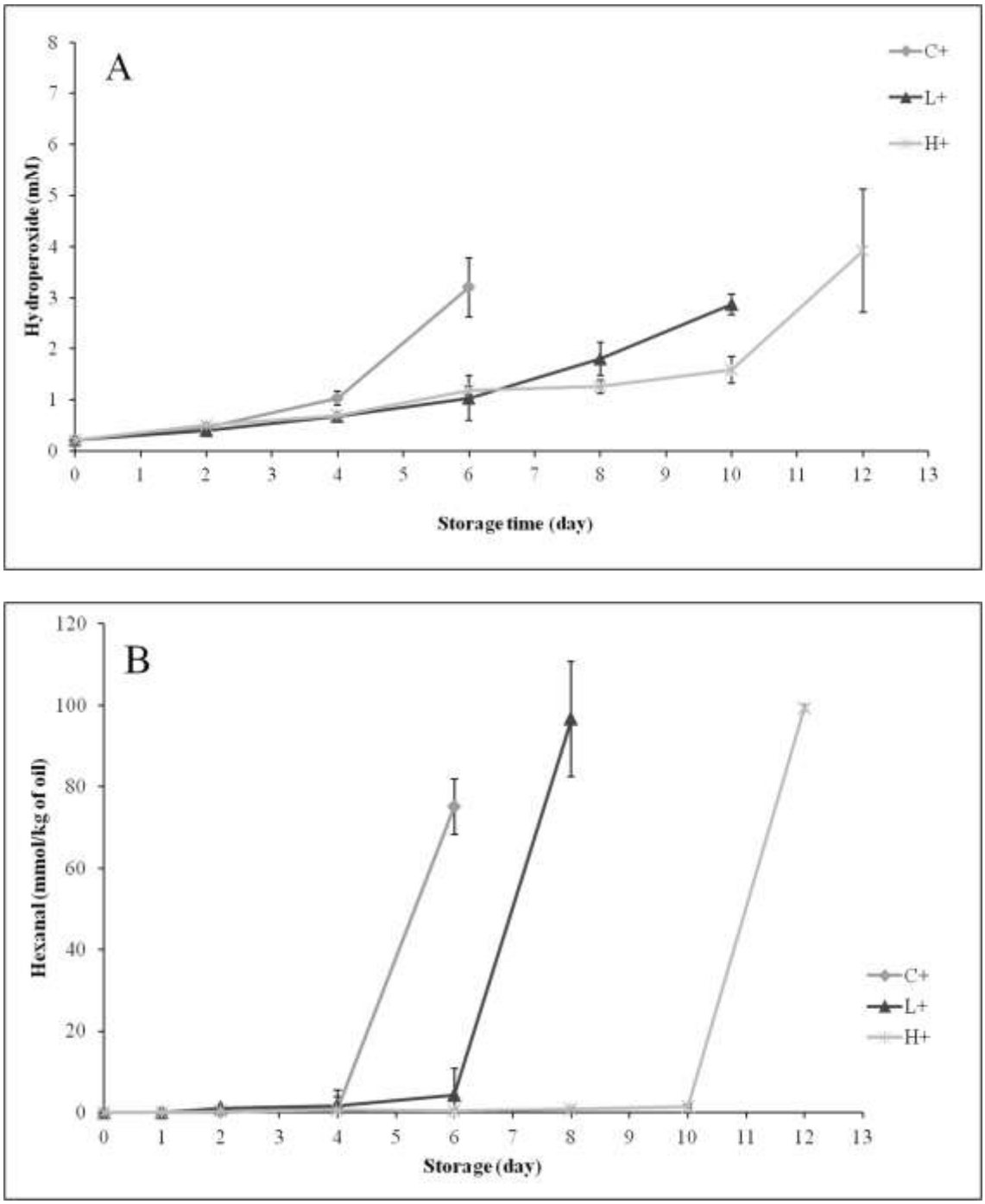


Figure 3. Lipid hydroperoxide (A) and hexanal (B) formation in 0.5% stripped soybean oil-in-water emulsions with α-tocopherol (30 μM) during storage at 37°C. Different concentration of SDS were added: control (C+, 1.75 mM), low concentration (L+, 2.5 mM) and high concentration (H+, 6.0).

According to the "antioxidant polar paradox", the non polar antioxidant (such as α-tocopherol) are effective in oil-in-water emulsions because they are retained in the emulsion droplet and do not

readily partition into the aqueous phase where they cannot interact with the lipid. However, in presence of an excess of surfactant, the partitioning of α-tocopherol into the aqueous phase increases and yet it displays better antioxidant activity. This observation is in agreement with Kiralan et al. (2014) who reported that the antioxidant activity of δ and α-tocopherols was enhanced by adding Tween 20 in aqueous phase of O/W emulsions.

The degradation of α-tocopherol in the O/W emulsion in the presence of 2.5 and 6.0 mM of SDS during the storage at 37°C was also determined. At day 0, there was no significant difference in total α-tocopherol concentration (27-33 μM) between emulsion samples with varying levels of SDS (Figure 4). However, after one day of storage, a dramatic reduction in total α-tocopherol was observed in the emulsion with the highest concentration of SDS. The same decay trend was observed in the control emulsion and in the emulsion with 2.5 mM of SDS but the rapid decrease in α-tocopherol was one and two days later, respectively (Figure 4). α-Tocopherol disappeared starting from 4 days in the control emulsion, which allowed primary oxidation as reported in Figure 3. In the emulsions added with SDS, a slowly antioxidant degradation occurred. In particular, from 1^{th} to 4^{th} day of storage, the α-tocopherol level in the emulsion system containing 6.0 mM of SDS arose from 5 to 10 μM, which was the same concentration found in the emulsion system treatment with 2.5 mM of SDS. From the 4^{th} day of storage onwards, a similar decay trend was observed in both emulsion systems (plus 2.5 and 6.0 mM of SDS) .

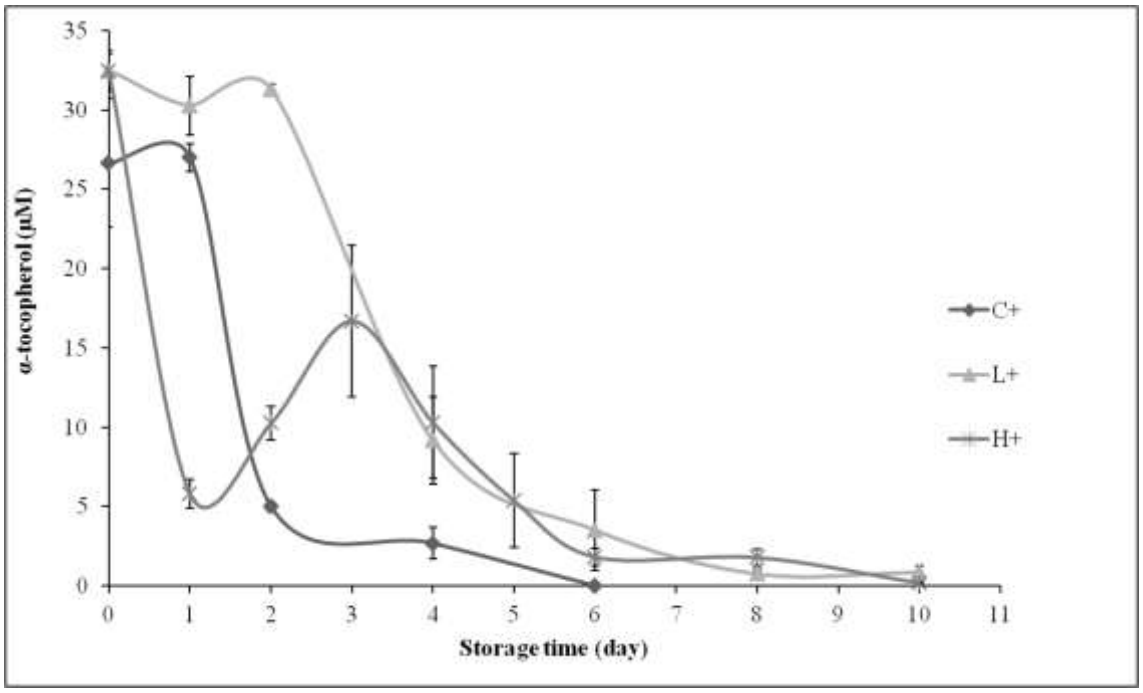


Figure 4. Evaluation of α-tocopherol degradation in 0.5% stripped soybean oil-in-water emulsions during storage at 37°C. Different concentration of SDS were added: control (C+, 1.75 mM), low concentration (L+, 2.5 mM) and high concentration (H+, 6.0).

The presence of α-tocopherol in the aqueous phase was also determined to understand the impact of anionic surfactant on tocopherol degradation and the oxidative stability of the O/W emulsions. A low concentration of α-tocopherol was detected in aqueous phase of the stripped soybean emulsion with a 1.75 and 2.5 mM of SDS, as in the partition study (see paragraph 3.2). It seems that the addition of 6.0 mM of SDS in the control emulsion and its arrangement in micelles increased the partition of antioxidant in the aqueous phase of O/W emulsion, as compared to other two emulsion systems prepared with 1.75 and 2.5 mM of SDS. Starting from day 0 a linear decrease of α-tocopherol was noticed up to 5 days of storage in the system containing 6 mM of SDS (Figure 5).

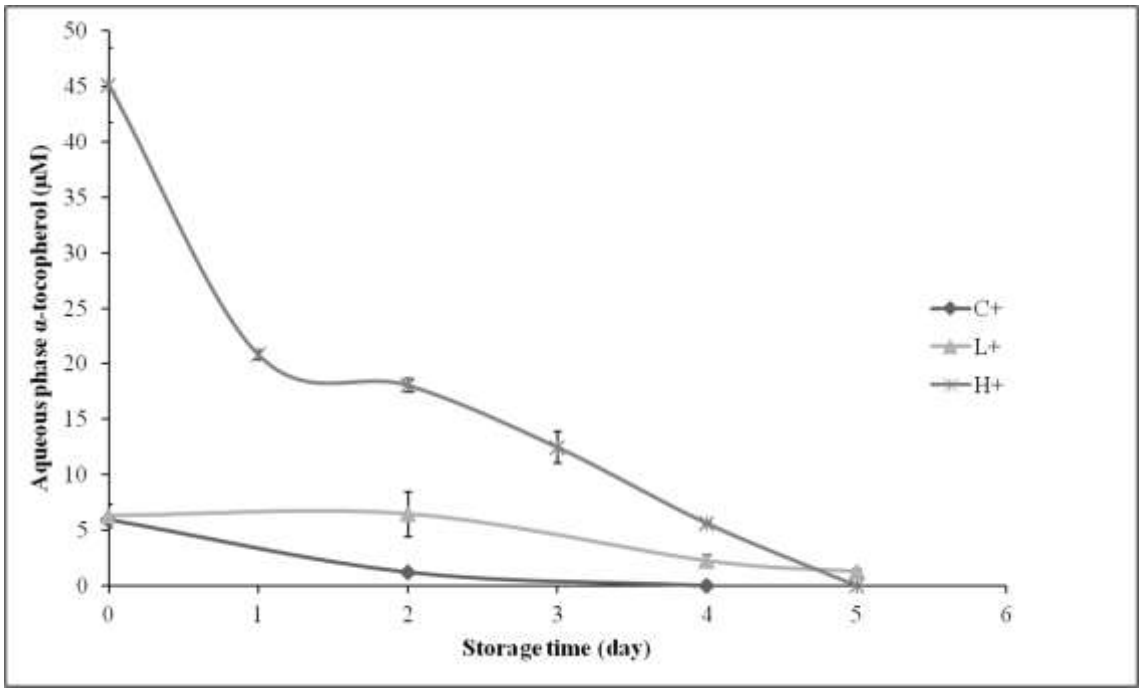


Figure 5. Evaluation of α-tocopherol degradation in aqueous phase during storage at 37°C. Different concentration of SDS were added: control (C+, 1.75 mM), low concentration (L+, 2.5 mM) and high concentration (H+, 6.0).

The physical location of antioxidants in food is strictly related to their chemical and physical properties, which also determine their effectiveness (Alamed et al., 2009). Lipid oxidation in O/W emulsion primarily occurs in the lipid droplet or at the lipid-water interface (McClements et al., 2000). Lipid oxidation is also promoted when prooxidants and lipid substrate are in close proximity; this is enhanced especially in emulsions with negatively charged oil droplet surface. In this study, lipid oxidation in O/W emulsion system with 6 mM of SDS containing α-tocopherol, was slowed. In general, when the SDS is in the concentration below a CMC, all molecules form a hydrophilic shell around the droplet oil, placing the prooxidants in close proximity to lipids. In our study, the O/W emulsion with 6 mM of SDS formed anionic surfactant micelles in aqueous phase that could dilute the prooxidants, interacting with lipids.

This trend could be explained by the formation of anionic surfactant micelles in the aqueous phase of O/W emulsion, which could separate the pro-oxidant agents (such as metal) from lipid

substrate in the emulsions droplet. Other studies on lipid oxidation had highlight the ability of nonionic micelles of surfactant to chelate or solubilized metal ions, scavenger free radicals and isolate the hydroperoxides from the oil droplets (Nuchi et al., 2002; Richards et al., 2002).

As reported by Kiralan et al. (2014), the surfactant micelles helped to solubilize the lipophilic antioxidant in the aqueous phase, thus increasing its effectiveness. Berton-Carabin et al. (2013) also stated that surfactant micelles can form an alternative environment for hydrophobic small molecules and could affect the reactivity of lipophilic molecules in emulsion systems. Due to partitioning of 4-phenyl-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl nitroxide from the lipid droplets to SDS micelles in the aqueous phase, a change in the probe's reduction rate was actually observed. In addition, lipid hydroperoxides could be displaced in the surfactant micelles, decreasing free radicals in the droplets (Nuchi et al., 2002; Waraho et al., 2011).

In conclusion, the SDS micelles could play a key role as a carrier, increasing the surface accessibility of liposoluble antioxidant. The stripped soybean oil-in-water emulsion system stabilized with a 6 mM of SDS allowed α-tocopherol to act as free radical scavenger, thus increasing the stability oxidative of the O/W emulsion.

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5 Conclusions

5. Conclusions

Minor components are of particular interest because of their antioxidant and biological properties. They may be natural food components (e.g. oils, eggs, milk, vegetables, etc.), or they may be added as specific functional ingredients (e.g. ω-3 fatty acid, phytosterols and α-tocopherol). An example of this last application is the production of phytosterols-enriched foods, which have become popular for their ability to decrease total and low-density lipoprotein "bad" cholesterol.

In PS-enriched food products, the percentage of sterols varies up to 8%, highly over the concentration range of edible oils or food vegetable extracts, for which conventional GC-FID is the most used analytical method. For this reason, in the first part of this thesis work, a Fast GC-MS method for plant sterols/stanols analysis was set up. A silylated mixture of the main plant sterols/stanols standards (β-sitosterol, campesterol, stigmasterol, campestanol, sitostanol) was well separated in 1.5 min, with a good peak resolution (>1.4, determined on a critical chromatographic peak pair (β-sitosterol and sitostanol)), repeatability (<13%) and sensitivity (<0.017 ng/mL). Each phytosterol was recognized using its characteristic mass fragmentation pattern produced by electron impact. The suitability of this Fast chromatography method was tested on plant sterols/stanols-enriched dairy products (yogurt and milk), which were subjected to lipid extraction, cold saponification and silylation prior to injection. The analytical performance (sensitivity <0.256 ng/mL and repeatability <10.36%) and significant reduction of the analysis time and consumables, demonstrates that Fast GC-MS could be also employed for the plant sterols/stanols analysis in functional dairy products.

Since sterols can oxidize and generate a series of oxidation products (such as phytosterol oxidation products (POPs)) with adverse biological effects (atherogenic, inflammatory and cytotoxic), it is important to understand how cooking techniques can impact phytosterol oxidation

in model systems, oils and food matrices. In particular, microwave ovens are widely used for fast cooking and reheating of food, but the latter reaches high temperatures during microwaving and thus can oxidize. Therefore, it would be interesting to study more in depth the effects of microwave heating on edible oils and lipid-containing food, especially considering that there are no available data on phytosterol oxidation during microwaving. The thermoxidative behavior of phytosterols in two different model systems (PSF and PS+TAG) was evaluated during MW heating. Different oxidative stability was observed depending on the model system. In particular, PS degradation fitted a first order kinetics, being faster in PSF than in PS+TAG model. In both systems, a marked PS degradation was noted in the first 1.5 min of microwaving, followed by a different degradation trend, which resulted in a higher extent of degradation in PSF than in the PS+TAG system. POPs were identified and quantified by Fast GC-MS, being the 7-keto derivates the most abundant POPs in both systems. POPs formed from 12 min of microwaving onwards and their highest levels was observed at different heating times, depending on the system. In particular, the POPs content at 12 min of MW reached a higher level in PSF than in PS+TAG. On the contrary, at the end of the MW treatment (30 min), the highest POPs content was found in PS+TAG system. These results confirm the important "protective/dilution" role of triolein towards PS oxidation, despite the prooxidizing effect of temperature detected in PSF. Furthermore, POPs formation in the PSF system was directly correlated with the temperature reached during microwaving, while no correlation was found in the PS+TAG system. Hence, the extent of phytosterol degradation depended on both heating time and the surrounding medium, which could impact the quality and safety of the food product destined to microwave heating/cooking.

Many minor lipid components are included in emulsion systems and can affect the rate of lipid oxidation. Previous research stated that the rate of phytosterols oxidation in O/W emulsions is higher than in bulk oil, since oxidation occurs at the droplet interface. Therefore, the oxidative

stability of functional emulsions containing plant sterol esters, ω-3 fatty acids and phenolic compounds was evaluated by using oxidative markers (hydroperoxides, thiobarbituric acid reactive substances, malondialdehyde, hexanal and POPs). The emulsion prepared with *Echium* oil as omega 3 fatty acids (ω-3 FA) source, and PSE were analyzed at the beginning, after heating (90°C for 45 min) and after a 14-day storage at room temperature. Eleven phenolic compounds extracted from red propolis (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans,trans*-farnesol, rutin, gallic acid or sinapic acid) were tested. TBHQ and a mixture containing ascorbic acid and $FeSO_4$ (ASCIR) were applied as negative and positive controls of the oxidation. The samples containing water-soluble compounds as ASCIR, resulted in a significant increase of all oxidative markers evaluated and had the highest content of total POPs. Likewise the results shown by other oxidation markers, ASCIR emulsions presented higher values of 7-ketosterols than the other emulsions. The oxidative damage observed in the functional emulsion was higher when ascorbic acid and iron were present, regardless of the temperature; considering this fact, plant sterols with a surface active character, could be particularly prone to oxidation when they are incorporated in these emulsions, as they are more exposed to these water-soluble prooxidant. Furthermore, among 11 natural phenolic compounds extracted from red propolis, sinapic acid and rutin hydrate were the most efficient antioxidants, being able to react with part of the radicals and transition metals in the aqueous phase or in the oil-water interface, inhibiting fatty acids and plant sterols oxidation. Therefore, these two compounds represent a potential alternative as natural antioxidants to be applied in a functional food emulsion containing ω-3 FA and plant sterols as bioactive lipids.

Finally, some minor lipophilic components may increase oxidative stability of food systems due to their antioxidant activity. However, a series of critical factors (physical location, partitioning, and distribution of minor compounds between water, oil and interface regions) are to be considered

when dealing with antioxidant strategies. Therefore, its effectiveness could be impacted mainly by displacing the antioxidant to a different region of the emulsion, which could be in turn affected by the emulsifier. The emulsifier in O/W emulsion functions as stabilizer and, once it has saturated the droplet surface, the excess emulsifier tends to partition into the aqueous phase. In this last part of the Ph.D. research, the α-tocopherol partitioning into aqueous phase and antioxidant activity was determined in the presence of sodium dodecyl sulfate (SDS, above and below its critical micelle concentration) in stripped soybean oil-in-water emulsions. At 37°C, the oxidative degradation of α-tocopherol in the control emulsion occurred faster than in the emulsion with SDS micelles. The surfactant-antioxidant co-micelles also increased the oxidation lag phase by 5 days as compared to the control. Considering these results, surfactant micelles could play a key role as an antioxidant carrier, potentially by increasing the accessibility of hydrophobic antioxidant to the emulsion droplet interface. Considering that real foods are complex systems in which the antioxidant activity can be increased or decreased depending upon other compounds present therein, in this context the polarity alone cannot explain the antioxidant behavior. For this reason, more research is needed to specifically determine the context in which health-promoting minor lipophilic compounds can impact the oxidative stability of foods.

6 Dissemination of results

Publications:

- Inchingolo R., Sezer Kiralan S., Rodriguez-Estrada M. T., Decker E. A. Increasing antioxidant activity by partitioning α-tocopherol into SDS-micelles in oil-in-water emulsions. To be submitted to *Food Research International.*

- Leal-Castañeda E. J., Inchingolo R., Cardenia V., Hernandez-Becerra J. A., Romani S., Rodriguez-Estrada M. T., García-Galindo H.S. Effects of microwave heating on phytosterol oxidation. Submitted to *Journal Agricultural and Food Chemistry (2015).*

- Rainho Espinosa R., Inchingolo R., Matias Alencar S., Rodriguez-Estrada M.T., Alves Castro I. (2015). Antioxidant activity of phenolic compounds added to a functional emulsion containing omega 3 fatty acids and plant sterols esters. *Food Chemistry (Accepted).* DOI:10.1016/j. foodchem.2015.02.130

- Inchingolo R., Cardenia V., Rodriguez-Estrada M. T. (2014). Analysis of phytosterols and phytostanols in enriched dairy products by Fast gas chromatography with mass spectrometry. *Journal of Separation Science, 37*, 2911-2919.

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Poster communication in Congress and Conferences:

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- Inchingolo R. (2013). Study of the effect of minor compounds on the oxidative stability and physical properties of oil-in-water emulsions and food emulsions. Proc. of the 18th Workshop on the Development in the Italian PhD Research on Food Science, Technology and Biotechnology, pp. 261-262.

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- Inchingolo R. (2012). Study of the effect of minor compounds on the oxidative stability and physical properties of oil-in-water emulsions and food emulsions. Proc. of the 17th Workshop on the Development in the Italian PhD Research on Food Science, Technology and Biotechnology, pp. 321-322.