Emulsions as delivery systems of unsaturated lipids: oxidative stability and applications

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Memoria presentada por **Dña. Mª Candelaria Poyato Aledo** para aspirar al grado de Doctor por la Universidad de Navarra.

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En Pamplona, septiembre de 2015

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Contents

Abstract

The formulation of healthier and functional foods sometimes implies the incorporation of bioactive compounds, and therefore the design and development of technological strategies to facilitate an adequate integration of the new substances into the final product.

Several studies highlight the advantages of emulsified oils for achieving nutritional improvements in the lipid fraction of food products. Thus, in the present work, the design of formulations as new delivery systems was proposed, aiming to obtain more complex and stable emulsions for food applications. In this sense, the use of hydrophilic polymers (carrageenan) allowed obtaining both multiple emulsions (W/O/W) and gelled emulsions for food use, physically stable and with a variable fat content, able to serve as delivery systems for bioactive compounds in the formulation of healthier foods. One type of bioactive compounds was unsaturated fatty acids, known by their benefitial health effects. Furthemore, due to the high oxidative susceptibility, the incorporation of antioxidant plant extracts (*Melissa officinalis, Fucus vesiculosus* and *Lavandula latifolia*) allowed to increase the oxidative stability of the emulsion systems studied in this work (conventional, multiple and gelled emulsions). Additionally, to improve the evaluation and monitoring lipid oxidation, a regression model was carried out by using lipid profile and a spectrophotometric technique as principal variables, to estimate the amount of aldehydes formed at high temperatures, which are interesting secondary oxidation compounds.

Among the systems studied, a gelled emulsion (40 % oil, 1.5 % of carrageenan and a surfactant/oil ratio of 0.003) was selected to be used as fat replacer in meat products, due to its adequate physicochemical properties. The application of the gelled emulsion in fresh and cooked meat products resulted in technologically feasible and sensory acceptable products. Thus, meat products resulting from the partial or total

replacement of animal fat for a highly unsaturated gelled emulsion, showed a healthier lipid profile, lower fat and cholesterol content and lower energy value. These nutritional characteristics allowed to establish, in some of the developed formulations, nutrition and health claims according to the current European legislation.

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Oral communication

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Poster communication

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Abbreviations

AAPH	2,2-azobis-(2-aminopropane) dihydrochloride
AE	acetone extract
ALA	α-linolenic acid
α-TOH	α-tocopherol
AO	algae oil
BHA	butyl-hydroxy-anisole
BOE	Boletín Oficial del Estado
CD	conjugated dienes
COP	cholesterol oxidation product
CVD	cardiovascular diseases
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPA	eicosapentaenoic acid
EU	European Union
EVO	extra-virgin olive oil
FA	fatty acids
GAE	gallic acid equivalents
GC	gas chromatography
GRAS	generally recognized as safe
HOSF	high-oleic sunflower oil
HPLC	high-performance liquid chromatography
IS	internal standard
LA	linoleic acid

LOD	limit of detection
LOQ	limit of quantification
L-ORAC	lipophilic oxygen radical absorbance capacity
MDA	malondialdehyde
MUFA	monounsaturated fatty acids
ORAC	oxygen radical absorbance capacity
O/W	oil-in-water emulsion
PCA	principal component analysis
PUFA	polyunsaturated fatty acids
PV	peroxide value
RD	Real Decreto
RF	reference
RO	refined olive oil
RSM	response surface methodology
SF	sunflower oil
SFA	saturated fatty acids
SOR	surfactant oil ratio
SPME	solid-phase microextraction
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TE	trolox equivalent
TPA	texture profile analysis
TPC	total phenolic compounds
TTC	total tocopherol content
UFA	unsaturated fatty acids
VC	volatile compounds
VL	virgin linseed oil
WE	water extract
W/O/W	water-in-oil-in-water emulsion

Part I
Introduction

1
Introduction to emulsions

Generally, an emulsion consists of at least two immiscible liquids (usually oil and water), with one of the liquids being dispersed as small spherical droplets in the other (Zorba, Ozdemir & Gokalp, 1998; McClements, Decker & Weiss, 2007; Katsuda, McClements, Miglioranza & Decker, 2008; Djordjevic, Cercaci, Alamed, McClements & Decker, 2008).

Many natural and processed foods exist either partly or wholly as emulsions, or have been in an emulsified state at some time during their existence, e.g. milk, mayonnaise, salad dressing, cream, ice cream, butter, and margarine. Food emulsions contain a variety of ingredients, including water, lipids, proteins, carbohydrates, minerals, sugars, and small-molecule surfactants (Dickinson, 1992; McClements, 2002). By a combination of covalent and physical interactions, these ingredients form the individual phases and structural components that give the final product its characteristic physicochemical properties (Eads, 1994; Dickinson, 2007). The bulk physicochemical properties of food emulsions, such as appearance, texture, and stability, depend ultimately on the type of molecules the food contains and their interactions among them.

In the food industry, kinetic stability is usually engineered into products by incorporating substances known as stabilizers such as emulsifiers, texture modifiers, weighting agents, and ripening retarders (McClements, 2010a). Emulsion technology is particularly suited for the design and fabrication of different types of delivery systems for bioactive compounds. Each of these delivery systems could be produced from food-grade (GRAS) ingredients using simple processing operations (for example,

mixing, homogenizing and thermal processing). In this sense, the design of emulsions as delivery systems for bioactive compounds is a great technological challenge, which can be related to instability of the bioactive ingredient itself or to unwanted interactions with other ingredients in the food products (Velikov, 2012). Nevertheless, the application of colloidal dispersions, such as emulsions, in the design of functional foods is an emerging area that is rapidly growing.

In general, there are two ways to introduce a functional ingredient into an emulsion: as soluble or as an insoluble component, depending on the compounds's characteristics. In emulsion systems, due to the presence of two phases, it is common that oil-soluble ingredients, such as certain vitamins, coloring agents, antioxidants, and surfactants are mixed with the oil, while water-soluble ingredients, such as proteins, polysaccharides, sugars, salts, and some vitamins, antioxidants, and surfactants are mixed with the water. Thus, the need to address specific molecular and physicochemical concerns associated with each component leads to different delivery systems (McClements, Decker, Park & Weiss, 2009).

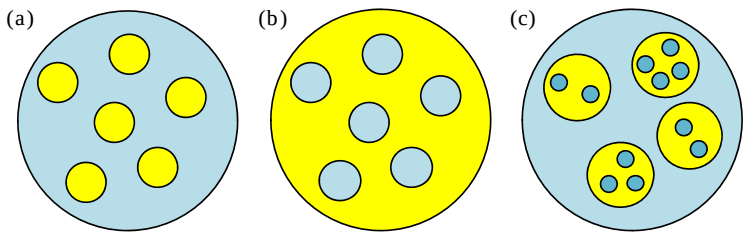


Fig. 1. Different types of delivery systems that can be created based on emulsion technology. (a) Oil in Water emulsion. (b) Water in Oil emulsion. (c) Water in Oil in Water emulsion.

Emulsions are typically classified according to the spatial distribution of the oil and water phases relative to each other. A system that consists of oil droplets dispersed within an aqueous phase is called an oil-in-water (O/W) emulsion, whereas a system that consists of water droplets dispersed in an oily phase is called water-in-oil (W/O) emulsion (**Fig. 1**). The liquid that makes up the droplets is usually called as dispersed, discontinuous or internal phase, whereas the surrounding liquid is referred to as the continuous or external phase. There are a large number of different emulsions

based delivery systems that could be used to deliver bioactive compounds, so it is important to select an appropriate system for a particular application.

1.1. Conventional emulsions

Conventional oil-in-water (O/W) emulsions are usually the first system considered for delivering of bioactive lipids because of their relative ease of preparation, compared to more sophisticated systems. Thus, they are the most common form of emulsion currently used in the food industry and they can be found in a variety of food products, including milk, cream, beverages, dressings, mayonnaise, dips, sausages and desserts.

The formation of conventional emulsions may involve a single step or a number of consecutive steps, depending on the nature of the starting material, the desired properties of the final product, and the instrument used to create it (McClements, 2002). The process of converting two immiscible liquids to an emulsion is known as homogenization, and a mechanical device designed to carry out this process is called a homogenizer. These emulsions are prepared by homogenizing an oil phase and an aqueous phase in the presence of a water-soluble emulsifier. The creation of an emulsion directly from two separate liquids is referred to as primary homogenization, whereas the reduction of the droplets size in an existing emulsion is referred to as secondary homogenization (**Fig. 2**). A variety of different homogenizers are available, including high shear mixers, and high-pressure, ultrasonic and membrane homogenizers (McClements, 2005; McClements et al., 2007).

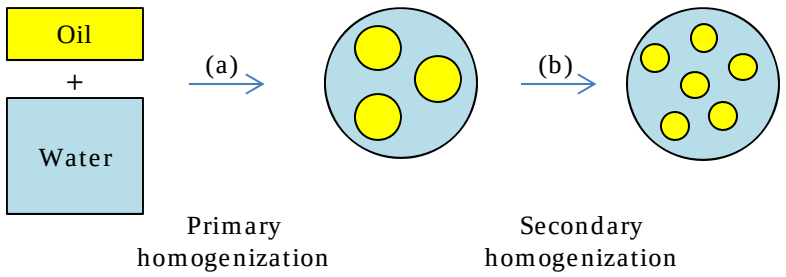


Fig 2. The homogenization process can be divided into two steps, (a) primary homogenization, creating an emulsion from two separated phases and (b) secondary homogenization, reducing the droplet size in an existing emulsion.

If the functional component intended to be incorporated into the emulsion is susceptible to chemical degradation (e. g. ω-3 fatty acids (FA), conjugated linoleic (CLA) and carotenoids), then it might be necessary to carefully control homogenization conditions to avoid exposure to factors that increase the degradation rate (e. g., high temperatures, oxygen, light or presence of transition metals).

Moreover, to form a stable emulsion, one must prevent the droplets from merging after they have been formed. This is achieved by having a sufficient high concentration of a surface-active substance, known as emulsifiers, present during the homogenization process. Two of the most important roles of emulsifiers during homogenization are (1) their ability to decrease the interfacial tension between oil and water and (2) their ability to form a protective membrane that prevents droplets from aggregating with other droplets during a collision (McClements, 2002). The selection of the most appropriate stabilizer to use in an emulsion is one of the most important things that a food manufacturer can do to control the self life and physicochemical properties of the final product (McClements, 2010b).

In this sense, one of the potential disadvantages in emulsions based delivery systems is that there are only a limited number of food-grade emulsifiers available to stabilize emulsions, which limits the potential for engineering novel or improved functional performance into products. Moreover they are often prone to physical instability, especially when exposed to environmental stresses such as heating, chilling, freezing, drying, extreme pH and high mineral concentrations (McClements, 2010a). Thus, despite they are relatively easy and inexpensive to prepare, other types of emulsion-based delivery systems are needed for other purposes.

1.2. Multiple emulsions

Multiple emulsions are structurally more complex versions of conventional emulsions and can be thought as emulsified emulsions. The most common forms are water-in-oil-in-water ($W_1/O/W_2$) (Benichou, Aserin & Garti, 2004; van der Graaf, Schroen & Boom, 2005; Muschiolik, 2007) that consists of tiny water particles (W_1) dispersed inside fat globules (O), which are dispersed in turn in a continuous aqueous

phase (W_2). Two types of interphases are present and stabilized by means of hydrophilic and lipophilic surfactants (Jiménez-Colmenero, 2013).

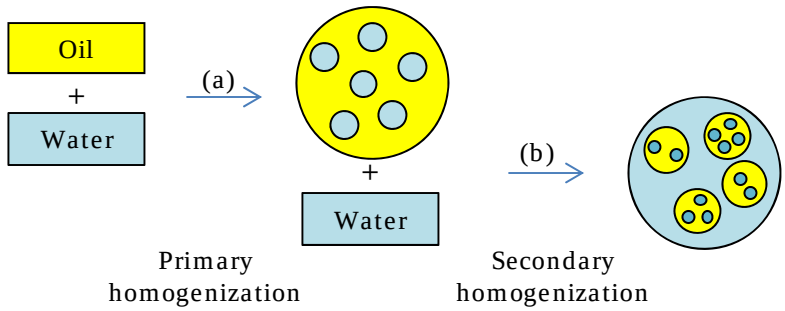


Fig. 3. Two-step procedure to produce W/O/W emulsions. (a) Primary homogenization: oil and aqueous phases are homogenized together in the presence of an oil-soluble emulsifier to form a W/O emulsion. (b) Secondary homogenization: the W/O emulsion is homogenized with a water phase in the presence of a water-soluble emulsifier to form a W/O/W emulsion.

There are several methods for preparing multiple emulsions, but most of them include a two-step emulsifying process (**Fig. 3**). There is a first stage in which a water-in-oil emulsion (W_1/O) is formed by homogenizing an aqueous phase (W_1) and a lipid phase (O) in the presence of a lipophilic emulsifying agent. In the second stage the W_1/O emulsion is homogenized with a new aqueous phase (W_2) with the help of a hydrophilic emulsifying agent, thus producing a double emulsion ($W_1/O/W_2$). Various types of protein emulsifiers have been used for this purpose, such as whey proteins, sodium caseinate, etc., and also some emulsifying polysaccharides such as gum arabic, xanthan gum, modified starch, etc. The homogenizing conditions are more energetic in the primary emulsion (W_1/O), whereas the second step is carried out with less shear in order to avoid rupturing the internal droplets (W_1/O) (Garti, 1997; Jiménez-Colmenero, 2013).

The location of bioactive compounds in multiple emulsions must be considered in order to understand their potential applications, since the target can be located in a number of different environments within a $W_1/O/W_2$ emulsion (McClements et al., 2009). On one hand, water-soluble compounds (minerals,

vitamins, amino acids, peptides, fibers, etc.) can be included both in the inner aqueous phase (W_1), or/and in the outer phase (W_2). On the other hand, oil soluble components (ω-3 FA, conjugated linoleic acid, carotenoids, antioxidants, etc.) can be incorporated by dispersion in the oil phase (McClements et al., 2009).

These emulsions may offer some advantages for food applications as compared to conventional emulsions, since they have been found to be a potentially useful strategy for producing low calorie and reduced fat products, preventing oxidation, improving sensory characteristics of foods, and controlling the release of bioactive compounds and protecting labile ingredients (Benichou et al., 2004; McClements et al., 2007; Muschiolik, 2007; Dickinson, 2011). Since multiple emulsions offer the opportunity to enclose nutritional and bioactive compounds, and these emulsions could be used as food ingredients, they offer an interesting approach among the technological strategies used to include dietary active components in new food systems such as functional foods.

However, despite the above-mentioned possibilities, there are very few examples of current application, probably due to their thermodynamic instability. Multiple emulsions are highly susceptible to breakdown during storage or when exposed to environmental stresses commonly occurring in the food industry, such as mechanical forces, thermal processing, and chilling, or freezing (McClements et al., 2009).

1.3. Gelled emulsions

One important mechanism for developing structures using biopolymers is gelation. Some biopolymers can be used as ingredients in emulsion-based delivery systems because of their ability to cause the water phase to gel. A gelled emulsion is a conventional emulsion in which the water phase consists of a three-dimensional network of aggregated biopolymers that entraps a large volume of water, giving the whole structure some solid-like characteristics. The gel formation contributes to reach a desirable textural and sensory attributes, as well as preventing emulsions destabilization. The appearance, texture, water holding capacity, reversibility, and gelation temperature of biopolymer gels depends on the structure and interactions of

the molecules they contain (McClements, 2002). Through gelation, the polymeric molecules aggregate and cross-link into three dimensional solid networks, entrapping the liquid solvent and resulting in different textural attributes (Norton, Espinosa, Watson, Spyropoulos & Norton, 2015).

Frequently, gums, starches, pectins, agar-agar and proteins including zein from corn, and several animal proteins like gelatin and whey proteins, are used as gelling agents, and can be broadly classified into polysaccharides and proteins. Most gelling polysaccharides and proteins used in food applications are found in nature, and their sources are also different including plant, animal, and microbial origin.

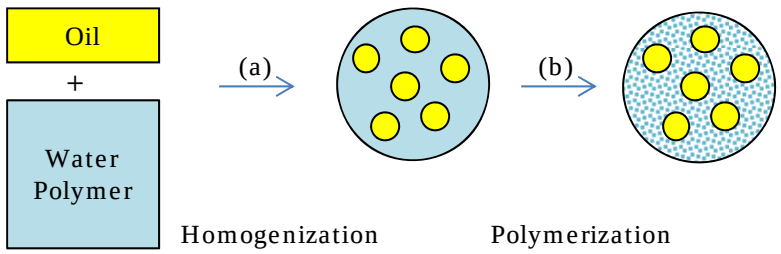


Fig. 4. Gelled emulsions producing procedure, (a) oil and aqueous phases are homogenized together in the presence of an oil-soluble emulsifier to form an O/W emulsion. (b) Polymerization, depending on the nature of the gelling agent induced by temperature, ionic strength, pH changes or adding enzymes.

The gel formation is a spontaneous process from simple polymer dispersion, or particulate suspension, and externally controllable conditions of temperature or solution composition. The formation of gels can be classified broadly as physically induced (heat, pressure) and chemically induced (acid, ionic, enzymatic) gelation reactions. The mechanism of gelation depends on the nature of the gelling agent and on the conditions of gel formation like the temperature, ionic strength, pH, adding enzymes and the concentration of gelling agents (**Fig. 4**) (Banerjee & Bhattacharya, 2012). Molecular characteristics of biopolymers, such as molecular weight, conformation, flexibility, and polarity, ultimately determine the properties of

biopolymer solutions (McClements, 2002). Thus, the selection of the most appropriate ingredient is often the key to succeed with a modified food products.

1.4. Multilayer emulsions

Multilayer emulsions consist of small oil or water droplets dispersed in the continuous phase, with each droplet being surrounded by a laminated interfacial layer, which usually consists of emulsifier and biopolymer molecules (McClements, 2010a). The particle size distribution and concentration of the core-shell particles in multilayer emulsions can be controlled through the characteristics of the interfacial layer coating the droplets, e.g., composition, thickness, charge, permeability, and environmental responsiveness. In this sense, the major advantage of the multilayer technology is that interfacial layers with specific physicochemical properties can be rationally designed to achieve particular functional performances.

An oil-in-water multilayer emulsion is prepared by homogenizing an oil and aqueous phase together in the presence of an ionized hydrophilic emulsifier. The resulting primary emulsion consists of small electrically charged oil droplets dispersed in an aqueous phase. An oppositely charged polyelectrolyte is then added to the system so that it adsorbs around the droplets and produces a secondary emulsion consisting of oil droplets coated by a two-layer emulsifier-polyelectrolyte shell. This procedure can be repeated a number of times to form oil droplets coated by nano-laminated shells consisting of three or more layers (McClements, 2005; Guzey & McClements 2006; McClements, 2010b). Thus, the fact that the thickness and permeability of the interfacial coating surrounding the oil droplets can be controlled means that it is possible to control the release rate of encapsulated functional agents (McClements, 2010b)

Moreover, the control of the composition and properties of nano-laminated coatings can be used to increase the physical and chemical stability of emulsified oils to environmental stresses (such as pH, salt, thermal processing, chilling, freezing, dehydration, and mechanical agitation) and by minimizing interactions between the encapsulated lipids and chemically reactive aqueous phase components (e.g., the oxidative stability of ω-3 fatty acids can be improved by preventing transition metals

from coming into close contact with them) (McClements & Decker 2000; Klinkesorn, Sophanodora, Chinachoti, Decker & McClements, 2005; Guzey & McClements, 2007).

Additional ingredients (e.g., biopolymers for coatings) and processing steps (e.g., mixing) are required to prepare multilayer emulsions compared with conventional emulsions. Consequently, this type of system is more expensive and difficult to prepare.

1.5. Solid lipid particles

Solid lipid particles (SLP) emulsions consist of emulsifier-coated lipid droplets where the lipid phase is either fully or partially solidified (Waraho & Decker, 2011).

SLP emulsions are usually created by homogenizing an oil phase and an aqueous phase together in the presence of a water-soluble emulsifier at a temperature above the melting point of the lipid phase (McClements, 2010a). The emulsion initially formed at these elevated temperatures contains liquid droplets, but when it is then cooled under controlled conditions, so that a portion or the entirety of the lipid phase crystallizes. The stability of the produced lipid droplets is usually controlled by careful selection of the number and type of lipids present, the nature of the surfactant used, the initial droplet size and concentration, and the cooling conditions (McClements et al., 2009; McClements, 2010b; Wharaho & Decker, 2011). Theoretically, it is possible to create a variety of different internal structures within solid lipid particles, e.g., homogeneous crystal structure, core-shell structures, or crystal dispersions.

Some advantages of SLP over conventional emulsions applications are the ability to improve the stability of chemically labile lipophilic components by trapping them within a structured solid matrix and the ability to create stable emulsions containing crystalline lipophilic components (McClements et al., 2009; McClements, 2010a). Moreover, a solid lipid phase could be designed to melt at a particular temperature, thereby releasing an encapsulated functional lipid component.

A major limitation of SLP emulsions is that they must be prepared at elevated temperatures to avoid crystallization of the lipid phase during homogenization. Consequently, the high temperatures used may cause chemical degradation of

heat-sensitive lipophilic components and should therefore be limited as much as possible (McClements et al., 2009; McClements, 2010a). Moreover, highly saturated fats are usually used due to their high melting point, which may have an adverse impact on health.

1.6. Filled hydrogel particles

Filled hydrogel particles emulsions consist of oil droplets contained within hydrogel particles that are dispersed within an aqueous continuous phase. This type of system can therefore be considered to be an oil-in-water-in-water (O/W1/W2) emulsion, where W1 and W2 refer to two aqueous phases with different composition (Matalanis, Jones & McClements, 2011). Typically, the inner water phase (W1) is a hydrocolloid gel, whereas the outer water phase (W2) is a liquid that may or may not contain hydrocolloids. These emulsions have been used to encapsulate and protect ω-3 fatty acids (Salcedo-Sandoval, Cofrades, Ruiz-Capillas, Matalanis, McClements, Decker & Jiménez-Colmenero, 2015) and flavor oils (Zhang, Zhang, Decker & McClements, 2015). The encapsulation of lipids within hydrogel particles may be beneficial for the protection and delivery of a variety of lipophilic bioactive components in foods. This kind of system is widely used in the pharmaceutical industry for the delivery of drugs, and it is likely to gain increasing utilization within the food industry once suitable formulations and preparation conditions have been identified (McClements, Decker & Weiss, 2007). A filled hydrogel particle can then be created by combining this O/W emulsion with an appropriate biopolymer solution and then adjusting the solution or environmental conditions to promote hydrogel particle formation.

Filled hydrogel particles can be prepared using a variety of different approaches. Most of these methods initially involve the formation of an oil-in-water emulsion by homogenizing an oil phase together with an aqueous phase containing a water-soluble emulsifier. The size, concentration, and charge of the droplets in these emulsions can be controlled by selecting an appropriate emulsifier (type and concentration) and homogenization procedure (homogenizer type and operating conditions). A lipophilic compound can be encapsulated within the lipid phase prior to homogenization. A filled hydrogel particle can then be created by combining this O/W

emulsion with an appropriate biopolymer solution and then adjusting the solution or environmental conditions to promote hydrogel particle formation.

The composition, dimensions, permeability, and environmental responsiveness of the hydrogel particles could be designed to control the release of encapsulated components, e.g., flavors or nutraceuticals (Matalanis et al., 2011). This could be achieved by altering the swelling/shrinking of the hydrogel particle or promoting its degradation/dissolution under different temperature, pH, salt, or enzyme conditions (McClements et al., 2009; McClements, 2010a).

Filled hydrogel particles are more difficult to prepare than conventional emulsions, requiring additional ingredients (e.g., proteins, polysaccharides, and cross-linkers) and additional processing operations (such as careful mixing, pH control, and cross-linking conditions). Consequently, their production is likely to be more expensive and more prone to failure.

2
Physical properties of emulsions

To produce a high quality foodstuff a good evaluation of the factors that determine the properties of the final product must be achieved (McClements, 1999) (e.g., appearance, texture, mouthfeel, taste, shelf life). These physicochemical and sensory properties are determined, in the case of emulsions, by molecular and colloidal properties such as dispersed volume fraction, droplet size distribution, droplet–droplet interactions, and interfacial properties. Consequently, a wide variety of experimental techniques have been developed to characterize the molecular, colloidal, microscopic, and macroscopic properties of emulsions (McClements, 1999). The characterization of the physical properties of emulsions when they are being used to develop food products is necessary to assess the properties of foods during processing, to ensure the quality of the final product.

2.1. Factors affecting physical properties

Droplet size and the relative proportion of oil and water in an emulsion, both determine many of the physicochemical and sensory properties of emulsions, such as appearance, rheology, taste and stability. In this sense, for example emulsions tend to become more turbid and to have a higher viscosity when the concentration of droplets is increased (McClements, 1999; McClements, 2002).

Moreover, the physical state of the components of an emulsion has a pronounced influence on its overall properties (McClements, 1999). For example, the addition of a gelling agent as food additive modifies the rheological properties of the

emulsion due to the formation of a molecular network, changing the viscoelasticity with the development of solid characteristics (Banerjee & Bhattacharya, 2012).

Finally, despite comprising only a small fraction of the total volume of an emulsion, the interfacial region that separates the oil from the aqueous phase plays major role in determining physicochemical properties. The most important properties of the interface are the concentration of emulsifiers, and the thickness, viscoelasticity, electrical charge, and interfacial tension of the interface. Thus, the nature and concentration of adsorbed emulsifiers and the physical chemistry of the aqueous phase (pH, ionic strength, and ions) are important factors to be taken into consideration when preparing emulsions. These parameters are deeply involved in the physical stability of emulsions.

2.2. Mechanisms of emulsion instability

Emulsions are thermodynamically unstable, so the emulsion stability refers to the ability of an emulsion to resist this breakdown, as a result of a variety of physicochemical mechanisms, including gravitational separation, flocculation and coalescence (**Fig. 5**) (McClements, 2007; McClements, 2010a).

Gravitational separation is one of the most common forms of instability in emulsions, and it may occur either by creaming or by sedimentation, depending on the relative densities of the dispersed and continuous phases. Creaming is the upward movement of droplets due to the fact that they have a lower density than the surrounding liquid, whereas sedimentation is the downward movement of droplets due to the fact that they have a higher density than the surrounding liquid. Liquid edible oils normally have lower densities than liquid water, so creaming is more prevalent in oil-in-water emulsions, whereas sedimentation is more prevalent in water-in-oil emulsions. Nevertheless, this may not be the case in emulsions that contain fully or partially crystalline lipids, because the density of oils usually increases when crystallization occurs. In addition, in structured emulsions, the lipid droplets may be surrounded by dense biopolymer coatings (multilayer emulsions) or embedded in dense biopolymer particles (filled biopolymer particles), which may impact their trend to cream or sediment.

Other mechanisms are derivate of the continuous motion of the droplets in emulsions, which produces collision with their neighbours. After a collision, emulsions droplets may either move apart or remain aggregated. Flocculation and coalescence are two types of aggregation commonly observed in emulsions. In flocculation, two or more droplets come together to form an aggregate, whereas in coalescence, the droplets merge together to form a single large droplet.

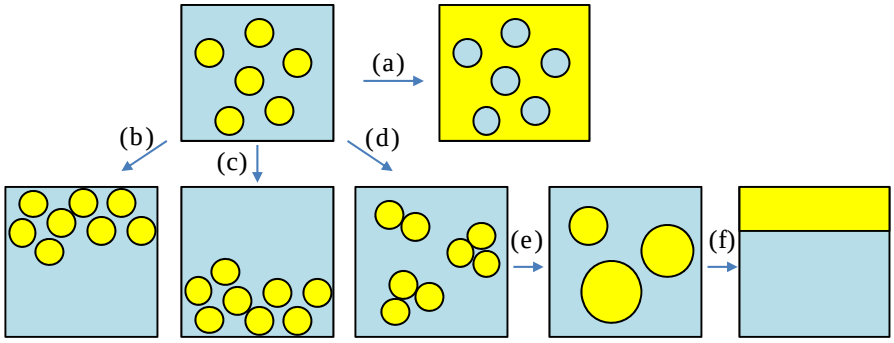


Fig. 5. Diagram of most common instability mechanisms that occur in food emulsions, (a) Phase inversion, (b) Creaming, (c) Sedimentation, (d) Flocculation, (e) Coalescence, (f) Phase separation.

3
Understanding oxidation processes in emulsions

Oil-in-water emulsions such as milk, infant formula, salad dressing, mayonnaise, sauces, soups, beverages, cream, and some desserts are one of the most common forms of lipids in foods (McClements, 2005; Waraho & Decker, 2011). The oxidative degradation of lipids in foods is recognized as one of the most important causes of deterioration of oils and fats, which does not only reduce shelf life, sensory acceptance and the nutritional value of food, but also produces harmful compounds to health (Esteubauer, Schaur & Zollner, 1991).

The mechanism for lipid oxidation in oil-in-water emulsions differs from bulk lipids because emulsions have an aqueous phase which contains both prooxidants and antioxidants and oil-in-water interface that impact interactions between oil and water components (Frankel, Huang, Kanner & German, 1994; McClements & Decker, 2000; Waraho & Decker, 2011). Moreover, there are other many factors that can potentially influence the rate of lipid oxidation in oil-in-water emulsions, such as fatty acid composition, aqueous phase pH and ionic composition, type and concentration of antioxidants and prooxidantes, oxygen concentration, lipid droplet characteristics such as particle size, concentration and physical state, and emulsion droplet interfacial properties such as thickness, charge, rheology, and permeability (Waraho & Decker, 2011). As a consequence, lipid autooxidation can rapidly occur and it is greatly influenced by the nature of the interface, being larger surface areas the cause that facilitates interactions between the lipids and prooxidants.

3.1. Lipid oxidation process

Autooxidation is a general term that is used to describe a complex sequence of chemical changes that result from the interaction between lipids with molecular oxygen. The precise mechanism of lipid oxidation in a particular food depends on the nature of the reactive species present and their physicochemical environment (Fritsch, 1994; Coupland & McClements, 1996; Erickson & Sista, 1997; Decker, 1998).

Lipid oxidation can be conveniently divided into three distinct stages: initiation, propagation, and termination (Frankel, 1998). These processes often consist of a complex sequential free radical chain-reaction mechanism.

The formation of the first radicals by the reaction of oxygen with lipidic compounds must be catalyzed, due to the relatively high activation energy (146 kJ/mol). It has been proposed that the initiation process could be produced due to hydroperoxide decomposition by metal catalysis or light exposure. In the presence of initiators, unsaturated lipids lose hydrogen radicals to form lipid free radicals.

After that, the oxidation is propagated due to the rapid reaction of lipid free radicals with molecular oxygen to form peroxyl radicals, which then react with unsaturated lipids to form hydroperoxides. This is the most widely occurring oxidation and describes the first stages of the oxidation of unsaturated lipids, producing hydroperoxides as the fundamental primary products.

At the last stages of oxidation, after reaching a maximum, the oxidation rate decreases, the peroxyl radicals react among them to form non-radical products. Moreover, the hydroperoxides formed can undergo thermal or metal-catalysed decomposition generating peroxyl radicals and alkoxyl radicals, leading either to additional radicals that continue the chain or to non-radical end-products (Frankel, 1998). Alkoxyl radicals can thus either react with unsaturated lipids to form stable and innocuous alcohols, or undergo fragmentation into unsaturated aldehydes and other unstable products causing rancidity in highly polyunsaturated lipid (PUFA) matrices. These unsaturated aldehydes can react with oxygen as well leading to secondary oxidation products, such as hydrocarbons, alcohols, ketones and aldehydes (e.g. malonaldehyde) under various conditions of elevated temperatures and in the

presence of metal catalysts. Due to their low odour threshold, the presence of certain volatile secondary oxidation products, even at low concentrations, significantly decreases the sensory quality of oil containing emulsions (Jacobsen, 2011; Petersen, Kleeberg, Jahreis, Stockfisch & Fritsche, 2012).

3.2. Factors affecting lipid oxidation in emulsions

3.2.1. Unsaturation degree of fat

Oxidative susceptibility of lipids depends primarily on the composition of their fatty acids, more specifically, their degree of unsaturation. The oxidation rate of the fatty acid series stearic, oleic, linoleic and linolenic acid, for instance, was reported to be in the ratio of 1: 100: 1200: 2500 (de Man, 1999).

Emulsions with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation than more saturated oils or fats. The high susceptibility of polyunsaturated lipids to lipid oxidation has restricted their incorporation into many food products, which is unfortunated because greater consumption of polyunsaturated lipids is beneficial to health and it is currently recommended in dietary guidelines (Givens & Gibbs, 2008; Anderson & Ma; 2009; EFSA, 2009; WHO, 2009)

3.2.2. Oxygen concentration

Lipid oxidation involves the reaction between unsaturated lipids and oxygen. Oxygen is about three times more soluble in oils than in water (Ke & Ackman, 1973) and so there is always likely to be sufficient oxygen present in the oily phase to fuel lipid oxidation, unless specific measures are taken to exclude it (Sun, Wang, Chen & Li, 2011). At low oxygen concentrations, it was observed that the rate-limiting step for lipid oxidation was the diffusion of oxygen through the aqueous phase (Lue, Chen & Xia, 2008; Sun et al., 2011), so the oxidation rate increased with some mechanical processes such as agitation and cooling. So the effective method to retard oxidation is to decrease the concentration of oxygen, for example, by packing the food under vacuum or nitrogen. The amount of oxygen needed to cause oxidation is generally very small and by reducing oxygen to less than 2 %, oxidative stability can be greater

enhanced (Johnson & Decker, 2015) Nevertheless, exclusion of oxygen from food products during processing and storage is often not practical, and once a product is opened to the atmosphere it will become susceptible to lipid oxidation (McClements & Decker, 2000).

3.2.3. Storage conditions

Environmental factors to which lipids are exposed during processing and storage may also affect their oxidation rate, such as exposure to high temperatures. Temperature is the most important factor to be considered in evaluating the oxidative stability of unsaturated fats, because the mechanism of oxidation changes with the temperature (Frankel, 1998). Lipids may also undergo oxidative changes during packaging and upon storage of emulsions, as well as during processing of the emulsion-containing products. It is well known that oxidation is accelerated as temperature increases (Almajano & Gordon, 2004). How storage-temperature can affect the development of lipid oxidation in these systems would also be of significant technological importance for the development of highly unsaturated emulsions.

For practical purposes, the use of high temperatures (accelerated storage conditions) to estimate oxidative stability or susceptibility of emulsions to oxidation is a widely used method.

3.2.4. Interfacial and droplet characteristics

The physicochemical properties of emulsions are highly dependent on the properties of the droplets that they contain (McClements, 2005; Sun et al., 2011).

The droplets in most emulsions are coated by a layer of adsorbed species in order to protect them from aggregation as it has been already mentioned in the previous chapter (for example, emulsifiers or biopolymers). The presence of surfactant micelles in oil-in-water emulsions can inhibit lipid oxidation (Richard, Chaiyasit, McClements & Decker, 2002), e. g. solubilizing iron and removing it from emulsion droplets (Cho, McClements & Decker, 2002). However, surfactant micelles can also solubilize antioxidant out of emulsion droplet, which would be expected to decrease oxidative stability.

The properties of the interfacial region are determined by the type, concentration, and interactions of any surface-active species present during homogenization (Dickinson, 2003; Chaiyasit, Elias, McClements & Decker, 2007). Since lipid oxidation rates are often influenced by the interactions of aqueous phase prooxidants with lipid phase oxidizable substrates (i.e. hydroperoxides), the physical properties of the emulsion droplet interface strongly influence oxidation kinetics (Waraho & Decker, 2011). Interfacial membrane may be able to act as physical and chemical barrier that separates lipid substrates from pro-oxidants in the aqueous phase and may act as a chemical barrier to lipid oxidation. In this sense, adsorbed emulsifiers are likely to be particularly effective at retarding lipid oxidation because of their high local concentration and close proximity to the oxidation substrate (McClements & Decker, 2000).

On the other hand, the particle size of emulsion droplets could impact lipid oxidation rates because smaller particle sizes result in larger surface area and thus greater possibility for lipid-aqueous phase prooxidant interactions (Hu, McClements & Decker, 2003).

3.2.5. Interactions among emulsion components

Food emulsions might contain ingredients in the aqueous phase that could directly trigger lipid oxidation, or interact with other molecular species involved in the lipid oxidation reaction. Some sugars and phenolic compounds in aqueous solution are capable of scavenging free radicals, thus retarding lipid oxidation. Some proteins are more readily oxidized than lipids (Sun, Wang, Chen & Li, 2011). Huang et al. (1996) reported that oxidation of corn oil-in-phosphate buffer emulsions, preceded faster when pH increased from 3 to 7. Increased formation of primary oxidation products may be associated with the partitioning of iron and other transition metals, which is affected by pH (Dimakou, Kiokias, Tsaprouni & Oreopoulou, 2007). However, Faraji, McClements and Decker (2004) reported that proteins in the continuous phase of menhaden oil-in-water emulsions could inhibit lipid oxidation at pH 7.0 through a combination of free radical scavenging and metal chelation. This would greatly decrease the oxidation rate of unsaturated lipid in emulsions as long as it is reduced the

metal content through the addition of EDTA, inositol hexaphosphate, etc., to chelate transition metal ions (Yoshida & Niki, 1992; Kanner, 2010).

Complexity of the composition of these varied physicochemical systems makes difficult the evaluation of the mechanism and factors involved in their oxidation susceptibility.

4

Synthetic and natural antioxidants in emulsion stabilization

The most common method to increase the oxidative stability of foods is probably to use antioxidants. This is the reason why antioxidants are usually added to emulsions. The oxidative stability of foods is dependent on the concentrations, composition and activity of prooxidants and antioxidants. Shifting the balance between these factors is the key to designing oxidative stable foods (Decker, 1998). The ability of antioxidants to inhibit lipid oxidation in emulsions depends on factors such as antioxidant concentration, reactivity, partitioning between oil, water and interfacial phases, interactions with other food components, and environmental conditions such as pH, ionic strength and temperature (Frankel, 1998; McClements & Decker, 2000; Waraho, McClements & Decker, 2011).

Antioxidants significantly delay or inhibit oxidation of oxidizable substrates at low concentrations, compared to the higher contents of lipids and proteins in foods (Gutteridge & Halliwell, 2010). The effectiveness of antioxidants depends on several factors such as their polarity, lipid substrate, non-lipid constituents, pH, ionic strength and temperature, concentration of antioxidants, the presence of metal ions, and the physical properties of the food (McClements & Decker, 2000).

Antioxidants interrupt the chain reactions involved in oxidation in two different ways, by protecting target lipids from oxidation initiators or by delaying the propagation phase. Antioxidants can be broadly classified by the mechanism of action as primary antioxidants (chain-breaking antioxidants) and secondary antioxidants (preventive antioxidants). Primary antioxidants are free-radical acceptors that delay the

initiation step or interrupt the propagation step of autooxidation through reaction with lipid and peroxy radicals and convert them to more stable, non-radical products. The most commonly used primary antioxidants in foods are synthetic compounds such as phenolic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ). However, a few natural primary antioxidants such as phenolic compounds, tocopherols, flavonoids, as well as carotenoids are commonly added to foods. The activity of phenolic antioxidants is often lost at high concentrations; therefore, they may become pro-oxidative, due to their involvement in the initiation phase (Salcedo & Nazareno, 2015).

Secondary antioxidants act through numerous possible. They can hinder reactive oxygen species (ROS) formation or scavenge species responsible for oxidation initiation (O_2^-, $^1O_2$, etc.). There are many different preventive antioxidation pathways including chelation of transition metals, singlet oxygen deactivation, enzymatic ROS detoxification, UV filtration, inhibition of prooxidant enzymes, antioxidant enzyme cofactors, etc. Moreover, they can act as reducing agent and regenerate primary antioxidants, decompose hydroperoxides to nonradical species and act as oxygen scavengers. Due to the fact that these secondary antioxidants can enhance the antioxidant activity of primary antioxidants, they are also called synergists.

As food products are predominantly multiphase systems, the solubility characteristics of the antioxidants in relation to the oxidation site must also be considered. According to the so-called polar paradox hypothesis, the efficacy of an antioxidant in emulsions is highly affected by its polarity and thereby location in the different phases (Decker, 1998; Sorensen, Nielsen, Decker, Let, Xu & Jacobsen, 2011). Whereas hydrophilic antioxidants may be more effective than hydrophobic antioxidants in bulk oils, hydrophobic antioxidants may be more effective in emulsified oils (Poter, 1993; Shahidi & Zhong, 2011; Sorensen et al., 2011; Sun et al., 2011). This observation was attributed to the ability of polar antioxidants to concentrate at the oil-air interface of bulk oils where oxidation was most prevalent, and the ability of non-polar antioxidants to concentrate in the lipid phase of emulsions, whereas polar antioxidant partitioned in both the lipid and water phases. However

recent results showed that not all antioxidants behave in the manner proposed by this hypothesis in oil and emulsions systems, so the polar paradox is a particular case of more global phenomenon. In bulk oil, new evidences have been brought to demonstrate that the crucial site of oxidation is not the air-oil interface, as postulated by the polar paradox, but association colloids formed with traces of water and surface active molecules such as phospholipids. In oil-in water emulsion, it has been recently discovered that there is non linear influence of the hydrophobicity on antioxidant capacity (Laguerre, Bayrasy, Panya, Weiss, McClements, Lecomte, Decker & Villeneuve, 2014). In this sense, that to date no rule yet emerges to rationalize the antioxidant behaviour in lipid-based foods.

5
Potential applications of fat replacers in healthier meat products

The influence of the diet on the human health is strongly accepted, as demonstrated by evidences in the knowledge of the relation between the dietary components and many chronic diseases or health problems (WHO, 2003; Donaldson, 2004; Geraldo & Alfenas, 2008; Jew et al., 2009; Guzmán, 2010). Whereas in high-income societies a shift occurred towards diets with more fat, sugar, processed foods and less fibre, leading to an increase of nutritional diseases, increasing evidence nowadays suggests that a next shift is occurring, following a behavioural change to consuming higher-quality fats, more whole grains, fruit and vegetables, and particularly less meat.

The role of meat and, more generally, animal-based foodstuffs in human consumption has changed over time. While meat consumption tends to increase with economic development, the environmental and human health implications of too high meat consumption are causing increasing controversy in developed societies (Tilman & Clark, 2014; Mathijs, 2015). However, meat and meat products are recognized in the Western diet as the main source of essential aminoacids, high biological value proteins, bioavailable iron, other minerals and vitamins of the B group (Hill, 2002; Biesalski, 2005; Williamson et al., 2005; McAfee et al., 2010). Therefore, consumer's willingness to healthier meat products and to the functional food acceptance has increased, although the lack of knowledge and a worse taste perception of these food products have been described as the main limitation factors for the complete success of these products (Nayga & Capps, 1999; Verbeke, 2006; Wim et al., 2006; Diekman & Malcolm, 2009; Ares et al., 2008). In this sense, food technologists have been making

great efforts to develop novel meat products (**Fig. 6**), in which the new formulation ensures the stability of the added ingredients to maintain their nutritional properties during the processing and distribution of the final product. So, the aim in the development of functional or healthier meat products should be improving the human health or reducing the risk of any diseases by formulating long self-life food products to meet consumers' expectations.

Fig. 6. Frecuent modifications in meat products.

Fat content and lipid profile	Minerals and salt
Reduced fat and cholesterol content Modified and healthier fatty acids profile. Higher MUFA and PUFA content.	Reduced sodium levels. Meat products with added iodine calcium and selenium.
Plant based derivates	**Fermented meat products**
Dietary antioxidants like vitamins A, C and E, polyphenols, flavonoids and terpenoids. Plan fibres as functional ingredient. Extracts and essential oils of herbs and spices as antimicrobial and antifungical compounds.	Good source of probiotic bacteria like *Lactobacillus* and *Bifidobacteria sp.*

One of the areas in which major attention has been paid regarding potentially food products is related to the modifications of the lipid fraction. Increasing the supply of UFA in meat and meat products can be performed by means of two potential strategies: feeding animals with UFA enriched diets, so that these animal nutrition practices can enhance the natural omega-3 content in eggs, fish, meat, etc., or including ingredients rich in UFA in the formulation of processed foods. In the case of processed meats, this second strategy has been widely investigated along the last decade, using different ingredients and by means of several technological practices.

In particular, the use of vegetable-derived ingredients to reformulate healthier meat products has been explored and includes a wide variety of possibilities. The most studied ones include the use of (1) vegetable oils to improve the lipid profile, (2) natural antioxidant and antimicrobial extracts to improve the stability of the new products and (3) natural fibres to reduce the energy and fat content.

In contrast to vegetable oils, the fat in the meat and meat products in general contains high amount of saturated fatty acids (SFA) and cholesterol. The

recommendations of health organizations to increase the intake of healthier fats and to improve certain lipid ratios (ω-6/ω-3, PUFA/SFA, MUFA+PUFA/SFA) in the diet support the development of healthier meat and meat products by replacing animal fat with vegetable oils. Thus, the enrichment of meat products with healthier lipid fractions such as omega-3 and omega-6 polyunsaturated fatty acids will enhance their nutritional quality.

The main technological approaches are the direct addition nuts or oils (vegetable, fish and algae oils) and, especially in the last years, the use of emulsified oils has become a good technological alternative. Most of the times the novel ingredients partially substitute the traditional animal fat in the processed meats formulations, and sometimes, a combination of partial substitution of fat and fat reduction is also aimed.

O/W emulsions have been shown to be suitable systems to incorporate ω-3 fatty acids from fish and algae oils, into food products including sausages, hams and meat patties (Salminem et al., 2013). The total or partial replacement of pork fat with emulsified oils (e. g. olive, linseed, corn oil, among others) in several studies resulted in lower content of fat, mainly SFA, and higher levels of UFA (Valencia, Ansorena & Astiasarán, 2006; García-Íñiguez de Ciriano et al., 2010; Berasategi et al., 2011; Delgado-Pando, Cofrades, Ruiz-Capillas, Triki & Jiménez-Colmenero, 2012; Salcedo-Sandoval, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2013; Berasategi et al., 2014a; Barriuso, Ansorena, Calvo, Cavero & Astiasarán, 2015).

However, at the same time, the stability of these vegetable oils enriched products might be compromised, as they are prone to faster lipid oxidation.

Hence, herbs and spices extracts have been used to stabilize these functional ingredients in meat products. The increasing interest of meat industry in natural antioxidants led to extensive research on the utilization of this type of extracts as lipid oxidation inhibitors. The role of vegetal extracts, including rosemary, oregano, lemon balm, clove, thyme and citrus fruits have been studied due to their antioxidant potential in cooked, fermented and irradiated meat products (Rodríguez Vaquero, Tomassini Serravalle, Manca de Nadra & Strasser de Saad, 2010; Hygreeva, Pandey & Radhakrishna, 2014).

The use of both vegetable oils and plant antioxidant extracts in meat products not only help improving the fatty acid profiles but also help in increasing product stability in terms of lower lipid oxidation. Estévez et al. (2007) reported that addition of sage and rosemary essential oils to liver patties significantly reduced the PUFA loss, decreased the total amount of volatile compounds and delayed lipid oxidation when compared with BHT. Berasategi et al. (2011) showed that the incorporation of a *Melissa officinalis* extract in Bolonga-type sausages enriched with ω-3 polyunsaturated fatty acids, improved both the oxidative stability and the nutritional properties of the reformulated product.

It is important to highlight that fat is an essential component in meat and meat products responsible for such quality characteristics as meaty flavour, juiciness, texture, cooking yield (Choi et al., 2013). In this sense, the use of emulsified oils into meat product, sometimes lead to textural changes due to their different structural properties compared to animal fat.

Many studies have successfully utilized plant dietary fibres as partial fat replacers, and reported that incorporation of dietary fibres into meat products may also enhance the binding properties, cooking yield and textural characteristics of meat products, such as meat balls, patties, sausages and bolognas (Borderías et al., 2005; Fernández-Ginés et al., 2004). Moreover, plant fibres are regarded as functional ingredients in meat products in two ways, by decreasing the fat and caloric content and by increasing the complex carbohydrate content, which is low in meat products. Jiménez-Colmenero et al. (2013) replaced pork back fat with a mixture of konjac flour, cornstarch and *ι*-carrageenan and developed reduced fat dry fermented sausages, with an important fat and energy reduction as compared to a conventional product. Schmiele et al. (2015) included amorphous cellulose fibre and obtained a meat model system with good technological properties and a significant improvement in the nutritional and cooking characteristics of the product.

Recently, new delivery systems are being developed towards the use of vegetable polymers to stabilize these emulsions systems. The combination of high unsaturated oils and vegetable fibres to perform more complex emulsion based delivery systems as fat replacers is giving good results. Pintado et al. (2015) developed

O/W emulsion gels stabilized with chia and alginate, with adequate technological and nutritional properties for use as a healthier fat ingredient. Salcedo-Sandoval et al. (2015) used pectin to perform more complexity-structured emulsion ($W_1/W_2/O$) named filled hydrogel particles, which resulted more oxidative stable than the conventional PUFA delivery system (O/W).

The inclusion of vegetable polymers in emulsion gels, in addition to the technological properties, may open up interesting possibilities in the development of healthier products as it contributes a variety of bioactive compounds (α-linolenic fatty acids, valuable proteins, antioxidants, dietary fiber, etc.).

Part II
Objectives

General objective

To develop emulsion-based delivery systems with a high content of unsaturated lipids, in order to produce physicochemically stable, sensory viable and technologically suitable products.

Specific objectives

1. To design and optimize physically stable emulsions for delivering unsaturated fatty acids, to be used as animal fat replacers in healthier reformulated food products.
2. To evaluate the oxidative stability under diverse storage conditions of the fatty acids included in the emulsion systems developed.
3. To study the influence of the lipid composition on the oxidation compounds formation induced by heat treatment.
4. To study the technological and sensory viability of the emulsions as new ingredients in reduced-fat and highly unsaturated reformulated meat products (fresh and cooked).
5. To characterize the nutritional value of the developed meat products in order to define their possible nutrition and health claims.

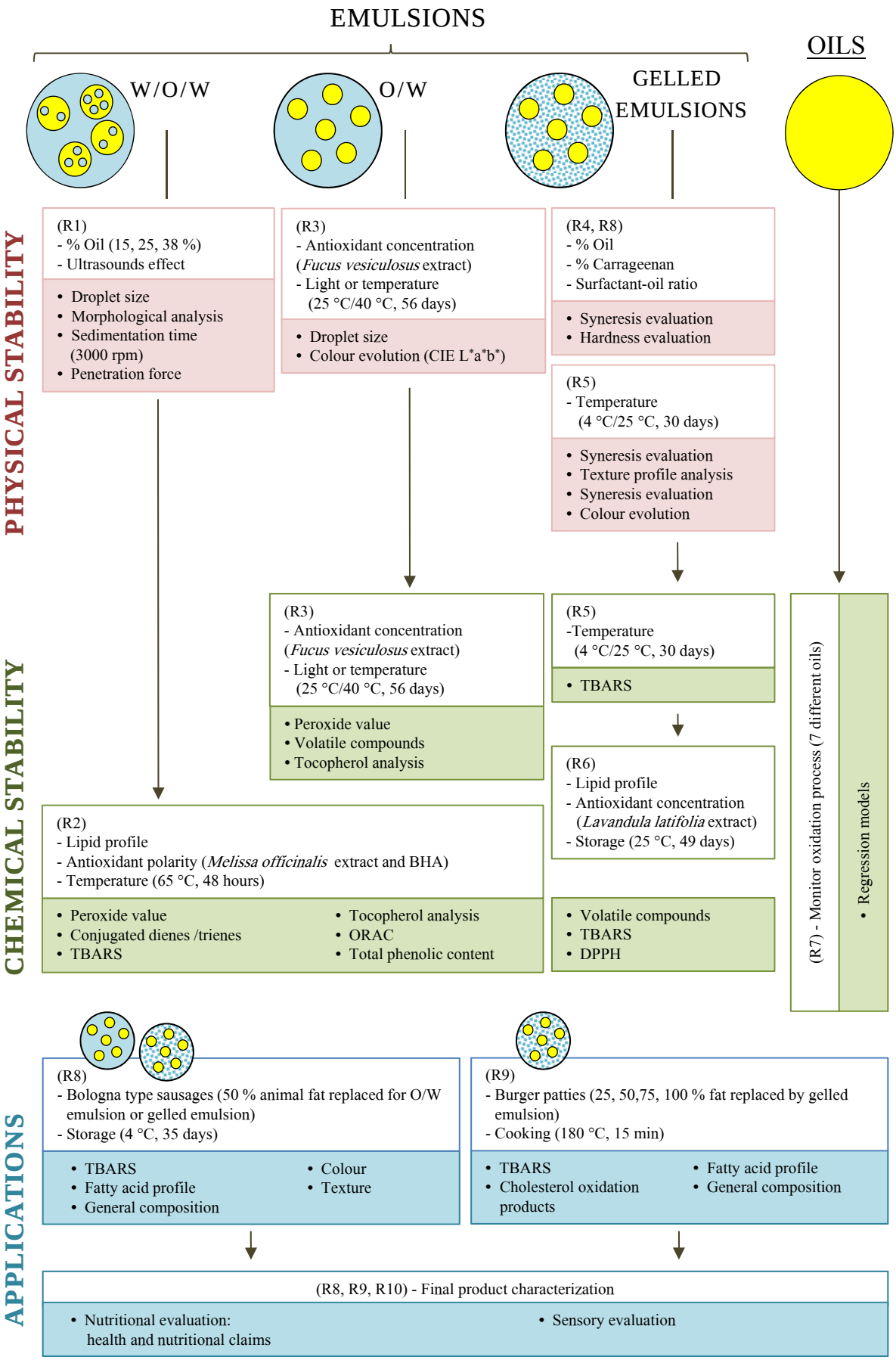
Objetivo general

Desarrollar sistemas basados en emulsiones para la vehiculización de lípidos altamente insaturados, de modo que los productos resultantes muestren estabilidad y viabilidad tecnológica y sensorial.

Objetivos específicos

1. Desarrollar y optimizar el diseño de sistemas de emulsión, físicamente estables, como vehículo de ácidos grasos altamente insaturados, que puedan ser utilizados como sustitutos de grasa animal en la reformulación de alimentos.
2. Evaluar la estabilidad oxidativa bajo distintas condiciones de almacenamiento, de los lípidos incluidos en los sistemas emulsión desarrollados.
3. Estudiar la influencia de la composición de la matriz lipídica en la evolución de la oxidación inducida por tratamiento térmico.
4. Estudiar la viabilidad tecnológica y sensorial de emulsiones como ingredientes en la reformulación de productos cárnicos (crudos y cocidos) reducidos en grasa y enriquecidos en ácidos grasos insaturados.
5. Realizar una caracterización nutricional completa de los productos cárnicos desarrollados, que permita definir las declaraciones nutricionales y/o saludables correspondientes.

Part III
Experimental design



(R) Result. The number corresponds to the chapter in the report.

Part IV
Methods

Physical properties

1. Colour

Colour coordinates Cie L*a*b*. Digital colorimeter, Chromameter-2 CR-200, Minolta (Osaka, Japan) [R3, R5, R8].

2. Droplet size determination

Microscopic image analysis. Nikon E-800 (Kawasaki, Japan) [R1, R2].

Laser difraction. Mastersizer 2000 (Malvern Ins., Worcestershire, UK) [R3].

3. Phases separation

Time of sedimentation after centrifugation at 3000 rpm. Nahita, angular rotor 2660 (La Rioja, Spain) [R1].

4. Syneresis

Weight loss after 3 days at 25 °C [R4, R5, R8].

5. Texture profile analysis

Penetration force under a 10 g load cell and 10 mm in depth. Cilinder P/25, ANAME Texture Analyser, TA-TX2i (Stable Micro Systems, Surrey, UK). Modified from "Penetration force comparision test of traditional and low fat mayonnesa" [R1].

Compression force under a 5 kg load cell at deformation rate 30 %. Cilinder P0.5R, ANAME Texture Analyser, TA-TX2i (Stable Micro Systems, Surrey, UK) [R5, R8].

Oxidative analysis

1. Cholesterol oxidation products

Purification with amino-propyl cartridges and derivatization to trimethyl silyl ethers and analysis by GC-MS were performed as described in Ansorena et al. (2013). GC-MS, Agilent 6890N equipped with an Agilent 5975 Mass Selective Detector [R9].

2. Conjugated dienes and trienes

Absorbance measured at 234 and 280 nm using a Lambda 5 UV–Vis Spectrophotometer (Perkin Elmer, Paris, France). Method described by Frankel, Huang, Aeschbach and Prior (1996) with slight modifications [R2].

3. Oil extraction

Folch, Lees and Stanley (1957).

Bligh and Dyer (1959) method modified by Iverson, Lang and Cooper (2001).

4. Peroxide value

Iodine titration with sodium thiosulphate. Method by AOAC, "Determination of peroxide content". 965.33.12. (2002) [R2].

Iron-thiocyanate complex measurement at 500 nm. Shimadzu UV1800 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Method by Shantha and Decker (1994) [R3].

5. Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances. FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). Method described by Masqsood and Benjakul (2010) with slight modifications [R2, R5, R6, R8, R9].

6. Volatile compounds

Volatile aldehydes by HS-SPME-GC-MS method. Fiber coat DVB/CAR/PDMS (Supelco). GC-MS, HP 6890 Series, equipped with a HP Mass Selective Detector 5973 (Hewlett Packard). Method described by Guillén and Uriarte (2012) with slight modifications [R6, R7].

Volatile aldehydes by HS-SPME-GC-MS method. Tenax GR™ packed tubes, automatic thermal desorber ATD-400 Perkin-Elmer (Norwalk, CT). GC-MS, Agilent 5890 IIA equipped with a HP 5972 Mass Selective Detector (Hewlett Packard) [R3].

Antioxidant capacity

1. DPPH assay

Inhibition percentage at 516 nm. FLUOStar Omega spectrofluorometric analyser, (BMG Labtechnologies, Offenburg, Germany). DPPH method was performed as described by García-Herreros et al. (2010) [R2, R6].

2. Lipophilic oxygen radical absorbance capacity (L-ORAC$_{FL}$) assay

Fluorescence evolution. FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). Method described by Prior et al. (2003) with slight modification [R2].

3. Tocopherol analysis

α-tocopherol by HPLC-UV analysis. Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA (Perkin Elmer, Paris, France). Method described by Berasategi, Barriuso, Ansorena and Astiasarán (2012) [R2].

α-, β-, γ- and δ-tocopherol by HPLC-FL. Agilent HPLC 1100 Series (Agilent Technology). Method according to AOCS (1998) to quantify the contents of α-, β-, γ- and δ-tocopherols [R3].

4. Total phenolic content

Folin-Ciocalteu colorimetric method measured at 765 nm. Lambda 5 UV–Vis spectrophotometer (Perkin Elmer, Paris, France). Method by Herchi et al. (2011) with slight modifications [R2].

Folin-Ciocalteu colorimetric method measured at 765 nm. FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). Method by Herchi et al. (2011) with slight modifications [R6].

General composition

1. Fat content

Fat content. Soxhlet. AOAC. (2002). Fat (crude) or ether extract in meat. 960.39. In W. Horwitz (Ed.), Official method of analysis. (17th ed., pp. 12-13). Gaithersburg, Maryland: Association of Official Analytical Chemists [R8, R9, R10].

2. Fatty acid profile

Fatty acids profile. GC-FID, Perkin-Elmer Clarus 500 gas chromatograph (Paris, France). AOAC. (2002). Methyl esteres of fatty acids in oils and fats. 969.33. In Official methods of analysis. (pp. 19-20). Gaithersburg, Maryland: Association of Official Analytical Chemists [R2, R3, R6, R8, R9, R10].

3. Moisture

Moisture content. AOAC. (2002). Determination of moisture content. 950.46. In W. Horwitz (Ed.), Official method of analysis. (17th ed., pp. 12-13). Gaithersburg, Maryland: Association of Official Analytical Chemists [R8, R9].

4. Protein determination

Protein. Kjeldahl. AOAC. (2002). Crude protein in meat. 981.10. In W. Horwitz (Ed.), Official method of analysis. (17th ed., pp. 7-8). Gaithersburg, Maryland: Association of Official Analytical Chemists [R8, R9].

5. Cholesterol

Derivatization to trimethyl silyl ethers and analysis by GC-FID. Perkin-Elmer Autosystem XL gas chromatograph [R9].

Sensory analysis

Triangle test. 21 semi-trained panellists. Spanish norm UNE 87-006-92 (1992). Test in appendix I [R8].

Multiple comparison test. Eleven trained panellists, scores (1. very much less; 2. much less; 3. considerably less; 4. slightly less; 5. not differences; 6. slightly more; 7. considerably more; 8. much more; 9. very much more). Anzaldúa-Morales (1994). Test in appendix II [R9].

Hedonic test. 33 non-trained panellist. 7-point scale for scoring the samples (3. I really like; 2. I like; 1. I slightly like; 0. I rather like or dislike; -1. I slightly dislike; -2. I dislike; -3. I really dislike). Anzaldúa-Morales (1994). Test in appendix III [R9].

Part V
Results

1

Estabilidad de emulsiones W/O/W reducidas en grasa como vehículo de compuestos bioactivos

C. Poyato, I. Astiasarán, D. Ansorena

Resumen: Se estudió la estabilidad de 3 tipos de emulsiones dobles (W/O/W) preparadas con distintas proporciones de aceite:agua (en presencia de carragenato 2 %, BHA 200 ppm y Tween 80 como surfactante) y sometidas a distintos tiempos de ultrasonidos (0, 15 y 30 min.). Se midió la textura mediante test de penetración, la estabilidad mediante resistencia a separación por centrifugación, y se observó la estructura por microscopía óptica. Se obtuvieron emulsiones con contenido total de grasa de 38, 25 y 15 %. La aplicación de ultrasonidos incrementó en todos los casos la consistencia de las emulsiones, reduciendo el tamaño de las gotas de grasa y el de las gotículas de la fase interna acuosa. En el caso de las emulsiones con menor porcentaje de grasa (15 %) solo se consiguió la homogeneidad con la aplicación de ultrasonidos durante 30min. Las emulsiones con 25 % de grasa resultaron las más inestables.

Palabras clave: emulsiones múltiples, aceite de lino, textura, micrografía.

1. Introducción

Una emulsión water-in-oil-in-water (W/O/W) es un sistema de emulsión doble en el cual la fase dispersa es una emulsión water-in-oil (W/O), y la fase continua es de naturaleza acuosa (McClements, 2000). Entre las interesantes características que presentan estas emulsiones destacan la alta capacidad de incorporar compuestos hidrofílicos, diversas sustancias incompatibles entre sí dentro el mismo sistema, la mejora del rendimiento de compuestos bioactivos o la protección y liberación prolongada de los compuestos atrapados en la fase acuosa interna (Leal-Calderón et al., 2007). En la industria alimentaria la utilización de este tipo de sistemas ha cobrado en los últimos años una gran importancia debido a la capacidad que tienen de atrapar, en

el compartimento interno, sustancias solubles en agua como vitaminas (Benichou et al., 2004) o minerales (Bonnet et al., 2004). La encapsulación de dichos compuestos puede ser interesante para la formulación de alimentos funcionales en la medida que pueden utilizarse para el enriquecimiento de productos alimenticios en compuestos bioactivos. Otra de las aplicaciones prometedoras en este campo es la de producir productos que cumplan con propósitos nutricionales, como los alimentos reducidos en grasas en general o grasas saturadas en particular.

Los problemas asociados con la estabilidad de las emulsiones múltiples han sido ampliamente estudiados durante las pasadas dos décadas (Leal-Calderón et al., 2007). Las gotas de aceite en las emulsiones W/O/W son susceptibles de sufrir procesos de formación de crema, floculación o coalescencia. Las emulsiones dobles presentan generalmente dos emulsificantes de solubilidad opuesta (hidrofílicos e hidrofóbicos), lo cual favorece los procesos de coalescencia, que pueden ocurrir de diversas maneras: (1) entre las pequeñas gotas internas; (2) entre los grandes glóbulos; (3) entre la fase acuosa externa y la interna. Algunas de las estrategias que se utilizan para superar los problemas asociados con la preparación de emulsiones múltiples estables son: una correcta elección de los emulsificantes, la incorporación de biopolímeros en la fase acuosa interna, la solidificación de la fase oleosa y la regulación del balance osmótico. Algunos de los parámetros que afectan a la estabilidad, tanto física como química, de las emulsiones son: concentración de las gotas, tamaño, carga, estado físico, características interfaciales y distribución molecular (McClements, 2010a).

El desarrollo de alimentos funcionales plantea dos grandes retos: (1) tecnológico, para garantizar la adecuada biodisponibilidad de los ingredientes bioactivos y (2) sensorial, para conseguir productos con adecuadas propiedades organolépticas (Ritzoulis et al., 2010). En este trabajo se pretendió desarrollar emulsiones múltiples (water-in-oil-in-water, W/O/W) como sistema de vehiculización de compuestos bioactivos, ácidos grasos omega-3 y antioxidantes, que puedan ser incluidos en productos reducidos en grasa, con propiedades físico-químicas y sensoriales similares a los productos originales. Se estudió la influencia que ejercen

factores como la proporción de grasa y la aplicación o no de ultrasonidos, sobre la estabilidad final del sistema.

2. Material y métodos

2.1. Preparación de las emulsiones

Se prepararon 9 emulsiones en función de dos factores, el porcentaje de grasa y el tiempo de aplicación de ultrasonidos.

Las emulsiones water-in-oil-in-water se prepararon en dos etapas consecutivas en todos los casos. Primero se realizaron 3 emulsiones W/O en las que las relaciones agua/aceite de lino fueron 0.4, 1.1 y 2.5. Para ello se calentó tanto el aceite de lino, el cual contenía Tween 80, como el agua (2 % carragenatos) a 70 °C. Posteriormente se homogeneizó la fase acuosa, a la cual se le incorporó el BHA, sobre la fase oleosa en un agitador magnético a 1500 rpm. Seguidamente, se dividió la emulsión en 3 partes iguales, dos de las cuales se sometieron a ultrasonidos en un baño termostatizado a 70 °C (Selecta, Ultrasons-H), durante 15 y 30 min. La emulsión se dejó enfriar a temperatura ambiente permitiendo la polimerización de los carragenatos. En la segunda etapa se homogeneizó la emulsión previamente descrita con una mezcla de proteína de soja y agua, en proporciones 10:1:8 respectivamente, durante 3 min a 6500 rpm (Ultra-Turrax® T25basic, IKA®WERKE, Germany).

Se procedió de igual manera en todas las emulsiones, cambiando las proporciones de aceite, agua y Tween 80, según se indica en la **tabla 1**. Las emulsiones se realizaron por duplicado.

Tabla 1. Composición de las emulsiones por 100 g.

	Emulsión 1	**Emulsión 2**	**Emulsión 3**
	Etapa 1: Water-in-oil		
Aceite de lino (g)	38,6	25	15
Agua (2 % carragenatos) (g)	14,04	27,6	37,6
Tween 80 (mg)	24	49	66
BHA (mg)	14,67		
	Etapa 2. Water-in-oil-in-water		
Fase dispersa, W/O emulsion (g)	52,6		
Proteína de soja (g)	5,3		
Agua (g)	42,1		

2.2. Ensayo de estabilidad

Las emulsiones fueron sometidas a centrifugación (Nahita, Rotor angular Modelo 2660, La Rioja, España) con el objetivo de evaluar su resistencia a la separación de fases en cada tipo de emulsión. Para ello se pesaron 5 gramos de emulsión en un tubo de centrífuga (1,5 cm) y se sometieron a 3000 rpm. Se realizó un seguimiento de la separación de fases en intervalos de 5 minutos.

2.3. Análisis morfológico mediante microscopía

Para la verificación de la estructura W/O/W, se observaron las emulsiones en microscopio óptico (NIKON Eclipse E800, Kawasaki, Japón) tras 24 horas de refrigeración a 4 °C. Para ello se realizó una preparación de una pequeña cantidad de la emulsión sobre una gota de agua, previamente depositada sobre un portaobjetos (76×26 mm). Tras la extensión de la misma se colocó un cubre (24×32 mm). Se midieron los diámetros de las gotas predominantes en cada tipo de emulsión. Las emulsiones se clasificaron atendiendo al número y tamaño de gotículas internas (Pal, 2008). Las emulsiones tipo-A se caracterizan por presentar una única gotícula interna, las tipo-B por presentar varias gotículas internas pequeñas y las emulsiones tipo-C por presentar un gran número de gotículas internas.

2.4. Textura

Para comparar la consistencia de las distintas emulsiones se utilizó un Texturómetro (ANAME Texture Analyser, TA-TX2i) aplicando el "Test de comparación de fuerzas de penetración y consistencia de mayonesas tradicionales y reducidas en grasa utilizando una sonda cilíndrica" con ligeras modificaciones. Se introdujeron 95 g de muestra en un frasco de 5,5 cm de diámetro. Las muestras se midieron tras 24 horas de refrigeración a 4 °C. Se midió la fuerza de compresión utilizando el accesorio cilíndrico P/25 (25 mm), con un pre-test Speed y un test speed de 1 mm/s, la profundidad de penetración se fijó en 10 mm y la fuerza de penetración del cilindro en 10 g. El test se realizó por decuplicado.

3. Resultados y discusión

El trabajo consistió en producir emulsiones múltiples W/O/W, con una gran capacidad de encapsulación y mínimo porcentaje de aceite posible, además de estudiar su estabilidad física. Se obtuvieron emulsiones con un contenido en grasa final de 38, 25 y 15 %.

En el estudio realizado mediante microscopía (**Fig. 1**) se observó que en las emulsiones que no fueron sometidas a ultrasonidos, presentaron diferencias tanto en el tamaño de las gotas de aceite como en el de las gotículas acuosas internas mostrando una distribución que se podría describir como heterogénea. Estas diferencias de tamaño fueron tanto mayores cuanto menor fue la fracción acuosa interna.

La aplicación de ultrasonidos favoreció la homogeneidad en el tamaño de partícula de las gotas de aceite de las emulsiones en todos los casos (**Tabla 2**). En las emulsiones con 38 y 25 % de grasa la homogeneidad se consiguió con 15 min de aplicación de ultrasonidos, mientras que en las de 15 % de grasa sólo se consiguieron emulsiones homogéneas tras 30 min de ultrasonidos. El empleo de ultrasonidos consiguió reducir el tamaño de las gotas de aceite así como el de las gotículas de fase acuosa interna, incrementando así la cantidad de las mismas por gota. Esta distribución del tamaño de gotículas ejerció una influencia significativa sobre la consistencia de las emulsiones tal y como se pondrá de manifiesto con los resultados de textura.

Para el estudio de la estabilidad física de las emulsiones, se sometieron a centrifugación con el objetivo de romper las emulsiones, considerándose más estables aquellas emulsiones que tardaban más en separar sus fases. El tiempo de sedimentación, tras centrifugar a 3000 rpm, de las emulsiones con 25 % de grasa fue más corto que el de las emulsiones con 15 y 38 % de grasa, y por tanto puede decirse que son las emulsiones más inestables (**Tabla 2**). Este comportamiento puede ser debido al incremento en la relación de volumen de fases iniciales, por el cual conforme se reduce la proporción de aceite, se va desestabilizando el sistema. En el caso de las emulsiones con un 15 % de grasa la proporción de agua que está incluida en las gotas de aceite es mucho mayor, lo que propicia una inversión de fases parcial que conlleva un incremento de la estabilidad del sistema (Aranberri et al., 2006). Por otro lado, la mayor cantidad de carragenatos que incorporan estas emulsiones en su fase interna

(emulsión O/W) es otro factor a tener en cuenta en su elevada resistencia a la separación de fases. Al mismo tiempo se puede observar que la aplicación de ultrasonidos favoreció la estabilidad en los 3 tipos de emulsiones, lo que puede deberse a una reducción en el tamaño de las gotas de aceite, lo que incrementó la estabilidad termodinámica del sistema (Cho & Park, 2003).

Tabla 2. Descripción cualitativa de cada emulsión en función del porcentaje de grasa y el tiempo de ultrasonicación.

	38 %	25 %	15 %
0 min	- Heterogénea - Predominio M - Tipo A - t= 10 min	- Heterogénea - S,M - Tipo B - t= 5 min	- Heterogénea - XS, S, M, L - Tipo C - t= 15 min
15 min	- Homogénea - S - Tipo B - t= 10 min	- Homogénea - M - Tipo C - t= 10 min	- Heterogénea - S, M - Tipo C - t= 40 min
30 min	- Homogénea - S (alguna L) - Tipo C - t= 15 min	- Homogénea - XS - Tipo B - t= 10 min	- Homogénea - XS, S - Tipo C - t= 25 min

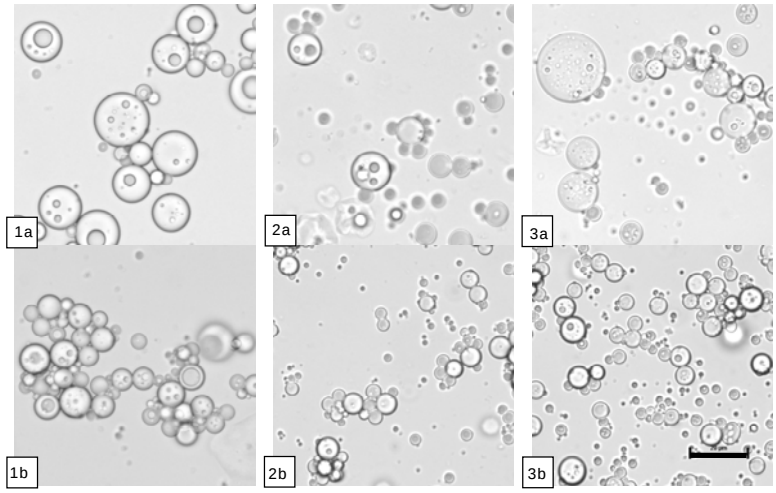


Fig. 1. Micrografías de emulsiones W/O/W. Los números indican el contenido en aceite (1, 38 %; 2, 25 %; 3, 15 %) y las letras el tiempo sometido a ultrasonidos (a, 0 min; b, 30 min). La barra equivale a 20 µm.

Los resultados del test de textura (**Fig. 2**) pusieron de manifiesto, en todos los casos, que la aplicación de ultrasonidos incrementó la consistencia de las emulsiones, probablemente debido a que la reducción en el tamaño de las gotas de aceite incrementa la viscosidad del sistema. Las emulsiones con un 25 % de grasa fueron las menos resistentes, independientemente del tiempo de ultrasonidos al cual fueron sometidas. Las emulsiones con un 15 % ó 38 % de grasa no mostraron diferencias significativas en su consistencia con 15 ó 30 min de ultrasonidos.

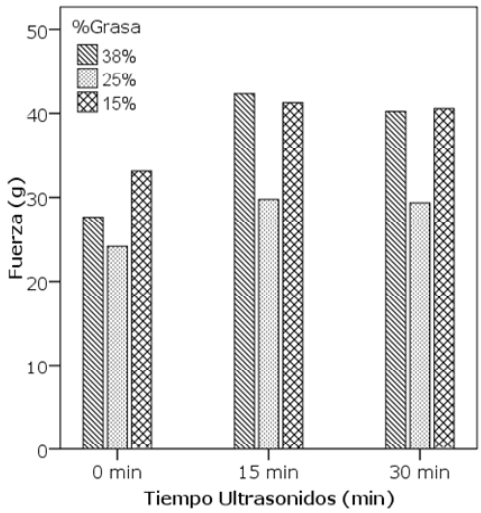


Fig 2. Fuerza de penetración resultante para cada tipo de emulsión. Las letras mayúsculas indican diferencias significativas ($p<0,05$) para una misma proporción de grasa y distintos tiempos de ultrasonidos. Las letras minúsculas indican diferencias significativas ($p<0,05$) para un mismo tiempo de ultrasonidos entre las distintas proporciones de grasa.

4. Conclusiones

Es posible generar emulsiones W/O/W estables con bajo porcentaje de grasa gracias a la polimerización de la fase acuosa interna mediada por carragenatos. La estabilidad de las emulsiones múltiples estabilizadas mediante polímeros hidrofílicos puede verse favorecida por el uso de ultrasonidos. La utilización de ultrasonidos en las etapas iniciales de elaboración de las emulsiones, incrementa la consistencia de las

emulsiones y reduce el tamaño tanto de las gotas de aceite como de las gotículas acuosas internas.

5. Agradecimientos

Programa Consolider-Ingenio 2010 CARNISENUSA CSD 2007-00016, " Proyecto AGL2008-01099/ALI" (Ministerio de Ciencia e Innovación), y Plan “Investigador de la Universidad de Navarra” (PIUNA) por su contribución y ayuda financiera. C. Poyato agradece al “Grupo Santander-Universidad de Navarra”, “Cátedra Tomás Pascual Sanz-Universidad de Navarra” y “Asociación de Amigos de la Universidad de Navarra” por las becas recibidas.

ESTABILIDAD DE EMULSIONES W/O/W REDUCIDAS EN GRASA COMO VEHÍCULO DE COMPUESTOS BIOACTIVOS

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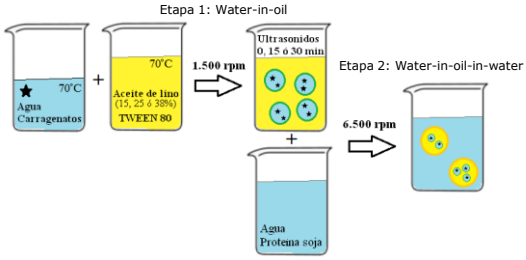
Objetivos

1. **Estudiar la influencia de la proporción grasa/agua sobre la estabilidad en un sistema de emulsión doble ($W_1/O/W_2$).**
2. **Valorar el efecto de la aplicación de ultrasonidos en la fase inicial de la preparación de una emulsión doble sobre la estabilidad final del sistema.**

Preparación de las emulsiones

Se prepararon emulsiones dobles (W/O/W) de 3 tipos (EM1, EM2, EM3) con diferente contenido graso (38, 25 y 15%). De cada tipo se realizaron 3 emulsiones sometidas a distintos tiempos de ultrasonido (0, 15 y 30 min).



COMPOSICIÓN POR 100 g	Emulsión 1	Emulsión 2	Emulsión 3
	Etapa 1: Water-in-oil		
Aceite de lino (g)	38,6	25	15
Agua (2% carragenatos) (g)	14,04	27,6	37,6
Tween 80 (mg)	24	49	66
BHA (mg)		14,67	
	Etapa 2. Water-in-oil-in-water		
Fase dispersa (g)		52,6	
Proteína de soja (g)		5,3	
Agua (g)		42,1	

Conclusiones

- **Es posible generar emulsiones W/O/W estables con un bajo porcentaje de grasa gracias a la polimerización de la fase acuosa interna mediada por carragenatos.**
- **La utilización de ultrasonidos en las etapas iniciales de elaboración de las emulsiones, incrementa la consistencia de las emulsiones y reduce el tamaño tanto de las gotas de aceite como de las gotículas acuosas internas.**

Agradecimientos

Programa Consolider-Ingenio 2010 CARNISENUSA CSD 2007-00016 y Plan "Investigador de la Universidad de Navarra" (PIUNA) por su contribución y ayuda financiera. C. Poyato agradece al "Grupo Santander-Universidad de Navarra", "Cátedra Tomás Pascual Sanz-Universidad de Navarra" y "Asociación de Amigos de la Universidad de Navarra" por las becas recibidas.

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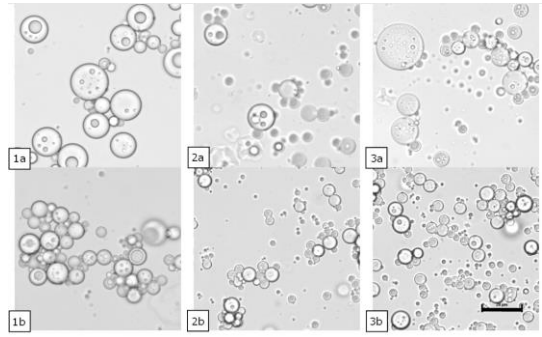
Resultados

Micrografía

Análisis morfológico (Microscopía de campo claro, NIKON Eclipse E800, Kawasaki)



Micrografías de emulsiones water-in-oil-in-water. Los números indican el contenido en aceite (1: 38%; 2: 25%; 3: 15%) y las letras el tiempo sometido a ultrasonidos (a: 0 min; b: 30 min).

Ensayo de estabilidad

Centrifugación de 5 g de muestra a 3000 rpm: medida del tiempo de sedimentación.

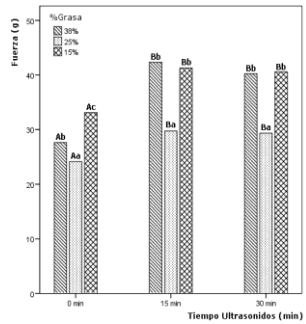
Tamaño de gota medido por microscopía.

	38%	25%	15%
0 min	- Predominio M - t= 10 min	- S,M - t= 5 min	- XS, S, M, L - t= 15 min
15 min	- S - t= 10 min	- M - t= 10 min	- S, M - t= 40 min
30 min	- S (alguna L) - t= 15 min	- XS - t= 10 min	- XS, S - t= 25 min

t= tiempo de sedimentación por centrifugación a 3000 rpm.
Diámetro de gota: XS< 4 µm; S= 4-7 µm; M= 7-10 µm; L> 10 µm.

Textura

Fuerza de penetración resultante para cada tipo de emulsión (ANAME Texture Analyser, TA-TX2i)



Mayúsculas: diferencias significativas ($p<0,05$) para una misma proporción de grasa y distintos tiempos de ultrasonidos.

Minúsculas: diferencias significativas ($p<0,05$) para un mismo tiempo de ultrasonidos entre las distintas proporciones de grasa.

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2

Oxidative stability of O/W and W/O/W emulsions: effect of lipid composition and antioxidant polarity

C. Poyato, I. Navarro-Blasco, M. I. Calvo, R. Y. Cavero, I. Astiasarán, D. Ansorena

Abstract: The effect of storage temperature (65 °C, 48 hours) on the oxidative stability of a food-grade water-in-oil-in-water (W/O/W) emulsion was studied by comparison with an oil-in-water (O/W) emulsion. The emulsions were prepared with linseed oil or olive oil, and in each case, two antioxidants were evaluated, an aqueous *Melissa* lyophilized extract and BHA. Emulsions were characterized using brightfield light microscopy and the oxidation was monitored by measuring the lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), conjugated dienes (CD) and trienes (CT), alpha-tocopherol and Lipophilic Oxygen Radical Absorbance Capacity (L-ORAC$_{FL}$) Assay.

A great stability of olive oil emulsions was observed, without noticing differences between antioxidants or type of emulsion. This behaviour was not observed in linseed oil emulsions. In this case the lipophilic antioxidant (BHA) seemed to be more efficient delaying the lipid oxidation in W/O/W emulsions than the water *Melissa* extract while the opposite occurs in the O/W emulsion. The type of antioxidant is a key factor in controlling oxidation in W/O/W and O/W emulsions which are prepared with highly polyunsaturated oils, but not in the case of highly monounsaturated ones.

Key words: linseed oil, olive oil, antioxidant, multiple emulsion, lipid oxidation, lemon balm.

1. Introduction

The use of vegetable oils as functional ingredients in food lipids emulsions might be complex from a technological point of view due to the high oxidation susceptibility of these unsaturated oils (Jacobsen, Timm & Meyer, 2001; Taherian, Britten, Sabik & Fustier, 2011). It is known that oxidation may lead to production of rancid odours, unpleasant flavours and even compromise the safety of foods because of the formation of harmful compounds. Lipid oxidation can occur rapidly in emulsions due to their large surface area that facilitates interactions between the lipids and the water-soluble prooxidants. There are many factors that can potentially influence the physical and oxidative stability of emulsions: fatty acid composition, pH and ionic composition, type and concentration of antioxidants and prooxidants, emulsion droplet and interfacial properties, lipid droplet characteristics, concentration and physical state (McClements & Decker, 2000; Raikos, 2010; Naji & Karazhiyan, 2012; Xu, Wang, Jiang, Yuan & Gao, 2012).

Several studies have been carried out to elucidate lipid oxidation mechanisms in oils, providing important insights into the factors that influence lipid oxidation and strategies to control it (Waraho, McClements & Decker, 2011). The most common method to increase the oxidative stability of emulsion systems is probably the use of antioxidants (Gutteridge & Halliwell, 2010). Antioxidants are potentially able to be located in a number of different physical environments within a conventional oil-in-water emulsion (O/W) or in a multiple water-in-oil-in-water emulsion (W/O/W). Hydrophilic components can be incorporated by dispersing them in the external water phase in an O/W emulsion or into the internal aqueous phase in a W/O/W emulsion. Lipophilic components can be incorporated into the oil droplets by dispersing them in the oil phase (McClements, 2010a). Porter (1993) first described the “antioxidant paradox” as a phenomenon where hydrophilic antioxidants were more effective than lipophilic antioxidants in bulk oils while lipophilic antioxidants were more effective in emulsified ones. This observation was attributed to the ability of non-polar antioxidants to concentrate in the lipid phase of emulsions, whereas polar antioxidant partitioned in both the lipid and water phases (Laguerre et al., 2010; Shahidi & Zhong, 2011; Sorensen et al., 2011). According to this hypothesis, the efficacy of an

antioxidant contained by the emulsion is affected by its polarity and its location in the different phases (Sorensen et al., 2011). Although the theory has been generally accepted, new evidence from more comprehensive assessments has more recently emerged and disagrees with the polar paradox, hence requiring a revaluation to this hypothesis (Shahidi et al., 2011). Several recent studies have shown that not all antioxidants behave according to the polar paradox hypothesis, indicating that antioxidant activity in complex systems is more complicated than previously assumed (Laguerre, Lecomte, Figueroa-Espinoza & Barea, 2009).

Synthetic phenolic antioxidants, such as Butylated Hydroxyanisole (BHA), have been used as effective additives to control lipid oxidation in high fat content foods. However, an increasing concern about their safety has resulted in a preferential research on natural antioxidants (Lee & Kunz, 2005). A number of studies deal with the use of natural antioxidant extracts such as grape seed, rosemary, blackseeds, green tea, among others, in order to protect emulsions and other foods from oxidation (Samotyja & Malecka, 2007; Ramful et al., 2011; Gibis & Weiss, 2012). *Melissa officinalis* aqueous extracts have shown antioxidant activity tested in O/W emulsion made with linseed and algae oil (García-Íñiguez de Ciriano et al., 2010) but the hypothetical advantages to minimize lipid oxidation using W/O/W *versus* O/W emulsions systems has not previously been reported. Moreover, it has been said that the use of W/O/W systems for food applications is further limited by lack of suitable food-grade emulsifiers and stabilizers for the inner and outer emulsions (Sapei, Ali Naqvi & Rousseau, 2012). According to these authors, shelf-life stability of these emulsions at high temperatures must be ascertained prior to their successful usage in foods.

The aim of this paper was to evaluate the oxidative stability of W/O/W emulsions compared to O/W ones, under the use of a lyophilized water extract of *Melissa officinalis* and BHA as natural and synthetic antioxidants respectively. The type of antioxidant used and the phase in which they are placed might be relevant factors that can affect their effectiveness in different emulsion systems. Furthermore, taking into account the different oxidation susceptibility of oils depending on their unsaturation degree, two different oils were evaluated (linseed and olive oil).

2. Materials and methods

2.1. Materials

The oils used in this study were Extra Virgin Olive Oil (Aceites del Sur, Coosur S.A., Jaén, Spain) and Linseed Oil (Biolasi Productos Naturales S.L., Guipúzcoa, Spain), which were obtained using the cold-pressed method. The lipid profile of both oils was analyzed by gas chromatography (**Table 1**). 2-thiobarbituric acid, α-tocopherol acetate 98 %, α-tocopherol 97 %, tetraethoxypropane, fatty acid methyl esters, gallic acid monohydrated >98 %, fluorescein sodium salt and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97 %) were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). Boron trifluoride/methanol, Folin-Ciocalteu's phenol reagents, butylated hydroxyanysole (BHA) and butylated hydroxytoluene (BHT) were obtained from Merck (Barcelona, Spain). Potassium hydroxide, hexane, cyclohexanone, methanol, hydrochloric acid, trichloroacetic acid, ammonium sulphate, monopotassium phosphate, dipotassium phosphate, Polysorbate 80, sodium carbonate and acetone were supplied by Panreac (Barcelona, Spain). Ethanol was purchased from Oppac (Navarra, Spain) and HPLC grade methanol from Scharlab (Barcelona, Spain). *k*-carrageenan and soya protein were from Cargill (San Sebastian, Spain) and ANVISA (Madrid, Spain) respectively. AAPH (2,2'-Azobis(2-methylpropionamidine) dihydrochloride 98 %) was from Across Organic (New Jersey, USA). RMCD (randomly methylated β-cyclodextrin) was purchased from Ciclolab R&D Dltd. (Budapest, Hungary).

Melissa officinalis dried leaves were purchased from Plantaron S.L. (Barcelona, Spain) and the aqueous *Melissa* extract was obtained following the method described by García-Íñiguez de Ciriano et al. (2010). Chemical characterization of this extract was done, giving rise to a DPPH value of 552.13 mg trolox equivalent/g lyophilized extract and to an ORAC value of 1728 mg trolox equivalent/g lyophilized. A more detailed characterization of water and hydroalcoholic lyophilized extracts of *Melissa* was presented in Encalada et al. (2011).

2.2. Emulsion preparation

8 types of emulsions were prepared in the 2×2×2 experiment that was designed in this paper. Linseed oil (L) and olive oil (O) were used to prepare two types of emulsions, an oil-in-water (O/W) and a water-in-oil-in-water (W/O/W). Two antioxidants, aqueous *Melissa* extract (Mel) and butylated hydroxyanysole (BHA), were tested for each one of the emulsions.

The corresponding codes for the 8 types of emulsions were: L:Mel:O/W, L:Mel:W/O/W, L:BHA:O/W, L:BHA:W/O/W, O:Mel:O/W, O:Mel:W/O/W, O:BHA:O/W and O:BHA:W/O/W. Amounts of each ingredient shown in the following lines are referred to 100g of emulsion.

The O/W emulsions were prepared by mixing 42.1 g of water and 5.3 g of isolated soya protein (2 min). Afterwards 52.63 g of oil were slowly added and gently stirred in order to obtain a stable emulsion (3 min, 16.000 rpm, Ultra-Turrax® T25basic, IKA®WERKE, Germany). Two different batches were prepared depending on the antioxidant: a BHA batch in which 20 mg of the synthetic antioxidant was added into the oily fraction and another one in which 47.7 mg of *Melissa* was added into the water. The concentration of *Melissa* is equivalent to tenfold the antioxidant activity of BHA measured by the DPPH method. This amount was successfully used in a previous experiment in which the stability of the O/W emulsions that were rich in unsaturated fatty acids was tested at room temperature. (García-Íñiguez de Ciriano et al., 2010).

The W/O/W emulsions were carried out by a two step protocol that consisted on the preparation of a W/O simple emulsion and a further addition of this emulsion to a second aqueous phase. The simple W/O emulsion was prepared by adding the aqueous phase (14.04 g, that included 1 % carrageenan and the antioxidants: 34.99 mg of *Melissa* or 14.67 mg BHA) to the oil phase (38.61 g, that contained the hydrophobic surfactant: 0.03 % Polysorbate 80). Both phases were previously heated separately to 70 °C and then mixed. After the homogenization process (2 min, 16.000 rpm, Ultra-Turrax® T25basic) the emulsions were cooled down to room temperature, allowing the k-carrageenan to polymerize. In the second step, 42.1 g of water and 5.3 g of isolated soya protein were mixed (2 min). Afterwards 52.6 g of the previously W/O emulsion

were incorporated. The W/O emulsion was slowly added and gently stirred in order to obtain a stable W/O/W emulsion (3 min, 16.000 rpm, Ultra-Turrax® T25basic).

In W/O/W emulsions the oil/antioxidant ratio was the same as in the O/W emulsion.

2.3. Microscopic image analysis

The patterns of the emulsions were checked by a Nikon E-800 (Kawasaki, Japan) brightfield light microscopy with 40× magnification. The emulsions were observed after 24 hours of refrigeration at 4 °C. 4.0 µL of the emulsion was trickled through microsyringe over a drop of water (3 µL) previously deposited on a slide (76×26 mm) and covered after the extension with a coverslip (24×32 mm). The images were monitored and captured by digital Nikon DXM-1200. The analyses were performed in triplicate, and the particle sizes were determined from the images using the application image analySIS^D 5.0 Olympus BioSystems GmbH (Soft Imaging System GmbH). The distribution of particles sizes was calculated for diameters lower than 4 µm, between 4-7 µm, 7-10 µm and higher than 10 µm.

2.4. Accelerated oxidation study

To study the susceptibility of the emulsions to oxidation, the samples were subjected to an accelerated oxidation test under standardized conditions. Emulsions (60 g) were placed in 125 mL flasks which were kept sealed during 48 h under Schaal oven test conditions (65 °C). Samples were taken every 24 h for analysis. It has been previously established that 1 day of storage under this condition is equivalent to 1 month of storage at room temperature (Abou-Gharbia, Shehata, Youssef & Shahidi, 1996). The experiment was performed in duplicate.

2.5. Oil extraction

The method stated by Folch, Lees and Stanley (1957) was used for the extraction of the lipid fraction from the emulsions heated during 48 h. This lipid fraction was used for the analysis of the lipid profile, peroxide value, α-tocopherol and L-ORAC.

2.6. Lipid fraction analysis

Fatty acid profile was obtained by gas chromatography FID detection, previous preparation of the fatty acid methyl esters derivatives. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (AOAC, 2002a). A Perkin-Elmer Clarus 500 gas chromatograph (Madrid, Spain), equipped with a split-splitless injector, automatic autosampler, and coupled to a computerized system for data adquisition (TotalChrom, version 6.2.1) was used. It was fitted with a capillary column SP™-2560 (100 m×0.25 mm×0.2 μm; Sigma-Aldrich). The temperature of the injection port was 250 °C and detector was 260 °C, the oven temperature was programmed to increase from 170 to 200 °C at a rate of 10.0 °C/min and then at rate of 4.0 °C/min to 220 °C. The carrier gas was hydrogen, 30.0 psi. The sample size was 0.5 μL and the split ratio was 120. The quantification of individual fatty acids used heptadecanoic acid methyl ester as internal standard. The identification of the fatty acids was done by comparison of their retention times with those of pure fatty acid methyl esters.

2.7. Oxidative analysis

2.7.1. Peroxide value

Peroxide value (PV) was analyzed in the extracted fat according to the AOAC Official Method (AOAC, 2002b). Results were expressed in meq O_2/kg fat.

2.7.2. Conjugated dienes and trienes

A modification of the method described by Frankel, Huang, Aeschbach and Prior (1996) was used for the determination of conjugated dienes (CD) hydroperoxides. The lipid extract of the emulsion sample (0.1 g) was dissolved in 5.0 mL of methanol and vortexed for 30 seconds. The absorbance was measured at 234 nm using a Lambda 5 UV–Vis Spectrophotometer (Perkin Elmer, Paris, France). A filtration through 0.20 μm filter (Syringe-driven Filter Unit, Millex®) was applied just before the measurement to remove the protein fraction from the oil extracted sample and thereby diminish its spectrum interference in this region (Dimakou, Kiokias, Tsaprouni & Oreopoulou, 2007). The trienes were measured according to the method previously mentioned. The absorbance was measured at 280 nm. Results were measured as the increase in absorbance value.

2.7.3. TBARS (Thiobarbituric acid value)

TBARS values were determined on the emulsions according to the method described by Masqsood and Benjakul (2010) with slight modifications. Briefly, the TBARS reagent was prepared by mixing 15 % w/v trichloroacetic acid, 0.375 % w/v 2-thiobarbituric acid in 0.25 N hydrochloric acid. The emulsion sample (0.5 g), 0.5 mL of distillate water, 20 µL of BHT (1 %) and the TBARS reagent (2 mL) were vortexed in a centrifuge tube for 30 s, placed in a boiling water bath for exactly 15 min and then cooled down in an ice bath to room temperature. Cyclohexanone (4 mL) and ammonium sulphate (1 mL, 4 M) were added to the mixture and were vortexed for 30 s. The mixture was centrifuged at room temperature at 4000 rpm for 10 minutes. The supernatant was collected and the absorbance was measured at 532 nm. A calibration curve TEP (tetraethoxypropane) was done for quantification purposes, using the same procedure as with the sample. Results were expressed in mg of malondialdehyde (MDA) equivalents/kg oil.

2.8. Antioxidant capacity

2.8.1. α-tocopherol analysis

The α-tocopherol (α-TOH) content was determined by HPLC-UV analysis according to the method described by Berasategi, Barriuso, Ansorena and Astiasarán (2012). 0.1 g of the lipid extract of the emulsion sample and 0.1 mL of internal standard (α-tocopherol acetate 10 mg/mL solved in methanol) were filled up to 10 mL with previously warmed (30 °C) supergradient HPLC grade methanol. Dilution was vortexed for 30 s and filtered with 0.20 µm filter (Syringe-driven Filter Unit, Millex®). UV spectra were recorded on a Perkin Elmer UV-Vis Lambda 200 Series equipped with a photodiode array detector Series 200 PDA, using an analytical precolumn (3.8 mm × 8 mm with 4 mm × 3 mm of C18 cartridges, Phenomenex, California, USA) and a Supercosil LC18 column (25 mm × 4.6 mm, 5 µm particle size; Perkin Elmer Brownlee columns, Massachusetts, USA). A total of 10 µL of the sample was injected into the HPLC system and an isocratic elution with methanol/water (97:3) at 1.5 mL/min flow was performed. The UV acquisition was recorded at 292 nm for a 12 min run. Identification of α-tocopherol was done using the retention time of the pure standard compound (RT= 4.5 min) (Vitamin E 97 %) and its characteristic UV spectra.

The quantification was performed using a calibration curve previously plotted with tocopherol acetate (RT = 7.5 min) (Vitamin E acetate 98 %).

2.8.2. Determination of total phenolic content (TPC)

TPC was determined spectrophotometrically following the Folin-Ciocalteu colorimetric method described by Herchi et al. (2011) with slight modifications. A one-gram oil sample was weighed, dissolved in 10 mL hexane and transferred to a separatory funnel. Then, 20 mL of a methanol-water mixture (80:10 v/v) were added. After 3 min of shaking, the lower methanol-water layer was removed. The extraction was repeated twice and the methanol-water phases were combined. The methanol-water extract was driven to dryness in a rotary evaporator under vacuum at 40 °C. The dry residue was then dissolved in 1 mL of methanol. The composition of the reaction mixture was of 0.1 mL of suitable diluted sample, 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu´s reagent, and 1.5 mL of 20 % sodium carbonate anhydrous solution (added 2 min after the Folin-Ciocalteu´s reagent). After the initial mixing, the tubes stood at room temperature for 2 hours in the dark. The optical density of the blue-colored resulting solution was measured at 765 nm using a Lambda 5-UV-VIS spectrophotometer. The total phenolic content was expressed as mg gallic acid/kg oil, using the corresponding calibration curve. Absorbance measurements were made in duplicate for each diluted solution.

2.8.3. Lipophilic oxygen radical absorbance capacity (L-ORAC$_{FL}$) assay

L-ORAC$_{FL}$ assays were performed similarly to those described by Prior et al. (2003). The lipid extract of the emulsion sample (3-5 mg) was dissolved in 400 µL acetone and then diluted with 4.6 mL of a 7 % RMCD solution (1:1, acetone/water, v/v). Samples were shaken at room temperature on an orbital shaker operating at 180 rpm for 1 hour before use. A 0.5 M stock solution of Trolox was prepared in 10 mM phosphate buffer, and divided into 1 mL aliquots, which were stored at -20 °C until use. A new set stock Trolox vials were taken from the freezer daily for the preparation of the samples on order to accomplish the standard-addition procedure (0, 5, 12, 20 µM) and the quality controls (12.5 and 50 µM). The 7 % RMCD solution was used as blank, to dissolve the Trolox quality controls and to prepare the samples. To conduct the L-ORAC$_{FL}$ assay, 40 µL of the lipophilic solution and 120 µL of the fluorescein solution (132.5 nM)

were added to the 96 well black plate. The microplate was equilibrated (5 min, 37 °C) then the reaction was initiated by the addition of AAPH (40 µL, 150 mM); readings were obtained immediately, in a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The antioxidant capacity was expressed as mols Trolox equivalent/100 g oil.

2.9. Data analysis

Mean and standard deviation of all replicates were calculated. For each parameter, one factor ANOVA with Tuckey-b *post hoc* multiple comparisons was used in order to evaluate the significant differences through time. The correlation between peroxide value and conjugated dienes in linseed oil were evaluated by Pearson's correlation test. Within each type of oil and type of emulsion the differences between antioxidants were evaluated by Student t-test.

The statistical analysis of data was done using the SPSS 15.0 program (SPSS, INC., Chicago, IL, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

3. Results

The microscopic images of the two types of emulsions confirmed the adequate structure of both the simple (O/W) and the double emulsion (W/O/W) (**Fig. 1**). In the case of the double emulsion, a combination of single inner droplets and several inner droplets was found, as described by Florence and Whitehill (1981) for this type of emulsions. The mean particle diameter in the O/W emulsions ranged from 2.92-12.71 µm, while the diameter of W/O/W emulsions ranged from 2.76-13.06 µm. The size distribution of the oil droplets was, in the O/W emulsions, 11.79 % (<4 µm), 34.96 % (4-7 µm), 29.27 % (7-10 µm) and 23.98 % (>10 µm). In the W/O/W emulsions the size distribution was 21.79 % (<4 µm), 42.31 % (4-7 µm), 8.97 % (7-10 µm) and 26.92 % (>10 µm). With this distribution the physical stability was maintained along the experiment and no syneresis was noticed during the whole accelerated oxidation test.

The mean value for total fat content of the emulsions was 52.6 g/100 g and 38.6 g/100 g for O/W and W/O/W samples emulsions, respectively. The fatty acid

profile of the emulsions after 48 h at 65 °C was compared to the profile of the oils in order to elucidate potential undergoing changes during the accelerated oxidation study. The α-linolenic and linoleic acid were the predominant PUFA of the total fatty acid for the linseed oil, accounting for 52.9 and 14.8 g/100 g oil respectively. On the other hand, oleic acid (78.3 g/100 g oil), as expected was the most prevalent MUFA in olive oil. No significant modifications were found in the two representative fatty acids of both emulsion oils after the accelerated oxidation study compared to those in the unheated oil (data not shown).

Primary oxidation products were measured by PV (**Fig. 2**). The initial oxidation status of the 8 types of emulsions showed significant higher PV for olive oil containing samples compared to the linseed oil ones, i.e. 14.28 meq O_2/kg oil and 2.86 meq O_2/kg oil for BHA:O/W emulsions with olive oil and linseed oil, respectively. PV values in olive oil emulsions (including O/W and W/O/W) kept their values between 11.7–17.6 meq O_2/kg oil without showing statistical differences at the end of the treatment between the two antioxidants ($p<0.05$). In linseed oil emulsions, gradual increases in PV were observed, with different behaviour depending on the antioxidant and the type of emulsion. In O/W emulsions, aqueous *Melissa* extract seemed to be more effective than BHA ($p<0.05$) delaying the increase in PV. Only a slight increase was noticed after 48 h of treatment (up to 6.32 meq O_2/kg oil), while emulsions with BHA showed 14.14 meq O_2/kg oil. Regarding W/O/W emulsions, *Melissa* extract showed significantly higher values at 48 h (10.61 meq O_2/kg oil) than those with BHA (8.57 meq O_2/kg oil) ($p<0.05$) for linseed oil emulsions.

The analysis of primary oxidation products was completed with the conjugated dienes and trienes measurement (**Table 2**). Results showed that CD value tended to increase with storage time in all cases ($p<0.05$). Moreover, W/O/W emulsions showed lower CD increments during the storage than their O/W counterparts, whatever the antioxidant was. CD behaved similarly in linseed oil emulsions (with increments during storage of 1.05-1.11 for O/W and 0.76-0.79 for W/O/W ones) and olive oil emulsions (increments O/W: 1.07-1.11; W/O/W: 0.48-0.72). Increases in CT were also observed in all emulsions during storage. BHA emulsions showed higher increments in

the CT values than the *Melissa* emulsions in linseed oil samples in both types of systems (O/W: 0.14 and 0.06; W/O/W: 0.14 0 and 0.09).

Secondary oxidation products were monitored by the TBARS test (**Fig. 3**). As expected, very different behaviour of TBARS depending on the type of oil and emulsion was detected. In olive oil emulsions, TBARS value was stable and low in O/W emulsions, with no significant differences between *Melissa* and BHA throughout the accelerated oxidation study. In W/O/W emulsions, a trend toward higher values compared to O/W emulsions was noticed, without differences between antioxidants. Additionally, in linseed oil emulsions TBARS values gradually increased during the storage period, being the increase faster in the first 24 hours in O/W emulsions than in W/O/W emulsions. However, after 48 h of storage, Mel:O/W stabilized their TBA value, while the other three types of emulsions continued increasing. A maximum of 19.79±1.22 mg/kg was found for BHA:O/W, which was 1.74-fold higher than Mel:O/W, 1.35-fold higher than BHA:W/O/W and no significant differences in Mel:W/O/W.

The initial antioxidant capacity measured by L-ORAC (**Fig. 4**) showed higher values for linseed oil emulsions containing samples (8315 μmol trolox equivalent/100 g oil on average) than those for olive oil (1978 μmol trolox equivalent/100 g oil). Storage significantly decreased the antioxidant capacity of all the emulsions particularly in the case of linseed oil. Data indicated that the antioxidant capacity of different emulsions differs depending on the type of antioxidant. Moreover, in linseed oil emulsions, *Melissa* extract showed a faster decrease in the antioxidant capacity than BHA during storage, in contrast to olive oil emulsions. In W/O/W emulsions, the decrease in the antioxidant capacity was slower than what was observed in O/W emulsions.

Results for α-TOH are shown in **Fig. 5** Linseed oil emulsions were naturally higher in α-TOH (64.93 mg α-tocopherol/100 g oil on average) compared to olive oil ones (31.52 mg α-tocopherol/100 g oil on average). Once again, in linseed oil, a gradual decrease was observed in all the emulsions with different behaviour depending on the antioxidant used and the type of emulsion. Higher α-TOH content was detected in *Melissa* extract containing samples compared to BHA. No changes or a slight trend

to decrease was noticed during time. In olive oil, the two antioxidants behaved similarly during time, maintaining constant values in the W/O/W emulsions and slightly increasing in the O/W emulsions.

4. Discussion

W/O/W emulsions contain much lower amount of fat than O/W ones (14 g less of fat per 100 g emulsion), which is an aspect to be taken into account when they are used as ingredients for functional foods. Although there is widespread recognition of the perceived value of W/O/W emulsions in contributing to the development of reduced-fat products and as vehicles for the delivery of nutrients, the potential of double emulsions in the food technology has yet to be fully elucidated (Dickinson, 2011).

The amount of fat in functional foods is a relevant factor for nutritional purposes and also its fatty acid profile. The lack of differences in the fatty acid profile of all emulsions during the treatment (0-48 h, 65 °C) pointed out that the nutritional value of the oil was not affected by the heat treatment, regardless of the type of emulsion.

Furthermore, both antioxidants were effective in keeping the supply of the main fatty acids in both types of emulsions, α-linolenic and oleic acid in linseed oil and olive oil emulsions, respectively. Consequently, irrespective of the type of emulsion and antioxidant used, both oils provided an interesting fatty acid profile, leading to excellent ratios from the nutritional standpoint.

García-Íñiguez de Ciriano et al. (2010) in a previous work concluded about the need of the use of antioxidants in highly unsaturated O/W emulsions in order to control lipid oxidation. Regarding the oxidation status, a distinct behaviour through the accelerated oxidation study was noticed between the two oils, as it was expected, due to their different profile. Thus, their response to the two antioxidants and the type of emulsion applied was studied independently. Whereas olive oil emulsions tend to remain unaltered during heating irrespective of the antioxidant and type of emulsion applied, linseed oil emulsions were more sensitive to these two variables under study. Discussion of data will take into account these relevant aspects.

4.1. Olive oil emulsions

In olive oil emulsions, the analysis of primary oxidation products pointed out that PV remained, in every condition, below the maximum acceptable level by the Commission Regulation (EC) No 1989/2003 set at <20 meq O_2/kg oil. Dienes increased linearly during storage in all cases, without differences between antioxidants during the heating treatment. Trienes and TBARS, however, showed higher values for W/O/W emulsions compared to O/W ones. This finding can indeed be attributed to the different procedures applied to obtain the emulsions. The preparation of the W/O/W emulsions includes a 70 °C heating treatment of the oil, which could have contributed to promote a slight degree of lipid oxidation.

It is worthy to mention that in general CD, CT and TBARS are inherently insensitive to MUFA, as oleic acid hydroperoxides contain less than two double bonds. In general, significant amounts of TBARS are only formed when fatty acids with 3 or more double bonds are involved (Fennema, 1996). This fact could lead to underestimation of oxidation in highly monounsaturated lipids (Waraho, Cardenia, Decker & McClements, 2010). In our study, these three parameters were able to reveal differences among emulsions.

Unexpectedly, the α-TOH content did not decrease during the accelerated oxidation study of olive oil emulsions, moreover a slightly increase of the content was noticed in the case of O/W emulsions. It could be related to a synergism process among different antioxidants, between α-TOH and polyphenols in which olive oil is rich. A number of studies describe a synergic effect between α-TOH and polyphenols (Zhu, Huang, Tsang & Chen, 1999; Pedrielli & Skibsted, 2002), whereby the regeneration of α-TOH from its one-electron-oxidized form by some flavonoids was suggested, in analogy to the well-known synergism between α-TOH and ascorbate (Niki, 1987; Bisby & Parker, 1995). This process could have also occurred in O:W/O/W emulsions, but it would have been masked by the fact that these emulsions were subjected to higher oxidative processes, which are more antioxidant demanding.

However, total antioxidant capacity measured by L-ORAC, showed a slight decrease in both types of emulsions (O/W and W/O/W), probably in response to the

increased oxidation trend that was observed during heating by dienes and trienes analysis. It points to the fact that the total antioxidant capacity of olive oil is dependent on other types of compounds, different from α-TOH, which was hardly modified during heating. In fact, phenolic compounds greatly contribute to olive oil stability, even more than other compounds (Pellegrini, Visioli, Buratti & Brighenti, 2001; Farhoosh, Sharif & Rafire, 2011). The presence of tyrosol, hydroxytyrosol and catechol has been well described in olive oil (Choe & Min, 2009), and a high polyphenol concentration was detected in the olive oil used in this work (253.7 mg gallic acid/kg oil).

4.2. Linseed oil emulsions

As previously stated, linseed oil containing emulsions were far more sensitive than olive oil emulsions with respect to the use of different antioxidants and emulsion system during the accelerated oxidation study.

Although the PV and TBARS pointed out a favorable initial oxidation state compared to olive oil, a rapid increase in these parameters was observed during the storage due to the high content in polyunsaturated fatty acids, which are more likely to form hydroperoxides. It has been reported that the rate of oxidation of α-linolenic and linoleic acid is 20 and 10-fold the value exhibited by oleic acid, respectively (Fennema, 1996). Furthermore, a positive correlation was found between PV and CD (R^2= 0.915), in agreement to the results found by Marmesat, Morales, Velasco, Ruiz-Mendez and Dobarganes (2009). The water *Melissa* extract was far more effective in lagging the increase in PV in O/W, whereas BHA was more efficient in W/O/W emulsions. *Melissa* extract is rich in rosmarinic acid (García-Íñiguez de Ciriano et al., 2010), a polar molecule known by its antioxidant activity, which effectively delays the formation of oxidation products in O/W emulsions. As a chain-breaking antioxidant, it maintained a lag-phase during which the substrate was not substantially oxidized, that continued until the antioxidant is completely consumed. However BHA since it is placed in the oily phase, due to its lipophilic character, was not able to do so on the O/W emulsions.

In the case of W/O/W emulsions it was only after 48 h heating when there was a different behaviour among both antioxidants. BHA was dispersed in the water internal phase and it is hypothesised that is slowly released into the oily phase. As the oil phase of W/O/W emulsion is commonly regarded as a liquid membrane separating the internal and external aqueous phases (Dickinson, 2011), BHA could efficiently exert its antioxidant activity in the oily phase along its slow release. In this case, *Melissa* extract rich in rosmarinic acid, exerts directly its antioxidant activity in the oily/aqueous interface. A linear increment of TBARS and PV was observed from the first day of heating until the end. This finding confirmed the effectiveness of *Melissa* extract protecting the oil droplets when the antioxidant is located in the aqueous environment.

α-TOH and L-ORAC data at initial time were significantly higher in linseed oil compare to olive oil containing emulsions (2 fold and 4 fold, respectively). The increasing oxidation status shown by primary and secondary oxidation products during heating was reflected in a decrease of L-ORAC, particularly evident in *Melissa* containing emulsions. However, the tocopherol loss during the treatment was hardly appreciated. Thus, it seemed that the compounds that accounted for the main L-ORAC activity were different from tocopherol, which should be confirmed in further experiments.

5. Conclusions

Olive oil emulsions remained unaltered during a 48 h accelerated oxidation study and a low influence of the type of antioxidant and location was detected. Linseed oil emulsions showed a high initial antioxidant status, although they were significantly affected by the accelerated oxidation study. In that case, differences were found in the effectiveness of the used antioxidants depending on the emulsions: the hydrophilic antioxidant (*Melissa*) was more efficient in O/W emulsions, whereas the lipophilic antioxidant (BHA) was more effective in W/O/W ones. No clear distinction was observed for the stability of W/O/W emulsions compared to O/W ones for each antioxidant used.

6. Acknowledgements

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Table 1. Lipid profile of the oils used in the emulsions (g fatty acids/100 g oil mean ± standard deviation).

	Linseed Oil	Olive Oil
Caprilic C8:0	nd	nd
Capric C10:0	nd	nd
Lauric C12:0	0.02 ± 0.01	nd
Myristic C14:0	0.06 ± 0.01	0.04 ±0.02
Palmitic C16:0	5.88 ± 0.15	9.85 ± 0.11
t-palmitoleic C16:1	nd	0.12 ± 0.01
Palmitoleic C16:1	0.10 ± 0.01	0.58 ± 0.09
Stearic C18:0	4.62 ± 0.09	2.91 ± 0.04
Elaidic C18:1	0.11 ± 0.03	0.29 ± 0.03
Oleic C18:1 (ω-9)	20.1 ± 0.46	78.3 ± 0.02
Vaccenic C18:1 (ω-7)	0.80 ± 0.02	2.44 ± 0.06
t- linoleic C18:2	0.01 ± 0.01	0.04 ± 0.01
c,t- linoleic C18:2	0.06 ± 0.01	0.03 ± 0.01
t,c- linoleic C18:2	0.10 ± 0.01	0.05 ± 0.01
Linoleic C18:2 (ω-6)	14.8 ± 0.07	3.93 ± 0.04
Arachidic C20:0	0.17 ± 0.01	0.26 ± 0.02
γ-linolenic C18:3 (ω-6)	0.22 ± 0.01	0.16 ± 0.01
Eicosenoic C20:1 (ω-9)	nd	nd
α-linolenic C18:3 (ω-3)	52.9 ± 0.74	0.49 ± 0.02
Behenic C22:0	nd	0.06 ± 0.01
Brassidic C20:1	nd	nd
Erucic C22:1	nd	nd
Eicosatrienoic C20:3 (ω-3)	nd	nd
Arachidonic C20:4 (ω-6)	nd	0.55 ± 0.03
Eicosapentaenoic C22:5 (ω-3)	nd	0.05 ± 0.01
Nervonic C24:1 (ω-9)	nd	0.02 ± 0.01
Docosatrienoic C22:3 (ω-3)	nd	nd
Docosapentaenoic C22:5 (ω-3)	nd	0.11 ± 0.01
Lignoceric C24:0	nd	0.05 ± 0.01
SFA	10.8 ± 0.22	13.1 ± 0.15
MUFA	20.9 ± 0.51	81.3 ± 0.26
PUFA	68.0 ± 0.79	5.29 ± 0.01
ω-3	52.9 ± 0.20	0.53 ± 0.02
ω-6	15.1 ± 0.15	4.75 ± 0.01
ω-6/ω-3	0.28 ± 0.01	8.89 ± 0.34
trans	0.28 ± 0.04	0.53 ± 0.03

Table 2. Effect of accelerated storage conditions (65 °C for 48 hours) in O/W and W/O/W emulsions, made with olive oil and linseed oil, for *Melissa* extract and BHA containing samples on conjugated dienes (ABS_{235}) and trienes (ABS_{280}).

		O/W		W/O/W	
	Time	*Melissa*	BHA	*Melissa*	BHA
		DIENES (ABS_{235})			
LINSEED OIL	0 h	2.38 ± 0.10^{Aa}	2.41 ± 0.07^{Aa}	2.54 ± 0.11^{Aa}	2.57 ± 0.19^{Aa}
	24 h	3.41 ± 0.01^{Bc}	2.88 ± 0.05^{Bab}	2.73 ± 0.12^{Aa}	2.92 ± 0.06^{Bb}
	48 h	3.43 ± 0.02^{Ba}	3.52 ± 0.31^{Ca}	3.30 ± 0.05^{Ba}	3.36 ± 0.02^{Ca}
OLIVE OIL	0 h	2.15 ± 0.03^{Aa}	2.30 ± 0.09^{Aa}	2.50 ± 0.08^{Ab}	2.64 ± 0.06^{Ab}
	24 h	3.17 ± 0.27^{Ba}	2.82 ± 0.14^{Ba}	2.90 ± 0.08^{Ba}	2.82 ± 0.09^{ABa}
	48 h	3.26 ± 0.18^{Ba}	3.37 ± 0.06^{Ca}	3.22 ± 0.04^{Ca}	3.12 ± 0.24^{Ca}
		TRIENES (ABS_{280})			
LINSEED OIL	0 h	0.41 ± 0.02^{Aa}	0.49 ± 0.02^{Bc}	0.41 ± 0.01^{Aab}	0.45 ± 0.02^{Ab}
	24 h	0.43 ± 0.02^{ABa}	0.44 ± 0.01^{Aa}	0.48 ± 0.04^{Ba}	0.47 ± 0.01^{Aa}
	48 h	0.47 ± 0.02^{Ba}	0.58 ± 0.01^{Cb}	0.50 ± 0.02^{Ba}	0.59 ± 0.00^{Bb}
OLIVE OIL	0 h	0.44 ± 0.10^{Aa}	0.68 ± 0.09^{Aab}	0.67 ± 0.17^{Aab}	0.77 ± 0.04^{Ab}
	24 h	0.45 ± 0.04^{Aa}	0.67 ± 0.01^{Ab}	0.73 ± 0.14^{Ab}	0.95 ± 0.10^{Bc}
	48 h	0.69 ± 0.09^{Ba}	0.86 ± 0.06^{Bb}	0.74 ± 0.04^{Aab}	1.09 ± 0.05^{Bc}

Fig. 1. Brigtfield optical microscopy images of (a) O/W emulsion and (b) W/O/W emusion. Bar equals to 20 µm.

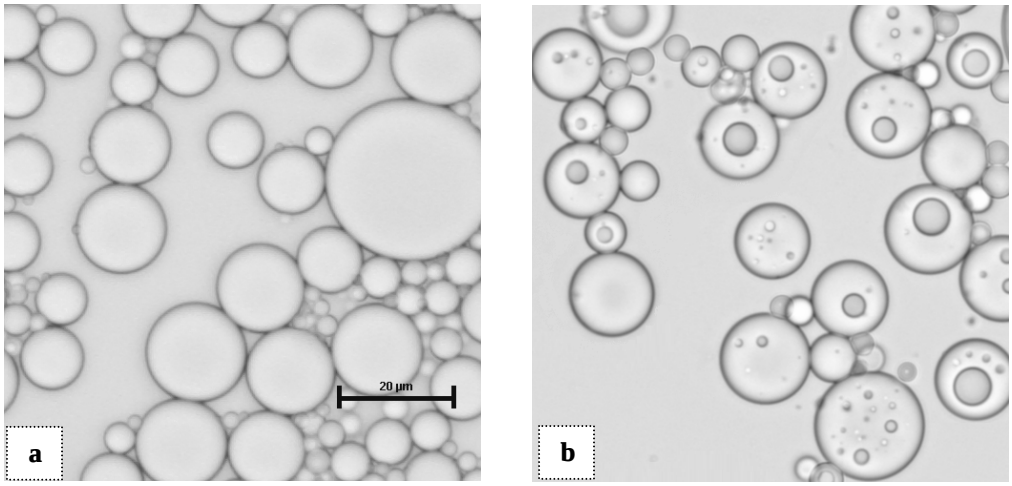


Fig. 2. Effect of adding *Melissa* extract and BHA on PV (meq O_2/kg oil) of O/W and W/O/W emulsions made with olive oil and linseed oil under accelerated storage conditions (65 °C for 48 h). Different capital letters denote significant differences for *Melissa* extract containing samples and different small letters denote significant differences for BHA containing emulsions ($p<0.05$). Level of significance for the Student t-test comparing antioxidants: ns= not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

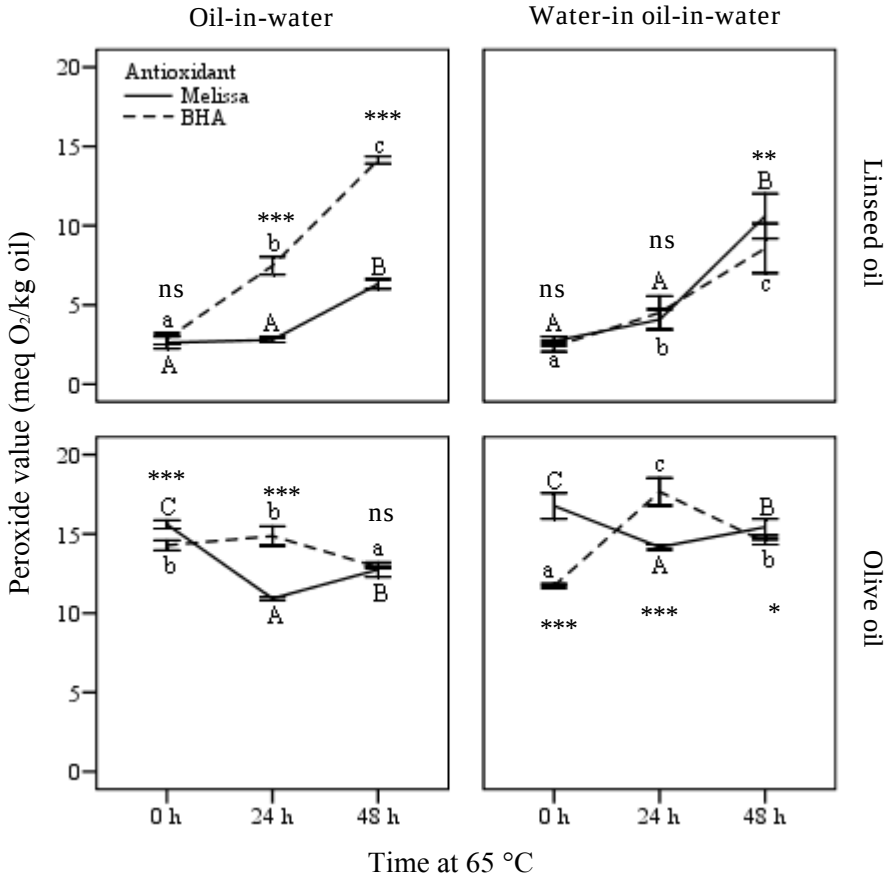


Fig. 3. Effect of adding *Melissa* extract and BHA on TBARS (mg MDA/kg oil) of O/W and W/O/W emulsions made with olive oil and linseed oil under accelerated storage conditions (65 °C for 48 h). Different capital letters denote significant differences for *Melissa* extract containing samples and different small letters denote significant differences for BHA containing emulsions ($p<0.05$). Level of significance for the Student t-test comparing antioxidants: ns= not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

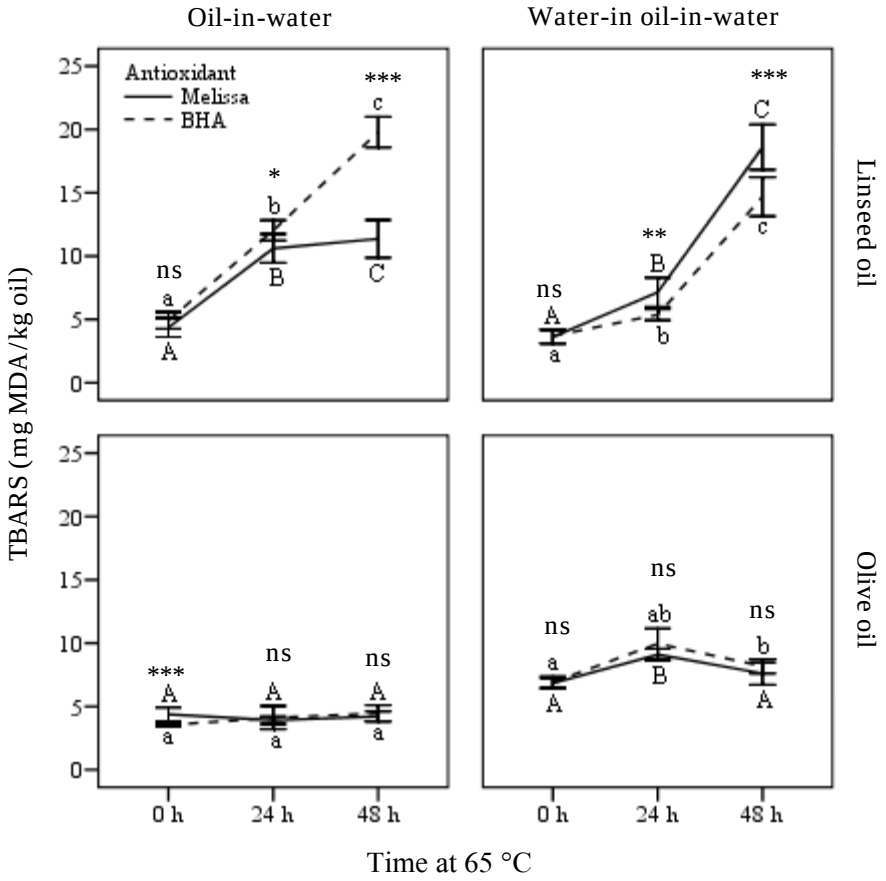


Fig. 4. Effect of adding *Melissa* extract and BHA on ORAC (µmol Trolox eq/100 g oil) of O/W and W/O/W emulsions made with olive oil and linseed oil under accelerated storage conditions (65 °C for 48 h). Different capital letters denote significant differences for *Melissa* extract containing samples and different small letters denote significant differences for BHA containing emulsions ($p<0.05$). Level of significance for the Student t-test comparing antioxidants: ns= not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

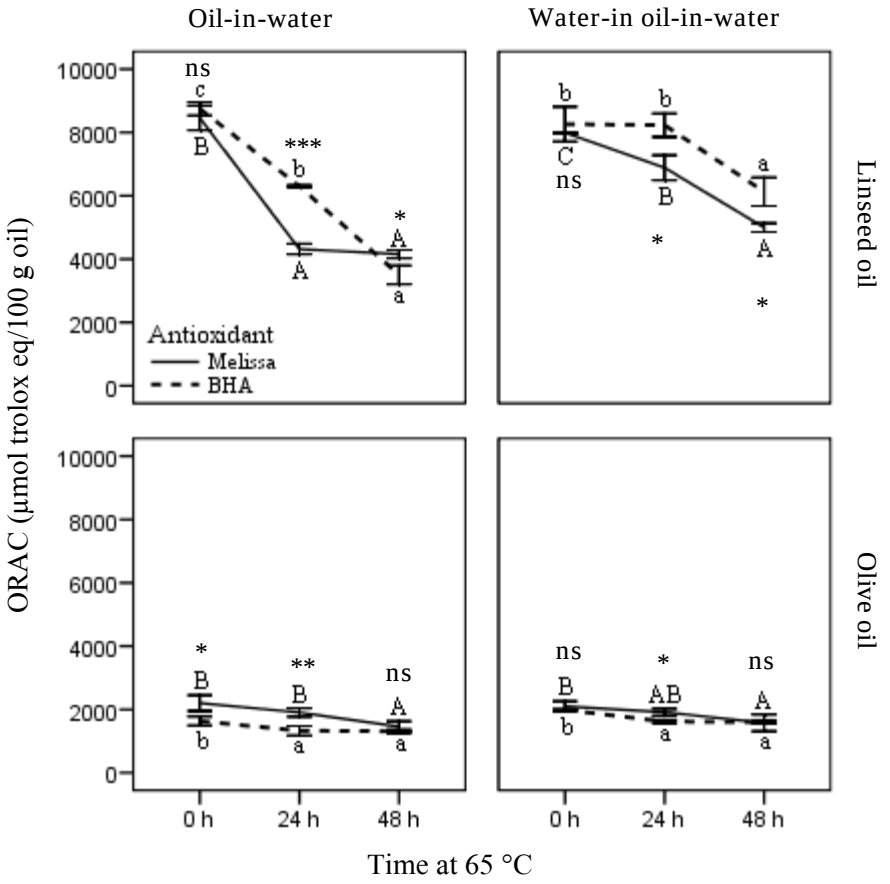
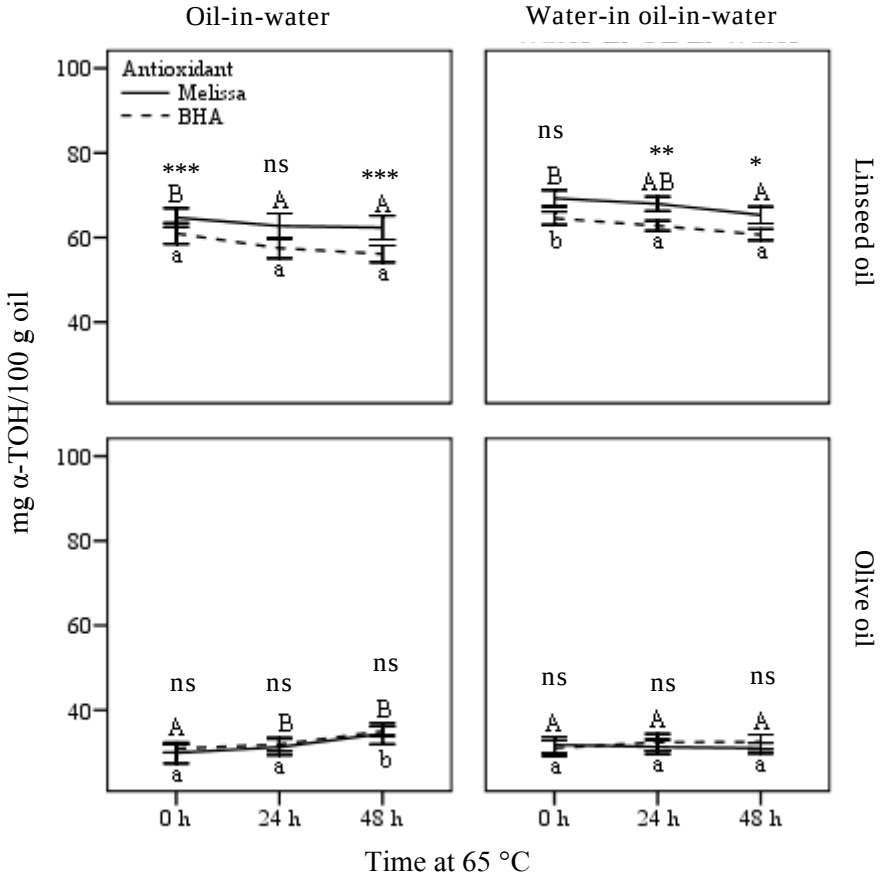


Fig. 5. Effect of adding *Melissa* extract and BHA on α-tocopherol (mg α-TOH/100 g oil) of O/W and W/O/W emulsions made with olive oil and linseed oil under accelerated storage conditions (65 °C for 48 h). Different capital letters denote significant differences for *Melissa* extract containing samples and different small letters denote significant differences for BHA containing emulsions ($p<0.05$). Level of significance for the Student t-test comparing antioxidants: ns= not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.



In preparation

3

Antioxidant effect of water and acetonic extracts of *Fucus vesiculosus* on oxidative stability of skin care emulsions

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Abstract: A water and an acetonic extract of *Fucus vesiculosus* were evaluated as potential natural antioxidant source in skin care emulsions. To assess the efficacy to inhibit lipid oxidation against photo- and thermoxidation, they were stored in darkness and room temperature as control conditions, and compared to those stored under accelerated conditions (light and room temperature, or darkness and 40 °C). The presence of extracts in the skin care emulsions induced remarkable colour changes in the presence of light, but mainly because of the high temperature. High temperature also caused higher increments in the droplet size of the emulsions. Tocopherol content, peroxide value and volatile compounds were evaluated during the storage and, whereas both water and acetonic extracts, showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the water one showed antioxidant activity against photooxidation.

Key words: cosmetic emulsion, brown algae, lipid oxidation, skin care emulsion.

1. Introduction

Natural derived ingredients combined with carrier agents, preservatives, surfactants, humectants and emulsifiers are commonly used in skin care products. A natural ingredient is based on using botanically sourced ingredients currently existing in nature (such as herbs, roots, essential oils and flowers), in order to reduce synthetic compounds in the final product. Nowadays, there is an increasing interest in natural

ingredients (Balboa, Soto, Nogueira, González-López, Conde, Moure & Domínguez, 2014) because of the negative perception of the synthetic ones. Thus, the evolution of the cosmetic industry to adapt products to the trends of the XXI century consumer has given rise to new challenges, and to the need of considering the consequences that may imply some of the novel proposals.

Emulsions are the most common type of delivery system used in cosmetics, with creams and lotions being the best-known ones. Skin care emulsions enable a wide variety of active ingredients to be quickly delivered to skin. In this sense, there are many factors that can potentially influence in the physical and oxidative stability of these emulsions, such as fatty acid and ionic composition, type and concentration of antioxidants and prooxidants, emulsion droplet size and interfacial properties (McClements & Decker, 2000; Raikos, 2010; Naji & Karazhiyan, 2012; Xu, Wang, Jiang, Yuan & Gao, 2012).

Lipid oxidation can occur in skin care emulsions (Thomsen et al., 2015) and can be triggered or enhanced by light and/or high temperatures. Moreover, the high content of vegetable oils in skin care emulsions´ formulations might contribute to induce lipid oxidation, causing unpleasant odours, colour changes and in consequence, low quality products (Thomsen et al., 2015).

Therefore, it's important to limit lipid oxidation and extend the shelf life of skin care products using natural antioxidants. In addition, some natural antioxidants can give the skin product added functional value. It has recently been suggested that the use of natural antioxidants, such as vitamins A and E, in skin care formulations could provide a preventive therapy for skin photoaging (Briganti & Picardo, 2003; Mukherjee et al., 2011).

Vitamin E is one of the most used natural antioxidants in skin care products, usually added due to its radical scavenging activity (Mukherjee et al., 2011). However, in highly complex matrices containing metals, such as cosmetic products (Borowska & Malgorzata, 2014), other antioxidant properties such as metal chelating ability might be of relevance. Therefore, to stabilise lipid rich skin care products, extra addition of antioxidants might be necessary.

Natural derived antioxidants from various plants and marine algae have shown great potential in improving oxidative stability of these kinds of products. The high variety of bioactive compounds, such as pigments, sulphated polysaccharides, proteins and polyphenols, have been described for different types of brown and red algae by Farvin and Jacobsen (2013). Especially, the high content of phlorotannins, the major polyphenolic compounds in brown algae, has been related to high antioxidant activity and these compounds can work both as radical scavengers and metal chelators (Chkhikvishvili & Ramazanov, 2000; Wang et al., 2010). Furthermore, phlorotannins have been shown to possess biological activity of potential medicinal value making them valuable in development of nutraceutical, pharmaceutical and cosmetic products (Mancini-Filho et al., 2009; Balboa et al., 2013; Thomas & Kim, 2013). Balboa et al. (2014) successfully used a *Sargassum muticum* extract to improve the oxidative stability of oil-in-water model emulsions with cosmetic purposes. Farvin and Jacobsen (2013) found that compared to other brown algae, *Fucus vesiculosus* had higher phenolic content and exhibited the highest antioxidant activity *in vitro*. Wang et al. (2009; 2012) found that the high *in vitro* antioxidant activity of *F. vesiculosus* extracts were related to a high phenolic content and identified the phlorotannin tetramer, fucodiphloroethol E, to be the main contributor to this activity. Moreover, Hermund et al. (2015) evaluated *F. vesiculosus* extract as potential antioxidant against lipid oxidation in fish-oil-enriched food emulsions, obtaining promising results.

Whereas the *in vitro* antioxidant properties of *F. vesiculosus* have been widely studied (Wang et al., 2010; Farvin et al., 2015), applied studies on the antioxidant activity of *F. vesiculosus* extracts to hinder lipid oxidation are sparse (Habeebullah, Nielsen & Jacobsen, 2010; Hermund, et al., 2015). The aim of this study was to evaluate the antioxidant properties of two extracts obtained from Icelandic brown algae *F. vesiculosus* (water and acetone extract) in terms of assessing their efficacy to inhibit lipid oxidation during storage of skin care emulsions, at room temperature in darkness and under accelerated conditions (photo- and thermooxidation).

2. Materials and methods

2.1. Materials

The ingredients for the formulation of the skin care emulsion were purchased from Urtegaarden (Allingåbro, Denmark). All solvents used were of high-performance liquid chromatography (HPLC) grade and purchased from Lab-Scan (Dublin, Ireland). External standards were purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Extraction

The two extracts used in this study were provided by Matís in Iceland and have been used in previous studies (water extract previously used by Hermund et al. (2015) and the acetone extract by Honold et al. (2015)).

The extractions were carried out according to Wang et al. (2009, 2010, 2012). The seaweed was collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in 2011. At the collecting spot the seaweed was washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were rinsed with tap water and wiped with paper towel. The samples were freeze-dried, pulverised into powder and stored at -80 °C prior to extraction.

The extracts were produced as follows: Five grams of the algal powder was mixed with 100 mL of distilled water or 70 % aqueous acetone (v/v), Hereafter these were incubated on a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2168 g for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. Each extraction was conducted in duplicate and stored at -80 °C until use.

The water extract (WE) composition was as follows: phenolic content, 14.73 g gallic acid equivalent/100 g extract; chlorophylls, 0.46 μg/mg extract; xanthophylls, 2.17 μg/mg extract; carotenes, 1.72 μg/mg extract; iron, 4.39 μg/mg extract and copper, 0.91 μg/mg extract. The acetone extract (AE) composition was as follows: phenolic content, 18.55 g gallic acid equivalent/100 g extract; chlorophylls, 0.85 μg/mg extract; xanthophylls, 0.75 μg/mg extract; iron, 9.53 μg/mg extract and copper, 1.21 μg/mg extract.

2.3. Skin care emulsion production and storage conditions

The two *F. vesiculosus* extracts, water (WE) and acetone extract (AE) were applied to the skin care emulsion in two concentrations (1 and 2 mg/g of skin care emulsion). These amounts were successfully used in a previous experiment in which the stability of a fish-oil-enriched milk and mayonnaise were tested under different storage conditions (Hermund et al., 2015). Thus, five different types of skin care emulsions were finally obtained: RF (reference, without extract), WE1, WE2, AE1, AE2. **Table 1** shows all the ingredients for the fat phase and the aqueous phase (including the extract, when used). They were weighted in individual pots and heated to 70-75 °C. The oily phase was slowly poured into the water phase under powerful steering (9.500 rpm, Ultra-Turrax® T25basic). After the homogenization process, the emulsions were cooled to room temperature. The skin care emulsions were packed in transparent 50 mL containers. Then the samples were stored at different conditions: room temperature (21.2±0.7 °C) and darkness (A0), room temperature (24.4±0.3 °C) and light (A+) and high temperature (42.3±1.5 °C) and darkness (H0). Five different batches were prepared depending on the type and concentration of the extract, and stored at three different conditions. The samples were analysed at different storage times (0, 7, 21, 35 and 56 days).

2.4. Lipid extraction

Lipids were extracted from the skin care emulsions according to the method described by Iverson, Lang and Cooper (2001) based on the method of Bligh and Dyer (1959). For each sample, two oil extractions were performed and analyzed independently. Resulting lipid extracts were used as starting material for the analysis of peroxides, fatty acid composition and tocopherol content.

2.5. Fatty acid composition (fatty acid methyl esters, FAME)

The fatty acid composition of the oil phases was determined after fatty acid methylation and analysis by GC-FID. The Bligh and Dyer (1959) lipid extract from skin care emulsion, corresponding to 30-60 mg lipid, were weighted in vials. 100 µL toluene, 200 µL heptane with 0.01 % (v/v) BHT and 100 µL internal standard (C23:0)

(2 % w/v) were added. One mL of BF3 in methanol was added to the lipid extract mixture and the lipids were methylated in a one-step procedure using a microwave oven (Multiwave3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave were 5 min at 500 Watt followed by 10 min cooling. The fatty acid methyl esters (FAMEs) were washed with 1 mL saturated NaCl and 0.7 mL heptane with 0.01 % (v/v) BHT. The heptane phase was transferred to a GC vial and FAMEs were analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to AOCS (1998). For separation DB127-7012 column (10 m × ID 0.1 mm × 0.1 µm film thickness, Agilent Technologies, Palo Alto, CA, USA) was used. Injection volume was 0.2 µL in split mode (1:50). The initial temperature of the GC-oven was 160 °C. The temperature was set to increase gradually being as follows: 160-200 °C (10.6 °C/min), 200 °C kept for 0.3 min, 200-220 °C (10.6 °C/min), 220 °C kept for 1 min, 220-240 °C (10.6 °C/min) and kept at 240 °C for 3.8 min. The measurements were performed at storage day 0 and 56, in duplicates, and the results were given in % area.

2.6. Tocopherol content

The lipid extracts from the skin care emulsions were evaporated under nitrogen and dissolved in heptane. The samples were analysed by HPLC (Agilent 1100 Series, Agilent Technology) according to AOCS (1998) to quantify the contents of α-, β-, γ- and δ-tocopherols. These tocopherol homologues were separated using a silica column (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 µm silica film). A stock solution added 10 mg tocopherols (mixture of α-, β-, γ- and δ-tocopherols) per litre was prepared and used for quantification. The analyses were done in duplicate and results were reported as µg tocopherol/g skin care emulsion.

2.7. Peroxide value (PV)

PVs of the lipid extract of the skin care emulsions were determined at all sampling points. This was done according to the method by Shantha and Decker (1994), based on the formation of an iron-thiocyanate complex. The coloured complex was measured spectrophotometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific

Instruments, Columbia, MD, USA). The analyses were done in duplicate and the results were expressed in milliequivalents oxygen per kg oil (meq O_2/kg oil). In addition, for every sample, oxidation rates were calculated as follows:

$$Oxidation\ \ rate\,(\%) = \frac{\left(PV_{day35or56} - PV_{day1}\right)}{PV_{day1}} \times 100$$

2.8. Volatile compounds (VC)

Tenax GR™ packed tubes were used to collect volatile compounds by dynamic headspace. The collection of the volatile compounds was carried out using 4 g of emulsion (incl. 30 mg int. std. (30 µg/g of 4-methyl-1-pentanol in ethanol)) and 20 mL of distilled water. The volatile secondary oxidation products were collected at 45 °C under purging with nitrogen (flow of 150 mL/min for 30 min), followed by flushing the Tenax GR™ packed tube with nitrogen (flow of 50 mL/min for 5 min) to remove water. The trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin- Elmer, Norwalk, CT) connected to an Agilent 5890 IIA model gas chromatograph equipped with a HP 5972 mass selective detector. The settings for the MS were: electron ionization mode, 70 eV, mass to charge ratio (*m/z*) scan between 30 and 250. Chromatographic separation of volatile compounds was performed on a DB1701 column (30m × ID 0.25 mm × 0.5 µm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min).

The temperature programme for was as follows: 3 min at 35 °C, 3 °C/min from 35 to 120 °C, 7 °C/min to 120-160 °C, 15 °C/min 160-200 °C and hold for 4 min at 200 °C.

The auto sampler collectors setting details are: 9.2 psi, outlet split: 5.0 mL/min, desorption flow: 60 mL/min. The analysis was performed in triplicate in all sampling points and the results are given in ng/g of emulsion.

The quantification of the different volatiles was done by the use of a calibration curve prepared from the following external standards dissolved in ethanol: pentanal (calibration range, c.r: 0.007-3.77 mg/g), hexanal (c.r: 0.005-2.69 mg/g), heptanal (c.r: 0.008-4.15 mg/g), *trans*-2-heptenal (c.r: 0.005-2.95 mg/g), octanal

(c.r: 0.006-3.11 mg/g), *trans*-2-octenal (c.r: 0.005-2.91), 1-octen-3-ol (c.r: 0.006-3.01 mg/g) and 2-ethyl-1-hexanol (c.r: 0.006-3.19 mg/g). 1 µL of every solution prepared at different concentrations, was added to a Tenax GR™ tube and flushed with nitrogen (flow of 50 mL/min for 5 min) to remove the solvent. Then, the volatiles were analyzed in the same way as for the samples. Results for each compound were expressed as ng/g of extract, and oxidation rates were calculated as follows:

$$Oxidation\ \ rate\,(\%) = \frac{\left(VC_{day\,35or\,56} - VC_{day\,1}\right)}{VC_{day\,1}} \times 100$$

2.9. Droplet size distribution

The size of fat globules in the O/W emulsion systems was determined by laser diffraction using a Mastersizer 2000 (Malvern Ins.,Worcestershire, UK).

The skin care emulsion was diluted 1:9 in SDS buffer (10 mM NaH_2PO_4, 5 mM SDS, pH 7) prior to analysis. Droplets of the diluted skin care emulsion was added to recirculation water (3000 rpm) reaching an obscuration of 12–14 %. The set-up used was the Fraunhofer method, which assumed that all sizes of particles scatter light with the same efficiency and that the particles are opaque and transmits no light. The refractive index (RI) of sunflower at 1.469 and water at 1.330 were used as particle and dispersant, respectively. Measurements were performed on day 0 and 56. The measurements were performed in triplicate. Results are given as surface area mean diameter D(0.9), this value indicates that 90 % of the volume of the oil droplets is smaller than this value.

2.10. Colour determination

Colour of skin care emulsion was measured using a digital colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) to obtain the colour coordinates L*, a* and b*. These values were used to calculate the euclidean distance value ($\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}$) that allowed both comparing samples with and without extracts, and also comparing the initial colour of every sample to that detected along the storage. The measurements were performed in triplicate.

2.11. Statistical processing

Mean and standard deviation of results obtained were calculated. For each parameter, one way ANOVA with Tukey-b *post hoc* multiple comparisons was used in order to evaluate the significant differences among samples and treatments. Within each type of sample, the differences between 0 and 56 days were evaluated by Student t-test.

The statistical analysis of data was done using the SPSS 15.0 program (SPSS, INC., Chicago, IL, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

3. Results and discussion

3.1. Physical changes

3.1.1. Droplet size determination

The distributions of oil droplets in the skin care emulsions were determined at the beginning and the end of all storage conditions (**Fig. 1**). D(0.9) value was selected to highlight the differences among samples and treatments. This value indicates that 90 % of the volume of droplets is smaller than this value. When an increment in this parameter is observed, a destabilization of the emulsion system is occurring. Regarding the accelerated storage conditions, there was a trend to increase D(0.9) in the presence of light (A+), but especially at high temperature (H0) a significant increase in D(0.9) was observed. Hence, in all emulsions stored at high temperature, these changes caused a large destabilization of the emulsions leading to an evident syneresis at the end of the storage (visual appreciation). Due to this observed syneresis effect in H0 stored samples at day 56, only samples stored up to 35 days were considered for further analysis in this case. Moreover, WE2 and AE2 showed the highest D(0.9) increments, highlighting that the presence of high doses of the extract in the skin care emulsions under high temperature, could influence the physical stability of the system. In the case of A+ stored samples, only the addition of 2 mg/g of AE extract seemed to decrease the stability of the skin care emulsions, although no syneresis was found.

3.1.2. Colour

In order to evaluate the influence of the presence of some pigments (carotenoids, xantophylls and chlorophylls), euclidean distance value (ΔE) was calculated before storage. When comparing colour between the extract containing samples and the RF at day 0, the calculated ΔE were 3.90, 6.80, 4.93 and 7.59 for WE1, WE2, AE1, and AE2, respectively. As these values were higher than 2 (Francis & Clydesdale, 1975) clear colour differences, were noticed between the extract containing samples and the references, with a strong influence of the concentration and extract type of *F. vesiculosus* used. The instrumental colour data confirmed that, whereas no differences in lightness (L*) and redness (a*) were found, yellowness (b*) was significantly higher ($p<0.05$) in the emulsion containing extract compared to the RF. These colour differences were dose dependent and higher in the AE containing emulsions as compared to WE ones. This colour differences pointed out that the use of seaweed extracts did not perfectly mimic the characteristic of conventional skin care emulsions.

Additionally, the evolution of the colour was also measured during the storage conditions (**Fig. 2**). When comparing each sample with their own colour at day 0, the five emulsions did not maintain the colour during storage, as observed by the ΔE increments in all cases. The increment was higher in the samples with higher amount of extract (WE2 and AE2). Particularly light, and mainly temperature (40 °C), induced remarkable colour changes in the extract containing samples, whereas no significant differences were found between storage conditions for the RF samples. These changes were a consequence of an increment in a* value and a reduction in the L* value (data not shown), as the samples went more brown over time. The adverse storage conditions could induce oxidative reactions that might affect pigments produce premature browning in the samples. However, this deserves more research.

3.2. Oxidative changes

Almond oil (*Prunus amygdalus*) is one of the most valuable skin care oils due to its penetrating, moisturising and restructuring properties, and high content of unsaturated fatty acids. It can be used for numerous skin problems because of their

anti-inflammatory, emollient, sclerosant and cicatrizing effects (Ahmad, 2010). Therefore, in the present study, almond oil was used in the formulation of the oil-in-water skin care emulsions. However, the susceptibility of the unsaturated lipids present in almond oil to oxidation might be a major cause of quality deterioration and rancidity in the skin care emulsion. The lipid profile of the samples was determined at the beginning and at the end of the storage in every sample, and it was observed that it remained unchanged during the storage period, with oleic acid as the major fatty acid, followed by linoleic, and the saturated ones, palmitic and stearic acid. On average, the fatty acid composition was as follows: 14:0, 0.27 %; 16:0, 12.0 %; 16:1 (ω-7), 0.11 %; 18:0, 12.1 %; 18:1 (ω-9), 53.8 %; 18:1 (ω-7), 0.66 %; 18:2 (ω-6), 18.4 %; 18:3 (ω-3), 0.08 %; 20:0, 0.28 %; 20:1 (ω-11), 0.47 %; 20:4 (ω-6), 0.31 %; 22:1 (ω-9), 0.17 %.

3.2.1. Tocopherol content during storage

Four tocopherol homologues were detected in the skin care emulsions, α-, β-, γ-, δ-tocopherol. The most abundant one at the beginning of the storage was gamma-tocopherol (4895±151 μg tocopherol/g skin care emulsion) followed by delta- (1657±94 μg tocopherol/g skin care emulsion), alpha- (1329±72 μg tocopherol/g skin care emulsion) and beta-tocopherol (116±10 μg tocopherol/g skin care emulsion), respectively. The addition of seaweed extract did not affect the content of tocopherols.

The sum of all of them was calculated, as similar changes were observed in the four homologues, and represented as the total tocopherol content (TTC) along the storage (**Fig. 3**). The TTC decreased in all samples during storage, and the highest rate of decrease was observed at the high temperature conditions. However, at this condition, the presence of the highest doses of both extracts (WE2 and specially AE2) contributed to maintain in higher TTC values as compared to the reference **Fig. 3c**). The same behaviour was observed for WE2, AE1 and AE2 in light storage conditions (**Fig. 3b**) and for AE1 and AE2 in dark storage samples at room temperature (**Fig. 3a**).

This protective effect of WE and AE on tocopherols could be attributed to the presence of metal-chelating properties described by Wang et al. (2009) and Farvin et al. (2013). It could be also hypothesized a synergistic effect between tocopherol and

phenolic compounds or pigments, contributing to the regeneration of tocopherol in skin care emulsions containing extracts.

3.2.2. Peroxide value (PV)

The autoxidation of unsaturated fatty acids is a chain process occurring autocatalytically through free radical intermediates, and can be accelerated during storage by exposure to light, temperature and in presence of redox metals. On that basis, the primary oxidation compounds, expressed as the peroxide content of the skin care emulsions stored in the different conditions were determined (**Table 2**).

At the beginning of the storage, WE2 and AE2 samples showed slightly higher PV values than RF samples ($p<0.05$). This could be consequence of the presence of trace metals in the algae extracts (iron and copper) which promoted, together with the temperature of processing (70-75 °C), oxidative reactions at an initial stage. During storage, significant increments in PV were found in all samples ($p<0.05$). In the case of high temperature conditions, similar values of PV were found in the samples with the highest concentrations of extracts (WE2 and AE2), in spite of their high initial PV that were expected to propagate lipid oxidation. It should be pointed out that a higher oxidation rate, between day 1 and 35, was found in RF (185 %), compared to WE2 (102 %) and AE2 (110 %). The high content of polyphenols in the extracts, with radical scavenger activity, could interfere in the lipid oxidation process slowing down fatty acid degradation. Moreover, regarding light storage conditions, WE1 and WE2 had lower PV, value than the RF one ($p<0.05$). This could be due to the higher content of carotenoids in this extract, which are well known inhibitors of free radical chain reactions caused by photooxidation process (Stahl & Sies, 2003).

Even though similar data were found during accelerated storage conditions (A+ and H0), in the A0 stored ones a particular behaviour was observed. During the storage of these samples an increase in the oxidation rate, between day 1 and 56, was found in WE2 (151 %) and AE2 (154 %) compared to RF (108 %). Regarding to this, several studies showed that *F. vesiculosus* extracts containing phlorotannins, which had good ferrous ion-chelating capacity (Wang et al., 2009; Farvin & Jacobsen, 2013). As it is well known, metal chelating capacity is claimed as one of the important mechanisms of antioxidant activity, because of the interactions between lipid

hydroperoxides and transition metals acts as precursors of volatile compounds (McClements et al., 2000; Let et al., 2007). In this sense, the presence of phlorotannins may form complexes with metals and inactivate their catalytic effects in promoting peroxide decomposition. Due to this antioxidant effect, an accumulation of peroxide compounds in the extract containing samples might take place, and consequently lead to a lower formation of volatile compounds, as will be discussed below.

3.2.3. Volatile compounds

Odour deterioration of lipid containing products is caused mainly by the presence of volatile lipid oxidation products, which have an impact on odour at extremely low concentrations. Peroxide derivate compounds formed during storage can either react with unsaturated lipids to form stable and innocuous alcohols, or undergo fragmentations into aldehydes and ketones causing rancidity in unsaturated matrices (Frankel, 1998). Major volatile compounds identified from the headspace of the fifteen samples throughout the storage were: 4 alkanals (pentanal, hexanal, heptanal and octanal), 2 alkenals (*trans*-2-heptenal and 2-octenal) and 2 alcohols (1-octen-3-ol and 2-ethyl-1-hexanol). These compounds represent groups of secondary oxidation products resulting mainly from the autooxidation of oleic, linoleic and α-linolenic acid (Belitz, Grosch & Schieberle, 2009; Guillén et al., 2012, Poyato et al., 2014). Hexanal and 2-octenal showed the highest initial concentrations (248±99 and 222±52 ng/g emulsion, respectively). However, others such as pentanal and heptanal showed greater differences among samples and also more evident variations during storage compared to their initial concentrations. This was why they were selected to follow their evolution during the whole storage (**Fig. 4**). During accelerated storage conditions (A+ and H0), the peroxides decomposition generated higher volatile amounts than in the A0 stored ones, so there was a higher transformation rate from hydroperoxides to secondary oxidation products due to thermo and photooxidation processes. Furthermore, results showed that temperature had significantly higher effect than light in the formation of volatile compounds, with higher absolute amounts of both aldehydes at the end of the storage.

Regarding the presence of antioxidants, on one hand, in the samples with the highest extract content (WE2 and AE2), pentanal showed significantly lower

concentrations in all samples compared to RF at the end of the storage (reduction up to 72 % in AE2 samples at A0 storage conditions), whereas heptanal amount was lower than RF only at the end of storage at high temperature (19 % reduction). On the other hand, the presence of antioxidant modified the timing of volatile formation. Thus, even though the presence of extract at the beginning of the storage resulted in higher amounts of pentanal and heptanal in all samples, lower oxidation rates were observed during storage in these samples. In particular, in the light stored samples, lower oxidation rates for pentanal and heptanal were found in WE2 (6.7 %, 69 %, respectively) and AE2 (-35.2 %, 40 %, respectively) compared to RF (144 %, 211 %, respectively). Moreover, at high temperature, AE2 showed the best results against the formation of pentanal and heptanal, with oxidation rates of 261 and 281 %, respectively, compared to the rates calculated for RF samples (1251 % and 1419 %). Finally, it is worth to notice that at A0 stored conditions, while RF samples showed an increment of pentanal (81 %) and heptanal (71 %) between day 1 and 56, the highest extract concentration samples lead to a reduction compared to their initial amounts. These results were in agreement with the accumulative effect observed in PV in these samples. The presence of the extract decreased the hydroperoxide decomposition rate to volatile compounds, at all storage conditions, being AE2 the best one. This information helps to elucidate the antioxidant mechanism of those extracts, which may influence in the protection of the peroxides decomposition to secondary oxidation products. However, more studies are needed to confirm.

4. Conclusions

The type of antioxidant remained a key factor in controlling oxidation processes influenced by light or temperature. Whereas both water and acetonic extract, showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the water one showed antioxidant activity against photooxidation. Therefore, both the polyphenols content (radical scavenging activity) and, in particular, the presence of phlorotannins (ion-chelating capacity), contributed to decrease the lipid oxidation process slowing down fatty acid degradation. Moreover, the higher carotenoids content in the water extract could inhibit free radical chain reactions caused by photooxidation process.

Fucus vesiculosus extracts, containing polyphenols, were effective in protecting highly-unsaturated skin care emulsions.

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Table 1. Formulation of the different samples.

Water phase	
Demineralized water	52.5 g/ 100 g
Aloe vera water	10.0 g/ 100 g
Glycerin	6.3 g/ 100 g
MF fat	3.6 g/ 100 g
Natriumbenzoat	0.6 g/ 100 g
F. vesiculosus extract (water or acetonic)	1 or 2 mg/g
Oily phase	
Almond oil	21.8 g/ 100 g
Lanette wax	2.0 g/ 100 g
VE fat	1.8 g/ 100 g
Vitamin E	0.9 g/ 100 g

Table 2. Effect of adding water or acetonic *F. vesiculosus* extract on PV (meq O_2/kg oil) of skin care emulsions under accelerated stored conditions or room temperature and dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); S.C, storage condition; A0, darkness and room temperature; A+, light and room temperature; H0, darkness and 40 °C).

	Peroxide value (meq O2/kg oil)					
	Day 0	S.C	Day 7	Day 21	Day 35	Day 56
RF	5.52 ± 0.51^{A}	A0	6.85 ± 0.29^{Ba}	7.31 ± 1.00^{Aa}	9.48 ± 0.45^{Aa}	11.62 ± 0.31^{A}
		A+	8.22 ± 0.52^{Ab}	11.71 ± 1.07^{Bb}	13.11 ± 1.19^{ABb}	18.93 ± 0.63^{B}***
		H0	11.49 ± 0.84^{ABc}	11.65 ± 0.29^{Ab}	15.01 ± 0.78^{ABb}	17.52 ± 0.49^{b}
WE1	5.19 ± 0.29^{A}	A0	6.09 ± 0.04^{ABa}	6.97 ± 0.45^{Aa}	8.53 ± 0.36^{Aa}	10.77 ± 1.25^{A}
		A+	8.11 ± 0.05^{Ab}	10.58 ± 0.59^{ABb}	12.37 ± 0.70^{ABb}	15.67 ± 1.29^{A}**
		H0	10.11 ± 1.11^{Ac}	13.08 ± 1.33^{Ac}	14.31 ± 0.07^{Ac}	21.74 ± 1.93^{c}
WE2	6.58 ± 0.69^{B}	A0	12.89 ± 0.67^{Da}	11.95 ± 0.22^{Ba}	13.96 ± 0.32^{Ca}	17.67 ± 1.38^{C}
		A+	14.08 ± 0.21^{Da}	12.40 ± 0.69^{Ba}	14.38 ± 1.28^{Ba}	16.42 ± 1.42^{A} ns
		H0	13.05 ± 1.60^{BCa}	11.38 ± 0.19^{Aa}	13.97 ± 0.19^{Aa}	15.80 ± 1.07^{a}
AE1	6.28 ± 0.65^{B}	A0	14.26 ± 0.29^{Ea}	13.36 ± 1.03^{Bb}	15.34 ± 0.35^{Db}	18.78 ± 0.71^{C}
		A+	13.16 ± 0.32^{Ca}	10.90 ± 1.15^{ABa}	10.34 ± 0.52^{Aa}	23.97 ± 0.49^{C}***
		H0	13.73 ± 1.71^{Ca}	16.58 ± 1.61^{Bc}	16.47 ± 1.73^{Bb}	19.43 ± 2.27^{a}
AE2	6.63 ± 0.75^{B}	A0	11.31 ± 0.28^{Cb}	17.03 ± 0.47^{Cb}	12.36 ± 0.87^{Ba}	15.14 ± 0.95^{B}
		A+	9.58 ± 0.23^{Ba}	9.43 ± 0.35^{Aa}	17.36 ± 1.99^{Cb}	18.89 ± 0.55^{B}**
		H0	10.94 ± 0.21^{ABb}	10.43 ± 0.78^{Aa}	14.05 ± 1.05^{ABab}	15.69 ± 0.88^{a}

Different capital letters in the same column denote significant differences between samples for each stored condition ($p<0.05$)
Different small letters in the same column denote significant differences among storage conditions for each sample ($p<0.05$)
Level of significance for the Student t test comparing storage conditions at day 56: ns= not significant ($p>0.05$); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Fig. 1. Droplet size distribution on skin care emulsions with or without *F. vesiculosus* extract (water or acetonic) after 56 days of storage under accelerated conditions or at room temperature and dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); A0, darkness and room temperature; A+, light and room temperature; H0, darkness and high temperature).

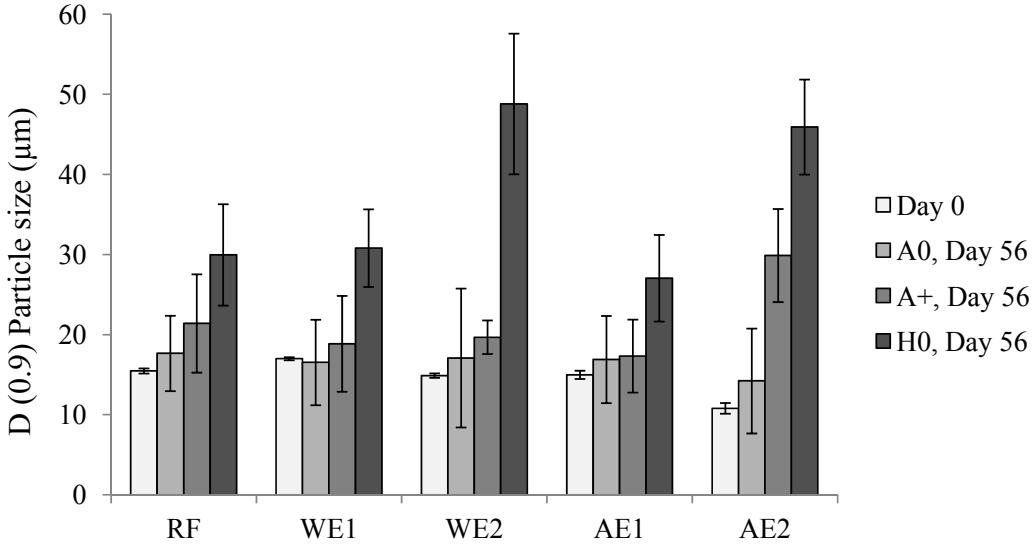


Fig. 2. Euclidean distance value of the skin care emulsions calculated along the storage. (a) Room temperature and darkness; (b) room temperature and light; (c) high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

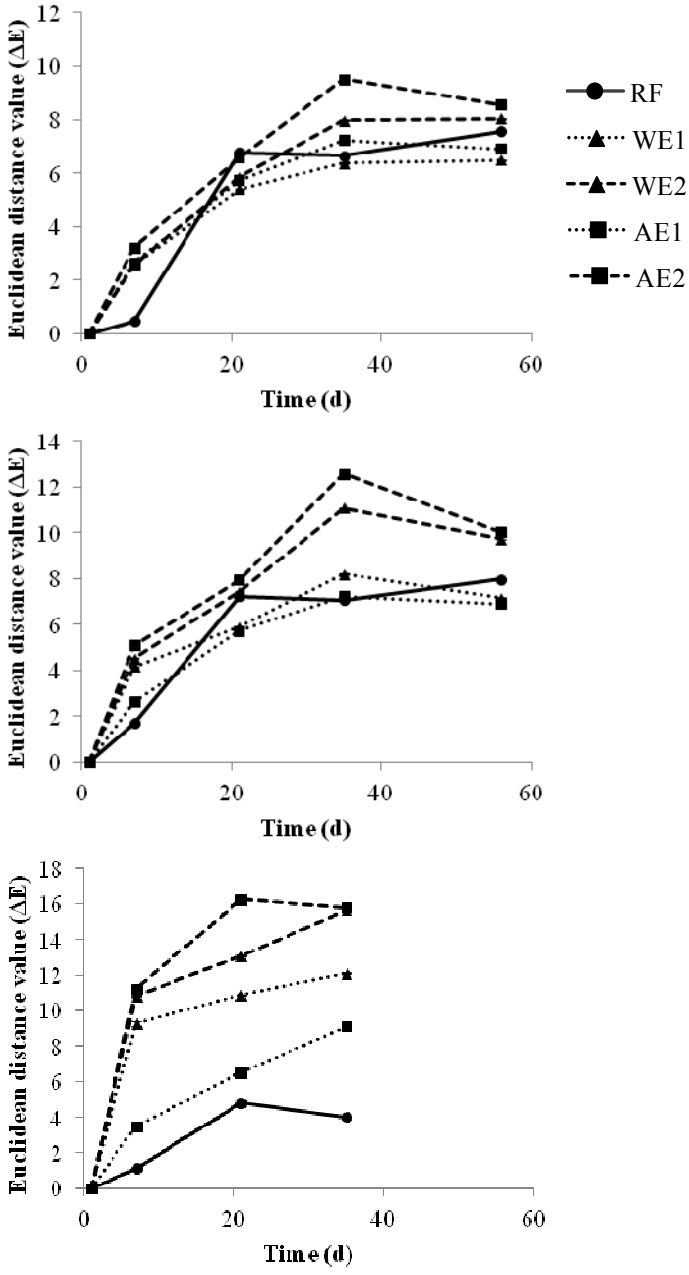


Fig. 3. Total tocopherol content (µg tocopherol/g emulsion) in skin care emulsions with WE or AE including a control without any extract during storage. (a) Room temperature and darkness; (b) room temperature and light; (c) high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

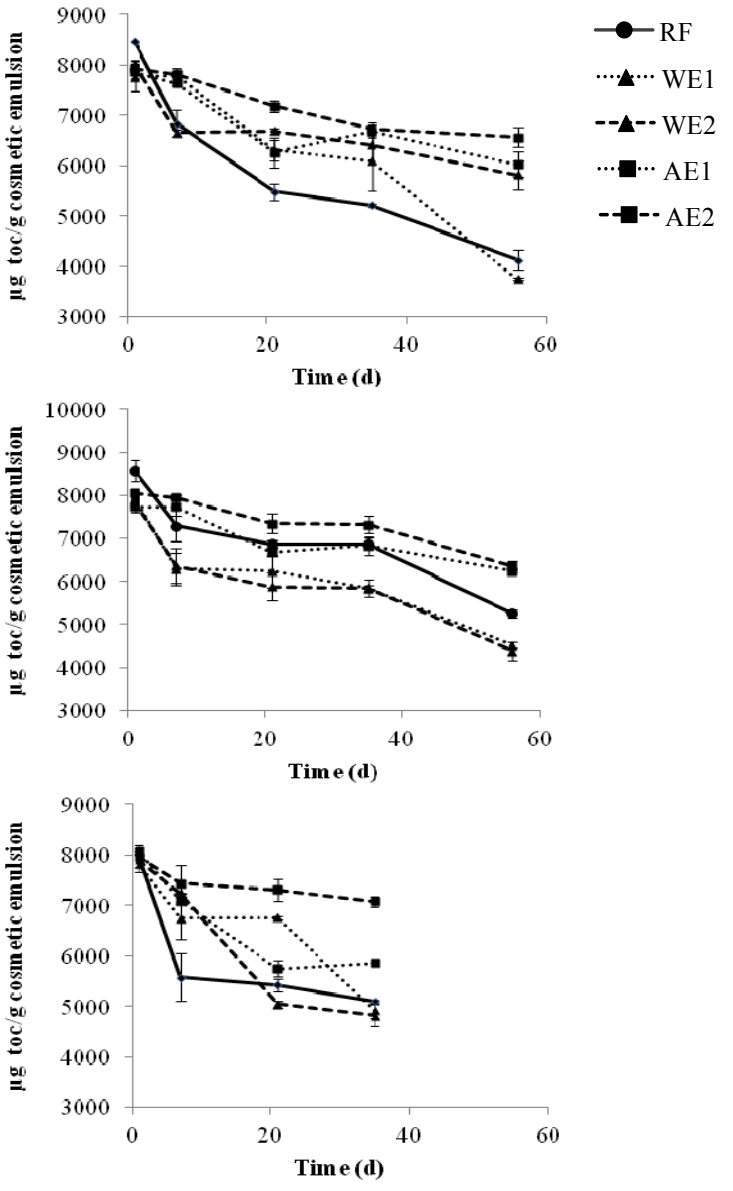
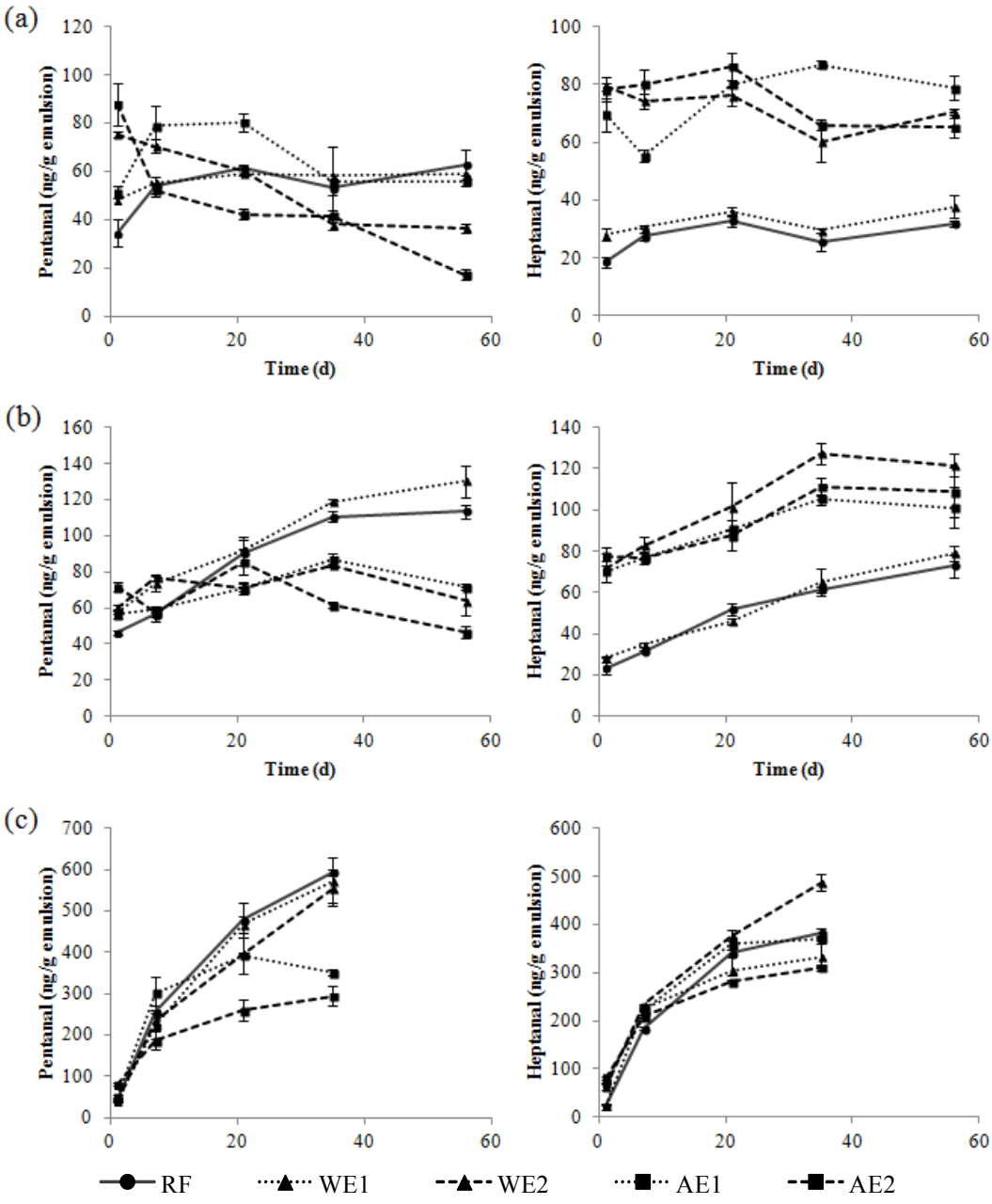


Fig. 4. Development of volatile compounds, pentanal and heptanal (ng/g emulsion), during the storage. (a) Room temperature and darkness; (b) room temperature and light; (c) high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.



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Pamplona, Spain

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Aplicación de RSM para la optimización de emulsiones gelificadas

C. Poyato, D. Ansorena, I. Navarro-Blasco, I. Astiasarán

Resumen: La Metodología de Superficie Respuesta (RSM) es un conjunto de técnicas matemáticas utilizadas para el tratamiento de problemas en los que una respuesta de interés está influida por varios factores de carácter cuantitativo.

En el presente trabajo se formularon distintas emulsiones gelificadas y se estudió la influencia de los factores "contenido en aceite (%)" y "contenido en carragenato (%)" sobre dos variables de respuesta: valores tecnológicamente aceptables de dureza y sinéresis.

Los intervalos seleccionados para el análisis factorial fueron: 40-70 % de aceite de lino y 0.5-1.5 % de carragenatos. Se utilizó un Diseño Compuesto Central (2^3 + star) obteniéndose 16 condiciones experimentales, de las cuales se analizaron experimentalmente los correspondientes valores de dureza y sinéresis. Los resultados obtenidos sea ajustaron a un modelo cuadrático y se obtuvieron los correspondientes gráficos de superficie respuesta. Las ecuaciones resultantes permitieron obtener los valores de los factores óptimos para lograr formulaciones de gel con la máxima dureza y la mínima sinéresis.

Los geles obtenidos constituyen un vehículo potencial de ingredientes funcionales a incorporar en distintos tipos de alimentos.

VII JORNADA DE INVESTIGACION EN CIENCIAS EXPERIMENTALES Y DE LA SALUD

APLICACIÓN DE RSM PARA LA OPTIMIZACIÓN DE EMULSIONES GELIFICADAS

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Introducción

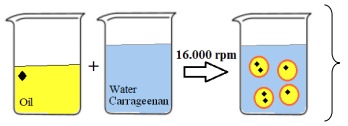
Los sistemas de liberación basados en emulsiones son particularmente adecuados para proteger y liberar lípidos bioactivos (omega-3). Dichas emulsiones han de poseer, para ser viables como ingredientes, ciertas características tecnológicas y sensoriales similares a las grasas animales que pretenden sustituir.

Objetivos

1. **Optimizar la formulación de una emulsión oil-in-water gelificada, preparada con aceite rico en ácidos grasos ω-3 (aceite de lino), carragenatos, surfactante y agua, con el fin de obtener un ingrediente funcional mediante un diseño factorial de superficie respuesta (RSM).**
2. **Obtener una emulsión gelificada de la máxima dureza, y mínima sinéresis, que la haga viable como sustituto del tocino empleado habitualmente en productos cárnicos.**

Preparación de las emulsiones

Se prepararon emulsiones gelificadas oil-in-water (O/W) con diferente contenido graso en aceite de lino y proporción de carragenato.



MEDIDA DE:
-DUREZA
-SINÉRESIS

Central composite design (2^3+ star; incluyendo 2 puntos centrales) para las tres variables y las respuestas observadas.

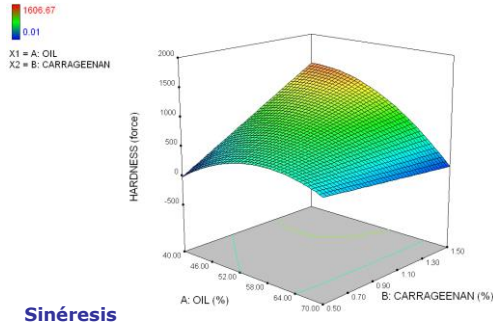
Run	Aceite de lino (%)	Carragenato (%)	SOR	Dureza[a] (g)	Sinéresis[a] (%)
1	35.69	1.00	0.0040	548	32.06
2	55.00	1.64	0.0040	1606	53.36
3	40.00	1.50	0.0050	1383	27.77
4	55.00	1.00	0.0040	822	32.88
5	55.00	1.00	0.0053	894	32.52
6	55.00	1.00	0.0027	873	36.78
7	74.31	1.00	0.0040	2.32	99.01
8	55.00	0.36	0.0040	310	31.93
9	40.00	1.50	0.0030	1364	19.90
10	40.00	0.50	0.0030	69.00	30.81
11	70.00	1.50	0.0050	0.01	98.65
12	40.00	0.50	0.0050	65.29	39.47
13	55.00	1.00	0.0040	874	38.97
14	70.00	1.50	0.0030	0.01	99.20
15	70.00	0.50	0.0030	376	53.11
16	70.00	0.50	0.0050	426	51.36

[a]Media de los triplicados
SOR: surfactant oil ratio

Resultados

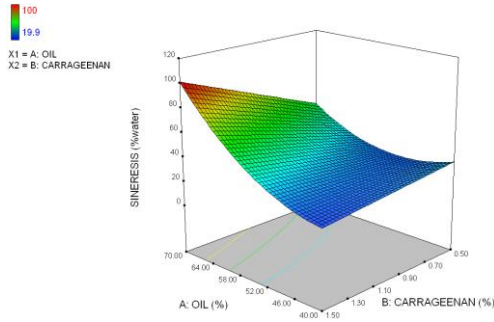
Dureza

Análisis de textura (Medida de la fuerza de compresión, TA-XT2i, Stable Micro Systems, Surrey, Reino Unido)



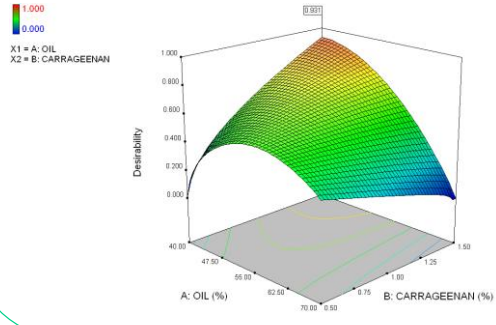
Sinéresis

Medida de la pérdida de agua durante el almacenamiento (3 días, 25 ºC). Sinéresis (%) = [(W0 – Wt)/C0] x 100



Optimización múltiple

Gráfico superficie respuesta resultante de la optimización de las respuestas: dureza y sinéresis (Design-Expert 9, stat-Ease, Inc., Minneapolis, USA)



Conclusiones

- **Es posible generar emulsiones gelificadas O/W tecnológicamente estables con un bajo porcentaje de grasa (40%, 1.5% carragenatos).**
- **La emulsión gelificada puede ser usada como ingrediente, siendo un sustituto parcial del tocino en productos cárnicos funcionales.**

Agradecimientos

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Carrageenan containing gelled emulsion: potential fat replacer for functional food

C. Poyato, D. Ansorena, I. Astiasarán

Abstract: The use of emulsion based delivery systems is a particularly suited technology for protecting and releasing bioactive lipids within the food industry. The characteristics of fat analogues for food industry are needed to be studied in order to achieve the appearance, technological, rheological and sensory properties required for use to replace animal fats.

The objective of this work was to present a gelled emulsion that can be potentially used as pork back-fat replacer, so that healthier foods might be formulated with it. Different presentations of this product are shown in **Fig. 1**.

The ingredients of an optimized formulation of the emulsion were: water, oil, carrageenan and surfactant. Technological parameters (colour, texture) and the oxidative stability (TBARS) of the gelled emulsion were measured (1 and 30 days, in products stored under vacuum at 4 °C and at 25 °C).

Proximate comparison showed 40 % fat and 1.5 % fiber, leading to 360 kcal/100 g approximately, significantly lower than 673 kcal/100 g of pork back fat. Depending on the oil used in the formulation, the lipid profile of the product can be easily modulated. Thus, values of MUFA up to 78-83 % can be reached if virgin olive oil or high-oleic sunflower oil are used, and values up to 54 % of ω-3 fatty acids can be obtained when using linseed oil. This implies a relevant nutritional advantage compared to pork back-fat.

From the technological standpoint, storage of the products during 30 days at 4 °C ensures colour and texture stability and maintenance of low TBARS values

(0.24 mg/100 g product). Storage at 25 °C might slightly increase oxidation susceptibility, without colour or texture modification.

The use of this ingredient in fresh and cooked meat products (**Fig. 2**) has demonstrated the sensory effectiveness of this new product as a partial or total pork back-fat replacer.

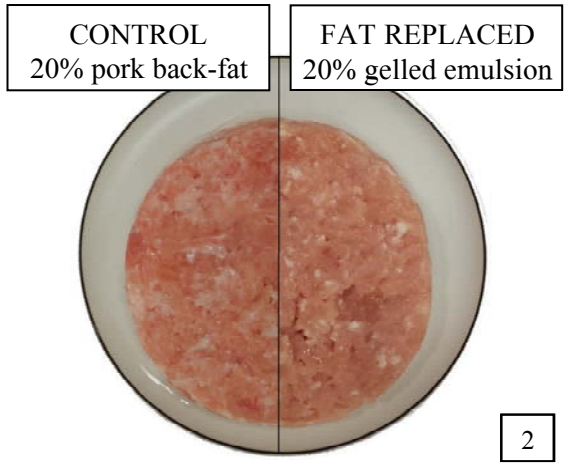

Fig. 1. Different presentations (dice and chop) of the gelled emulsion.

Fig. 2. Control burger and fat replaced burger.

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CARRAGEENAN CONTAINING GELLED EMULSION: POTENTIAL FAT REPLACER FOR FUNCTIONAL FOOD

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INTRODUCTION

Meat products are highly consumed worldwide. Improving their nutritional profile without compromising their sensory properties is aimed by the meat industry.

When animal fat replacers are used, the characteristics of these analogues are needed to be studied in order to achieve the appearance, technological, rheological and sensory properties typical from animal fat.

Our research group has developed an stable functional ingredient capable of incorporating vegetable oils in meat products to partially or totally replace the traditional pork back-fat present in these products.

OBJECTIVE

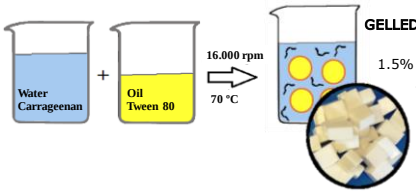
To present a gelled emulsion that can be potentially used as pork back-fat replacer, so that healthier foods might be formulated with it.

To assess the stability of this ingredient during storage.

EXPERIMENTAL DESIGN

Table 1. Amounts of sums of main lipid fractions (g fatty acids/100 g oil) and lipid ratios of pork back-fat, and in the three oils used in the gel formulation.

	Pork-back fat	Linseed oil	High-oleic Sunflower oil	Extra Virgin Olive oil
SFA	38.1	10.9	8.55	13.9
MUFA	47.6	20.6	83.0	78.1
PUFA	13.5	68.2	7.97	7.73
ω-3	0.13	54.1	0.51	0.77
ω-6	13.3	14.1	7.46	6.96
ω-6/ ω-3	103	0.26	14.5	9.01
PUFA/SFA	0.35	6.24	0.93	0.56
PUFA+MUFA/SFA	1.60	8.12	10.6	6.17
trans	0.77	0.25	0.51	0.28



- Chemical composition
- Physical and oxidative stability after 30 days (4 ºC and 25 ºC):
  - Syneresis (Poyato et al., 2014)
  - Texture (Poyato et al., 2014)
  - Colour (Berasategi et al., 2014)
  - TBARS (Poyato el al., 2013)
- Approach of application in meat patties

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RESULTS

Table 1. Chemical composition per 100 g of ingredient (pork back-fat and gelled emulsion).

	Pork-back fat	Gelled emulsion
Energy	2013 kJ/673 kcal	1504 kJ/360 kcal
Protein (g/100 g)	8.4	0
Carbohydrates (g/100 g)	0	0
Fat (g/100 g)	71	40
Fiber (g/100 g)	0	1.5
Water (g/100 g)	20.6	58.5
Cholesterol (mg/100 g)	57	0

A reduction of the **46.5%** of the **energy** supply could be obtained.

The gelled emulsion shows a **43.6%** lower amount of **fat**.

Figure 1. Amounts of main lipids (oleic, linoleic and linolenic acid; g fatty acids/100 g) of the three analyzed gelled emulsions and the pork back-fat.

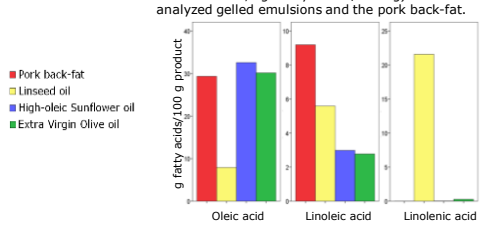


Table 2. Syneresis observed in the gelled emulsion† after one day of storage at 4 ºC and 25 ºC, with or without vacuum packaging.

	4 ºC		25 ºC	
	Not packaged	Packaged	Not packaged	Packaged
Syneresis (%)	**1.70**	**3.14***	**5.26**	**0.66***

†The oil used was high-oleic sulflower oil.
*Significance for the Student t test (p<0.05).

Table 3. Texture analysis of the gelled emulsion† during storage (day 1 and day 30) at 4 ºC and 25 ºC.

	4ºC		25ºC	
	Day 1	Day 30	Day 1	Day 30
Hardness	**1175**	**1248**	**1163**	**1260***
Fracturability	**1175**	**1248**	**1163**	**1260***
Adhesiveness	**-62**	**-62**	**-55**	**-83***
Springiness	**0.972**	**0.970**	**0.973**	**0.955**
Cohesiveness	**0.839**	**0.827**	**0.836**	**0.830**
Gumminess	**987**	**1033**	**973**	**1045**
Chewiness	**953**	**1001**	**935**	**998**
Resilience	**0.509**	**0.492**	**0.507**	**0.469**

†The oil used was high-oleic sunflower oil.
*Significance for the Student t test comparing time of storage (p<0.05).

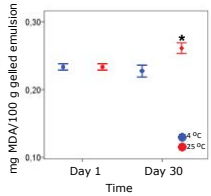
Table 4. Color coordinates of the gelled emulsion† during storage (day 1 and day 30) at 4 ºC and 25 ºC.

	4 ºC		25 ºC	
	Day 1	Day 30	Day 1	Day 30
L*	96.07	96.05	96.25	95.87
a*	-0.97	-1.08	-0.94	-1.15
b*	2.67	2.97	2.67	3.06
ªHue	-70.04	-69.98	-70.56	-69.42
ªChroma	2.84	3.16	2.83	3.26
ªΔE		0.32		0.59

†The oil used was high-oleic sulflower oil.
ªColor coordinates L*, a* and b* were used to calculate Hue, Chroma and the Euclidean distance value ($\Delta E = \sqrt{(L_2-L_1)^2+(a_2-a_1)^2+(b_2-b_1)^2}$)

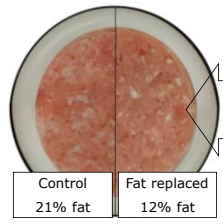
No color differences were observed during the storage, as ΔE was lower than 2 (Francis & Clydesdale, 1975)

Figure 2. TBARS (mg MDA/100 g gelled emulsion†) obtained for the days 1 and 30 of storage at 4 ºC and 25 ºC.



†The oil used was high-oleic sunflower oil.
*Significance for the Student t test comparing time of storage (p<0.05).

Figure 3. Meat patties samples: control and 100% animal fat replaced by the gelled emulsion.



The use of this ingredient in fresh and cooked meat products has demonstrated the sensory effectiveness of this new product as a partial or total pork back-fat replacer.

CONCLUSIONS

A "reduced fat" and "reduced energy" gelled emulsion can successfully be used as pork back fat replacer in meat products. Modification of its lipid profile can be achieved by using different lipid sources.

Storage of the gelled emulsion during 30 days at 4 ºC ensures colour and texture stability and maintenance of low TBARS values (0.24 mg/100 g product). Storage at 25 ºC might slightly increase oxidation susceptibility and texture, without colour modification.

In preparation

6

Physicochemical stability of highly-unsaturated gelled emulsions with *Lavandula latifolia* extract during storage

C. Poyato, L. Gayoso, D. Ansorena, M. I. Calvo, R. Y. Cavero, I. Astiasarán

Abstract: A water *Lavandula latifolia* extract was added to unsaturated gelled emulsions (65.5 or 131 mg of extract/g emulsion) and stored in darkness at 25 °C for 49 days. Three different oils were used as oily phase (sunflower oil, linseed oil and algae oil) so that the efficacy of the *L. latifolia* extract to inhibit lipid oxidation in different lipid profiles could be evaluated. To test the effect of the extract in the technological properties of the gelled emulsions, the texture analysis profile and colour determination were analysed during the storage.

Key words: lipid oxidation; TBARS; DPPH; volatile aldehydes; colour; texture.

1. Introduction

Emulsions have been widely described as a suitable delivery system of functional ingredients in the food industry (McClements, Decker & Weiss, 2007; McClements, 2010). Additionally, modification of the characteristics of these delivery systems allows improving the health properties of foodstuffs. In case these modifications imply increasing the PUFA/SFA ratio, the resulting products might be more susceptible to oxidation, and it could be necessary the use of extra antioxidants (Berasategi, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena, 2014; Cofrades, Santos-López, Freire, Benedí, Sánchez-Muniz & Jiménez-Colmenero, 2014; Poyato, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena, 2013).

The current growing demand for natural antioxidants justifies the search for new plant extracts as substitutes of synthetic antioxidants, which have been questioned due to health and safety reasons (Moure et al., 2001). The Lamiaceae family gathers several widely known plants, some of them with bioactive species. Among them, extracts from some lavender species (*L. angustifolia, L. viridis*) have been described as a potential source of antioxidants and other functional compounds (Costa, Gonçalves, Valentao, Andrade & Romano, 2013; Gallego, Gordon, Segovia, Skowyra & Almajano, 2013; Gonçalves, Gomes, Costa & Romano, 2013). *Lavandula latifolia*, commonly known as spike lavender, is naturally found in several regions in the East of Spain (Herraiz-Peñalver, Cases, Varela, Navarrete, Sánchez-Vioque & Usano-Alemany, 2013). Most of the research conducted with the spike lavender has been done with the essential oil, being less studied the antioxidant capacity of the extracts (López, Akerreta, Casanova, García-Mina, Cavero & Calvo, 2007).

Our research group developed a gelled emulsion enriched in PUFA that was successfully applied in fresh and cooked meat products, making possible to design potentially functional reduced fat products with healthier lipid profile (Poyato, Ansorena, Berasategi, Navarro-Blasco & Astiasarán, 2014; Poyato, Astiasarán, Barriuso & Ansorena, 2015). The study of the physicochemical stability of this ingredient during the storage could be a key factor due to the fact that lipid oxidation products are related to undesirable sensory and biological effects. Moreover, changes in colour and/or texture could contribute to reduce the self-life of the ingredient and in consequence the storage stability of the developed foods.

The present study is focused on the study of the stability during storage of the optimized gelled emulsion formulated with three different polyunsaturated oils (sunflower, linseed and algae oil), testing an aqueous extract from *Lavandula latifolia* as natural antioxidant.

2. Material and methods

2.1. Materials

The aerial parts of *L. latifolia* were collected during October 2012 in the Southern part of Navarra (Spain) and afterwards were dried at room temperature and stored in the laboratory of Pharmacognosy at the University of Navarra.

Gelled emulsion ingredients: sunflower oil (Urzante S.L., Navarra, Spain); linseed oil (Naturgreen, Laboratorios Almond S.L., Murcia, Spain), algae oil (Martek Biosciences Corporation, Columbia, USA); carrageenan (kappa-carrageenan) (kindly donated by Cargill, San Sebastián, Spain); Polysorbate 80 (Sigma-Aldrich, Steinheim, Germany).

Reagents: fatty acid methyl esters, NaCl, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 97 %), DPPH (2,2-diphenyl-1-picrylhydrazyl) and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Boron trifluoride/methanol and heptane were obtained from Merck (Whitehouse Station, NJ, USA). Folin-Ciocalteu reagent was supplied by from Panreac (Barcelona, Spain).

2.2. Aqueous *Lavandula latifolia* extract

2.2.1. Preparation of extract

The extraction of *L. latifolia* was done following a sequential extraction, using four different solvents of increasing polarity (dichloromethane, ethyl acetate, methanol and water) (López et al., 2007). The water extract was lyophilized (Cryodos-50, Telstar, Barcelona, Spain) and analyzed for antioxidant capacity.

2.2.2. Characterization of antioxidant capacity

Total phenolic compounds were determined spectrophotometrically following the Folin-Ciocalteu colorimetric method described in Herchi et al. (2011). The reaction mixture was done in a 96 well micro plate: 3 µL of diluted sample (1.5-2 mg/mL), 237 µL of distilled water, 15 µL of Folin-Ciocalteu´s reagent, and 45 µL of 20 % sodium carbonate anhydrous solution (adding 2 min after the Folin-Ciocalteu´s reagent). After 2 hours in the dark at room temperature, the absorbance was read at

765 nm using a FLUOStar Omega spectrofluorometric analyzer (BMG Labtechnologies, Offenburg, Germany). The amount of total phenolic compounds was expressed in µg gallic acid/mg lyophilized extract.

DPPH method was performed as described by García-Herreros et al. (2010). Briefly, a DPPH solution (0.04 mg/mL) was prepared in methanol and diluted to obtain an absorbance of 0.8 at 516 nm. Then, 750 µL of diluted extract (11-17.6 µg/mL) were mixed with 750 µL of DPPH solution. A control sample was prepared with 750 µL of methanol. After 30 min in the dark at room temperature, 200 µL of each solution were transferred into a 96 well micro-plate and the absorbance was measured at 516 nm (FLUOStar Omega spectrofluorometric analyser, BMG Labtechnologies, Offenburg, Germany). The average absorbance for each sample was calculated as the inhibition percentage (% I) calculated according to the formula:

$$\% I = (ABS_{control} - ABS_{sample})/ABS_{control} \times 100$$

where $Abs_{control}$ was the absorbance of the control after 30 min of reaction and Abs_{sample} was the absorbance of the sample after 30 min of reaction. The percentage of inhibition was plotted versus the concentration of the extracts. A calibration curve with Trolox was used for calculating the antioxidant capacity. Results were expressed as µg Trolox/mg lyophilized extract.

2.3. Gelled emulsion preparation

Sunflower, virgin linseed and algae oil were used to prepare three types of gelled emulsions in each case: control without antioxidant (CTRL), antioxidant 1 (AOX1) and antioxidant 2 (AOX2). AOX1 included 65.5 mg *L. latifolia* aqueous extract /100 g of gel and AOX2 included 131 mg *L. latifolia* aqueous extract /100 g of gel, which were added into the aqueous phase before the homogenization process. To establish the concentration of extract in AOX1 samples, we used an amount whose antioxidant capacity was that of *Melissa officinalis* aqueous extract (measured by the DPPH), which was successfully used in emulsions rich in unsaturated fatty acids (Poyato et al., 2013). The concentration of extract in AOX2 samples was twice the concentration of AOX1 samples, and it was used to study a potential dose-response effect.

The gel formulation used in this study was optimized in Poyato et al. (2014a): 40 % of oil, 1.5 % of carrageenan and 0.003 surfactant-oil ratio. The carrageenan and the lavender extract (when used) were dissolved in water and the surfactant (Polysorbate 80) was added to the oil phase. Both phases were heated separately up to 70 °C. Then, the oil phase was incorporated to the aqueous phase and the homogenization process was made with an Ultra-Turrax® T25 basic at 16.000 rpm.

2.4. Storage conditions

Gels prepared in sealed flasks were cooled to room temperature allowing the carrageenan to polymerize. After that, gels were kept in a climate chamber at 25 °C during 49 days of storage. The experiment was done in triplicate.

2.5. Analysis of gelled emulsions

The method of Folch, Lees and Stanley (1957) was used for the extraction of fat. The fatty acids were determined in the lipid extracts by gas chromatography FID detection according to the procedure described by Valencia et al. (2008). The identification of the fatty acid methyl esters was done by comparison of the retention times of the peaks in the sample with those of standard pure compounds and by spiking the sample with each standard individually. The quantification of individual fatty acids was based on the internal standard method, using heptadecanoic acid methyl ester. After the quantification of the individual fatty acids, the sums of saturated, SFA, (capric, lauric, myristic, palmitic, stearic, arachidic, and behenic acid), monounsaturated, MUFA, (palmitoleic, oleic, vaccenic, erucic, nervonic and eicosenoic acid), polyunsaturated, PUFA, (ω-3: α-linolenic, eicosadienoic, eicosatrienoic, docosapentaenoic, docosahexahenoic acid; ω-6: linoleic, γ-linoleic, arachidonic, docosapentaenoic) were calculated.

2.5.1. Thiobarbituric acid reactive substances (TBARS)

TBARS values were determined according to the method described by Maqsood and Benjakul (2010) with slight modifications. Briefly, the TBARS reagent was prepared by mixing 15 % w/v trichloroacetic acid, 0.375 % w/v 2-thiobarbituric acid in 0.25 N hydrochloric acid. The oil (0.3 g), distillate water (250 μL), solution 1 % BHT (20 μL)

and the TBARS reagent (1 mL) were vortexed in a centrifuge tube (20 s), placed in a boiling water bath for exactly 15 min and then cooled in an ice bath to room temperature. Cyclohexanone (2 mL) and 4 M ammonium sulphate (500 µL) were added to the mixture and were vortexed for 30 s. The mixture was centrifuged at room temperature at 4000 rpm for 10 min to allow separation of phases. In the case that solid particles were formed in the interface, the TBARS reaction was repeated using lower amount of sample. After centrifugation, the supernatant was collected and the absorbance was measured at 390 and 532 nm (FLUOStar Omega spectrofluorometric analyzer, BMG Labtechnologies, Offenburg, Germany). Results were expressed in mmol 2,4-decadienal/100 g gelled emulsion for $TBARS_{390}$ and in mg MDA/100 g gelled emulsion for $TBARS_{532}$.

2.5.2. Volatile compounds (VC)

Volatile compounds were analyzed by headspace solid phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) as described in Gayoso et al. (2015). The SPME fiber coating used was divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (50/30 µm film thickness, Supelco). The gelled emulsion (2 g) was weighted into a 25 mL headspace vial and before sealing the air was replaced with nitrogen to eliminate air contaminants. The sample was equilibrated at 50 °C during 15 min and the adsoption time, with the fiber exposed to the headspace of the sample, was 60 min at the same temperature. Desorption time for the fiber in the injection port of the gas chromatograph was 30 min. The GC-MS instrumentation used was a Hewlett Packard 6890 coupled to a mass selective Hewlett Packard 5973 detector. Volatiles were separated using a capillary column HP-5MS, 5 % phenyl methyl siloxane (30 m long × 0.25 mm inner diameter × 0.25 µm film thickness, Agilent Technologies). Chromatographic conditions were as follows: the oven temperature was held for 5 min at 42 °C, then increased to 120 °C at 3 °C/min and to 250 °C at 10 °C/min (5 min hold); injector temperature, 250 °C; detector temperature 280 °C; ion source temperature, 230 °C; quadrupole mass analyzer temperature, 150 °C. Helium was used as carrier gas at 1 mL/min. The mass spectrometer was operated by electronic impact at 70 eV and ions were scanned over the m/z range of 33-350 at a rate of 4.43 scan/s.

The identification of each peak was made taking into account Kovats Index (KI) reported in the literature (Kondjoyan & Berdagué, 1996; Roldán, Ruiz, Sánchez del Pulgar, Pérez-Palacios & Antequera, 2015) and comparing their mass spectra with the spectra of a commercial library (Wiley 275.L, Mass Spectral Database). In the case of overlapping peaks, the quantification of the corresponding compound was done by a specific ion and taking into account the relative proportion in which this ion is present in each compound. Results are expressed in area $\times 10^3$/g of gelled emulsion. Samples were analyzed in triplicate.

2.5.3. DPPH determination

DPPH was determined spectrophotometrically as described by García-Herreros et al. (2010) with slight modifications described in Gayoso et al. (2015). An antioxidant extraction was performed before DPPH determination to follow the evolution of the antioxidant capacity in the oily and in the aqueous phase of the gelled emulsions. Five-gram emulsion sample was weighed, dissolved in 30 mL hexane and homogenized with an Ultra-Turrax® T25 basic (14 min, 16.000 rpm). The sample was filtered before transferred to a separatory funnel. The extraction was repeated twice and the hexane phases were combined. 20 mL of water was added and after 1 min of shaking, the lower aqueous layer was removed. The extraction was repeated twice and the aqueous phases were combined. The aqueous extract was lyophilized (Cryodos-50, Telstar, Barcelona, Spain) and analyzed for antioxidant capacity.

Then, 50 mL of a methanol-water mixture (80:10 v/v; 30 °C) were added to the hexane phase. After 3 min of shaking, the lower methanol-water layer was removed and the extraction was repeated. Another two extractions with 30 mL of methanol each were carried out (30 °C) and the phases were combined. The methanol-water extract was driven to dryness in a rotary evaporator under vacuum at 40 °C. The dry residue was then dissolved in 10 mL of methanol and analyzed for antioxidant capacity. Results were expressed as µg Trolox/mg extract.

2.5.4. Texture analysis profile

The texture (TPA) was measured using a Universal TA-XT2i texture analyzer. Gel samples were cut into cylinders (D= 2.8 cm, h= 1 cm) and placed under the probe (P 0.5R probe), underwent compression under a 5 kg load cell at a deformation rate of

30 %. Force-time curves were recorded at a crosshead speed of 0.5 mm/s. Ten measurements were performed in each type of sample.

2.5.5. Colour determination

Colour of skin care emulsion was measured using a digital colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) to obtain the colour coordinates L*, a* and b*. These values were used to calculate the euclidean distance value ($\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}$) that allowed both comparing samples with and without extracts, and also comparing the initial colour of every sample to that detected along the storage. The measurements were performed in triplicates.

2.6. Data analysis

Significant differences in volatile chemical classes between control, AOX1 and AOX2 samples in the same storage conditions for each oil were determined using one-way analysis of variance (ANOVA) in combination with Tukey-b post-hoc test ($p<0.05$). Student-t test was applied to determine significant differences in volatile chemical classes between samples at day 1 and 49.

The statistical analysis of data was done using the SPSS 15.0 program (SPSS, INC., Chicago, IL, USA). Significance level of $p\leq0.05$ was used for all evaluations.

3. Results

Table 1 showed the lipid profile of the three oils used in the formulation of the gelled emulsions.

The presence of extracts in gelled emulsion induced remarkable colour changes (**Fig. 1**) with different behaviour depending on the lipid profile. However no effects were observed in texture profile analysis (**Table 2**).

Thiobarbituric acid reactive substances (TBARS) and volatile compounds were followed during the storage. Whereas the presence of extract showed protective effect against volatile compounds formation (**Fig.2**), no effect was found delaying the formation of TBARS (**Table 3**).

To evaluate the influence of the antioxidant polarity against lipid oxidation, the antioxidant capacity of the hydrophilic and the lipophilic compounds present in the emulsion system was evaluated by DPPH assay (**Table 4**). Despite the polar fraction of the gelled emulsion had higher DPPH values, higher antioxidant loss were observed in the apolar fraction. In sunflower and linseed oil, lower antioxidant losses were observed in the presence of *L. Latifolia* extract.

These preliminary results pointed out the protective effect of *L. latifolia* extract against volatile formation and their protective effect on the antioxidants present in the gelled emulsions. A deeper analysis will be done in the future.

4. Acknowledgements

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Table 1. Lipid profile of the oils used in the gelled emulsion (g fatty acids/100g oil mean ± standard deviation).

	Sunflower oil	Linseed oil	Algae oil
Caprilic C8:0	nd	nd	0.45 ± 0.01
Capric C10:0	nd	nd	1.46 ± 0.02
Lauric C12:0	nd	nd	5.27 ± 0.06
Myristic C14:0	0.08 ± 0.01	0.05 ± 0.01	12.51 ± 0.13
Palmitic C16:0	6.51 ± 0.03	5.13 ± 0.08	8.84 ± 0.11
t-palmitoleic C16:1	nd	nd	0.02 ± 0.01
Palmitoleic C16:1	0.14 ± 0.01	0.06 ± 0.01	2.60 ± 0.02
Stearic C18:0	3.28 ± 0.01	3.06 ± 0.01	0.74 ± 0.02
Elaidic C18:1	0.08 ± 0.02	0.06 ± 0.01	0.17 ± 0.01
Oleic C18:1 (ω-9)	31.47 ± 0.03	15.76 ± 0.06	22.91 ± 0.19
Vaccenic C18:1 (ω-7)	0.81 ± 0.01	0.61 ± 0.01	0.19 ± 0.01
t-linoleic C18:2	0.02 ± 0.01	nd	nd
c,t-linoleic C18:2	0.13 ± 0.01	0.07 ± 0.01	0.04 ± 0.01
t,c-linoleic C18:2	0.11 ± 0.01	nd	0.06 ± 0.01
Linoleic C18:2 (ω-6)	56.56 ± 0.04	15.96 ± 0.03	1.24 ± 0.01
Arachidic C20:0	0.13 ± 0.02	nd	nd
γ-linolenic C18:3 (ω-6)	nd	0.21 ± 0.01	nd
Eicosenoic C20:1 (ω-9)	0.10 ± 0.01	nd	0.07 ± 0.01
α-linolenic C18:3 (ω-3)	0.09 ± 0.01	58.98 ± 0.11	0.07 ± 0.01
Behenic C22:0	0.49 ± 0.01	0.06 ± 0.01	0.20 ± 0.01
Eicosapentaenoic C22:5 (ω-3)	nd	nd	0.15 ± 0.01
Docosapentaenoic C22:5 (ω-3)	nd	nd	0.61 ± 0.01
Docosahexaenoic C22:6 (ω-3)	nd	nd	42.40 ± 0.57
SFA	10.5 ± 0.08	8.30 ± 0.08	29.48 ± 0.34
MUFA	32.5 ± 0.05	16.43 ± 0.05	25.77 ± 0.22
PUFA	56.6 ± 0.05	75.14 ± 0.10	44.47 ± 0.56
ω-3	0.09 ± 0.01	58.98 ± 0.11	43.23 ± 0.56
ω-6	56.6 ± 0.04	16.17 ± 0.02	1.24 ± 0.01
ω-6/ω-3	651	0.27	0.03
trans	0.34 ± 0.04	0.13 ± 0.02	0.29 ± 0.01

Table 2. Texture analysis of the nine gelled-emulsion formulations during the storage (SF, sunflower oil; VL, virgin Linseed oil; AO, algae oil; CTRL, control without extra antioxidant; AOX1, 65.5 mg *L. dentata* aqueous extract/100 g gelled emulsion; AOX2, 131 mg *L. dentata* aqueous extract/100 g gelled emulsion).

		Time (days)							
		1	7	14	21	28	35	42	49
Hardness/Fracturability									
	CTRL	927	927	986	932	912	995	972	955
SF	AOX1	1172	1172	1132	1075	1126	1110	1270	1107
	AOX2	1247	1247	1236	1146	1235	1208	1255	1222
	CTRL	980	980	1040	1010	1017	1064	1017	1069
VL	AOX1	1156	1156	1178	1229	1269	1301	1233	1069
	AOX2	1163	1163	1190	1380	1266	1325	1337	1408
	CTRL	999	999	960	970	999	1017	1047	925
AO	AOX1	1194	1194	1130	1386	1370	1405	1284	1235
	AOX2	1323	1323	1259	1320	1457	1457	1459	1422
Adhesiveness									
	CTRL	-64	-64	-73	-66	-50	-89	-59	-70
SF	AOX1	-93	-93	-64	-106	-59	-88	-115	-59
	AOX2	-85	-85	-106	-95	-109	-113	-103	-93
	CTRL	-82	-82	-103	-80	-72	-75	-69	-84
VL	AOX1	-72	-72	-81	-77	-111	-74	-59	-84
	AOX2	-89	-89	-87	-76	-84	-113	-96	-52
	CTRL	-84	-84	-65	-59	-75	-70	-73	-60
AO	AOX1	-98	-98	-85	-84	-74	-71	-118	-41
	AOX2	-93	-93	-70	-70	-85	-47	-90	-87
Springiness									
	CTRL	0.981	0.981	0.964	0.961	0.965	0.971	0.959	0.974
SF	AOX1	0.952	0.952	0.975	0.961	0.970	0.949	0.965	0.964
	AOX2	0.969	0.969	0.954	0.954	0.967	0.961	0.952	0.956
	CTRL	0.956	0.956	0.959	0.959	0.958	0.953	0.967	0.968
VL	AOX1	0.961	0.961	0.955	0.957	0.949	0.962	0.953	0.968
	AOX2	0.957	0.957	0.964	0.973	0.960	0.963	0.971	0.970
	CTRL	0.955	0.955	0.959	0.964	0.963	0.963	0.965	0.955
AO	AOX1	0.958	0.958	0.968	0.965	0.966	0.958	0.959	0.956
	AOX2	0.950	0.950	0.961	0.957	0.965	0.954	0.967	0.951
Cohesiveness									
	CTRL	0.831	0.831	0.832	0.839	0.839	0.830	0.830	0.824
SF	AOX1	0.804	0.804	0.806	0.788	0.811	0.792	0.786	0.776
	AOX2	0.787	0.787	0.791	0.765	0.770	0.749	0.772	0.750
	CTRL	0.824	0.824	0.820	0.811	0.829	0.818	0.818	0.815
VL	AOX1	0.798	0.798	0.806	0.788	0.802	0.780	0.777	0.815
	AOX2	0.774	0.774	0.768	0.765	0.791	0.775	0.803	0.768
	CTRL	0.827	0.827	0.828	0.826	0.828	0.819	0.827	0.823
AO	AOX1	0.801	0.801	0.801	0.792	0.804	0.804	0.788	0.818
	AOX2	0.785	0.785	0.778	0.785	0.782	0.801	0.785	0.794

(continuation table 4)

		Time (days)							
		1	7	14	21	28	35	42	49
Gumminess									
	CTRL	770	770	820	753	765	824	806	785
SF	AOX1	942	942	912	848	891	877	997	859
	AOX2	980	980	970	907	974	904	969	915
	CTRL	807	807	853	819	843	869	831	871
VL	AOX1	921	921	944	968	1016	1015	958	871
	AOX2	897	897	914	1056	995	1026	1077	1080
	CTRL	826	826	794	800	826	833	866	761
AO	AOX1	933	933	906	1089	1098	1130	1011	988
	AOX2	1010	1010	979	1034	1141	1166	1144	1128
Chewiness									
	CTRL	753	753	818	723	739	800	773	764
SF	AOX1	897	897	888	798	864	833	962	828
	AOX2	950	950	925	838	941	868	922	876
	CTRL	771	771	818	784	808	829	803	843
VL	AOX1	891	891	902	927	965	977	913	843
	AOX2	858	858	881	1028	955	989	1044	1048
	CTRL	826	826	794	800	826	833	866	761
AO	AOX1	933	933	906	1089	1098	1130	1011	988
	AOX2	1010	1010	979	1034	1141	1166	1144	1128
Resilence									
	CTRL	0.516	0.516	0.519	0.532	0.523	0.505	0.507	0.525
SF	AOX1	0.459	0.459	0.457	0.459	0.466	0.458	0.449	0.453
	AOX2	0.443	0.443	0.437	0.419	0.436	0.414	0.433	0.422
	CTRL	0.488	0.488	0.484	0.479	0.491	0.489	0.494	0.498
VL	AOX1	0.459	0.459	0.464	0.456	0.474	0.452	0.451	0.498
	AOX2	0.418	0.418	0.420	0.423	0.455	0.435	0.467	0.442
	CTRL	0.498	0.498	0.500	0.504	0.509	0.492	0.505	0.509
AO	AOX1	0.455	0.455	0.475	0.469	0.476	0.470	0.453	0.475
	AOX2	0.459	0.459	0.444	0.461	0.457	0.459	0.448	0.510

Table 3. $TBARS_{390}$ (mmol 2,4-decadienal/100 g gelled emulsion) and $TBARS_{532}$ (mg MDA/100 g gelled emulsion) ± standard deviation obtained for the nine different formulation during the storage (49 days) (SF, sunflower oil; VL, virgin Linseed oil; AO, algae oil; CTRL, control without extra antioxidant; AOX1, 65.5 mg *L. dentata* aqueous extract/100 g gelled emulsion; AOX2, 131 mg *L. dentata* aqueous extract/100 g gelled emulsion).

			Time (days)							
			1	7	14	21	28	35	42	49
390 nm	SF	CTRL	4.04 ± 0.18	5.54 ± 0.70	7.73 ± 0.31	6.99 ± 0.19	5.97 ± 0.28	7.22 ± 0.26	6.53 ± 0.76	6.46 ± 0.16
		AOX1	3.07 ± 0.36	5.86 ± 0.21	7.68 ± 0.24	7.59 ± 0.06	6.76 ± 0.15	7.60 ± 0.08	6.52 ± 0.40	6.73 ± 0.55
		AOX2	3.83 ± 0.27	5.60 ± 0.25	7.83 ± 0.05	7.03 ± 0.55	6.59 ± 0.23	7.43 ± 0.15	6.75 ± 0.30	6.56 ± 0.04
	VL	CTRL	3.82 ± 0.12	6.10 ± 0.14	7.61 ± 0.09	7.78 ± 0.55	6.41 ± 0.14	7.07 ± 0.12	6.66 ± 0.30	6.76 ± 0.61
		AOX1	3.61 ± 0.26	6.22 ± 0.28	7.36 ± 0.14	7.40 ± 0.28	6.90 ± 0.07	7.78 ± 0.12	7.10 ± 0.13	7.27 ± 0.75
		AOX2	3.49 ± 0.55	5.84 ± 0.19	7.50 ± 0.24	7.42 ± 0.19	7.11 ± 0.01	7.10 ± 0.05	7.62 ± 0.47	7.17 ± 0.53
	AO	CTRL	4.09 ± 0.60	6.82 ± 0.64	8.01 ± 0.25	8.04 ± 0.19	7.02 ± 0.17	7.55 ± 0.57	7.18 ± 0.93	7.21 ± 0.40
		AOX1	3.91 ± 0.19	6.53 ± 0.37	8.38 ± 0.21	8.92 ± 0.10	7.63 ± 0.30	7.87 ± 0.60	7.42 ± 1.13	7.46 ± 0.88
		AOX2	4.55 ± 0.15	6.84 ± 0.53	8.60 ± 0.14	8.60 ± 0.09	7.15 ± 0.13	8.02 ± 0.03	7.35 ± 0.17	7.09 ± 0.15
532 nm	SF	CTRL	0.46 ± 0.03	0.31 ± 0.03	0.48 ± 0.01	0.44 ± 0.01	0.38 ± 0.01	0.44 ± 0.03	0.34 ± 0.04	0.38 ± 0.01
		AOX1	0.41 ± 0.02	0.32 ± 0.01	0.48 ± 0.03	0.43 ± 0.01	0.41 ± 0.01	0.44 ± 0.01	0.32 ± 0.01	0.38 ± 0.02
		AOX2	0.41 ± 0.03	0.29 ± 0.01	0.46 ± 0.01	0.41 ± 0.03	0.38 ± 0.01	0.42 ± 0.01	0.33 ± 0.01	0.35 ± 0.01
	VL	CTRL	1.46 ± 0.04	0.83 ± 0.05	1.29 ± 0.12	1.61 ± 0.23	1.33 ± 0.08	1.18 ± 0.04	0.90 ± 0.02	1.10 ± 0.07
		AOX1	0.51 ± 0.01	0.63 ± 0.03	0.80 ± 0.05	0.81 ± 0.02	0.89 ± 0.05	1.03 ± 0.13	0.81 ± 0.09	0.88 ± 0.04
		AOX2	0.79 ± 0.14	0.70 ± 0.01	0.81 ± 0.08	0.91 ± 0.06	0.83 ± 0.04	0.64 ± 0.13	0.66 ± 0.07	0.81 ± 0.06
	AO	CTRL	1.95 ± 0.45	1.48 ± 0.14	2.11 ± 0.23	2.33 ± 0.34	1.81 ± 0.09	1.70 ± 0.41	1.62 ± 0.05	1.55 ± 0.36
		AOX1	1.62 ± 0.26	1.43 ± 0.01	1.67 ± 0.02	1.91 ± 0.05	1.79 ± 0.06	1.77 ± 0.10	1.71 ± 0.15	1.48 ± 0.10
		AOX2	1.86 ± 0.21	1.22 ± 0.01	1.87 ± 0.15	2.06 ± 0.02	1.67 ± 0.01	1.63 ± 0.33	1.68 ± 0.13	1.56 ± 0.08

Table 4. DPPH (mg Trolox equivalent/g gelled emulsion) ± standard deviation of the aqueous and oily phase of the gelled emulsion obtained for the nine different formulations at the beginning and at the end of the storage (days 1 and 49) (SF, sunflower oil; VL, virgin Linseed oil; AO, algae oil; CTRL, control without extra antioxidant; AOX1, 65.5 mg *L. dentata* aqueous extract/100 g gelled emulsion; AOX2, 131 mg *L. dentata* aqueous extract/100 g gelled emulsion).

		DPPH Polar fraction			DPPH Apolar fraction			Total DPPH		
		Day 1	Day 49	Antioxidant loss (%)	Day 1	Day 49	Antioxidant loss (%)	Day 1	Day 49	Overall loss (%)
SF	CTRL	-	-	-	32.5±0.6	12.1±0.4	62.6	32.5±0.6	12.2±0.4	62.6
	AOX1	223± 57	178± 15	20.2	22.9±0.9	9.4±0.3	59.1	245±57	187±15	23.8
	AOX2	504± 53	501± 25	0.6	22.6±0.9	16.3±0.6	28.2	526±52	517±24	1.83
VL	CTRL	-	-	-	23.9±1.3	8.9±0.2	62.8	23.9±1.3	8.91±0.15	62.8
	AOX1	372± 20	311± 26	16.3	26.8±2.2	12.1±0.8	54.9	398±4	323±27	18.9
	AOX2	732± 33	624± 28	14.8	22.8±2.0	13.4±0.2	41.2	754±34	637±28	15.6
AO	CTRL	-	-	-	11.3±0.4	11.7±0.9	-3.93	11.3±0.4	11.7±0.9	-3.93
	AOX1	234± 22	270± 21	-15.4	12.1±1.1	14.9±1.8	-22.9	246±21	284±22	-15.7
	AOX2	562± 41	628± 86	-11.6	17.4±1.4	14.1±0.5	18.9	579±40	641±87	-10.7

Fig. 1. Volatile compounds detected at the beginning and at the end of the storage (day 1 and 49). Results are expressed in area counts $\times 10^3$ per gram of gel (CTRL, control without extra antioxidant; AOX1, 65.5 mg *L. dentata* aqueous extract/100 g gelled emulsion; AOX2, 131 mg *L. dentata* aqueous extract/100 g gelled emulsion).

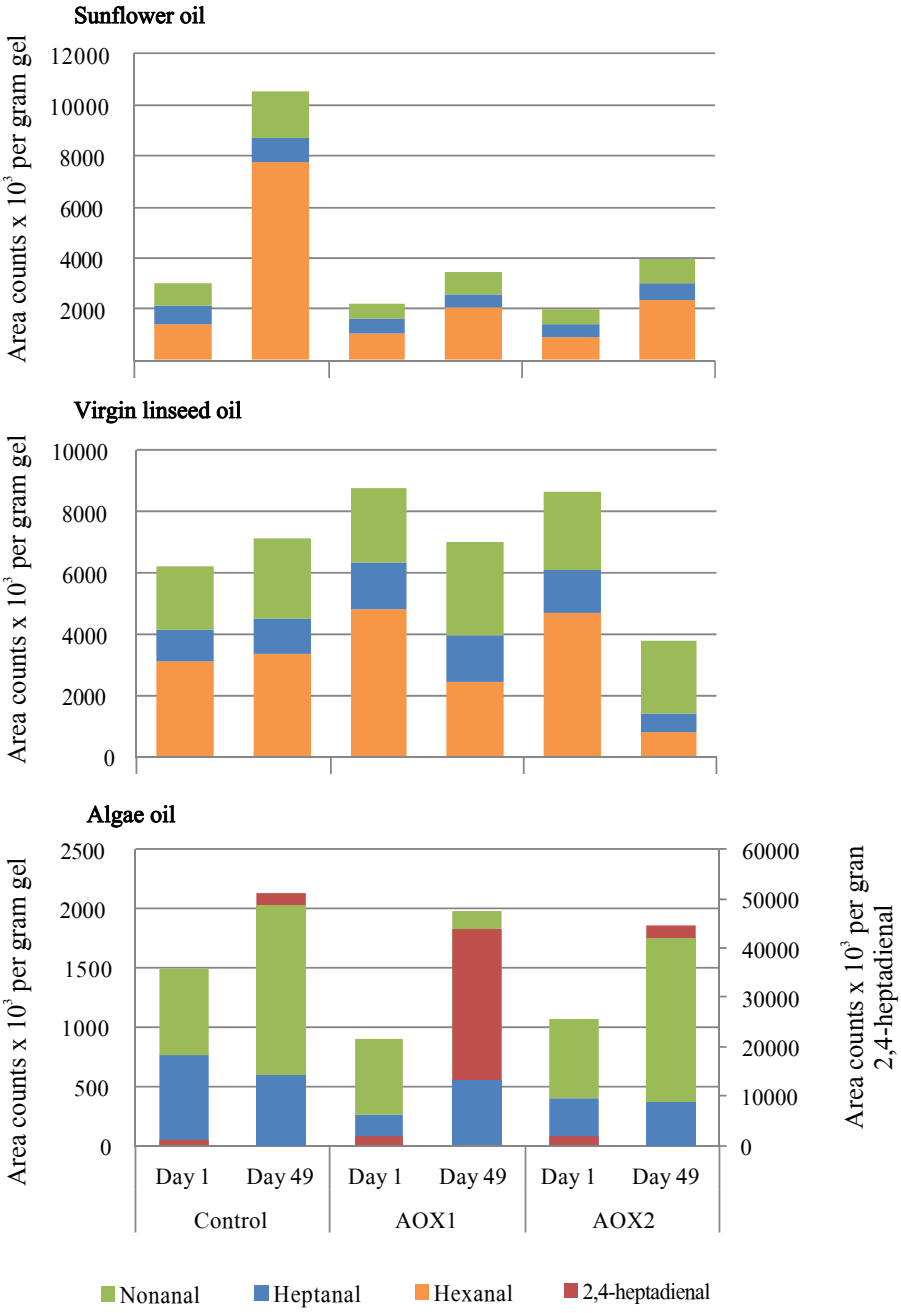
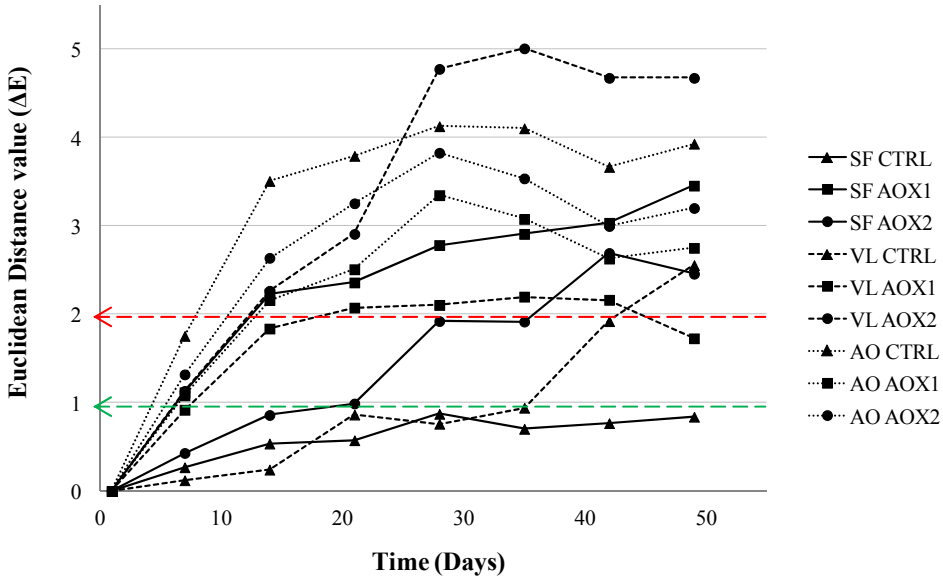


Fig. 2. Euclidean distance value of the nine types of gelled emulsion along the storage. ΔE values less than 1 are considered as imperceptible changes and values less than 2 as minimum changes (SF, sunflower oil; VL, virgin Linseed oil; AO, algae oil; CTRL, control without extra antioxidant; AOX1, 65.6 mg *L. dentata* aqueous extract/100 g gelled emulsion; AOX2, 131 mg *L. dentata* aqueous extract/100 g gelled emulsion).



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A novel approach to monitor the oxidation process of different types of heated oils by using chemometric tools

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Abstract: The oxidative stability of seven oils with different fatty acid profiles was assessed. Oxidation at 0, 2 and 4 hours at 180 °C was monitored by measuring the absorbance of thiobarbituric acid reactive substances (TBARS) along the absorption spectrum (300-600 nm), the volatile aldehydes (HS-SPME-GC-MS) and the fatty acid profile (FID-GC).

TBARS absorption spectrum behavior depended on the lipid composition of heated oils. Higher absorbance increments during heating were noticed at 390 nm compared to 532 nm (from 2 to 21 fold higher depending on the oil), pointing to its better sensitivity to detect oxidation. Furthermore, a close relationship between ABS_{390}, the loss of polyunsaturated fatty acids and their corresponding oxidation compounds (volatile aldehydes) was revealed by Principal Component Analysis

Multiparametric equations allowed predicting the formation of volatile aldehydes of heated oils by measuring only two parameters: $TBARS_{390}$ during their heating, and the lipid profile in unheated oils (MUFA, ω-3 and ω-6). Results pointed out the interest of choosing the ABS_{390} when the oxidative evolution of vegetable oils under heating is assessed by the TBARS test.

Key words: lipid oxidation; SPME; TBARS; volatile aldehydes; fatty acids; oil composition.

1. Introduction

The most important cause of deterioration of oils and fats is oxidation, which does not only reduce shelf life, sensory acceptance and the nutritional value of food, but also produces toxic compounds. Unsaturated and especially polyunsaturated fatty acids are firstly oxidized to form odorless and tasteless hydroperoxides, which are further decomposed to form secondary oxidation products (Papastergiadis, Mubiru, Van Langenhove & De Meulenaer, 2012). Most of them are mainly volatile aldehydes with different unsaturation degree and characterized by low threshold values (Petersen, Kleeberg, Jahreis & Fritsche, 2012). It is well-known that, by oxidation, oleic acid can give rise to heptanal, octanal, nonanal, decanal, (*E*)-2-decenal and (*E*)-2-undecenal; linoleic acid leads to hexanal, 2-heptenal, 2-octenal, (*E,Z*)-2,4-decadienal and (*E,E*)-2,4-decadienal and, lastly linolenic acid produces a greater proportion of (*E,Z*)-2,4-heptadienal and (*E,E*)-2,4-heptadienal (Belitz, Grosch & Schieberle, 2009).

Malondialdehyde (MDA) has been used as representative of non-volatile secondary oxidation products and its determination by the TBARS test measuring the absorbance at 532 nm has been widely used due to its simplicity (Barriuso, Astiasarán & Ansorena, 2013). However, this method exhibits some drawbacks. It has been stated that the TBARS assay is nearly worthless with heat-treated oils and fats (Guillén-Sans & Guzmán-Chozas, 1998) and that this test is reliable only when applied for the determination of MDA in unprocessed foods (Papastergiadis et al., 2012). According to these last authors (Guillén-Sans & Guzmán-Chozas, 1998; Papastergiadis et al., 2012) in thermally treated food, the $TBARS_{532}$ test overestimates the content of MDA because of the interference of other compounds, probably due to the presence of other secondary oxidation products, such as alkadienals. Besides, the overestimation of MDA can also be due to the fact that other types of compounds (carbohydrates, amino acids and nucleic acids) can react with TBA and absorb at 532 nm (Salih, Smith, Price & Dawson, 1987).

On the other hand, the measurement of MDA is inherently insensitive to monounsaturated fatty acids (MUFA), as oleic acid hydroperoxides contain less than two double bonds. This fact leads to underestimation of oxidation in highly monounsaturated lipids when TBARS test is used (Waraho, Cardenia, Rodríguez-

Estrada, McClements & Decker, 2009; Poyato, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena, 2013). Consequently, MDA measurements at 532 nm may not be true measure of oxidative deterioration in certain foods. A better option must be found to easily monitor the evolution of secondary oxidation products, especially during thermal treatments.

It has been reported that, at least, some aldehydes from lipid oxidation may also react with TBA to produce not only red (λ_{max}= 532 nm), but also yellow (λ_{max}= 455 nm) and orange pigments (λ_{max}= 495 nm) (Kosugi, Kato & Kikugawa, 1987). Many alkanals, alkenals and alkadienals produce a yellow pigment with TBA (Guillén-Sans & Guzmán-Chozas, 1998), depending on the time-temperature conditions in which the reaction is forced (Marcuse & Johansson, 1973). In addition, from the safety standpoint, these aldehydes other than MDA are also considered to be important lipid oxidation products due to their toxic effects, and should be considered more than just lipid oxidation markers (Stevens & Meier, 2008; Guillén & Uriarte, 2012).

In this context, the aim of this work was to optimize conditions to follow the oxidation rate of different edible oils subjected to an intense heating by (i) measuring volatile aldehydes by gas chromatography and, (ii) correlating them with the measurement of TBARS at a range of wavelengths (300-600 nm) in order to choose the highest sensitivity for detecting and predicting oxidation intensity. Seven oils with very different lipid profiles were chosen for the study, to evaluate the influence of the different composition on both parameters.

2. Materials and methods

2.1. Materials

The oils used in this study were Virgin Linseed oil, VL, (Biolasi Productos Naturales S.L.,Guipúzcoa, Spain); Algae oil, AO, (Martek Biosciences Corporation, Columbia, USA); Sunflower oil, SF, (Urzante S.L, Navarra, Spain); High-oleic Sunflower oil, HOSF, (Titan, Sos Corporación Alimentaria, S.A, Madrid, Spain); Extra Virgin Olive oil, EVO1, and Refined Olive oil, RO, (Koipe, Sos Corporación Alimentaria, S.A, Madrid, Spain); and Extra Virgin Olive oil, EVO2, (Carbonell, Sos Corporación Alimentaria S.A, Madrid, Spain).

2-thiobarbituric acid, tetraethoxypropane and fatty acid methyl esters were purchased from Sigma–Aldrich Chemical (Steinheim, Germany). Boron trifluoride/methanol and Butylated hydroxytoluene (BHT) were obtained from Merck (Whitehouse Station, NJ, USA). Potassium hydroxide, hexane, cyclohexanone, hydrochoric acid, trichloroacetic acid and ammonium sulphate were from Panreac (Barcelona, Spain).

2.2. Heating study

One gram of oil was weighed into a 25 mL glass vial. Before sealing, the air was replaced with nitrogen in order to control the lipid oxidation process. The test vials were placed in an orbital shaker (JP Selecta S.A., Rotaterm, Barcelona, Spain) previously stabilized at 180 °C. The septum was pierced by a needle allowing air exchange during heating. The vials were removed from the hotplate at different heating times (2 and 4 h). Then, the samples were introduced in an ice bath for 15 min for cooling and stored in the freezer (-20 °C) until analysis. Seven types of oils were tested. From each type, three independent batches were heated at 0, 2 and 4 hours. The 63 different samples were analyzed in triplicate.

2.3. Oil analysis

2.3.1. Fatty acid profile

Fatty acids (FA) were determined in the assayed oils by gas chromatography FID detection, previous preparation of the fatty acid methyl esters derivatives. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (AOAC, 2002a). A Perkin-Elmer Clarus 500 gas chromatograph, equipped with a split-splitless injector, automatic autosampler, and coupled to a computerized system for data acquisition (TotalChrom, version 6.2.1) was used. It was fitted with a capillary column SPTM-2560 (100 m × 0.25 mm × 0.2 μm). The temperature of the injection port was 250 °C and 260 °C for the detector. The oven temperature was programmed to increase from 170 to 200 °C at a rate of 10.0 °C/min and then at rate of 4.0 °C/min to 220 °C. The carrier gas was hydrogen, 2.15 mL/min. The sample size was 0.5 μL and the split ratio was 120:1. The identification 30 fatty acids analyzed was done by comparison of their retention times with those of pure fatty acid methyl esters and the quantification

used heptadecanoic acid methyl ester as internal standard. After the quantification of the individual FA, the sums of saturated, SFA, (capric, lauric, miristic, palmitic, stearic, arachidic, and behenic acid), monounsaturated, MUFA, (palmitoleic, oleic, vaccenic, erucic, nervonic and eicosenoic acid), polyunsaturated, PUFA, (linoleic, γ-linoleic, α-linoleic, eicosadienoic, eicosatrienoic, arachidonic, eicosapentaenoic, docosatrienoic, docosapentaenoic (ω-3), docosapentaenoic (ω-6) and docosahexahenoic acid), trans (*t*-palmitoleic, elaidic, *t*-linoleic, *c,t*-linoleic, *t,c*-linoleic and brassidic acid) and sums of ω-3 (α-linolenic, eicosadienoic, eicosatrienoic, docosapentaenoic, docosahexahenoic acid) and ω-6 (linoleic, γ-linoleic, arachidonic, docosapentaenoic) were calculated.

2.3.2. Determination of volatile aldehydes

The determination of the volatile aldehydes in the headspace of the oils throughout the heating time was carried out by a HS-SPME-GC-MS method. Vials containing 1 g of oil were placed into a water bath maintained at 50 °C. After a period of sample equilibration (15 min), a fiber coated with DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane, 50/30 μm film thickness, Supelco) was inserted into the headspace of the sample and maintained for 60 min (Guillén & Uriarte, 2012). The fiber was desorbed for 15 min in the injection port of a gas chromatograph model HP 6890 Series (Hewlett Packard), equipped with a HP Mass Selective Detector 5973. A fused-silica capillary column (30 m long × 0.25 mm inner diameter × 0.25 μm film thickness, from Agilent Technologies), coated with a non-polar stationary phase (HP-5MS, 5 % phenyl methyl siloxane) was used. The operating conditions were as follows: the oven temperature was set initially at 42 °C (5 min hold), increased to 120 °C at 3 °C/min and to 250 °C at 10 °C/min (5 min hold); the temperatures of the ion source and the quadrupole mass analyzer were kept at 230 °C and 150 °C, respectively. Helium was used as carrier gas at 1 mL/min; injector and detector temperatures were held at 250 °C and 280 °C, respectively. Mass spectra were recorded at 70 eV; using scan mode (amu range 33-350). Before performing every extraction, cleanness of the fiber was checked by running a blank and confirming the absence of peaks in the chromatogram. Compounds were identified by previous injection of standards, by their Kovats index and mass spectra or by matching with

mass spectra of a commercial library (Wiley 275.L, Mass Spectral Database). Total area for each compound was obtained on basis on the amount of a specific ion for each peak, and taking into account the relative ratio in which this ion is present in each compound (Petersen et al., 2012; Thomas, Mercier, Tournayre, Martin & Berdagué, 2013). Results are expressed in area $\times 10^5$/g.

2.3.3. TBARS value

TBARS values were determined according to the method described by Maqsood and Benjakul (2010) with slight modifications. Briefly, the TBARS reagent was prepared by mixing 15 % w/v trichloroacetic acid, 0.375 % w/v 2-thiobarbituric acid in 0.25 N hydrochloric acid. The oil (0.3 g), distillate water (250 µL), solution 1 % BHT (20 µL) and the TBARS reagent (1 mL) were vortexed in a centrifuge tube (20 s), placed in a boiling water bath for exactly 15 min and then cooled in an ice bath to room temperature. Cyclohexanone (2 mL) and 4 M ammonium sulphate (500 µL) were added to the mixture and were vortexed for 30 s. The mixture was centrifuged at room temperature at 4000 rpm for 10 min to allow separation of phases. In the case that solid particles were formed in the interface, the TBARS reaction was repeated using lower amount of sample. After centrifugation, the supernatant was collected and the absorbance was measured between 300 and 600 nm (FLUOStar Omega spectrofluorometric analyzer, BMG Labtechnologies, Offenburg, Germany). The spectra were collected with a resolution of 2 nm. Results were expressed in absorbance units corrected by sample weight.

2.4. Statistical data processing

ANOVA and Tukey b *post hoc* test were applied to compare the evolution of the lipid profile along the heating treatment with statistical significance set at $p<0.05$. Pearson correlation test was used to determine correlations among variables (fatty acids, aldehydes, and absorbances at every wavelength between 300-600 nm) and contributed to decide the wavelength selected.

A data matrix (63×27) whose rows were the different oil samples analysed (cases: 3 different batches from 7 types of oils at 3 different heating times) and whose columns were the selected analytical parameters assayed was built. A Principal

Component Analysis (PCA), was used to achieve a reduction of dimension, retaining the maximum amount of variability present in the experimental data in order to verify the existence of relationships between the analysed oil samples. The data were autoscaled before PCA in order to achieve independence on the different scale factors of the analytical parameters.

After studying the primary observation of principal components obtained, a linear regression model was applied in order to relate the production of volatile aldehydes with the TBARS test absorbances (390 and 532 nm). The variables that may affect the regression were introduced into the study following a forward stepwise model (inclusion criteria: $p< 0.05$; exclusion criteria $p> 0.10$). The selected variables were MUFA, ω-6, and ω-3 (in g/100 g oil) present in the unheated oils. The contribution of the variables was tested in the model for each absorbance (390 or 532 nm) and family of volatile compounds (alkanals, alkenals, alkadienals and total aldehydes). The significant interactions between variables were also taken into account to obtain the equations. A cross validation of the regression model was done by means of data splitting (Montgomery, Peck & Vining, 2006). The data set (n= 63) was split at random into an estimation set (n= 42) used to build the regression model, and a prediction set (n= 21) that studied the predictive ability. Cross validation estimates the prediction error of the obtained regression models. Several statistics were included to evaluate the quality of the diagnostic approach of the regression models: the coefficient of determination for the regression model (R^2), cross validation (R_{CV}^2) and prediction (R_{pred}^2), and root mean squared error of cross validation ($RMSE_{CV}$) or root mean squared error of prediction ($RMSE_P$), calculated as follows, based on the observed and predicted values of the analyzed parameters:

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y})^2}{n}} \qquad R^2 pred = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y})^2}{\sum_{i=1}^{n}(y_i - \bar{y})^2}$$

where y_i and $\hat{y}_i$ are the analytical parameter loads for observation i determined by experimental test and by estimated regression model, respectively; and $\bar{y}$ represents the mean of the observed data. Besides, the usefulness of regression models was evaluated using the ratio of prediction to deviation (RPD). This parameter expresses the ratio of

the standard deviation data set in relation to the root mean squared error of prediction. The regression model was considered an adequate good predictor of analyzed parameters when the RPD value was higher than 2, while a value below 1,5 indicates the useless of the model (Villar, Fernández, Gorritxategi, Ciria & Fernández, 2014; Kamruzzaman, ElMasry, Sun & Allen, 2012).

Finally, a double-cross validation by reversion of the roles of estimation and prediction data sets was carried out for confirmation purposes of the robustness of prediction regression models.

All statistical analyses were run using SPSS v. 15.0 for Windows program.

3. Results

3.1. Fatty acid profile

The fatty acid profile of the seven studied oils was analyzed throughout the heat treatment and sums of the different fractions were calculated (Table 1). Before heating, as expected, the EVO, RO and HOSF oils were rich in oleic acid (67-75 %), whereas SF, VL and AO oil were abundant in linoleic (58 %), α-linolenic acid (53 %) and DHA (35 %), respectively. Regardless the antioxidant amounts present in the oils (data not shown), the heat treatment caused a reduction in the fatty acids content (g FA/100 g oil) of all studied samples, which mainly affected the PUFA fraction. Thus, a large PUFA reduction (28.66 %) was noticed for algae oil, followed by olive oils (EVO1, 11.17 %; EVO2, 13.76 %; RO, 7.34 %), high-oleic sunflower oil (11.74 %) linseed oil (7.93 %), and sunflower oil (5.90 %). MUFA decreased only in the highly monounsaturated oils, being always this reduction lower than a 2.6 % of their initial content.

The evaluation of the absolute weight loss of fatty acids pointed out the DHA suffered the greatest decrease after the 4 h of treatment in AO (10.55 g/100 g oil), whereas in VL, it was α-linolenic who was mainly lost (4.66 g/100 g oil). Linoleic acid was the main PUFA affected by the heat treatment in SF oil (3.41 g/100 g oil). Only a slight reduction in the oleic acid content (1.7, 1.32, 0.76, 1.83 g/100 g oil) was noticed

in HOSF, EVO1, EVO2 and RO oils respectively, despite being the most abundant FA in these four oils.

3.2. Volatile aldehydes analysis

HS-SPMS-GC-MS data of major volatile aldehydes isolated from the headspace of the seven oils were followed throughout the heating treatment (**Table 2**). Five alkanals, nine alkenals and seven alkadienals were identified during the whole process. These compounds represent groups of secondary oxidation products resulting mainly from the autooxidation of oleic, linoleic, α-linolenic acid and DHA (Belitz, Grosch & Schieberle, 2009).

Before heating, only very low amounts of alkanals (hexanal and nonanal) in olive oils were found, while in VL only very low amounts of alkadienals (*(Z,E)*-2,4-heptadienal and *(E,E)*-2,4-heptadienal) were detected. No volatile aldehydes in AO, SF and HOSF oil before the heat treatment were obtained.

The thermal degradation of the fatty acids led to many other compounds that increased their concentrations in the headspace along the heating time, showing different aldehyde profiles depending on the type of oil. The highest increment measured as area counts ($\times 10^5$) was detected in SF followed by EVO oils, RO and VL. HOSF and particularly AO oil gave rise to the lowest amount of total aldehydes.

Alkenals were the main contributors to the total volatile aldehydes in EVO oils at the end of heating (up to 55 %) mainly due to the increase of those compounds with 10 or 11 carbon atoms. Alkenals and alkadienals were the predominant volatile compounds in AO (50 % each approximately), characterized by short chain compounds (alkenals of 3-5 carbon atoms and alkadienals of 6-7 carbon atoms). In both VL and SF, alkadienals represented more than 65 % of total aldehydes content, with (*E,E*)-2,4-decadienal and (*E,E*)-2,4-heptadienal being undoubtedly the principal responsible compounds, respectively. In the case of RO and HOSF oils, the distribution of both aldehydes was very similar, the alkenals around a 45 % and the alkanals to a 40 % of the total aldehydes, approximately.

3.3. TBARS

The full absorption spectra (300-600 nm) resulting from the TBA reaction of the different oils at the different sampling points are shown in **Fig. 1**. Oils characterized by a high PUFA content (VL, AO and SF) showed a substantial absorbance increase in the 350-450 nm region during heating. 390 nm was the wavelength in which the highest correlation coefficients between the measured yellow absorbances (350-420 nm) and the increments of the different families of volatile aldehydes and compositional variables was noticed. Moreover, the association between the reduction of fatty acids and the TBARS absorbance modification during treatment was higher at 390 nm than that observed at other wavelengths.

However, the values measured at the wavelength traditionally used for TBARS analysis (532 nm) were not so clearly modified. In fact, whereas the maximum values at 532 nm were lower than 2 absorbance units, results at the proposed target wavelength (390 nm) reached significantly higher values (up to 17 units in the case of SF and around 15 and 7 units for VL and AO, respectively). In SF and VL oils the absorbance increments during the 4 h treatment at 390 nm were around 21 fold the increments observed at 532 nm, whereas in the olive oils they were only 2-3 folds. Thus, the $TBARS_{390}$ produced during the heating process increased the absorbance in a greater extent than $TBARS_{532}$ along the 4 h treatment regardless of the type of oil tested. It should be also noted that, whereas the absorbance at 390 nm always increased along the process, a constant value or even a reduction in the absorbance at 532 nm was detected in the latest 2 hours (**Table 3**).

3.4. PCA and regressions

Prior to performing PCA, the suitability of autoscaled data for factor analysis was checked. **Table S1** (supplementary material) reports the correlation matrix (R) among the twenty seven variables selected. Inspection of this matrix revealed a great number of coefficients higher than 0.400. The determinant value (1.62×10^{-39}) of correlation matrix was low. All variable items showed a significant correlation with, at least, six other variables or more. The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.729, exceeding the recommended value of 0.6, and the Barlett's test of Sphericity

(value 4659) reached statistical significance ($p<0.001$), both data supporting the factorability of the correlation matrix. Moreover, the Anti-image Correlation matrix showed low values and the Measures of Sampling Adequacy (MSA) for each individual variable were from 0.541 to 0.902. The matrix was therefore, appropriate for PCA.

Four principal components with eigenvalues exceeding one were extracted according Kaiser Criterion which explained up to 90 % of the total variance (40.0 %, 23.5 %, 19.0 % and 7.5 %, respectively). The communalities (variance proportion of a variable involved in the PC space) of every descriptor were found higher than 0.75, except for oleic acid and absorbance at 532 nm (0.465 and 0.624, respectively).

The PC extracted correlation matrix was subjected to the Varimax rotation in order to clarify the assignment of experimental variables. The rotated factor matrix is shown in **Table 4**. After orthogonal rotation, easier interpretation of the factors was possible.

This four-factor model interpreted reasonably well the associations between the variation of certain fatty acids after 4 h (Δ FA) treatment, the ABS_{390} and the volatile aldehydes formed from every oil studied. Thereby, **figure 2** shows the associations obtained among the analyzed parameters in order to visualize the discriminating efficiency of principal factors. The first factor was characterized by high loadings for ABS_{390} (0.690) and also for those parameters (pentanal, hexanal, 2-heptenal, 2-octenal, 2,4-nonadienal and 2,4-decadienal) (loadings between 0.854-0.975) associated with linoleic acid loss (0.965). Heptanal, octanal, nonenal, 2-nonenal, 2-decenal and 2-undecenal, were the dominating variables (loadings higher than 0.926) in the second factor, followed by oleic acid loss (loading of 0.646), and ABS_{532} also correlating to a lower extent (0.428). The prevailing variables in the third factor, 2-butenal (0.965) and 2,4-heptadienal (0.966), mainly reflected the α-linolenic acid decline (0.948). Additionally, ABS_{390} and ABS_{532} had also significant positive loadings (0.647 and 0.505, respectively) related to this third factor. Finally, the fourth factor was related to DHA (0.961) and its oxidation products (2,4-hexadienal and 2-propenal with loadings 0.932 and 0.748, respectively). Also, PCA allows visualizing the discriminating efficiency of the rotated principal components in three-dimension

scores plot, making easy the observation and interpretation of the findings for the simple understanding. Scatterplot of loadings for assayed oil samples (**Fig. S1**, supplementary material) in the space defined by the principal factors show that different clusters are clearly distinguished according to the type of oil, setting a differentiation criterion. From visual inspection, a plain separation between linseed, sunflower and olive-based oils in **figure S1a**; and algae, sunflower and olive-based oils in **figure S1b**, was found.

After studying the primary observation of principal components obtained, the linear regression model applied showed no close relationship between the production of volatile aldehydes and the TBARS test absorbances (390 and 532 nm) using monoparametric equations, therefore a multiple regression model was tested. The **figure S2** (supplementary material) showed the multiparametric character of the relationship between the aldehydes and the absorbance at 390 nm. Numerous multiparametric equations establishing a relationship between the initial lipid profile of oils and the volatile aldehydes formed were calculated. Those in which the highest coefficients of correlation were achieved included the MUFA, ω-3 and ω-6 percentages (**Table 5**). In all cases, predictive models for volatile aldehydes were related with ABS_{390}. In the case of alkanals (equations 1) and alkenals (equations 2) the lineal model was related to % MUFA, aside from ABS_{390}. The adjustment of the regression was better for alkanals (0.907) in comparison with alkenals (0.789). In the case of alkadienals, the highest regression coefficient was obtained when the equation included the % ω-3 or % ω-6 ($R>0.9$). In these biparametric equations, the interaction between the lipidic fraction selected and the ABS_{390} was also included. The best prediction of total aldehydes was achieved by using a multiparametric equation that included the % MUFA as well as % ω-3 (equation 5; $R= 0.896$) or % ω-6 (equation 6; $R= 0.877$). As in the previous cases, the interaction between % MUFA and ABS_{390} was significant and included in the equation. On the contrary, predictive models for volatile aldehydes and ABS_{532} did not fit with the real behavior of volatiles concentration, basically due to the slight decrease of ABS_{532} observed during the last 2 h heating in some oils. Consequently, they were discarded and confirmed the suitability of ABS_{390} to evaluate lipid oxidation in heated oils.

The performance of regression models was evaluated by means of a double cross validation. **Table 5** provides also the performance statistics of multiple regression models obtained in comparison with those evaluated in cross validation. The calculated regression coefficients and determination coefficients exhibit a reasonable similarity for all estimated models. In addition, the RMSE of cross validation is close to the root mean squared error of regression model, deducing be a successful as a predictor. Besides the prediction determination coefficients showed that the quantitative prediction is possible for alkanals (R_{pred}^2= 0.821), alkadienals (R_{pred}^2= 0.877 and 0.874) and total aldehydes (R_{pred}^2= 0.800 and 0.786); supported at the same time by the values of RPD greater than 2. In a lesser extent, the alkenals regression model allows to establish a limited used for prediction (R_{pred}^2= 0.600, RPD>1.5), but explanatory purposes may be claimed. Finally, the little differences in predictive performance and estimated regression coefficients obtained in double-cross validation (data partially showed in **table 5**) prove the useful of the developed model to evaluate the analytical parameters in the different types of assayed oils.

4. Discussion

A significant oxidation of oils was evidenced during heating, giving rise to a decrease in FA, especially the highly unsaturated ones. Furthermore, the higher the unsaturation degree of the FA, the higher was its quantitative loss. These data are consistent with the lower oxidative stability of PUFA, and more specifically with the longer chain FA compared to that of MUFA (Belitz et al., 2009). In fact, polymerization can easily take place in high polyunsaturated oils, which leads to a decrease the fatty acids´ content, giving rise to polar compounds and polymers (Choe & Min, 2007).

The formation of volatile aldehydes during heating constitutes a good index of lipid oxidation intensity (Petersen et al., 2012; Ritter & Budge, 2012). A high association between the total volatile compounds formed and the loss of MUFA and PUFA was observed. In particular, high inverse correlations were especially found between the loss of ω-6 fatty acids and alkadienals (R= -0.968) and total volatile aldehydes (R= -0.853). Aldehydes produced were substantially different in quantity and type (alkanal, alkenal or alkadienal) depending on the composition of the lipid

matrix. These findings were in agreement with those obtained by Guillén & Uriarte (2012) after the analysis of different edible oils heated at high temperatures for long periods of treatment. The individual volatile aldehydes formed were in accordance with the main FA loss detected in each oil. Oils rich in ω-3 FA (VL and AO) showed (*E,E*)-2,4-heptadienal as the main aldehyde formed. SF, rich in ω-6 showed the highest value for (*E,E*)-2,4-decadienal, and oils rich in MUFA (HOSF, EVO1, EVO2 and RO) produced high amounts of 2-decenal and nonanal.

The secondary oxidation compounds formed colored compounds with TBA, whose absorbance was measured at different wavelengths (300-600 nm) and correlated with the increments of the different families of volatile aldehydes. In the yellow area (350-450nm), the highest correlation coefficients between the measured absorbances and the increments of the different families of volatile aldehydes and compositional variable were found at 390 nm. This wavelength was chosen as the best representative parameter for the assessment of the yellow pigment formed from the reaction between the volatile aldehydes and TBA. Moreover, the sensitivity of the measure was higher at ABS_{390} than at ABS_{532} due to the larger increments of absorbance values at 390 nm throughout the same period of heating. In addition, within each type of oil, results showed that a higher correlation was found between total aldehydes and ABS_{390} compared to ABS_{532}, showing the better suitability of the absorbance measured at 390 nm.

Although increases in total aldehydes were found for all analyzed oils during the last 2 hours of heating, it is worthy to be mentioned that no absorbance increases at 532 nm were noticed in this period for AO, HOSF, EVO1 and RO using $TBARS_{532}$. This fact means that this parameter was not able to reveal that an oxidation process was taking place during that period, whereas according to the aldehydes analysis and ABS_{390}, the oxidation increased significantly.

Also, the association between the reduction of ω-6 fatty acids and the TBARS absorbance modification during treatment was higher at 390 nm (R= -0.748) than that observed at 532 nm (R= -0.223).

Chemometric techniques have been used to discriminate among experimental data on edible oils (Giacomelli, Mattea & Ceballos, 2006; Cordella, Tekye, Rutledge &

Leardi, 2012; Vaclavik, Belkova, Reblova, Riddellova & Hajslova, 2013; Tsiaka, Christodouleas & Calokerinos, 2013). In our case, PCA evidenced the associations found between the decrease of individual fatty acids, the absorbances (390 and 532 nm) and the volatile aldehydes produced during the heat treatment. The high association of ABS_{390} to the first and the third components, indicated that the major volatile TBA-reactive compounds that gives rise to yellow chromophores, proceed basically from the oxidation of linoleic (highly associated to the first component) and α-linolenic acid (highly associated to the third component). On the contrary, no associations were found between the first component and ABS_{532}. These data are consistent with previous studies in which it was proved that MDA is not always formed in some oxidized systems. Esterbauer and Cheeseman (1990) proposed that linoleic acid is a poor precursor of MDA, explaining the fact that the decline of this fatty acid is not properly detectable at 532 nm.

PCA also showed a low association between ABS_{390} and the amount of oleic acid (second component). However, in olive oils, the increases of ABS_{390} were also significant during heating because the greater FA decrease was linked to PUFA, not to MUFA, as it has been previously stated. The association of ABS_{532} to the second component was higher than expected. It has been described that TBARS test is insensitive to MUFA oxidation. However, alkenals and alkanals (highly associated to the second component), can also form red pigments in the TBA reaction (Kosugi et al., 1987) and contributed to this association. For these reasons, it seems that the ABS_{390} measurement is more appropriate than the traditional wavelength (ABS_{532}) to follow the oxidation rate of oxidized lipids, at least under the conditions assayed in the present study.

An interesting possibility regarding the estimation of the intensity of lipid oxidation is to find a mathematical model that could be able to monitor it by finding solid relationships among different parameters related to this process. Richards, Wijesundera and Salisbury (2005) found that PV were well associated with the formation of two volatile compounds (hexanal and (*E,E*)-2,4 heptadienal) during accelerated oxidation of canola oil, developing a suitable model to predict the PV from the amounts of those compounds. Petersen, Kleeberg, Jahreis, Busch-Stockfisch and

Fritsche (2012) identified key volatile aldehydes (propanal, *(E,E)*-2,4-hexadienal and *E*-2-heptenal) to differentiate rapeseed oil samples with different oxidative properties. In our study relationships between ABS_{390} and the MUFA amount, ω-3 and ω-6 in the unheated oils were able to predict the amount of different families of aldehydes (alkanals, alkenals and alkadienals) and also total volatile aldehydes and, in consequence, to estimate the intensity of the oxidation process.

The developed equations showed that the amount of alkanals and alkenals were highly dependent on the initial MUFA content of the oils, whereas in the case of alkadienals, regression was mainly related to the initial PUFA content. This finding is in agreement with previous studies in which the PUFA content had also been described as a good predictor of lipid oxidation (Maestre, Pazos & Medina, 2011; Guillén & Uriarte, 2012).

Our regression models are an easy, simple and good tool to predict the formation and semi-quantification of volatile aldehydes, from the fatty acids profile of oils and the evaluation of TBARS absorbance at 390 nm, particularly when it is carried out under accelerated oxidation conditions (180 °C-4 h).

We proposed that measuring TBARS absorbance at 390 nm resulted more sensitive than the measurement making use of 532 nm to monitor lipid oxidation in heated oils, regardless of the lipid profile of the oil evaluated. The formation of volatile aldehydes from lipid oxidation during heating can be predicted from two simple and routine parameters, by measuring the absorbance at proposed wavelength (390 nm) through the TBARS test and assessing the lipid profile of the oil samples.

Our results confirm the validity of using TBARS for assessing oxidation in heated oils if wavelength used is 390 nm.

5. Acknowledgements

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Table 1. Amounts of sums of main lipid fractions (g fatty acids/100 g oil) of the seven analyzed oils during the heating treatment (180°C for 0, 2 and 4 hours). (VL, virgin linseed oil; AO, algae oil; SF, sunflower oil; HOSF, high-oleic sunflower oil; EVO, extra virgin olive oil; RO, refined olive oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-3 fatty acids).

		SFA	MUFA	PUFA	ω-3	ω-6	Trans
VL	0 h	10.56 ± 0.11^{a}	19.95 ± 0.11^{a}	66.63 ± 0.25^{b}	52.87 ± 0.26^{b}	13.76 ± 0.01^{b}	0.20 ± 0.03^{a}
	2 h	10.41 ± 0.01^{a}	19.96 ± 0.04^{a}	61.11 ± 0.03^{a}	47.90 ± 0.01^{a}	13.21 ± 0.04^{a}	0.25 ± 0.01^{a}
	4 h	10.55 ± 0.52^{a}	19.61 ± 0.40^{a}	61.34 ± 0.45^{a}	48.26 ± 0.44^{a}	13.08 ± 0.13^{a}	0.24 ± 0.01^{a}
AO	0 h	24.56 ± 0.34^{b}	21.47 ± 0.22^{a}	37.05 ± 0.55^{c}	36.01 ± 0.56^{c}	1.03 ± 0.01^{a}	0.24 ± 0.01^{c}
	2 h	24.35 ± 0.04^{b}	21.81 ± 0.03^{b}	34.86 ± 0.06^{b}	33.83 ± 0.07^{b}	1.03 ± 0.01^{a}	0.16 ± 0.01^{a}
	4 h	23.48 ± 0.22^{a}	22.29 ± 0.09^{c}	26.43 ± 0.33^{a}	25.40 ± 0.31^{a}	1.03 ± 0.01^{a}	0.21 ± 0.01^{b}
SF	0 h	10.54 ± 0.06^{a}	23.01 ± 0.05^{a}	58.03 ± 0.08^{c}	0.07 ± 0.01^{b}	57.96 ± 0.08^{c}	0.27 ± 0.01^{a}
	2 h	11.18 ± 0.09^{c}	23.77 ± 0.03^{c}	55.87 ± 0.09^{b}	0.06 ± 0.01^{a}	55.81 ± 0.09^{b}	0.42 ± 0.02^{c}
	4 h	10.95 ± 0.14^{b}	23.35 ± 0.05^{b}	54.61 ± 0.11^{a}	0.06 ± 0.01^{a}	54.55 ± 0.11^{a}	0.37 ± 0.02^{b}
HOSF	0 h	7.86 ± 0.05^{b}	76.83 ± 0.04^{b}	7.41 ± 0.02^{c}	0.47 ± 0.01^{c}	6.94 ± 0.02^{c}	0.47 ± 0.02^{c}
	2 h	7.88 ± 0.01^{b}	77.36 ± 0.02^{c}	7.07 ± 0.04^{b}	0.46 ± 0.01^{b}	6.62 ± 0.04^{b}	0.26 ± 0.01^{a}
	4 h	7.60 ± 0.03^{a}	75.13 ± 0.06^{a}	6.54 ± 0.03^{a}	0.36 ± 0.01^{a}	6.17 ± 0.03^{a}	0.37 ± 0.01^{b}
EVO 1	0 h	12.79 ± 0.02^{b}	71.64 ± 0.04^{b}	7.06 ± 0.02^{c}	0.69 ± 0.01^{b}	6.37 ± 0.01^{c}	0.32 ± 0.06^{ab}
	2 h	12.31 ± 0.02^{a}	72.21 ± 0.03^{c}	6.65 ± 0.06^{b}	0.59 ± 0.01^{a}	5.86 ± 0.01^{b}	0.21 ± 0.01^{a}
	4 h	13.41 ± 0.02^{c}	70.31 ± 0.06^{a}	6.27 ± 0.11^{a}	0.59 ± 0.02^{a}	5.68 ± 0.09^{a}	0.36 ± 0.05^{b}
EVO 2	0 h	13.71 ± 0.10^{a}	70.26 ± 0.07^{c}	8.14 ± 0.01^{c}	0.65 ± 0.01^{c}	7.48 ± 0.01^{c}	0.32 ± 0.03^{b}
	2 h	14.56 ± 0.20^{c}	70.14 ± 0.07^{b}	7.38 ± 0.18^{b}	0.57 ± 0.01^{b}	6.80 ± 0.17^{b}	0.12 ± 0.01^{a}
	4 h	14.16 ± 0.12^{b}	69.47 ± 0.06^{a}	7.02 ± 0.01^{a}	0.51 ± 0.02^{a}	6.51 ± 0.02^{a}	0.28 ± 0.01^{b}
RO	0 h	14.37 ± 0.12^{a}	74.45 ± 0.17^{b}	6.34 ± 0.10^{b}	0.59 ± 0.01^{b}	5.74 ± 0.09^{b}	0.49 ± 0.01^{c}
	2 h	14.71 ± 0.06^{b}	72.74 ± 0.29^{a}	5.79 ± 0.22^{a}	0.59 ± 0.02^{a}	5.55 ± 0.20^{a}	0.28 ± 0.01^{a}
	4 h	14.68 ± 0.04^{b}	72.52 ± 0.03^{a}	5.88 ± 0.01^{a}	0.54 ± 0.01^{a}	5.33 ± 0.01^{a}	0.32 ± 0.01^{b}

For each oil and fraction, different letters denote significant differences during the heating treatment ($p<0.05$).

Table 2. Volatile aldehydes detected along the heat treatment for the seven analyzed oils. Results are expressed in area counts $\times 10^5 \times g$ for each compound; between brackets it is indicated the ion used for quantification. (VL, virgin linseed oil; AO, algae oil; SF, sunflower oil; HOSF, high-oleic sunflower oil; EVO, extra virgin olive oil; RO, refined olive oil).

COMPOUND	hours	VL	AO	SF	HOSF	EVO 1	EVO 2	RO
Pentanal	0	-	-	-	-	-	-	-
(44)	2	-	-	34.6 ± 5.36	7.79 ± 0.06	-	20.2 ± 0.97	17.5 ± 2.15
	4	-	-	57.4 ± 4.86	16.6 ± 1.20	-	22.7 ± 0.16	28.8 ± 1.20
Hexanal	0	-	-	-	-	1.07 ± 0.11	-	0.57 ± 0.02
(56)	2	5.78 ± 0.79	-	82.3 ± 8.87	12.8 ± 1.06	35.4 ± 5.23	46.5 ± 7.48	39.5 ± 0.30
	4	14.5 ± 4.22	-	170 ± 0.53	41.6 ± 0.66	93.3 ± 4.55	61.1 ± 2.25	35.2 ± 2.34
Heptanal	0	-	-	-	-	-	-	-
(70)	2	1.14 ± 0.00	1.02 ± 0.14	5.19 ± 0.63	4.26 ± 0.06	15.1 ± 0.09	21.1 ± 3.06	18.8 ± 3.93
	4	2.61 ± 0.31	2.50 ± 0.06	12.2 ± 0.51	12.0 ± 0.22	43.1 ± 4.33	30.7 ± 1.30	28.0 ± 2.80
Octanal	0	-	-	-	-	-	-	-
(43)	2	1.53 ± 0.35	-	5.83 ± 0.58	5.69 ± 0.09	72.2 ± 3.05	30.3 ± 0.80	35.5 ± 7.28
	4	-	-	15.3 ± 2.77	23.5 ± 0.22	88.4 ± 1.45	49.0 ± 0.80	58.1 ± 1.48
Nonanal	0	-	-	-	-	2.56 ± 0.15	1.41 ± 0.28	1.60 ± 0.56
(57)	2	8.26 ± 0.55	3.00 ± 0.31	17.1 ± 1.44	32.1 ± 2.16	144 ± 5.78	46.5 ± 1.42	71.7 ± 2.45
	4	12.0 ± 1.40	5.28 ± 0.24	36.9 ± 2.84	50.3 ± 0.08	223 ± 25.0	92.1 ± 7.35	96.2 ± 1.94
(*E*)-2-propenal	0	-	-	-	-	-	-	-
(56)	2	45.8 ± 4.26	57.3 ± 9.40	26.1 ± 4.53	7.65 ± 0.70	10.6 ± 0.75	10.9 ± 0.79	10.5 ± 1.77
	4	54.5 ± 3.73	79.6 ± 3.56	27.0 ± 0.52	9.36 ± 1.22	8.60 ± 0.98	11.1 ± 1.85	14.4 ± 1.47
(*E*)-2-butenal	0	-	-	-	-	-	-	-
(70)	2	36.4 ± 1.86	12.5 ± 2.17	-	-	9.06 ± 0.24	5.32 ± 0.53	8.53 ± 0.15
	4	62.0 ± 4.31	20.8 ± 1.64	-	0.92 ± 0.07	8.26 ± 0.24	6.01 ± 0.93	7.73 ± 1.17
(*E*)-2-pentenal	0	-	-	-	-	-	-	-
(55)	2	14.61 ± 2.15	16.4 ± 2.35	-	-	4.10 ± 0.12	3.88 ± 0.31	3.88 ± 0.18
	4	26.20 ± 3.22	32.2 ± 0.75	-	-	5.09 ± 0.27	31.5 ± 0.02	4.15 ± 0.30

(Continuation table 2)

COMPOUND	hours	VL	AO	SF	HOSF	EVO 1	EVO 2	RO
(*E*)-2-hexenal	0	-	-	-	-	-	3.93 ± 0.71	-
(55)	2	2.25 ± 0.49	-	17.3 ± 3.20	2.60 ± 0.04	6.64 ± 0.24	6.27 ± 0.09	5.61 ± 0.28
	4	4.43 ± 0.43	1.57 ± 0.47	26.3 ± 2.92	5.10 ± 0.48	10.4 ± 1.42	8.07 ± 1.26	5.79 ± 0.34
(*E*)-2-heptenal	0	-	-	-	-	2.10 ± 0.21	-	-
(83)	2	6.58 ± 0.12	-	122 ± 12.3	16.3 ± 1.03	42.4 ± 1.37	35.0 ± 1.88	27.5 ± 0.55
	4	10.8 ± 1.11	-	193 ± 14.5	30.5 ± 5.63	64.2 ± 3.39	57.8 ± 1.87	25.8 ± 1.61
(*E*)-2-octenal	0	-	-	-	-	-	-	-
(70)	2	-	-	18.5 ± 1.46	3.02 ± 0.18	13.3 ± 3.97	10.3 ± 0.72	9.47 ± 0.69
	4	-	-	43.8 ± 2.73	6.87 ± 0.50	27.3 ± 2.00	17.9 ± 2.48	11.9 ± 0.18
(*E*)-2-nonenal	0	-	-	-	-	-	-	-
(70)	2	-	-	2.63 ± 0.58	2.41 ± 0.57	11.5 ± 1.58	9.38 ± 0.54	12.3 ± 0.08
	4	-	2.20 ± 0.28	7.55 ± 0.57	7.32 ± 1.82	35.9 ± 8.65	26.2 ± 8.08	13.4 ± 0.37
(*E*)-2-decenal	0	-	-	-	-	-	-	-
(70)	2	3.31 ± 0.08	-	13.9 ± 1.82	15.5 ± 0.28	94.7 ± 6.28	186 ± 3.25	92.6 ± 0.08
	4	12.5 ± 3.77	5.39 ± 0.95	73.3 ± 3.48	64.9 ± 8.32	374 ± 18.1	366 ± 6.19	103 ± 13.0
(*E*)-2-undecenal	0	-	-	-	-	1.35 ± 0.34	-	-
(70)	2	2.72 ± 0.59	-	6.77 ± 1.42	5.33 ± 0.09	68.8 ± 5.20	100 ± 1.73	77.0 ± 2.54
	4	8.45 ± 0.69	4.89 ± 0.68	52.8 ± 0.64	51.1 ± 2.27	357 ± 1.51	232 ± 1.04	113 ± 0.41
(*E,E*)-2,4-hexadienal	0	-	-	-	-	-	-	-
(81)	2	1.00 ± 0.23	3.39 ± 0.54	-	-	-	-	-
	4	1.93 ± 0.08	7.02 ± 0.08	-	-	0.59 ± 0.01	-	-
(*Z,E*)-2,4-heptadienal	0	0.41 ± 0.06	-	-	-	1.31 ± 0.14	-	-
(81)	2	44.0 ± 1.53	18.5 ± 3.89	2.31 ± 0.25	-	6.38 ± 0.50	4.84 ± 0.00	3.60 ± 0.15
	4	68.0 ± 6.01	29.2 ± 1.87	5.34 ± 0.30	-	6.42 ± 1.03	5.02 ± 1.13	4.45 ± 0.33
(*E,E*)-2,4-heptadienal	0	0.47 ± 0.05	-	-	-	-	-	-
(81)	2	166 ± 7.49	59.2 ± 3.75	2.46 ± 0.22	2.33 ± 0.18	27.3 ± 2.22	19.5 ± 2.26	14.3 ± 1.14
	4	267 ± 12.1	93.5 ± 5.61	1.97 ± 0.65	1.60 ± 0.07	21.4 ± 0.87	21.6 ± 2.11	16.9 ± 1.85

(Continuation table 2)

COMPOUND	hours	VL	AO	SF	HOSF	EVO 1	EVO 2	RO
(*Z,E*)-2,4-nonadienal	0	-	-	-	-	-	-	-
(81)	2	-	-	0.88 ± 0.02	-	-	-	-
	4	-	-	2.45 ± 0.14	-	-	-	-
(*E,E*)-2,4-nonadienal	0	-	-	-	-	-	-	-
(81)	2	-	-	3.66 ± 0.42	-	-	-	-
	4	-	-	11.9 ± 0.13	-	4.28 ± 0.19	-	-
(*Z,E*)-2,4-decadienal	0	-	-	-	-	-	-	-
(81)	2	3.67 ± 0.66	-	35.6 ± 1.84	5.19 ± 0.33	33.0 ± 0.28	17.6 ± 0.35	11.4 ± 2.32
	4	9.47 ± 0.71	-	163 ± 3.44	8.67 ± 0.71	33.9 ± 1.56	31.4 ± 6.11	19.5 ± 0.27
(*E,E*)-2,4-decadienal	0	-	-	-	-	-	-	-
(81)	2	23.5 ± 3.66	-	204 ± 7.34	28.1 ± 0.84	179 ± 5.13	132 ± 4.10	32.9 ± 1.13
	4	73.5 ± 5.65	-	1173 ± 22	60.5 ± 7.55	215 ± 14.9	223 ± 1.45	62.0 ± 5.80
ALKANALS	0	-	-	-	-	3.65 ± 0.05	1.42 ± 0.28	2.18 ± 0.60
	2	16.72 ± 1.06	3.86 ± 0.01	145 ± 16.0	62.7 ± 1.74	267 ± 5.44	165 ± 12.5	183 ± 15.5
	4	29.2 ± 5.10	7.79 ± 0.20	292 ± 4.68	144 ± 2.96	462 ± 10.9	256 ± 4.43	246 ± 4.47
ALKENALS	0	-	-	-	-	3.45 ± 0.48	3.94 ± 0.72	-
	2	111 ± 2.20	86.2 ± 13.8	207 ± 21.1	52.8 ± 2.25	261 ± 8.26	369 ± 7.77	248 ± 0.80
	4	178 ± 7.30	146 ± 4.17	427 ± 20.2	176 ± 20.3	892 ± 23.7	757 ± 16.7	300 ± 12.0
ALKADIENALS	0	0.88 ± 0.12	-	-	-	1.30 ± 0.14	-	-
	2	238 ± 5.45	75.2 ± 15.37	239 ± 14.6	35.6 ± 1.36	246 ± 6.50	175 ± 4.25	62.4 ± 4.76
	4	415 ± 21.3	130 ± 7.53	1357 ± 25.8	65.8 ± 15.4	282 ± 14.6	281 ± 7.90	103 ± 5.05
TOTAL ALDEHYDES	0	0.88 ± 0.12	-	-	-	8.39 ± 0.57	5.35 ± 0.98	2.18 ± 0.60
	2	367 ± 7.31	181 ± 5.77	592 ± 32.0	151 ± 5.14	775 ± 15.1	708 ± 22.9	493 ± 11.6
	4	623 ± 32.6	284 ± 11.5	2077 ± 50.6	386 ± 1.95	1636 ± 2.52	1294 ± 13.2	650 ± 17.4

Table 3. Absorbance units (au) and increments at 390 nm and 532 nm of the seven oils studied during the heating process (180 °C, 0, 2 and 4 hours). (VL, virgin linseed oil; AO, algae oil; SF, sunflower oil; HOSF, high-oleic sunflower oil; EVO, extra virgin olive oil; RO, refined olive oil).

	Time (h)	ABS_{390} (au)	Δ ABS_{390}	ABS_{532} (au)	Δ ABS_{532}
VL	0	0.73 ± 0.03		1.08 ± 0.09	
	2	10.8 ± 0.11	10.154	0.41 ± 0.10	0.033
	4	14.7 ± 0.43	3.815	1.72 ± 0.16	0.607
AO	0	4.11 ± 0.03		0.92 ± 0.09	
	2	5.77 ± 0.41	1.656	1.60 ± 0.46	0.673
	4	7.66 ± 0.41	1.892	1.53 ± 0.07	-0.063
SF	0	0.37 ± 0.04		0.05 ± 0.01	
	2	8.44 ± 0.01	8.063	0.32 ± 0.01	0.267
	4	17.8 ± 1.20	9.398	0.89 ± 0.04	0.570
HOSF	0	0.25 ± 0.03		0.02 ± 0.01	
	2	1.47 ± 0.04	1.204	0.15 ± 0.02	0.123
	4	1.60 ± 0.03	0.147	0.14 ± 0.01	-0.010
EVO1	0	0.32 ± 0.03		0.17 ± 0.03	
	2	2.41 ± 0.18	2.084	1.23 ± 0.08	1.060
	4	2.90 ± 0.06	0.494	1.14 ± 0.10	-0.087
EVO2	0	0.38 ± 0.02		0.42 ± 0.02	
	2	2.18 ± 0.05	1.800	0.97 ± 0.47	0.527
	4	2.47 ± 0.16	0.287	1.57 ± 0.39	0.603
RO	0	0.33 ± 0.02		0.12 ± 0.01	
	2	2.61 ± 0.01	2.283	1.64 ± 0.06	1.523
	4	3.07 ± 0.08	0.461	1.30 ± 0.06	-0.333

Table 4. Varimax rotated factor matrix.

Factor	Principal Component			
	1	2	3	4
(*Z,E*)-2,4-nonadienal	0.975			
Linoleic acid	0.965			
(*Z,E*)-2,4-decadienal	0.962			
(*E,E*)-2,4-decadienal	0.958			
(*E*)-2-heptenal	0.957	0.208		
(*E,E*)-2,4-nonadienal	0.948			
(*E*)-2-hexenal	0.938	0.247		
Hexanal	0.902	0.404		
(*E*)-2-octenal	0.859	0.486		
Pentanal	0.854			
ABS 390 nm	0.690		0.647	0.232
Heptanal	0.200	0.960		
(*E*)-2-undecenal		0.957		
(*E*)-2-nonenal		0.953		
Octanal		0.935		
Nonanal		0.931		
(*E*)-2-decenal		0.926		
Oleic acid		0.646		
(*E,E*)-2,4-hHeptadienal			0.966	
(*E*)-2-butenal			0.965	
(*Z,E*)-2,4-heptadienal			0.950	0.271
α-linolenic acid			0.948	
ABS 532 nm		0.428	0.505	0.430
DHA				0.961
(*E,E*)-2,4-hexadienal			0.275	0.932
(*E*)-2-propenal			0.593	0.748
(*E*)-2-pentenal		0.236	0.578	0.636

Table 5. Coefficients (a, b, c, d and e) and Statistics (sample size, n, correlation coefficient, R, root mean squared errors, RMSE and ratio of prediction to deviation, RPD) of the equations obtained by fitting of ABS_{390} during the heating treatment and the proportion of monounsaturated (% MUFA), omega-6 (% ω-6) and omega-3 (% ω-3) in the unheated oils.

Eq.	Volatile Compounds	x_1	x_2		n	a $\times 10^5$	b $\times 10^5$	c $\times 10^5$	d $\times 10^5$	e $\times 10^5$	R	R^2	R^2_{pred}	RMSE	$RMSE_{pred}$	RPD_{pred}
1	Alkanals	MUFA		RM	63	-14.7	-36.3	-0.56	2.1		0.895	0.800		6.00×10^6		
				CV	42	-13.9	-35.2	-0.56	2.1		0.890	0.793	0.821	6.17×10^6	5.61×10^6	2.42
				DCV	21	-16.5	-28.4	-0.55	2.2		0.905	0.818	0.794			
2	Alkenals	MUFA		RM	63	25.5	-59.7	-1.59	3.6		0.758	0.617		1.53×10^7		
				CV	42	27.3	-59.6	-1.66	3.6		0.765	0.618	0.600	1.57×10^7	1.51×10^7	1.62
				DCV	21	20.6	-59.7	-1.41	3.6		0.784	0.615	0.618			
3	Alkadienals	ω-3		RM	63	-29.9	69.5	-2.00	-0.7		0.933	0.871		1.10×10^7		
				CV	42	-34.7	71.9	-1.83	-0.7		0.933	0.870	0.877	1.10×10^7	1.02×10^7	2.92
				DCV	21	-22.5	65.5	-2.15	-0.6		0.937	0.878	0.870			
4	Alkadienals	ω-6		RM	63	80.8	8.59	-4.03	1.2		0.930	0.865		1.10×10^7		
				CV	42	82.4	7.84	-4.19	1.3		0.929	0.863	0.874	1.12×10^7	1.04×10^7	2.88
				DCV	21	79.4	9.68	-3.94	1.1		0.935	0.874	0.863			
5	Total aldehydes	MUFA	ω-3	RM	63	141	-48.5	-4.30	6.3	-8.6	0.879	0.773		2.79×10^7		
				CV	42	170	-45.9	-4.70	6.2	-9.2	0.878	0.771	0.800	2.85×10^7	2.52×10^7	2.29
				DCV	21	75.2	-53	-3.40	6.4	-7.4	0.883	0.780	0.785			
6	Total aldehydes	MUFA	ω-6	RM	63	-397	-58	2.00	6.5	7.1	0.877	0.770		2.80×10^7		
				CV	42	-399	-56	2.00	6.5	7.5	0.875	0.766	0.786	2.89×10^7	2.61×10^7	2.22
				DCV	21	-397	-61	2.22	6.6	6.4	0.883	0.780	0.771			

RM: Regression model
CV: Cross Validation
DCV: Double-Cross Validation

Volatile Compound = $a + b \times ABS_{390} + c \times x_1 + (d \times ABS_{390} \times x_1) + e \times x_2$

Fig. 1. Absorption spectra of the seven oils studied. During the heating process (180 °C; 0, 2 and 4 hours) (VL, virgin linseed oil; AO, algae oil; SF, sunflower oil; HOSF, high-oleic sunflower oil; EVO, extra virgin olive oil; RO, refined olive oil).

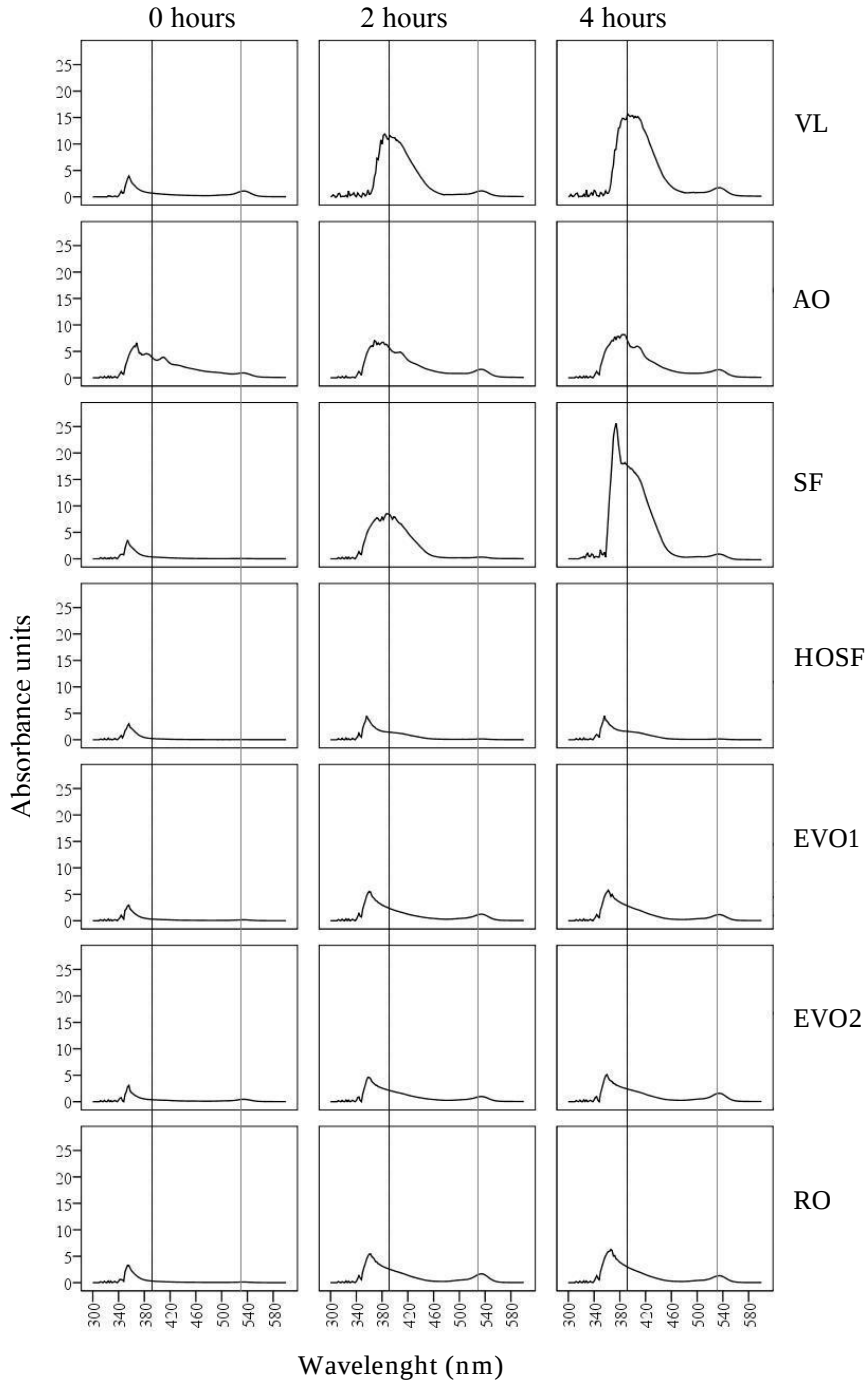
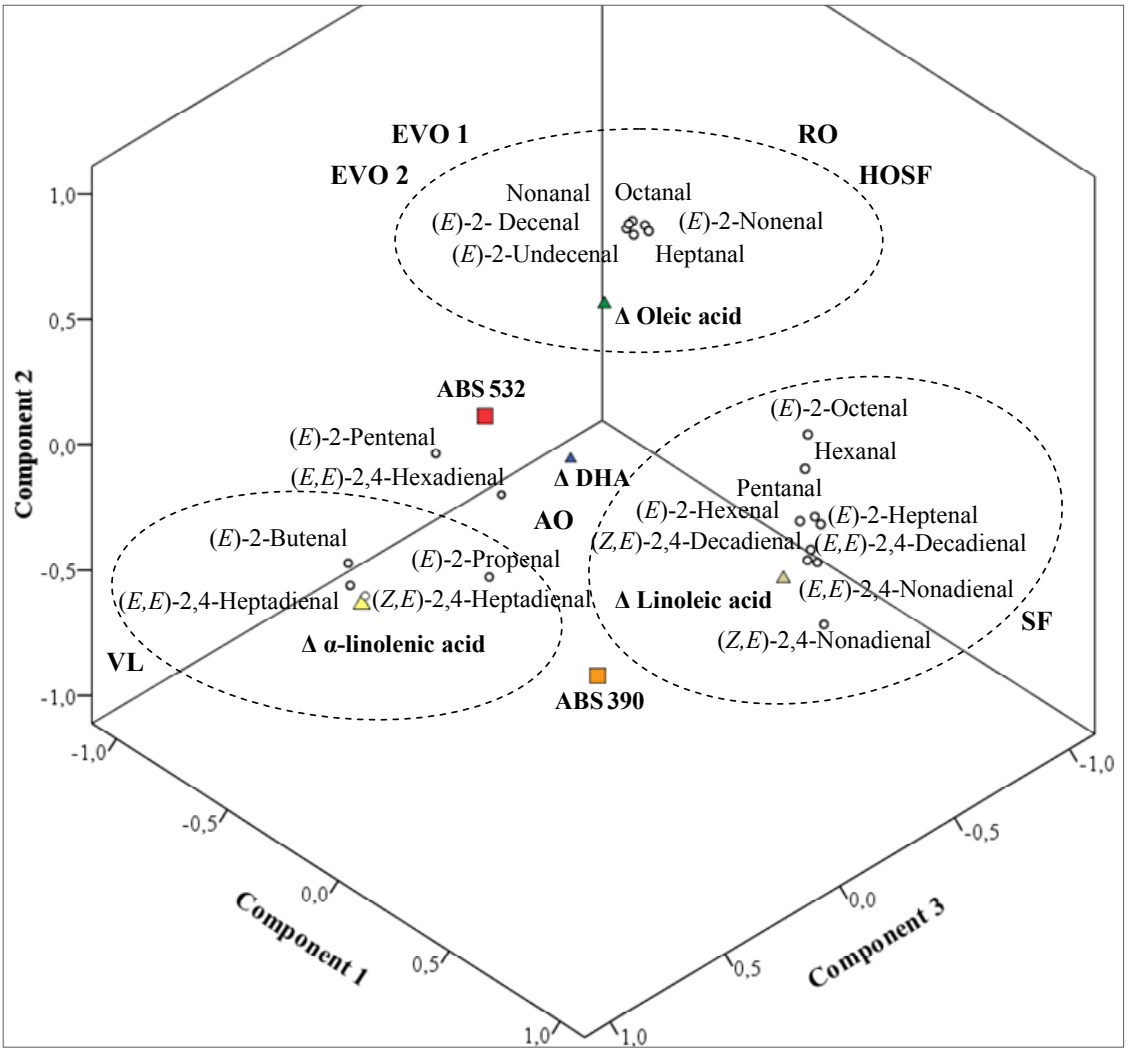


Fig. 2. Three dimensional representation of the principal component scores for volatile aldehydes, key absorbances and loss of fatty acids of the seven oils at 0, 2, 4 hours of heating. (VL, virgin linseed oil; AO, algae oil; SF, sunflower oil; HOSF, high-oleic sunflower oil; EVO, extra virgin olive oil; RO, refined olive oil).



Supplementary material

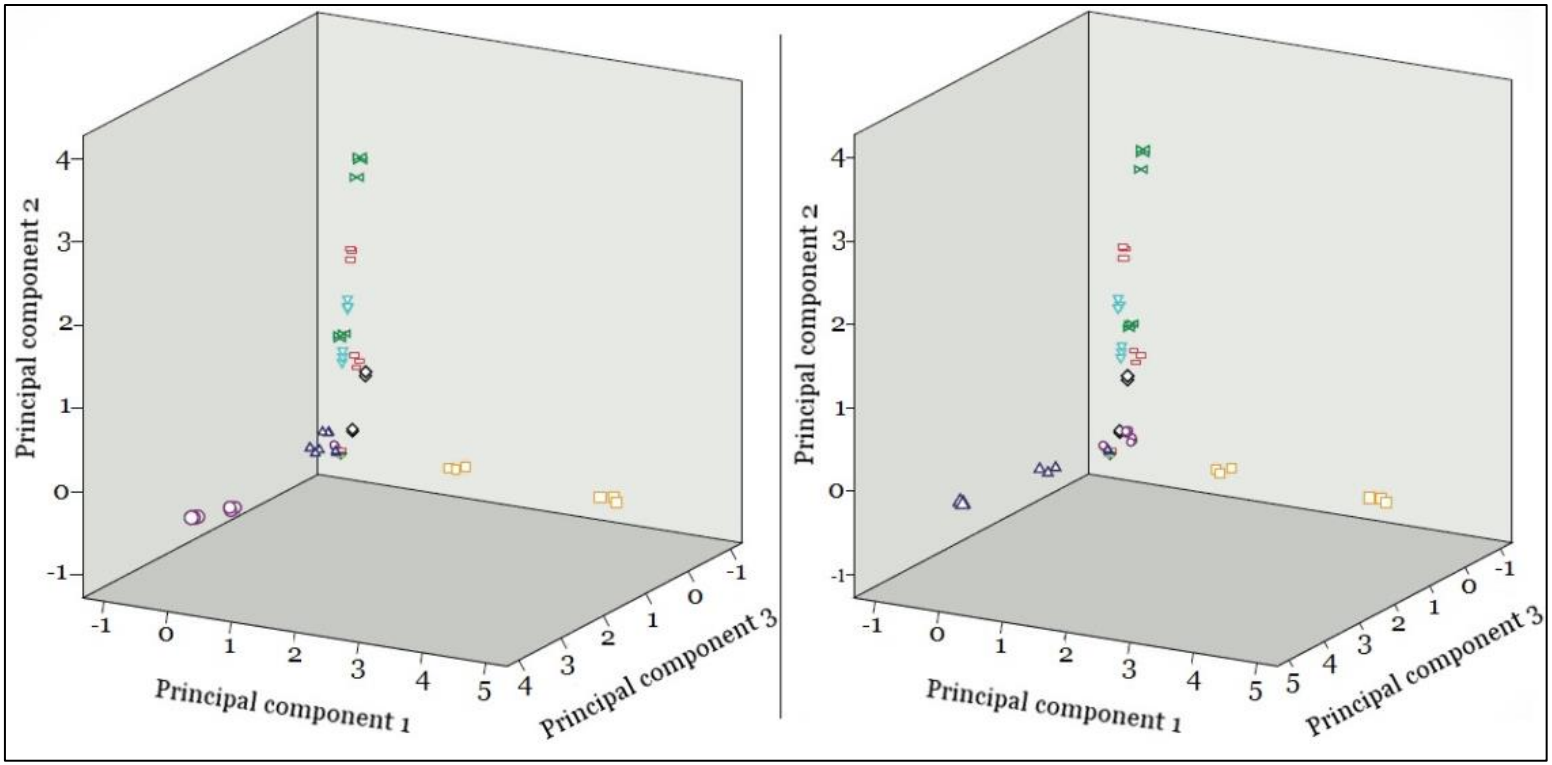
Table S1. Correlation matrix between analytical parameters in different samples of oil investigated.

Analytical parameter	*Pentanal*	*Hexanal*	*Heptanal*	*Octanal*	*Nonanal*	*(E)-2Propenal*	*(E)-2-butenal*	*(E)-2-pentenal*	*(E)-2-hexenal*	*(E)-2-heptenal*	*(E)-2-octenal*	*(E)-2-nonenal*	*(E)-2-decenal*	*(E)-2-undecenal*	*(E,E)-2,4-Hexadienal*	*(Z,E)-2,4-Heptadienal*	*(E,E)-2,4-Heptadienal*	*(Z,E)-2,4-Nonadienal*	*(E,E)-2,4-Nonadienal*	*(Z,E)-2,4-Decadienal)*	*(E,E)-2,4-Decadienal)*	*ABS 390nm*	*ABS 532nm*	*Oleic acid*	*Linoleic acid*	*α-linolenic acid*	*DHA*
Pentanal	1.000																									
Hexanal	0.829***	1.000																								
Heptanal	0.366**	0.576***	1.000																							
Octanal	0.207	0.471***	0.919***	1.000																						
Nonanal	0.131	0.491***	0.896***	0.969***	1.000																					
(E)-2-propenal	0.014	0.012	-0.131	-0.187	-0.153	1.000																				
(E)-2-butenal	-0.231*	-0.149	-0.058	-0.070	-0.032	0.685***	1.000																			
(E)-2-pentenal	-0.120	-0.090	0.133	0.036	0.035	0.742***	0.666***	1.000																		
(E)-2-hexenal	0.856***	0.954***	0.425***	0.339**	0.345**	0.089	-0.092	-0.075	1.000																	
(E)-2-heptenal	0.864***	0.961***	0.386**	0.304**	0.315**	0.052	-0.173	-0.118	0.978***	1.000																
(E)-2-octenal	0.785***	0.981***	0.641***	0.567***	0.579***	-0.032	-0.195	-0.091	0.921***	0.933***	1.000															
(E)-2-nonenal	0.255*	0.536***	0.935***	0.875***	0.891***	-0.142	-0.077	0.196	0.389**	0.363**	0.614***	1.000														
(E)-2-decenal	0.254*	0.523***	0.923***	0.814***	0.818***	-0.150	-0.061	0.262*	0.362**	0.344**	0.583***	0.947***	1.000													
(E)-2-undecenal	0.193	0.516***	0.944***	0.862***	0.896***	-0.148	-0.047	0.186	0.343**	0.319**	0.587***	0.964***	0.966***	1.000												
(E,E)-2,4-Hexadienal	-0.258*	-0.230*	-0.185	-0.220*	-0.172	0.861***	0.453**	0.701***	-0.193	-0.219*	-0.235*	-0.152	-0.170	-0.148	1.000											
(Z,E)-2,4-Heptadienal	-0.213*	-0.139	-0.153	-0.180	-0.130	0.768***	0.979**	0.689**	-0.070	-0.140	-0.195	-0.165	-0.137	-0.128	0.541***	1.000										
(E,E)-2,4-Heptadienal	-0.245*	-0.172	-0.140	-0.159	-0.114	0.711***	0.990**	0.668**	-0.106	-0.180	-0.227*	-0.152	-0.121	-0.116	0.478***	0.994***	1.000									
(Z,E)-2,4-Nonadienal	0.790***	0.805***	0.022	-0.060	-0.046	0.116	-0.166	-0.186	0.856***	0.888***	0.759***	-0.003	-0.020	-0.036	-0.117	-0.085	-0.142	1.000								
(E,E)-2,4-Nonadienal	0.709***	0.886***	0.231*	0.143	0.209*	0.078	-0.157	-0.186	0.874***	0.901***	0.863***	0.229*	0.194	0.228*	-0.113	-0.092	-0.148	0.939***	1.000							
(Z,E)-2,4-Decadienal	0.800***	0.912***	0.318**	0.260*	0.262*	0.068	-0.120	-0.098	0.901***	0.924***	0.904***	0.285*	0.273*	0.254*	-0.169	-0.079	-0.125	0.930***	0.940***	1.000						
(E,E)-2,4-Decadienal	0.779***	0.895***	0.277*	0.204	0.213*	0.077	-0.111	-0.077	0.881***	0.907***	0.879***	0.259*	0.260*	0.231*	-0.152	-0.062	-0.109	0.934***	0.941***	0.995***	1.000					
ABS 390 nm	0.455***	0.507***	-0.056	-0.142	-0.100	0.675***	0.589***	0.378**	0.595***	0.567***	0.434***	-0.089	-0.092	-0.093	0.325**	0.658***	0.613***	0.667***	0.619***	0.621***	0.631***	1.000				
ABS 532 nm	0.053	0.126	0.387**	0.357**	0.315**	0.556***	0.546***	0.667***	0.098	0.042	0.164	0.339**	0.351**	0.338**	0.449***	0.515***	0.501***	-0.060	-0.014	0.107	0.087	0.396**	1.000			
Oleic acid	0.306**	0.326**	0.649***	0.603***	0.584***	0.170	0.090	0.178	0.257*	0.208	0.337**	0.558***	0.463***	0.542***	0.103	0.036	0.038	-0.062	0.053	0.095	0.048	0.041	0.205	1.000		
Linoleic acid	0.865***	0.914***	0.272*	0.167	0.174	0.153	-0.019	-0.048	0.955***	0.967***	0.854***	0.233*	0.240*	0.197	-0.187	0.024	-0.013	0.902***	0.873***	0.905***	0.898***	0.693***	0.045	0.184	1.000	
α-ltinolenic acid	-0.202	-0.147	-0.177	-0.190	-0.148	0.464***	0.879**	0.420**	-0.082	-0.142	-0.209	-0.187	-0.149	-0.145	0.148	0.872***	0.895***	-0.098	-0.112	-0.099	-0.083	0.558***	0.291*	-0.051	0.049	1.000
DHA	-0.170	-0.193	-0.156	-0.184	-0.163	0.693***	0.197	0.574**	-0.156	-0.171	-0.183	-0.123	-0.152	-0.143	0.930***	0.284*	0.218*	-0.078	-0.093	-0.135	-0.123	0.168	0.302**	0.115	-0.180	-0.092

*** p<0.001; ** p<0.01; * p< 0.05

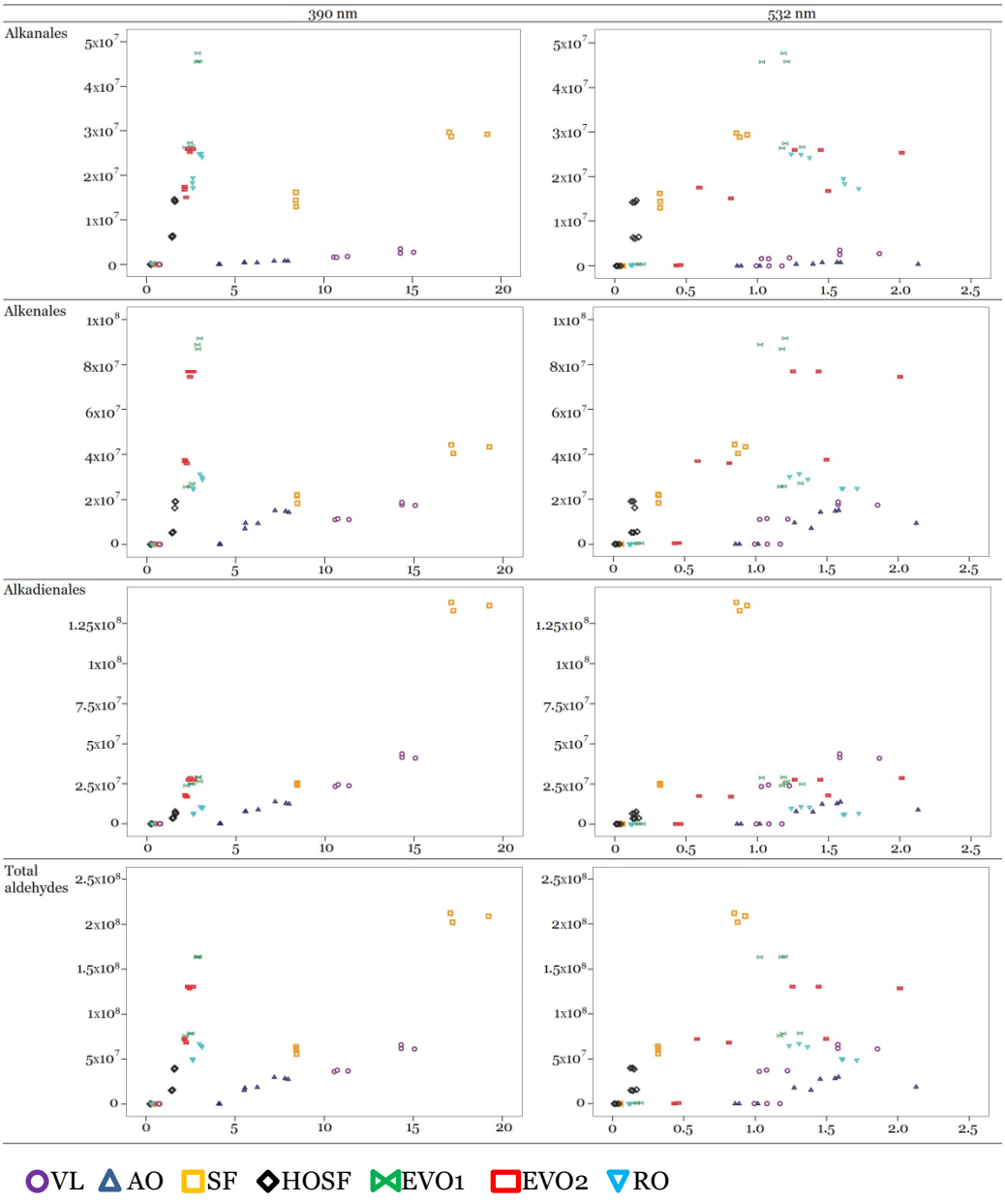
Supplementary material

Figure S1. Principal component scores for different types of oils analyzed



Supplementary material

Figure S2. Two-dimensional plots of the absorbance values at 390 and 532 nm *versus* the instrumental response corresponding to the different families of volatile aldehydes analysed.



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Optimization of a gelled emulsion intended to supply ω-3 fatty acids into meat products by means of Response Surface Methodology

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Abstract: The optimization of a gelled oil-in-water emulsion was performed for use as fat replacer in the formulation of ω-3 PUFA-enriched cooked meat products. The linseed oil content, carrageenan concentration and surfactant-oil ratio were properly combined in a surface response design for maximizing the hardness and minimizing the syneresis of the PUFA delivery system. The optimal formulation resulted in a gelled emulsion containing 40 % of oil and 1.5 % of carrageenan, keeping a surfactant-oil ratio of 0.003. The gel was applied as a partial fat replacer in a Bologna-type sausage and compared to the use of an O/W emulsion also enriched in ω-3. Both experimental sausages contributed with higher ω-3 PUFA content than the control. No sensory differences were found among formulations. The selected optimized gelled oil-in-water emulsion was demonstrated to be a suitable lipophilic delivery system for ω-3 PUFA compounds and applicable in food formulations as fat replacer.

Key words: fat replacer, hydrocolloids, delivery system, gelled emulsion.

1. Introduction

The characteristics of fat analogues intended to replace animal fats are needed in order to achieve the appearance and the technological, rheological and sensory properties required for use in the food industry (Tye, 1991). In fact, the use of fat replacers can cause, in some cases, technological problems due to the fact that fat has a great impact

on flavour, palatability and texture of foods (Hort & Cook, 2007; Delgado-Pando et al., 2010a; Horita, Morgano, Celeghini & Pollonio, 2011).

The use of emulsion based delivery systems is a suitable technology for protection and release of lipids in food (McClements, Decker & Weiss, 2007; Salminen, Herrmann & Weiss, 2013). There has been an increasing interest in improving the functional performance of foods using a wide variety of novel types of emulsion delivery systems, including solid lipid particles, filled hydrogel particles and conventional, multiple and multilayer emulsions (McClements, 2010a). These systems are able to incorporate lipophilic functional agents with beneficial health effects into food products (Valencia, O'Grady, Ansorena, Astiasarán & Kerry, 2008; Taneja & Singh, 2012; Chung, Degner & McClements, 2013; Nielsen & Jacobsen, 2013; Poyato, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena, 2013). Some of these emulsion delivery systems have been used as fat replacers to produce high ω-3 products for improving the nutritional quality of new products. In this sense, the potential development of functional meat products using reformulation strategies has been attempted with the aid of emulsion based systems. The substitution of pork back fat with pre-emulsified oils ω-3 type PUFA oils has been demonstrated to be a good strategy to achieve healthier lipid profiles in these products (García Íñiguez de Ciriano et al., 2010; Berasategi et al., 2011).

Recently, some papers (Triki, Herrero, Jiménez-Colmenero & Ruiz-Capillas, 2013a; Triki, Herrero, Rodríguez-Salas, Jiménez-Colmenero & Ruiz-Capillas, 2013b; Triki, Herrero, Jiménez-Colmenero & Ruiz-Capillas, 2013c; Jiménez-Colmenero, Triki, Herrero, Rodríguez-Salas & Ruiz-Capillas, 2013; Salcedo-Sandoval, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2013) have used konjac gel and oil stabilized in a complex konjac matrix as potential fat analogues to reduce or improve the lipid fraction of different meat products, obtaining good results.

In comparison to oil-in-water emulsions, gelled emulsions could be a better option to mimic hardness and water holding capacity of pork back fat used in most of the currently consumed meat products.

The objective of our research was to optimize the formulation of a gelled oil in-water emulsion prepared with oil rich in ω-3 fatty acids (linseed oil),

carrageenan, a surfactant and water, in order to obtain a successful functional ingredient by means of a factorial design of response surface. This optimized gelled oil-in-water emulsion was used as partial fat replacer in a meat product (Bologna type sausages), and its nutritional, sensory and technological properties were assessed.

2. Materials and methods

2.1. Materials

Fresh pork meat (shoulder and front leg) and back fat were obtained from a local meat market. The meat was trimmed of visible fat and connective tissue. Linseed oil (Biolasi Productos Naturales, Guipúzcoa, Spain) was obtained in a local market. BDRom Carne (a mixture of typical aromatic compounds) and the red colorant Carmin de Cochenille 50 % (E-120) were obtained from BDF Natural Ingredients S.L. (Girona, Spain). Carrageenan (kappa-carrageenan) was kindly donated by Cargill (San Sebastián, Spain) and Curavi (a mixture of curing agents: NaCl, E-250, E-252 and antioxidant E-331) BHA, polyphosphates, monosodium glutamate, sodium ascorbate and garlic were kindly donated by ANVISA (Arganda del Rey, Madrid, Spain). All the chemical reagents and Polysorbate 80 were obtained from Sigma-Aldrich Chemical Co. (MO, USA).

2.2. Gelled emulsion design

Response Surface Methodology (RSM) was applied to optimize the formulation of an oil-in-water gelled emulsion. The effect of three independent variables including oil concentration, carrageenan concentration and surfactant-oil ratio (SOR) were studied in order to maximize hardness and minimize syneresis of the obtained gels. The first approach for the optimization was the delimitation of the ranges for the three ingredients used in the preparation of the gels. The maximum oil concentration technologically able to produce a gelled oil-in-water emulsion was selected as the upper limit for this ingredient (70 %), whereas the lowest limit (40 %) was the minimum oil content needed for achieving a significant amount of fatty acids based on nutritional value. The lowest (0.5 %) and upper (1.5 %) limit for carrageenan concentration were the minimum and maximum carrageenan concentration able to

form a gelled oil-in-water emulsion with the lowest and highest amount of oil, respectively. In the case of polysorbate 80, the limits were expressed as the ratio between surfactant and oil amount (SOR). The lowest limit for SOR was that needed for obtaining a stable gelled oil-in-water emulsion (0.003), whereas the upper limit was the maximum concentration whose bitterness was not detected in the gelled emulsion formed (0.005).

Taking to account these limits, the application of the central composition design (2^3 + star, including 2 central points, Statgraphics Centurion XV software), resulted in a design of 16 experimental settings, which were carried out in triplicate, and in random order (**Table S1**, supplementary material).

2.3. Gelled emulsion preparation and analysis

50 mL of every 16 types of gelled emulsions were prepared as follows: the oil phase containing the hydrophobic surfactant (Polysorbate 80) was added to the aqueous phase that included the corresponding percentage of carrageenan and homogeneized. Both phases were previously heated separately to 70°C. After the homogenization process (16.000 rpm, Ultra-Turrax® T25basic), the emulsions were cooled to room temperature in a sealed flask, allowing the k-carrageenan to polymerize. The gels were kept overnight under refrigeration (4 °C) before analysis.

For the determination of hardness and syneresis, gel samples were cut into cylinders (D= 2.8 cm, h= 1 cm). Hardness was measured using a universal texture analyzer (TA-XT2i, Stable Micro Systems, Surrey, United Kingdom) with a P 0.5R probe to determine the textural characteristics of gels. Cylindrical samples were placed under the probe and underwent compression under a 5 kg load cell at a deformation rate of 30 %. Force-time curves were recorded at a crosshead speed of 0.5 mm/s. Ten measurements were performed in each type of sample.

For the determination of syneresis, each sample was weighed (W_0) inside Petri dishes, and placed in a cabinet at 25 °C for 3 days. The water that condensed on the container walls was removed before weighing the gels (W_t). The syneresis of the gels was calculated as follows: Syneresis (%) = $[(W_0 - W_t)/C_0] \times 100$, were C_0 is the initial water

content in the sample, expressed in percentage. The experiment was performed in triplicate.

The application of the Multiple Response Optimization to hardness and syneresis results let us to conclude that the optimum combination of the gel ingredients was: 40 % oil, 1.5 % carrageenan and 0.003 SOR. This was the gel used as partial fat replacer in Bologna type sausages elaborated in the second part of the work.

2.4. Sausage formulation and processing

Three different formulations (**Table S1**, supplementary material) of Bologna-type sausages were manufactured in a pilot plant according to the procedure described by Berasategi et al. (2011). Control products (Control) contained 16 % pork back fat, whereas in the two experimental batches, half of the pork back-fat was substituted by a conventional oil-in-water emulsion (Emulsion) or by the previously optimized gelled oil-in-water emulsion (Gel) rich in ω-3 fatty acids. The conventional oil-in-water (O/W) emulsion was prepared according to the procedure described by García-Íñiguez de Ciriano et al. (2010) and the gelled oil-in-water emulsion was prepared as previously described (Section 2.3). The conventional and gelled emulsions were kept under refrigeration until their use.

Previous experiments (Berasategi et al., 2011) demonstrated the need for the addition of extra antioxidants when cooked meat products contained high PUFA fat sources. Thus, in both experimental batches, 200 mg of BHA/kg meat batter were added in the mixture of all additives. The control type was manufactured free of extra antioxidants. The formulations were carried out in triplicate. Additionally, samples from every type of formulation were stored under refrigeration (4°C) for 35 days.

2.5. Analysis of sausages

Colour of sausages was measured using a digital colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) to obtain the colour coordinates L*, a* and b*. These values are used to calculate the euclidean distance value ($\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}$) of products along the storage. The

texture (TPA) was measured using a Universal TA-XT2i texture analyzer. Conditions applied for colour and texture were those described by Berasategi, Navarro-Blasco, Calvo, Cavero, Astiasarán & Ansorena (2014b).

The method of Folch, Lees & Stanley (1957) was used for the extraction of fat. In order to assess the oxidation status of the Bologna-type sausages, TBARS (Thiobarbituric acid value) value was determined in all three types of sausages over storage time, using 0.25 g fat, according to the method described by Masqsood & Benjakul (2010) with slight modifications (Poyato, Ansorena, Navarro-Blasco & Astiasarán, 2014). Results were expressed in mg of malondialdehyde (MDA) equivalents/kg sausage.

The fatty acids were determined in the lipid extracts by gas chromatography FID detection according to the procedure described by Valencia et al. (2008). Moisture, protein and fat content were analyzed using official methods (AOAC 2002c, 2002d, 2002e).

Fat extraction, TBARS, colour and texture were measured every 7 days of storage.

2.6. Sensory analysis of meat products

A triangle test was performed to determine the existence of perceptible sensory differences in hardness, taste and appearance between control and the gel containing products (Gel) and between the two experimental products (Emulsion and Gel). A total of 21 semi-trained panellists participated in the sessions. Three samples, of which two were identical, were presented to each panellist, and they were asked to indicate which sample differed from the others. The number of correct answers was collected. According to the Spanish norm UNE 87-006-92 (1992), for a 21-member panel, the difference among samples was significant if the number of correct answers were 12 (* $p<0.05$), 13 (** $p<0.01$) and 15 (*** $p<0.001$).

2.7. Statistical analysis

2.7.1. Gelled emulsion design and optimization

The experimental data (corresponding to the measures of hardness and syneresis) were analyzed by multiple regressions to the independent variables to a polynomial model with Expert Design 8 software. The goodness of fit of the model was evaluated by the determination coefficient (R^2), the adjusted determination coefficient (adjusted R^2), the coefficient of variation (CV) and the lack-of-fit. The value R^2 (0.9302 for hardness and 0.9810 for syneresis) indicated a good correlation between the experimental and the predicted values of the responses. In addition, the value of adjusted R^2 (0.9049 and 0.9741 for hardness and syneresis, respectively) suggested that a high percentage of the total variation (90.5 % and 97.4 % respectively) would be explained by the independent variables. The non-significant value for lack of fit ($p > 0.05$) revealed that the quadratic model was statistically significant for the response and therefore, its use was allowed in the study.

Also, analysis of variance (ANOVA) was performed to determine the statistically significant factors and their interactions in the regression model at the confidence level of 95 % ($\alpha = 0.05$) (**Table 1**). Stepwise regression was used to eliminate the insignificant model terms and final equations were proposed, which are discussed in results and discussion section. Finally, a Multiple Response Optimization was performed in order to determine the combination of experimental factors which simultaneously optimized both responses: maximizing the hardness and minimizing the syneresis.

2.7.2. Bologna type sausages

Means and standard deviations of data obtained from the analysis of sausages are shown in corresponding tables. A one-way ANOVA test and the Tukey-*b post hoc* test were used to determine significant differences in both the different types of Bologna-type sausages and the different times of storage. SPSS version 15.0 was used (SPSS inc. Chicago, Illinois, USA) for the evaluations at a significance level of $p \leq 0.05$.

3. Results

3.1. Optimization of the gelled emulsion

The 16 types of gel formulations characterized for hardness and syneresis revealed a wide dispersion of experimental data, with a clear interaction between the amount of oil and carrageenan (**Table S2**, supplementary material). For hardness, the maximum experimental value (response) obtained was 1606 g (corresponded to the 50 % of oil and 1.64 % of carrageenan formulation) whereas the minimum for syneresis was 19.90 % (for the 40 % of oil and 1.5 % of carrageenan formulation).

Among the different regression models tested to explain the behaviour of the parameters studied, hardness and syneresis, the quadratic polynomial model was found to be the best fit for the experimental data both for hardness (R^2= 0.834) and syneresis (R^2= 0.978).

The analysis of variance of the empirical model for each variable is listed in **Table 1**. ANOVA showed that there were 4 terms of the model that had p-values lower than 0.05, indicating a significant impact (95 % of confidence level) on the final responses. The four terms were: oil concentration (A), carrageenan concentration (B), interaction between oil and carrageenan concentration (AB) and quadratic term of oil concentration (A^2). Even though a minimum ratio of surfactant-oil (C term) is required to produce the gelled emulsion, this factor seems to have no influence on the responses at the studied range.

The polynomial equations in terms of coded factors that resulted from these models were:

(1) Hardness (g) = $838.7 – 245.8A + 307.5B – 426.9AB – 362.6A^2$

(2) Syneresis (%) = $37.34 + 24.22A + 8.88B + 14.77AB + 16.22A^2$

These models showed that hardness and syneresis were influenced in different ways by the same factors (A, B, AB and A^2), so significant interactions could be expected among them, as also shown by the high F-values obtained in the ANOVA test. According to the interaction plots among studied variables (**Fig. 1A** and **B**) hardness decreased when the oil content increased at high carrageenan concentration

(1.5 %). This fact could be because in *k*-carrageenan and in mixed *k/ι*-carrageenan gels, the emulsion droplets are not connected to the matrix and weaken the gel network when the amount of oil is too high (Sala et al., 2008). In contrast, at lower carrageenan concentration (0.5 %) the hardness increased to a maximum point after which a decrease was again observed. The syneresis increased when the oil content increased at high carrageenan concentrations as the oil was not retained by the system. On the other hand, the syneresis decreased, reaching a minimum at the lower carrageenan concentration before increasing again.

As the gel was intended to be used as partial pork fat replacer, the objective was to get a gel with a maximum hardness (simulating the texture of pork back fat) and minimum syneresis (to avoid technological problems during the meat product elaboration). For this, a RSM was performed, estimating the desirability as the combination of maximum hardness and minimum syneresis. According to Jung and Joo (2013), the desirability function approach is one of the most widely used methods for the optimization of the multiple response process. It is based on the idea that the quality of a product or process that has multiple quality characteristics is unacceptable when one of them stays out-side of some desired range. As it can be seen in **Fig. 1C**, the combination of factor levels which maximized the desirability on the studied led to values close to 40 % of oil, 1.5 % of carrageenan and 0.003 for the surfactant-oil ratio, resulting in a desirability value of 0.931. This combination was used for the gelled oil-in-water emulsion tested as partial fat replacer in the second part of the work.

3.2. Comparative study among Bologna type sausages

Once the optimum gelled emulsion formulation was achieved, a practical application was designed in order to confirm the usefulness of the new ingredient. It consisted on comparing a traditional meat product (control) with other two formulations enriched in ω-3 fatty acids by means of the incorporation of the developed gelled emulsion (gel) and also of conventional emulsions (emulsion) previously used in different works. These two ingredients were added for the replacement of 50 % the pork back fat.

Regarding the sensory results for texture, no significant differences were found in the triangle test between Control and the Gel-type products, or between the Gel-type

and Emulsion-type products, as panellist were not able to differentiate between samples ($p > 0.05$) (**Table S3**, supplementary material). TPA results (**Fig. 2**) revealed that during the first 10 days, hardness was similar between Control and Gel-type products, whereas emulsion-type showed lower values ($p<0.05$). These results led to conclude that the gelled emulsion ingredient could be more efficient to maintain the hardness of the original product. The three products showed an increase of hardness during storage, probably as a consequence of a slight water loss, and giving rise to similar values among the three products from the 15th day of storage. Similarly, other authors (Rubio et al., 2007; Ayadi, Kechaou, Makni & Attia, 2009; Cierach, Modzelewska-Kapitula & Szacilo, 2009; Triki et al., 2013a) reported increments in hardness during the storage of meat products in which partial fat replacements were done.

The triangle test led us to conclude that the use of the gelled emulsion did not show sensory problems related to odour, taste and juiciness, showing no significant differences when compared to Control products or the traditional Emulsion-type products ($p>0.05$).

Previous works have shown that colour differences are noticed in meat products when substituting pork back fat by a conventional oil-in-water emulsion (Jiménez-Colmenero, Herrero, Pintado, Solas & Ruiz-Capillas, 2010; Youssef & Barbut, 2011; Berasategi et al., 2013). As this finding was expected, sensory evaluation in this work was done under red light conditions, in order to avoid biased evaluation of the rest of parameters. Colour was not consequently included among the parameters assessed by panellists. In any case, the instrumental colour data of the three formulations (**Fig. 3**) confirmed that lightness (L*), yellowness (b*) and redness (a*) were significantly higher in the emulsion containing products compared to control ones, as expected. In comparing the gel containing products with the control, significant differences were also found for L*, a* and b* values pointing out that the use of the gel instead of the emulsion does not perfectly reproduce the colour contribution of lard to the new formulation. These colour modifications can be probably related to the much smaller oil globule diameter in emulsions, which reflect more light than the larger animal fat globules. Nevertheless, these differences do not

affect the evaluation of the general acceptability of the new products. Additionally, the three products maintained constant colour during storage, as ΔE in the three cases was lower than 2 (Francis & Clydesdale, 1975).

From the nutritional point of view, the use of the gelled emulsion gave the same advantages as the traditional emulsion when both modified formulations were compared to Control products. A significant decrease in total fat content was found as well as for every lipid fraction analyzed (**Table 2**). Despite the fact that the gel or the emulsion were added at a 8 % of the total formulation, a significant supply of α-linolenic acid, which is abundant in linseed oil, was noted for both modified products. This represents approximately 17-18-fold more fatty acid in the Emulsion- and Gel-type products compared to the control. This modification reduced significantly the ω-6/ω-3 ratio from 14 for control products to 0.75, on average, in the modified products. These α-linolenic amounts allowed claiming "high ω-3"for the products developed, which is set at 0.6 g α-linolenic per 100 g and 100 Kcal by EU Regulation (EFSA, 2009).

In order to monitor the potential oxidation of the new formulation, rich in PUFA, TBARS during storage were measured (**Fig. 4**). Both experimental products showed no lipid oxidation events during storage, having consistent values lower than 0.2 mg MDA/kg product, thus, confirming the effectiveness of BHA to control lipid oxidation in the gel containing product. On the contrary, control products showed incremental increases in TBARS from day 15, reaching values of approximately 0.27 mg MDA/kg product by day 20 and continuing to the end of the storage period. These results demonstrated the viability of modified products regarding oxidative stability despite their high content of unsaturated fatty acids.

In conclusion, the optimized gelled emulsion seems to be an effective ingredient as partial pork back fat replacer in cooked meat products, showing good technological properties, nutritional advantages and without negative influence on the sensory properties of the final product. More studies related to the stability of this ingredient and its efficiency when used at different concentrations as a fat replacer are needed. Evaluation of the bioavailability of the lipid compounds delivered by this product should be carried out to determine the efficacy of the nutrient delivery system.

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Table 1. ANOVA items of regression equations.

Source of variations	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
HARDNESS						
Model	3948911	9	438768	9.39	0.007**	significant
A-Oil	683754	1	683754	14.63	0.009**	
B-Carrageenan	1069946	1	1069946	22.89	0.003**	
C-Polysorbate 80	759	1	759	0.016	0.903	
AB	1458364	1	1458364	31.21	0.001***	
AC	148	1	148	0.003	0.957	
BC	91	1	91	0.002	0.966	
A^2	722156	1	722156	15.45	0.008**	
B^2	13566	1	13566	0.290	0.609	
C^2	131	1	131	0.003	0.959	
Residual	280367	6	46728			
Lack of Fit	249631	5	49926	1.62	0.5318	not significant
Pure Error	30735	1	30735			
Cor Total	4229278	15				
SYNERESIS						
Model	10830	9	1203	77.89	< 0.001***	significant
A-Oil	6635	1	6635	429	< 0.001***	
B-Carrageenan	892	1	892	57.79	< 0.001***	
C-Polysorbate 80	7.62	1	7.62	0.49	0.509	
AB	1744	1	1744	112	< 0.001***	
AC	41.78	1	41.78	2.70	0.151	
BC	0.12	1	0.12	0.01	0.934	
A^2	1443	1	1443	93.44	< 0.001***	
B^2	24.23	1	24.23	1.57	0.257	
C^2	40.71	1	40.71	2.63	0.156	
Residual	92.70	6	15.45			
Lack of Fit	74.14	5	14.83	0.80	0.686	not significant
Pure Error	18.57	1	18.57			
Cor Total	10923	15				

Table 2. Chemical composition (g/100 g product) of the different formulated Bologna-type sausages.

	Control	Emulsion	Gel
Moisture	69.09 ± 0.07[a]	70.87 ± 0.06[b]	71.48 ± 0.07[c]
Protein	15.06 ± 0.65[a]	13.91 ± 0.78[a]	14.88 ± 0.57[a]
Fat content	13.24 ± 0.15[b]	12.09 ± 0.15[a]	11.89 ± 0.04[a]
Caprilic C8:0	nd	nd	nd
Capric C10:0	0.03 ± 0.01[b]	0.02 ± 0.01[a]	0.02 ± 0.01[a]
Lauric C12:0	0.02 ± 0.01[b]	0.01 ± 0.01[a]	0.01 ± 0.01[a]
Myristic C14:0	0.17 ± 0.01[b]	0.09 ± 0.01[a]	0.10 ± 0.01[a]
Palmitic C16:0	3.08 ± 0.01[c]	2.11 ± 0.01[b]	2.06 ± 0.01[a]
t-palmitoleic C16:1	0.01 ± 0.01[b]	0.01 ± 0.01[a]	0.01 ± 0.01[a]
Palmitoleic C16:1	0.22 ± 0.01[c]	0.16 ± 0.01[b]	0.15 ± 0.01[a]
Stearic C18:0	1.67 ± 0.01[c]	1.16 ± 0.01[b]	1.12 ± 0.01[a]
Elaidic C18:1	0.07 ± 0.01[c]	0.04 ± 0.01[b]	0.03 ± 0.01[a]
Oleic C18:1 (ω-9)	5.10 ± 0.02[c]	4.29 ± 0.01[b]	4.00 ± 0.01[a]
c-vaccenic C18:1 (ω-7)	0.34 ± 0.01[c]	0.27 ± 0.01[b]	0.25 ± 0.01[a]
t-linoleic C18:2	nd	nd	nd
c,t-linoleic C18:2	0.01 ± 0.01[b]	0.01 ± 0.01[a]	0.01 ± 0.01[a]
t,c-linoleic C18:2	0.01 ± 0.01[b]	0.01 ± 0.01[a]	0.01 ± 0.01[a]
Linoleic C18:2 (ω-6)	2.23 ± 0.01[c]	1.90 ± 0.01[a]	1.93 ± 0.01[b]
Arachidic C20:0	nd	0.01 ± 0.01[a]	0.01 ± 0.01[a]
γ-linolenic C18:3 (ω-6)	0.01 ± 0.01[c]	0.01 ± 0.01[a]	0.01 ± 0.01[b]
Eicosenoic C20:1 (ω-9)	0.10 ± 0.01[c]	0.07 ± 0.01[b]	0.07 ± 0.01[a]
α-linolenic C18:3 (ω-3)	0.11 ± 0.01[a]	1.84 ± 0.01[b]	1.99 ± 0.01[c]
Behenic C22:0	nd	nd	nd
Brassidic C20:1	nd	nd	nd
Erucic C22:1	nd	nd	nd
Eicosatrienoic C20:3 (ω-3)	0.02 ± 0.01[a]	0.01 ± 0.01[a]	0.01 ± 0.01[a]
Arachidonic C20:4 (ω-6)	0.04 ± 0.01[a]	0.04 ± 0.01[a]	0.05 ± 0.01[b]
Eicosapentaenoic C22:5 (ω-3)	nd	0.01 ± 0.01[a]	0.01 ± 0.01[a]
Nervonic C24:1 (ω-9)	nd	nd	nd
Docosatrienoic C22:3 (ω-3)	nd	nd	nd
Docosapentaenoic C22:5 (ω-3)	nd	0.02 ± 0.01[a]	0.02 ± 0.01[a]
Lignoceric C24:0	nd	nd	nd
SFA	4.97 ± 0.02[c]	3.41 ± 0.01[b]	3.33 ± 0.01[a]
MUFA	5.76 ± 0.02[c]	4.79 ± 0.01[b]	4.48 ± 0.01[a]
PUFA	2.41 ± 0.01[a]	3.82 ± 0.02[b]	4.01 ± 0.01[c]
ω-3	0.12 ± 0.01[a]	1.87 ± 0.02[b]	2.03 ± 0.01[b]
ω-6	2.28 ± 0.01[c]	1.95 ± 0.01[a]	1.98 ± 0.01[b]
ω-6/ω-3	14.08[b]	0.78[a]	0.73[a]
trans	0.11 ± 0.01[b]	0.07 ± 0.01[a]	0.05 ± 0.01[a]

[1]Different letters in the same row denote significant differences among samples ($p<0.05$). nd: not detected

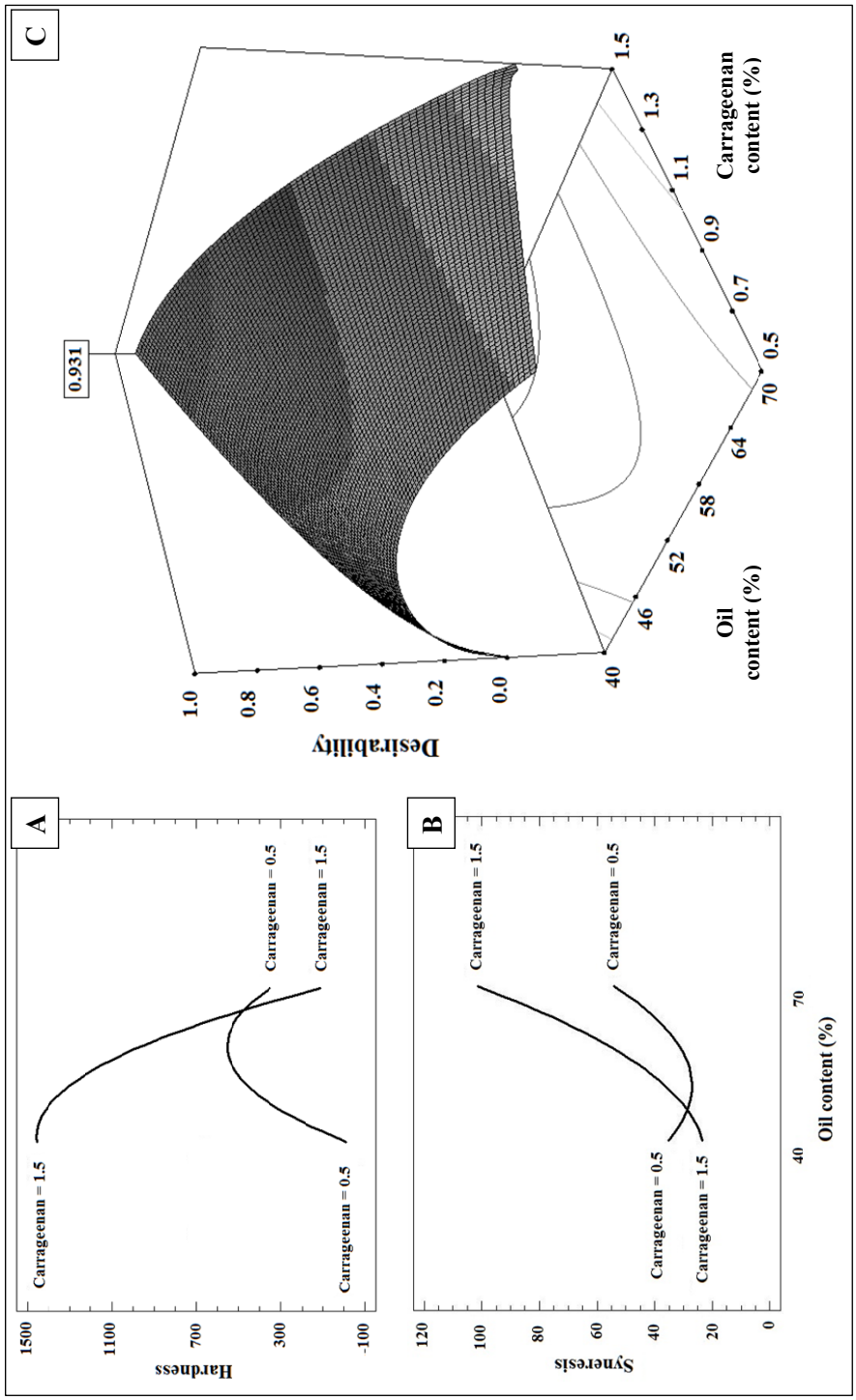
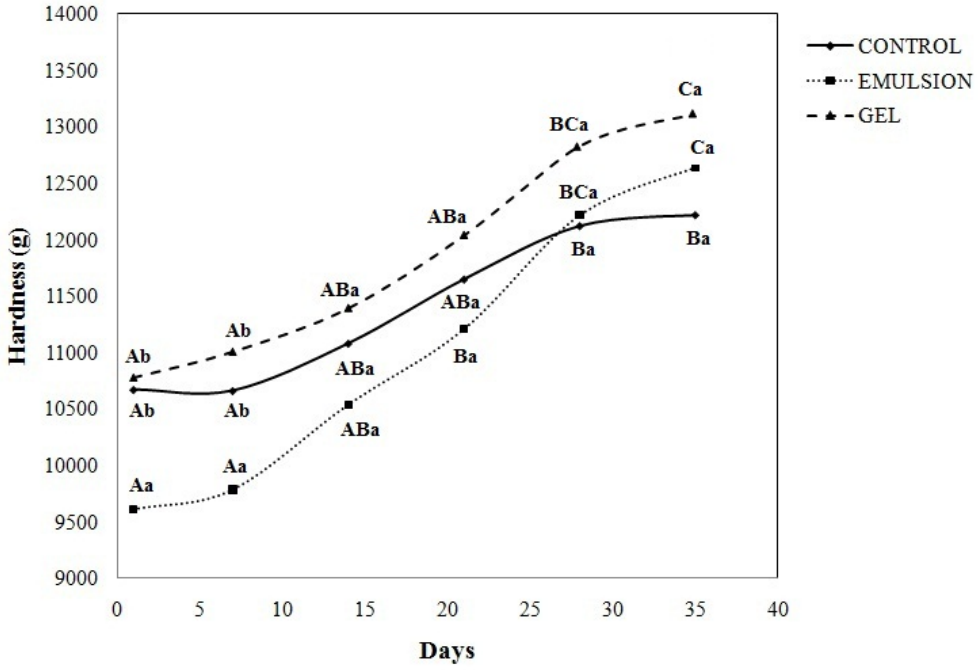


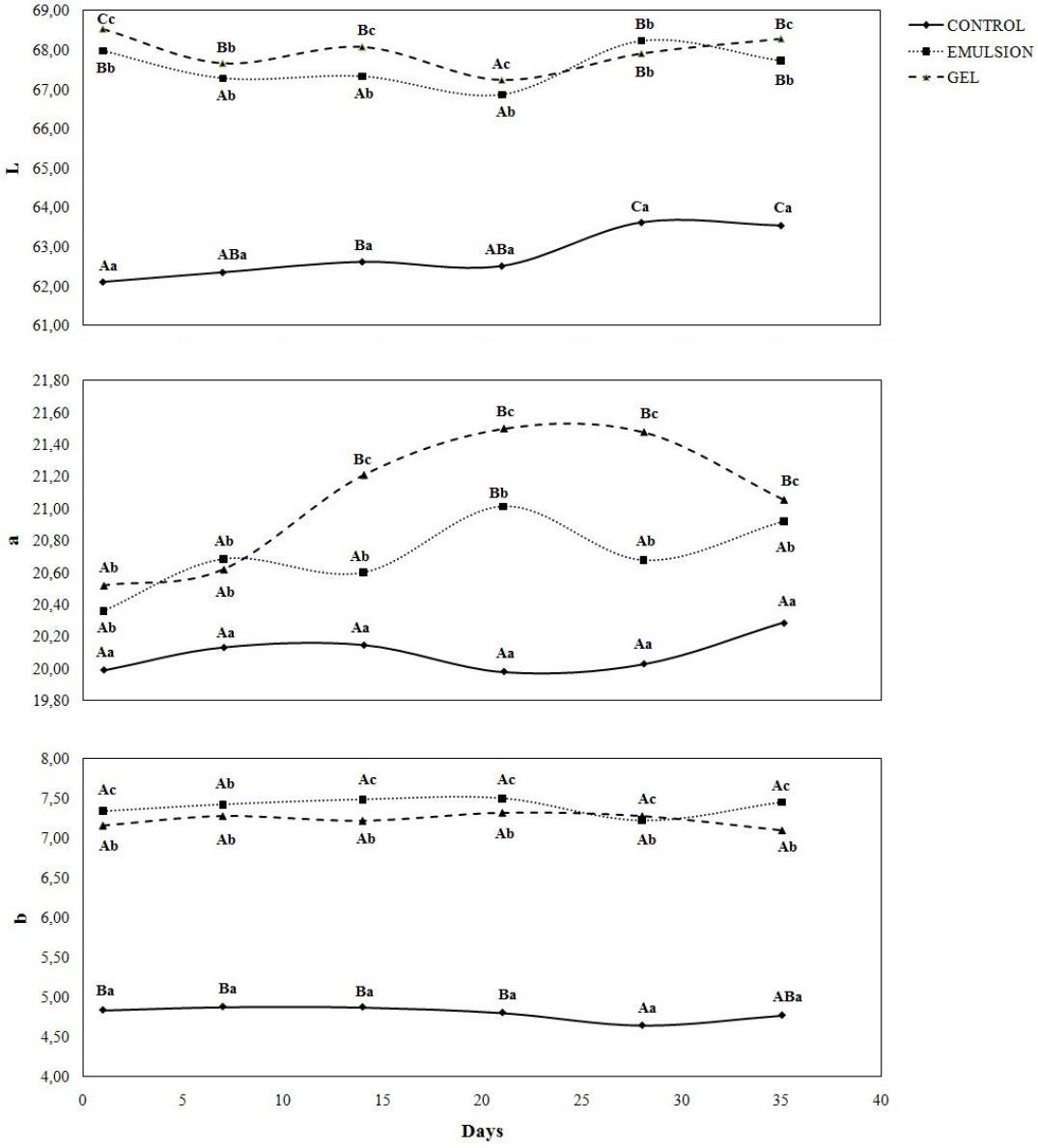
Fig. 1. (A) Interaction plots of the hardness (g) and (B) the syneresis (%). (C) Response surface plot of the multiple optimization of hardness and syneresis.

Fig. 2. Hardness values (g) obtained for the three formulations of Bologna-type sausages during the storage.



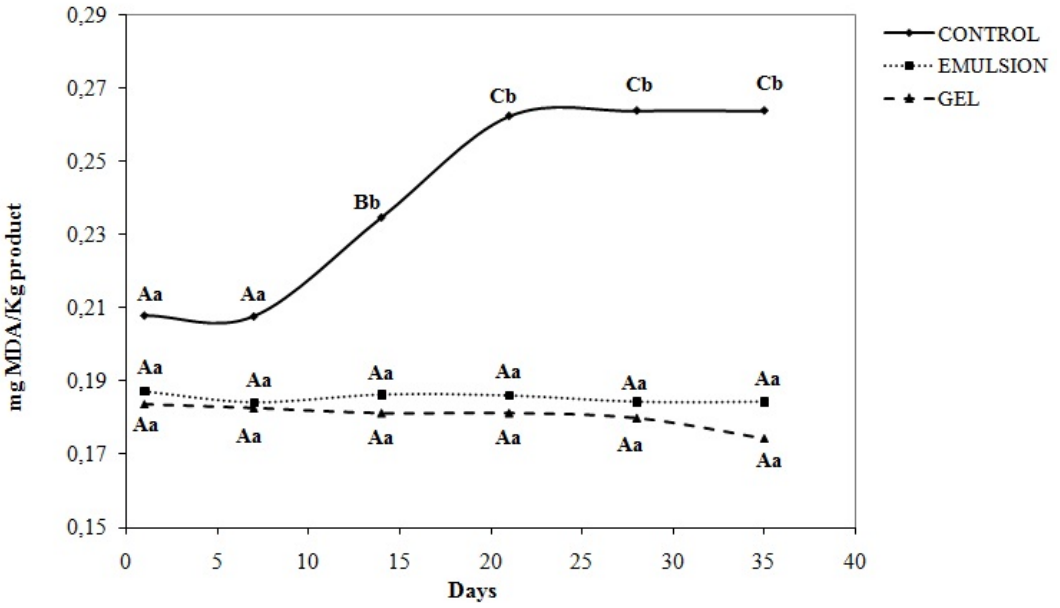
Within each type of formulation different capital letters denote significant differences along the storage, and within each sampling time, different small letters denote significant differences among types of formulation ($p < 0.05$).

Fig. 3. Colour coordinates of the three types of Bologna-type formulations along the storage.



Within each type of formulation different capital letters denote significant differences along the storage, and within each sampling time, different small letters denote significant differences among types of formulation ($p < 0.05$).

Fig. 4. TBARS (mg MDA/kg product) obtained for the three formulations of Bologna-type sausages during the storage.



Different capital letters denote significant differences for each type during storage and different small letters denote significant differences among types at each day ($p < 0.05$).

Supplementary material

Table S1. Formulation of the three types of Bologna-type sausages.

Ingredients	Modified products		
	Control	Emulsion	Gel
Pork meat (%)	55	55	55
Pork fat (%)	16	8	8
Ice (%)	29	29	29
Emulsion (%)	0	8	0
Gel (%)	0	0	8
BHA (mg/kg)	0	200	200
Iodized NaCl (g/kg of meat-fat batter)	26	26	26
Powdered milk (g/kg)	12	12	12
Garlic (g/kg)	3	3	3
Curavi[1] (g/kg)	3	3	3
Polyphosphates[2] (g/kg)	2	2	2
Sodium ascorbate (g/kg)	0.5	0.5	0.5
BDRom Carne (g/kg)	1	1	1
Monosodium glutamate (g/kg)	1	1	1
Carmin de Cochenille 50 % (E-120) (g/kg)	0.1	0.1	0.1

[1]Curavi: a mixture of curing agents: NaCl, E-250, E-252 and antioxidant E-331.
[2] Mixture of E-430i, E-454i and E-451i.

Supplementary material

Table S2. Central composition design (2^3+ star; including 2 central points) for the three variables and observed responses.

Run	Linseed oil (%)	Carrageenan (%)	SOR	Hardness[a] (g)	Syneresis[a] (%)
1	35.69	1.00	0.0040	548	32.06
2	55.00	1.64	0.0040	1606	53.36
3	40.00	1.50	0.0050	1383	27.77
4	55.00	1.00	0.0040	822	32.88
5	55.00	1.00	0.0053	894	32.52
6	55.00	1.00	0.0027	873	36.78
7	74.31	1.00	0.0040	2.3	99.01
8	55.00	0.36	0.0040	310	31.93
9	40.00	1.50	0.0030	1364	19.90
10	40.00	0.50	0.0030	69.0	30.81
11	70.00	1.50	0.0050	0.01	98.65
12	40.00	0.50	0.0050	65.3	39.47
13	55.00	1.00	0.0040	874	38.97
14	70.00	1.50	0.0030	0.01	99.20
15	70.00	0.50	0.0030	376	53.11
16	70.00	0.50	0.0050	426	51.36

[a] Average value of triplicate experiments
SOR: surfactant oil ratio

Supplementary material

Table S3. Scores obtained in the triangular sensory analysis.

	Odour	Taste	Juiciness	Texture
Control *versus* Gel				
Correct replies	10 ^{ns}	8 ^{ns}	7 ^{ns}	9 ^{ns}
Incorrect replies	11	13	14	12
Emulsion *versus* Gel				
Correct replies	2 ^{ns}	3 ^{ns}	3 ^{ns}	4 ^{ns}
Incorrect replies	19	18	18	17

For n=21, the difference between samples was significant if the number of correct answers was 12 (* = $p<0.05$), 13 (** = $p<0.01$) and 15 (*** = $p<0.001$), ns: not significant.

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A new polyunsaturated gelled emulsion as replacer of pork back-fat in burger patties: Effect on lipid composition, oxidative stability and sensory acceptability

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Abstract: A new gelled carrageenan containing emulsion developed as ingredient was used as fat replacer in burger patties. Increasing amounts (25, 50, 75 and 100 %) of this gelled emulsion were added into the product in order to reduce the fat content while improving its fatty acid profile. A 41 % reduction of the total fat content with an increment of the 74.5 % of the unsaturated fatty acids, and a significant decrease in cholesterol (47 %) and saturated fat (62 %) were achieved in the product with the highest level of substitution. These products showed significantly lower thiobarbituric acid reactive substances (TBARS) and cholesterol oxidation products (COPs) compared to control. Additionally, when samples were subjected to thermal treatment (180 °C, 15 min, oven conditions) higher lipid oxidation rates were found when increasing amounts of the gelled emulsion were incorporated into the new formulations, without impairment of their final sensory properties.

Key words: fat replacer, gelled emulsion, lipid oxidation, cholesterol oxidation, meat patties.

1. Introduction

The development of nutritionally improved meat products using reformulation strategies has been accomplished with the aid of emulsion based systems, being the improvement of the fat quality one of the most important goals. The substitution of pork back-fat with PUFA (polyunsaturated fatty acids) emulsified oils has been demonstrated to be a good strategy to achieve healthier lipid profiles in these products

(García-Íñiguez de Ciriano, Rehecho, Calvo, Cavero, Navarro, Astiasarán & Ansorena, 2010a; Berasategi, Legarra, García-Íñiguez de Ciriano, Rehecho, Calvo, Cavero, Navarro-Blasco, Ansorena & Astiasarán, 2011; Rodríguez-Carpena, Morcuende & Estévez, 2012). When using fat replacers a careful approach is needed in order to achieve the appearance and the technological, rheological and sensory properties required in the food industry (Tye, 1991). In this sense, polysaccharides can be used to create a variety of gel structures suitable for immobilizing oil droplets and forming gelled emulsions able to incorporate lipophilic agents with beneficial health effects into foodstuffs (Herrero, Carmona, Jiménez-Colmenero & Ruiz-Capillas, 2014; Poyato, Ansorena, Berasategi, Navarro-Blasco & Astiasarán, 2014a). Polysaccharidic biopolymers such as carrageenan, konjac, inulin, dextrin and alginate have been used as potential fat analogues to reduce or improve the lipid fraction of different meat products, obtaining good results (Triki, Herrero, Jiménez-Colmenero & Ruiz-Capillas, 2013a; Salcedo-Sandoval, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2013; Ruiz-Capillas, Carmona, Jiménez-Colmenero & Herrero, 2013; Utrilla, García-Ruiz & Soriano, 2014; Herrero, Carmona, Jiménez-Colmenero & Ruiz-Capillas, 2014). In comparison to conventional (oil-in-water) emulsions, gelled emulsions could be a better option to mimic hardness and water holding capacity of pork back fat used in most of the currently consumed meat products. Additionally, the use of gelled emulsion as fat replacer may be a suitable technology not only for delivering, but also for protecting lipids in food products. Recently, our group has optimized the processing conditions for obtaining a carrageenan containing gel rich in polyunsaturated fatty acids, which successfully served as fat replacer ingredient in a Bologna-type sausage (Poyato et al., 2014a). However, a clear understanding of the gelled emulsion behaviour under different processing conditions is needed in order to highlight its potential use as animal fat replacer in a variety of products. This work was focused on adapting the use of this novel ingredient in a fresh meat product and on the evaluation of the behaviour of the new product (burger patty) after usual cooking.

The behaviour of the fat replacer on the samples was studied in raw and cooked conditions, by assessing nutritional and sensory properties of the resulting products, and by evaluating their stability against oxidation.

2. Materials and methods

2.1. Materials

Fresh beef and pork meat (loin) and pork back fat were obtained from a local meat market. Sunflower oil (Urzante S.L, Navarra, Spain) was obtained in a local market. Carrageenan (kappa-carrageenan) was kindly donated by Cargill (San Sebastián, Spain). Cholesterol, 5α-cholestane, thiobarbituric acid, fatty acid methyl esters and Polysorbate 80 were purchased from Sigma-Aldrich Chemical (Steinheim, Germany). 19-hydroxycholesterol was obtained from Steraloids (Wilton, NH, USA). Tri-sil® reagent was obtained from Pierce (Rockford, IL, USA). Acetone, chloroform, ethyl acetate, methanol, hexane, 2-propanol, hydrochloric acid, cyclohexanone, trichloroacetic acid, potassium chloride, potassium hydroxide, anhydrous sodium sulphate and sodium phosphate were obtained from Panreac (Barcelona, Spain). Hexane for gas chromatography, dichloromethane for gas chromatography and boron trifluoride/methanol were from Merck (Whitehouse Station, NJ, USA). Strata NH_2 (55 μm, 70 A) 500 mg/3 mL Solid Phase Extraction cartridges were obtained from Phenomenex (Torrance, CA, USA).

2.2. Gelled emulsion preparation

The gelled emulsion was prepared according to the method described by Poyato et al. (2014a) using sunflower oil as the oil phase. The oil phase (40 g/100 g emulsion) containing the Polysorbate 80 as surfactant (0.12 g/100 g oil), was added to the aqueous phase (that included 1.5 g carrageenan/100 g emulsion) and homogenized. Both phases were previously heated separately to 70 °C. After the homogenization process (16.000 rpm, Ultra-Turrax® T25basic), the emulsion was cooled to room temperature in a sealed flask, allowing the κ-carrageenan to polymerize. The gel was kept overnight under refrigeration (4 °C) until being used (see supplementary material **Fig. S1**, showing the gel aspect). The physical appearance was maintained and no syneresis was noticed during the burger meat patties processing.

2.3. Meat patties formulation and processing

Five different formulations of meat patties were manufactured in a pilot plant. About 5 kg of loin (2.5 kg beef and 2.5 kg pork) was minced (10 mm plate) using a meat mincer. After mincing, the samples were assigned to one of the following five treatments (G0, G25, G50, G75 and G100). In the Control (G0) 20 g/100 g of pork back fat was added, whereas in the four experimental batches different percentages, 25 % (G25), 50 % (G50), 75 % (G75) and 100 % (G100), of the pork back-fat were substituted by the gelled emulsion. Salt (0.2 g/100 g) was also added in all batches, samples were thoroughly hand mixed. Minced meat patties (80 g portions) were formed compressing with the appropriate tool until a compacted and homogenized form was obtained (8.9 cm diameter and 1.5 cm thickness each patty). Half of the patties from each batch were randomly selected for being cooked in a hot air oven, (15 min at 180 °C). After cooling to room temperature, the patties were aerobically packaged and stored at -20 °C. The meat patties were analyzed in raw and cooked, in triplicate. The sensory evaluation was performed just after manufacturing the products on the first day. See supplementary material **Fig. S2**, showing the G0 and G100, raw and cooked samples.

2.4. Analysis of samples

Moisture, protein and fat content were analyzed using official methods (AOAC 2002c, 2002d, 2002e). The method of Folch, Lees & Stanley (1957) was used for the extraction of fat. The fatty acids were determined in the lipid extracts by gas chromatography FID detection according to the procedure described by Valencia et al. (2008). The identification of the fatty acid methyl esters was done by comparison of the retention times of the peaks in the sample with those of standard pure compounds and by spiking the sample with each standard individually. The quantification of individual fatty acids was based on the internal standard method, using heptadecanoic acid methyl ester. After the quantification of the individual fatty acids, the sums of saturated, SFA, (capric, lauric, myristic, palmitic, stearic, arachidic, and behenic acid), monounsaturated, MUFA, (palmitoleic, oleic, vaccenic, erucic, nervonic and eicosenoic acid), polyunsaturated, PUFA, (ω-3: α-linolenic, eicosadienoic,

eicosatrienoic, docosapentaenoic, docosahexahenoic acid; ω-6: linoleic, γ-linoleic, arachidonic, docosapentaenoic) were calculated.

2.4.1. TBARS value

TBARS values were determined on fat according to the method described by Maqsood and Benjakul (2010) with slight modifications. Briefly, the TBARS reagent was prepared by mixing trichloroacetic acid (15 g/100 mL), 2-thiobarbituric acid (0.375 g/100 mL) and hydrochloric acid (0.25 g/100 g). The fat (0.25 g), distillate water (250 μL), BHT (20 μL, 1 g /100 mL) and the TBARS reagent (1 mL) were vortexed in a centrifuge tube (20 s), placed in a boiling water bath for exactly 15 min and then cooled in an ice bath to room temperature. Cyclohexanone (2 mL) and 4 M ammonium sulphate (500 μL) were added to the mixture and were vortexed for 30 s. The mixture was centrifuged at room temperature at 4000 rpm for 10 min to allow separation of phases. After centrifugation, the supernatant was collected and the absorbance was measured between 300 and 600 nm (FLUOStar Omega spectrofluorometric analyzer, BMG Labtechnologies, Offenburg, Germany). The spectra were collected with a resolution of 2 nm. The quantification was performed using TEP (tetraethoxypropane) (calibration range: 2.6×10^{-6} – 8.30×1^{-5} mmol/g; LOD= 6.0×10^{-7} mmol/g; LOQ=1.8×10^{-6} mmol/g) and 2,4-Decadienal (calibration range: 6.9×10^{-3} – 2.6×10^{-2} mmol/g, LOD= 8.3×10^{-4}; LOQ= 1.8×10^{-3}) as external standards, measuring the absorbance at 532 nm and 390, nm respectively. Results were expressed in mg MDA/kg product and in mmol 2,4-decadienal/kg product, in each case.

2.4.2. Cholesterol determination

Meat patties (3 g) were added to 20 mL ethanol (95 %), 5 mL KOH (50 g/100 mL) and 1 mL 5α-cholestane as internal standard (2 mg/mL in chloroform), and heated at 50 °C for 1 h until complete saponification. Then, water (13 mL) was added and the unsaponifiable material was extracted with hexane (6×20 mL). After filtering through anhydrous sodium sulphate, organic solvent was evaporated using a rotatory vacuum evaporator at 30 °C. For derivatization purposes, Tri-Sil® reagent (400 μL) was added to each aliquot and they were kept at 60 °C for 45 min in a water bath. The solvent was evaporated under a stream of nitrogen and the TMS-ether derivates were solved in

hexane (400 μL) for gas chromatography. This solution was filtrated with a syringe and a filter (0.45 μm) and poured to a glass vial prior to GC-FID analysis. A Perkin-Elmer Autosystem gas chromatograph equipped with an HP1 column (30 m × 0.25 mm × 0.1 μm) was used. The oven temperature was maintained isothermally at 265 °C. The temperature of both the injection port and detector was 300 °C. The sample size was 1 μL.

Peak identification was based on comparison of the retention time of analytical standard. Quantification was made with calibration curve, with 5α-cholestane as the internal standard. Perkin-Elmer Turbochrom programme was used for the integration.

2.4.3. Cholesterol Oxidation Products (COP) determination

Approximately 0.5 g of the previously extracted fat was weighted in a flask containing 1M KOH in methanol and 1 mL 19-hydroxycholesterol (20 μg /mL in hexane:isopropanol 3:2) and kept at room temperature during 20 h to complete the cold saponification. The unsaponifiable material was extracted with diethyl ether (3×10 mL). The whole organic extract was washed with water (3×5 mL) and filtered through anhydrous sodium sulphate. Then it was recovered in a round-bottom flask, and the solvent was evaporated under a stream of nitrogen. Purification with amino-propyl cartridges and derivatization to trimethyl silyl ethers and analysis by GC-MS were performed as described in Ansorena et al. (2013). Quantification was done as described in Barriuso, Otaegui-Arrazola, Menéndez-Carreño, Astiasarán & Ansorena (2012). The results were expressed as μg/100 g product and also μg/100 g dry matter of the sample. The oxidation rate was also calculated as follows, using data on μg/100 g dry matter:

$$OxidationRate = \frac{\left(COP_{cooked} - COP_{raw}\right)}{COP_{raw}} \times 100$$

2.6. Sensory analysis of meat products

A hedonic test (Anzaldúa-Morales, 1994) was performed on raw samples. The degree of appearance acceptability was evaluated for 33 non-trained panellists. A 7-point scale was used for scoring the samples (3. I really like; 2. I like; 1. I slightly like; 0. I rather like or dislike; -1. I slightly dislike; -2. I dislike; -3. I really dislike).

In addition, a multiple comparison test (Anzaldúa-Morales, 1994) was performed on cooked products to determine the existence of perceptible sensory differences in colour, odour, taste, hardness, juiciness and fatness between the control and the gel containing products. This test was used to determine the effect of the possible changes caused by the substitution of an ingredient for another. A total of 11 trained panellists participated in the sessions to consider differences in the studied parameters and to assess the magnitude of the difference. Five samples were presented to each panellist, who were asked to indicate which sample differed from the control (G0). The scores (1. very much less; 2. much less; 3. considerably less; 4. slightly less; 5. not differences; 6. slightly more; 7. considerably more; 8. much more; 9. very much more) were collected and the statistical analysis of the results was carried out by an analysis of variance, in which numerical values were assigned to the descriptive terms of the questionnaire.

2.7. Statistical analysis

Mean and standard deviation of results obtained from the three replicates made per type of product were calculated. For each parameter, one factor ANOVA with Bonferroni *post hoc* multiple comparisons was used in order to evaluate the significant differences among samples. Within each type of sample, the differences between raw and cooked were evaluated by Student t-test.

Regarding the sensory tests the scores obtained in the multiple comparison tests and the hedonic test were evaluated by ANOVA. The effect of each variable and the variability among judges were evaluated. Pearson correlation test was used to determine correlations among variables.

The statistical analysis of data was done using the STATA/IC 12.1 program (StataCorp LP, Texas, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

3. Results and discussion

3.1. Sensory evaluation

The gelled emulsion developed in a previous work (Poyato et al., 2014a) showed good technological properties when added to Bologna-type sausages, where it did not cause sensory problems. Taking into account that sensory acceptability is a crucial factor when testing the incorporation of new ingredients in foodstuffs, another challenge was faced with this gel, being used in fresh meat product. A hedonic test was performed in raw samples in order to evaluate consumer acceptability of the new formulations. The scores reported by panellists for the gel containing products were higher than for the control one, meaning that the gel tend to improve the appearance of the new meat patties compared to the control, although no significant differences were found. After cooking, samples were subjected to multiple comparison tests between G0 and the gel-type products (G25, G50, G75 and G100) (**Table 1**). Results showed no significant differences ($p > 0.05$) for odour, colour, taste, hardness, juiciness and fatness between the experimental batches and the control in these cooked products and neither among the gel-containing samples, pointing out that the white colour and the consistence of the gel perfectly mimicked the appearance of lard in the new products. These results led to conclude that all replacements were satisfactory for the panellist, so that the gelled emulsion ingredient could be a good fat replacer for obtaining burger patties similar to the original product. Similarly, other authors (Gao, Zhang & Zhou, 2014) reported improvements or no significant differences in meat products in which partial fat replacements were done.

3.2. Overall nutritional value

Once the sensory acceptability was ensured, we evaluated the improvement of the nutritional quality of the developed products by the added gelled emulsion.

The use of the gelled emulsion significantly reduced the total fat content of reformulated products (up to 41 % in G100 raw samples compared to control ones) increasing at the same time the PUFA supply (up to 63 %) in a dose-dependant manner (**Table 2**). In fact, the higher the gel used, i.e., the lower the fat content, the higher the PUFA fraction ($r = 0.959^{**}$), and the lower the SFA one ($r = 0.957^{**}$). These

modifications resulted in improved PUFA/SFA and PUFA+MUFA/SFA ratios. These achievements were highly relevant from the nutritional point of view, because it has been demonstrated that an increased PUFA consumption as a replacement for SFA reduced the occurrence of coronary heart disease events (Mozaffarian, Micha & Wallace, 2010).

Moreover, a quantitative noticeable reduction in cholesterol content (up to 16.9 % decrease) was noticed when increasing the gel content in the product from G0 to G100. The combined action of the reduction of the fat content, SFA and cholesterol and the PUFA increment resulted in healthier products as long as more gel was incorporated.

As it was expected, the fat decrease was accompanied by a moisture increase, although these differences were reduced after the heat treatment due to the water loss. In fact, moisture loss during the heat treatment was slightly higher in the samples elaborated whit the gelled emulsion. No significant differences ($p > 0.05$) for protein content were noticed among the different formulations in raw products, and only significant differences ($p<0.05$) between G0 and G100 were detected in cooked samples. Additionally, net protein content increased with heat treatments as consequence of the water loss.

Table 3 gathered the potential nutrition claims that could be applied to the developed burger patties referred to raw products according to the EU Regulation (Regulation 1924/2006). A claim that a food is “high in protein” may be made in all products due to the fact that at least 20 % of the energy value of the product is provided by protein. Depending on the amount of gelled emulsion incorporated in the formulations several nutrition and health claims could be made. The reduction of 30 % in the energy supply in the G100 product compared to a conventional formulation could allow it to declare “energy reduced”. G75 and G100 products, in which the reduction in fat content was at least 30 % compared to the control (G0), can be declared as “reduced fat”. On the other hand, the claim "reduced saturated fat" can be made in the G50, G75 and G100 products because the sum of SFA and trans were, in all cases, at least 30 % less than in the G0; and the content in trans in the products was significantly lower ($p<0.05$) than in a conventional product. The PUFA+MUFA

content allowed claiming “high unsaturated fat content” for the G75 and G100 products, in which unsaturated fatty acids were at least 70 % of the fatty acids present in the product and provided more than 20 % of energy of the product. As it can be seen, the G100 product could be labelled with five nutrition claims: “energy reduced”, “high protein”, “reduced fat”, “reduced saturated fat”, and “high unsaturated fat”. Additionally, according to the Commission Regulation (EU) 432/2012, these nutritional modifications allowed to claim in G75 and G100 products that “Replacing saturated fats in the diet with unsaturated fats contributes to the maintenance of normal blood cholesterol levels”, “Linoleic acid contributes to the maintenance of normal blood cholesterol levels; the beneficial effect is obtained with a daily intake of 10 g of LA” and “Reducing consumption of saturated fat contributes to the maintenance of normal blood cholesterol levels”. This last claim could be also made for G50.

3.3. Oxidative stability

3.3.1. TBARS assessment

Increasing the PUFA content of products might enhance the oxidation susceptibility of the gelled emulsion containing formulations during the heating treatment (Poyato et al., 2014a). The most important cause of deterioration of oils and fats is oxidation, which does not only reduce shelf life, sensory acceptance and the nutritional value of food, but also produces toxic compounds. In order to monitor the oxidation status, TBARS at 390 nm (mmol 2,4-Decadienal/kg product) and at 532 nm (mg MDA/kg product) were measured, before and after the heating treatment (**Table 4**). A progressive reduction of the oxidation products in raw patties were observed at 390 and 532 nm, which was statistically significant in the products with the highest substitution level compared to the control one. This was caused probably as a merged action of the reduction of fat content and the lower oxidative status of the fat in these products. In fact, if the results were expressed over kg fat, significant differences were noted among formulations at 390 nm, and a trend in the reduction of the oxidation compounds at 532 nm was observed. Poyato et al. (2014b) reported that the sensitivity of the measure of the oxidation compounds, depending on the lipid profile, was higher at 390 than at 532, nm allowing us to detect these differences. As expected, cooking increased lipid oxidation in all products, having all burger patties higher values of mg MDA/kg

product and mmol 2,4-Decadienal/kg product in cooked samples compared to raw ones. Moreover, in cooked samples a dose dependent effect was noticed with the substitution level. Thus, the higher the amount of gel added (it means, the lower the fat present in the product), the higher the oxidation rate found. A negative correlation was found between fat content and mg MDA/g fat (-0.784*) or mmol 2,4-decadienal/kg fat (-0.843**) and a positive correlation between PUFA content and mg MDA/g fat (0.773**) or mmol 2,4-Decadienal/kg fat (0.834**) was noticed. Thus, compared to raw products, oxidation values increased with cooking a 19 % (G0) and a 95 % (G100), when analysing values at 390 nm and 71 % (G0) to 197 % (G100) at 532 nm. This is in accordance with other authors (Jacobsen, Timm & Meyer, 2001a; Taherian, Britten, Sabik & Fustier, 2011) who reported that the use of vegetable oils as functional ingredients in food lipid emulsions might be complex due to the high oxidation susceptibility of these unsaturated oils.

3.3.2. COPs assessment

As the selected food was from animal origin the study of the oxidative stability included also the cholesterol oxidation products (COPs) determination. These compounds have been used as a measure of markers of the oxidation process (Rodríguez-Estrada, Penazzi, Caboni, Bertacco & Lecker, 1997), besides their known role in some harmful effects for human health (Otaegui-Arrazola, Menéndez-Carreño, Ansorena & Astiasarán, 2010). In agreement to the lipid oxidation trend detected by the classical TBARS method, significantly lower total cholesterol oxidation compounds were observed in those products with the highest content of gelled emulsion (Pearson correlation between fat content and COP μg/100 g was 0.888** and correlation between cholesterol content and COPs was 0.707**). COPs were 2-fold lower in G100 samples than in the control patties (G0), in both raw and cooked samples when expressed per 100 g of product (**Table 5**). In order to avoid bias caused by water loss by cooking, results of COPs were also calculated on dry matter of the samples. These results pointed out that during heating, the cholesterol oxidation percentage gradually increased with the gelled emulsion incorporation from 15.3 % to 36.5 % in G0 and G100, respectively. The most abundant COPs, in all samples, were 5,6-β-cholesterol epoxide and 7-ketocholesterol. Rodríguez-Estrada et al. (1997)

reported that 7-ketocholesterol was used as a marker of the degree of cholesterol oxidation, due to its fast and continuous formation and its relatively high amounts with respect to the other oxidation products. However, the higher oxidation rate (referred to the amount in raw samples) was for 7α-hydroxycholesterol and 7β-hydroxycholesterol that gradually increased with the incorporation of gelled emulsion from 35.4 % (G0) to 88.9 % (G100) and from 49.2 % (G0) to 96.2 % (G100), respectively. These results were in agreement with the initial states of the thermal cholesterol oxidation in which 7α-hydroxycholesterol and 7β-hydroxycholesterol are two of the major oxidation products (Smith, 1987). Moreover epoxides significantly increased during the heating being the 5,6-β-cholesterol epoxide the predominating compound as expected (Lampi, Juntunen, Toivo & Piironen, 2002; Yen, Lu, Inbaraj & Chen, 2011; Ansorena et al., 2013). Finally, both epoxy compounds (5,6-β-cholesterol and 5,6-α-cholesterol) can give 3,5,6-cholestanetriol by hydration when the epoxy compounds had already started their decline (Ansorena, et al., 2013). Because of the short heat treatment applied to these samples (15 min) the 3,5,6-cholestanetriol had the lowest oxidation rate (from 8.1 (G0) to 18.6 (G100)), as this COP is formed at advanced stages of oxidation.

The results showed that higher PUFA content of the gel contributes to create a pro-oxidant environment for cholesterol causing the formation of COPs (Pearson correlation between COPs oxidation rate and PUFA content was 0.942**), despite the presence of antioxidant compounds in the sunflower oil such as tocopherols (72 mg/100 g, data obtained from the label). Other studies also showed that the higher unsaturation degree of the lipid matrix promotes cholesterol oxidation (Pignoli, Rodríguez-Estrada, Mandrioli, Barbantui, Rizzi & Lecker, 2009; Boselli, Rodríguez-Estrada, Ferioli, Caboni & Lecker, 2010) with the presence of free radicals and hydroperoxides (Ohshima, 2002).

4. Conclusions

In conclusion, the optimized gelled emulsion was an effective ingredient as partial or total pork back fat replacer in meat patties, showing nutritional advantages and without negative influence on the sensory properties of the final products. Additionally, results

also showed that cooking increased the susceptibility of the highly unsaturated ingredient to oxidation, being advisable to control this process in future applications.

5. Acknowledgements

We thank the PIUNA (Plan de Investigación de la Universidad de Navarra) for their contribution to the financial support of this work. C. Poyato is grateful to ‘‘Asociación de Amigos de la Universidad de Navarra’’ for the grants received.

Table 1. Scores ± standard deviation of a sensory multiple comparison tests for cooked samples between control (G0) and the different level of substitution samples. A total of 11 trained panellists participated.

	G25	G50	G75	G100	p-value
Odour	5.0 ± 1.0	4.4 ± 1.1	4.2 ± 1.5	4.4 ± 0.9	p>0.05
Colour	4.8 ± 0.6	4.7 ± 0.7	4.7 ± 0.7	4.8 ± 0.4	p>0.05
Taste	4.5 ± 0.8	4.5 ± 1.0	5.1 ± 1.1	4.8 ± 1.0	p>0.05
Hardness	4.6 ± 1.3	5.0 ± 1.2	4.6 ± 1.2	4.6 ± 0.8	p>0.05
Juiciness	4.5 ± 1.0	4.4 ± 1.0	4.6 ± 1.2	5.2 ± 1.4	p>0.05
Fatness	5.3 ± 0.9	5.0 ± 1.0	4.4 ± 1.0	4.6 ± 1.2	p>0.05

The p-value corresponds to ANOVA test among the substituted products.
Scores: 1. very much less; 2. much less; 3. considerably less; 4. slightly less; 5. not differences; 6. slightly more; 7. considerably more; 8. much more; 9. very much more.

Table 2. Chemical composition per 100 g product of the different formulated meat patties.

	G0			G25			G50			G75			G100		
	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p
Moisture (g)	60.99^{a}	58.52^{A}	0.020	62.96^{b}	59.51^{A}	0.001	64.59^{c}	60.59^{A}	<0.001	66.60^{d}	63.61^{B}	0.008	68.33^{e}	64.15^{B}	<0.001
Protein (g)	18.31^{a}	21.65^{A}	0.008	19.19^{a}	22.67^{AB}	0.002	18.34^{a}	23.16^{AB}	0.001	17.47^{a}	22.75^{AB}	<0.001	18.23^{a}	24.48^{B}	<0.001
Fat content (g)	21.01^{e}	18.92^{D}	0.005	18.04^{d}	18.12^{D}	0.895	16.18^{c}	15.97^{C}	0.598	14.40^{b}	13.42^{B}	0.020	12.32^{a}	10.09^{A}	0.001
SFA (g)	7.94^{e}	7.12^{E}	0.060	6.32^{d}	6.30^{D}	0.040	5.20^{c}	5.17^{C}	0.007	4.01^{b}	3.85^{B}	<0.001	3.02^{a}	2.52^{A}	0.002
MUFA (g)	9.93^{e}	8.94^{E}	0.863	8.20^{d}	8.22^{D}	0.148	6.92^{c}	6.78^{C}	<0.001	5.58^{b}	5.23^{B}	0.003	4.37^{a}	3.61^{A}	0.008
PUFA (g)	2.96^{a}	2.69^{A}	0.090	3.38^{b}	3.46^{B}	0.002	3.93^{e}	3.88^{C}	0.780	4.69^{d}	4.23^{D}	<0.001	4.83^{e}	3.88^{E}	0.002
ω-3 (g)	0.18^{e}	0.14^{E}	0.005	0.14^{d}	0.14^{D}	0.155	0.11^{c}	0.11^{C}	0.780	0.08^{b}	0.08^{B}	0.015	0.06^{a}	0.04^{A}	0.715
ω-6 (g)	2.78^{a}	2.55^{A}	0.005	3.23^{b}	3.31^{B}	0.003	3.81^{e}	3.76^{C}	0.760	4.61^{d}	4.15^{D}	<0.001	4.78^{e}	3.83^{E}	0.002
PUFA/SFA	0.37^{a}	0.38^{A}	0.083	0.53^{b}	0.54^{B}	0.006	0.75^{c}	0.74^{C}	0.033	1.17^{d}	1.10^{D}	<0.001	1.60^{e}	1.53^{E}	0.002
PUFA+MUFA/SFA	1.62^{a}	1.63^{A}	0.112	1.83^{b}	1.85^{B}	0.067	2.08^{e}	2.06^{C}	0.005	2.56^{d}	2.45^{D}	<0.001	3.04^{e}	2.97^{E}	0.002
trans (g)	0.16^{d}	0.15^{D}	0.234	0.12^{c}	0.13^{C}	0.242	0.11^{c}	0.12^{C}	0.029	0.10^{b}	0.09^{B}	0.014	0.08^{a}	0.07^{A}	0.079
Colesterol (mg)	60.61^{c}	68.17^{B}	<0.01	58.25^{cb}	67.21^{B}	<0.01	55.68^{b}	65.10^{AB}	<0.01	53.73^{ab}	64.18^{AB}	<0.001	50.31^{a}	61.45^{A}	<0.001

Different small letters in the same row denote significant differences among raw samples ($p<0.05$) obtained for the five formulations of meat patties.

Different capital letters in the same row denote significant differences among cooked samples ($p<0.05$) obtained for the five formulations of meat patties.

The p-value correspond to Student t test between raw and cooked products within each formulation.

Table 3. Parameters related to different nutritional claims in accordance with Regulation (EC) Nº 1924/2006.

	Energy decrease (%)	Energy provided by protein (%)	Fat decrease (%)	SFA+trans decrease (%)	PUFA+MUFA	
					Content (%)	Energy provided (%)
G0		27.9 [2]			61.4	44.2
G25	10.8	31.1 [2]	14.1	20.5	64.2	44.5
G50	16.5	33.5 [2]	22.9	34.4 [4]	67.1	44.6
G75	23.9	35.0 [2]	31.4 [3]	49.2 [4]	71.4	46.3 [5]
G100	30.0 [1]	26.6 [2]	41.4 [3]	61.7 [4]	74.5	45.1 [5]

Each superscript number refers to the nutritional claims listed below. [1]"Energy reduced", [2]"high protein", [3]"reduced fat", [4]"reduced saturated fat", and [5]"high unsaturated fat".

Table 4. $TBARS_{390}$ (mmol 2,4-Decadienal/kg) and $TBARS_{532}$ (mg MDA/kg) ± standard deviation obtained for the five formulations of meat patties.

g product		G0	G25	G50	G75	G100
90 nm	Raw	0.89 ± 0.01^{d}	0.79 ± 0.03^{cd}	0.74 ± 0.02^{bc}	0.67 ± 0.05^{b}	0.48 ± 0.04^{a}
	Cooked	0.96 ± 0.05^{b}	0.84 ± 0.01^{b}	0.92 ± 0.04^{b}	0.88 ± 0.07^{b}	0.65 ± 0.01^{a}
	p-value	0.08	0.04	0.003	0.01	0.001
32 nm	Raw	0.19 ± 0.60^{b}	0.14 ± 0.03^{ab}	0.10 ± 0.02^{ab}	0.09 ± 0.02^{a}	0.09 ± 0.01^{a}
	Cooked	0.29 ± 0.03^{b}	0.26 ± 0.02^{ab}	0.26 ± 0.02^{ab}	0.24 ± 0.04^{ab}	0.22 ± 0.01^{a}
	p-value	0.05	0.003	<0.001	0.005	<0.001
g fat						
90 nm	Raw	4.27 ± 0.02^{b}	4.40 ± 0.17^{b}	4.58 ± 0.14^{b}	5.48 ± 0.01^{c}	3.31 ± 0.01^{a}
	Cooked	5.10 ± 0.26^{ab}	4.66 ± 0.03^{a}	5.79 ± 0.29^{bc}	6.57 ± 0.46^{c}	6.46 ± 0.55^{c}
	p-value	0.006	0.06	0.06	0.06	<0.001
32 nm	Raw	0.91 ± 0.28^{a}	0.79 ± 0.15^{a}	0.62 ± 0.10^{a}	0.66 ± 0.11^{a}	0.74 ± 0.06^{a}
	Cooked	1.56 ± 0.18^{a}	1.44 ± 0.09^{a}	1.65 ± 0.12^{a}	1.76 ± 0.30^{ab}	2.21 ± 0.19^{b}
	p-value	0.02	0.003	0.001	0.004	<0.001

Different letters in the same row denote significant differences among samples ($p<0.05$). The p-value correspond to Student t test between raw and cooked products within each formulation.

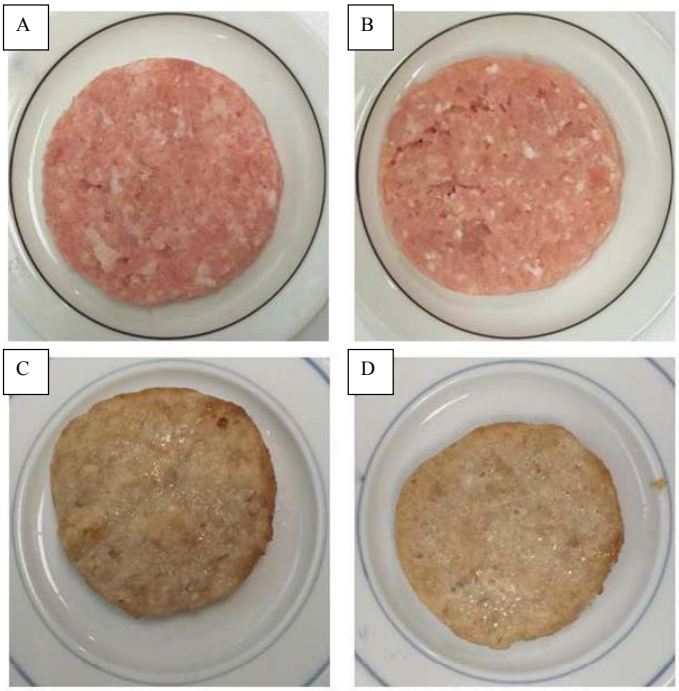
Table 5. Cholesterol oxidation compounds obtained for the five formulations of burger patties.

	G0			G25			G50			G75			G100		
µg/100 g product	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p	Raw	Cooked	p
7α-hydroxycholesterol	4.9^{d}	6.2^{C}	0.001	4.9^{d}	6.4^{C}	0.001	3.7^{e}	6.5^{C}	<0.001	3.1^{b}	5.4^{B}	<0.001	2.4^{a}	3.6^{A}	<0.001
7β-hydroxycholesterol	5.0^{d}	6.9^{BC}	<0.001	4.7^{e}	7.2^{C}	<0.001	4.4^{e}	6.7^{BC}	<0.001	3.6^{b}	6.3^{B}	<0.001	2.4^{a}	3.6^{A}	<0.001
5,6β-cholesterol epoxide	98.5^{e}	101.9^{D}	0.029	75.4^{d}	88.0^{C}	0.008	67.4^{e}	81.1^{C}	0.001	57.1^{b}	66.2^{B}	0.002	49.3^{a}	50.2^{A}	0.646
5,6α-cholesterol epoxide	14.1^{d}	15.6^{D}	0.001	12.0^{e}	16.3^{D}	<0.001	9.1^{b}	13.9^{C}	<0.001	8.4^{b}	10.7^{B}	<0.001	6.7^{a}	7.9^{A}	0.008
3,5,6-cholestanetriol	26.0^{d}	25.1^{D}	0.040	19.1^{e}	20.9^{C}	0.047	17.8^{e}	20.3^{C}	0.005	15.1^{b}	15.5^{B}	0.242	13.3^{a}	12.4^{A}	0.101
25-hydroxycholesterol	14.2^{e}	15.1^{C}	0.041	11.3^{b}	13.3^{B}	0.007	nd	nd	-	nd	nd	-	nd	nd	-
7-ketocholesterol	92.8^{b}	92.8^{D}	0.990	72.5^{b}	82.5^{C}	0.016	60.4^{b}	77.1^{C}	<0.001	50.9^{ab}	62.9^{B}	<0.001	43.4^{a}	48.4^{A}	0.028
Total	258^{e}	264^{E}	0.209	200^{b}	235^{D}	0.007	163^{e}	206^{C}	<0.001	138^{b}	167^{B}	<0.001	118^{a}	126^{A}	0.024
µg/100 g dry basis															
7α-hydroxycholesterol	13.0^{b}	17.6^{D}	<0.001	13.2^{ab}	17.3^{C}	<0.001	10.5^{a}	18.5^{BC}	<0.001	9.2^{a}	17.2^{B}	<0.001	7.6^{a}	14.3^{A}	<0.001
7β-hydroxycholesterol	13.1^{d}	19.6^{B}	<0.001	12.6^{cd}	19.5^{B}	<0.001	12.5^{e}	19.3^{B}	<0.001	10.9^{b}	20.2^{B}	<0.001	7.3^{a}	14.3^{A}	<0.001
5,6β-cholesterol epoxide	254^{e}	295^{D}	<0.001	203^{d}	236^{C}	<0.001	190^{e}	232^{C}	<0.001	171^{b}	211^{B}	<0.001	153^{a}	197^{A}	0.003
5,6α-cholesterol epoxide	36.0^{d}	44.9^{D}	<0.001	32.5^{e}	43.7^{D}	<0.001	25.7^{b}	39.9^{C}	<0.001	25.3^{b}	34.4^{B}	<0.001	20.7^{a}	31.0^{A}	<0.001
3,5,6-cholestanetriol	67.3^{d}	72.8^{C}	0.002	51.5^{e}	56.2^{B}	0.006	50.2^{e}	58.1^{B}	<0.001	45.1^{b}	49.6^{A}	0.001	41.3^{a}	49.0^{A}	0.019
25-hydroxycholesterol	42.7^{b}	43.5^{B}	0.148	30.4^{a}	25.6^{A}	<0.001	nd	nd	-	nd	nd	-	nd	nd	-
7-ketocholesterol	246^{e}	267^{D}	0.001	195^{d}	221^{C}	0.001	170^{e}	220^{C}	<0.001	152^{b}	201^{B}	<0.001	134^{a}	191^{A}	<0.001
Total	660^{e}	761^{E}	<0.001	539^{d}	630^{D}	<0.001	460^{e}	588^{C}	<0.001	414^{b}	534^{B}	<0.001	364^{a}	497^{A}	0.001

Different small letters in the same row denote significant differences among raw samples ($p<0.05$).Different capital letters in the same row denote significant differences among cooked samples ($p<0.05$). nd= not detected.
The p-value corresponds to t-student test between raw and cooked products within each formulation.

Fig. S1. Picture of the gelled emulsion.

Fig. S2. Pictures of representative raw and cooked burger patties. A: G0 raw. B: G100 raw. C: G0 cooked. D: G100 cooked.



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Pamplona, Spain

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Mejoras en el perfil lipídico de productos cárnicos incorporando aceite vegetal mediante diferentes ingredientes: emulsiones O/W y emulsiones gelificadas

C. Poyato, I. Berasategi, B. Barriuso, D. Ansorena I., Astiasarán

Introducción: Los productos cárnicos siguen siendo, a pesar de las recomendaciones nutricionales, alimentos de alto consumo en nuestro país. Por ello, resulta interesante mejorar su perfil nutricional, sin detrimento de sus propiedades sensoriales y sin incrementos significativos de su precio. Nuestro grupo investigador ha desarrollado 2 tipos de ingredientes funcionales susceptibles de incorporar aceites vegetales en productos cárnicos en sustitución parcial del tradicional tocino, que constituye una fuente significativa de colesterol y grasa saturada.

Objetivo: El presente trabajo pretende poner en evidencia las posibilidades tecnológicas de incluir aceites vegetales en la formulación de productos cárnicos (mortadela) con una significativa mejora de su perfil lipídico, y consiguiendo por tanto productos más saludables.

Material y métodos: Se desarrollan dos productos diferentes tipo mortadela empleando diferentes ingredientes:

1. Mortadela fabricada con una emulsión O/W elaborada con aceite de lino:agua:proteína de soja en proporciones 10:8:1 (Berasategi et al., 2014a).

2. Mortadela fabricada con una emulsión gelificada (40 % de aceite de lino, 1.5 % de carragenato y agua) (Poyato et al., 2014).

Resultados: Los dos tipos de productos fueron comparados con sus respectivos controles. En ambos casos, se sustituyó el 50 % del tocino por el respectivo sustituto (emulsión o gel). Se consiguieron reducciones de ácidos grasos saturados (AGS) del

orden de un 33-37 % e incrementos de PUFA de un 66.4 %. El aporte de ω-3, en el caso de emplear gel fue de 2.03 g/100g producto y para la emulsión de 1.31 g/100gproducto.

Conclusión: Los dos vehículos (emulsión y emulsión gelificada) proporcionan productos tecnológica y sensorialmente aceptables, con posibilidades de incluir declaraciones nutricionales relacionadas con la mejora de su fracción lipídica.

Mejoras en el perfil lipídico de productos cárnicos incorporando aceite vegetal mediante diferentes ingredientes: emulsiones O/W y emulsiones gelificadas

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INTRODUCCIÓN

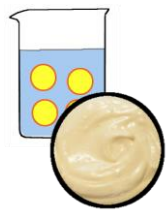
Los productos cárnicos, a pesar de las recomendaciones nutricionales, siguen siendo alimentos de alto consumo en nuestro país. Por lo que resulta interesante mejorar su perfil nutricional sin detrimento de sus propiedades sensoriales.

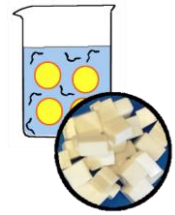
Nuestro grupo investigador ha desarrollado 2 tipos de ingredientes funcionales susceptibles de incorporar aceites vegetales en productos cárnicos en sustitución parcial del tradicional tocino.

OBJETIVO

Poner en evidencia las posibilidades tecnológicas de incluir aceites vegetales en la formulación de productos cárnicos cocidos con una mejora del perfil lipídico, consiguiendo productos más saludables.

DISEÑO EXPERIMENTAL





TOCINO DE CERDO	EMULSION OIL-in-WATER 10:8:1 Aceite:agua:prot. soja	TOCINO DE CERDO	EMULSION GELIFICADA 40% aceite 1.5% carragenato 58.5% agua

INCORPORACIÓN EN MORTADELA

CONTROL 1	MODIFICADO EMULSIÓN	CONTROL 2	MODIFICADO GEL
55% Magro cerdo	55% Magro cerdo	55% Magro cerdo	55% Magro cerdo
10% Tocino cerdo	5% Tocino cerdo	16% Tocino cerdo	8% Tocino cerdo
	5% Emulsión		8% Gel

RESULTADOS

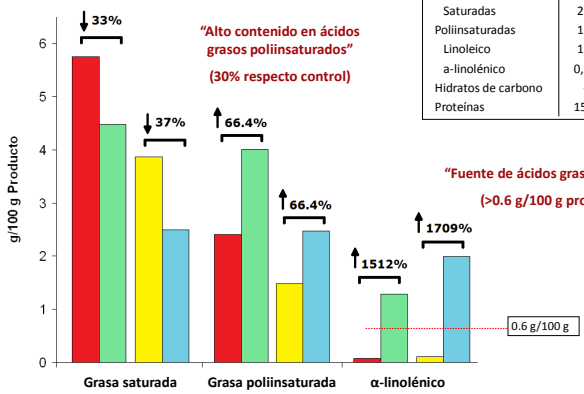
DECLARACIONES NUTRICIONALES

"Contenido reducido en grasas saturadas"

(30% respecto control)

"Alto contenido en ácidos grasos poliinsaturados"

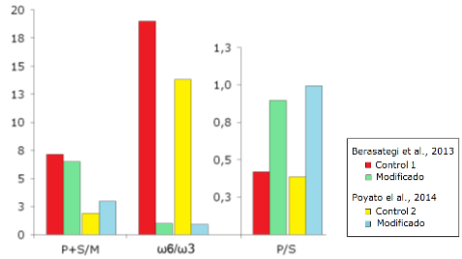
(30% respecto control)



	g/100 gramos de producto				Por ración (50 g)				% CDR con una ración			
	Control1	Emulsión	Control2	Gel	Control1	Emulsión	Control2	Gel	Control1	Emulsión	Control2	Gel
Valor energético (kcal)	133	123	179	166	66,5	61,5	89,7	83,3	3,3	3,1	4,5	4,2
Grasas, de las cuales	8,4	6,9	13,2	11,9	4,2	3,4	6,6	5,9	6,0	4,9	9,5	8,5
Saturadas	2,9	1,8	4,9	3,3	1,5	0,9	2,5	1,7	7,3	4,6	12,4	8,3
Poliinsaturadas	1,5	2,5	2,4	4,0	0,7	1,2	1,2	2,0	-	-	-	-
Linoleico	1,3	1,2	2,2	1,9	0,7	0,6	1,1	1,0	5,4	5,0	9,3	8,1
a-linolénico	0,08	**1,29**	0,11	**1,99**	0,0	0,6	0,1	1,0	3,64	**58,6**	5	**90,5**
Hidratos de carbono	-	-	-	-	-	-	-	-	-	-	-	-
Proteínas	15,1	13,9	15,1	14,9	7,5	7,0	7,5	7,4	16,4	15,1	16,4	16,2

"Fuente de ácidos grasos omega-3"

(>0.6 g/100 g producto)



CONCLUSIÓN

Los dos vehículos (emulsión y emulsión gelificada) proporcionan productos tecnológica y sensorialmente aceptables, con posibilidades de incluir declaraciones nutricionales relacionadas con la mejora de su fracción lipídica.

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Reglamento (CE) No 1924/2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos.

Reglamento (UE) 1169/2011 sobre la información alimentaria facilitada al consumidor.

Part V
General discussion

Development of emulsion based-delivery systems

The formulation of healthier and functional foods sometimes implies the incorporation of bioactive compounds, and therefore the design and development of technological strategies to facilitate an adequate integration of the new substances into the final product.

There are many food components that cannot be simply incorporated into foods in their regular form, and must be previously introduced into some kind of delivery system, such as emulsions. A number of reasons justify why some food components, such as lipids (e. g. unsaturated fatty acids, carotenoids, vitamins A and D, phytosterols), proteins (e. g. peptides), carbohydrates (e. g. prebiotics, dietary fibers) and minerals (e. g. calcium, iron, selenium) are previously included into emulsions before their addition into foods, (1) they can be successfully incorporated into the food matrix without adversely affecting the quality attributes, (2) to protect them against chemical, physical or biological degradation, (3) to mask off-flavours, (4) to control the release of the bioactive compounds, and (5) to extend their self life.

In this sense, several studies highlight the advantages of emulsified oils for achieving nutritional improvements in the lipid fraction of food products (Chen, McClements & Decker, 2013; Jiménez-Colmenero, 2013; Qiu, Zhao, Decker & McClements, 2015; Salminen, Herrmann & Weiss, 2013; Sanz, Laguna & Salvador, 2015; Walker, Decker & McClements, 2015). Most of the currently used delivery systems are structurally simple, such as the case of O/W emulsions, since they are inexpensive and easy to fabricate. However, more structurally complex food-grade delivery systems may have extended technological properties, worthy to be explored.

A number of studies report that some of these more complex systems, such as multiple emulsions, have great potential as delivery systems for functional food components (Morais, Rocha & Burgess, 2010; Li et al., 2012; Giroux et al., 2013), but

there are still a number of important issues that are needed to be addressed before this technology can be successfully applied in the food industry. Among them, it can be mentioned that their thermodynamic and kinetic instability and the lack of suitable food-grade emulsifiers and stabilizers, limit the use of certain complex emulsions for food applications (Lamba, Sathish & Sabikhi, 2015).

In the present work, the design of formulations for new delivery systems was proposed, aiming to obtain more complex and stable emulsions, with the following potential benefits compared to conventional ones: (1) increased unsaturated fatty acids (UFA) content, (2) reduced energy and fat content, (3) increased oxidative stability of the system (4) reduced technological problems, (5) sensory acceptable reformulated products.

Therefore, one of our challenges was to formulate lipid-based delivery systems from food-grade ingredients that were obtained by means of relatively simple processing operations, ensuring, at the same time, that they were stable within the final product. With this purpose, W/O/W emulsions and gelled emulsions were developed, in order to study the potential physicochemical and nutritional advantages of these delivery systems in comparison to more simple formulations. In both cases, highly unsaturated oils as oily phase, and carrageenan as principal stabilizing polymer were used.

Regarding the formulation of W/O/W emulsions, factors such as the influence of the oil/water ratio and the effect of ultrasounds on the droplet size reduction were taken into account for assessing the physical structure of the emulsion system. Thus, three different concentrations of linseed oil (rich in ω-3 FA) were used (38, 25 and 15 %), and ultrasounds were applied (0, 15 and 30 min). Knowing that physical stability greatly affects the shelf life of the emulsions, the evaluation of their appearance and functionality, and also of the suitability of the emulsions to be subsequently used as lipid delivery system were carried out. This was done through the assessment of the resistance to phase's separation, consistency, and the determination of the droplet size.

Preliminary results suggested that it was possible to prepare W/O/W emulsions with a wide fat range content (15, 25, 38 %), and that their stability could be increased

by means of the ultrasounds application during the processing, which reduced the droplet size of the emulsions. These smaller droplet sizes increased the consistency and the physical stability of these multiple emulsions.

Taking into account that most of the current studies that are related to multiple emulsions preparation deal with emulsions not suitable for being used in food applications (O'Regan & Mulvihill, 2010; Jiménez-Colmenero, 2013), the food-grade multiple emulsion developed in our work could be used to modify qualitative and quantitative aspects of the lipid material in foods, improving this fraction through two main approaches, (1) by reducing the fat content and (2) by providing healthier fatty acid profiles (Benichou et al., 2004; Dickinson, 2011; McClements et al., 2007; Muschiolik, 2007). Nevertheless, it should also be considered that the nature and concentration of the components used in their formation, (e. g. oil phase, the type of emulsifiers, or the composition of the inner and outer aqueous phases), could affect the oxidative stability of the emulsion system.

For these reasons, the effect of the storage under accelerated conditions (0-48 h, 65 °C) on the oxidative stability of a food-grade W/O/W emulsion was studied, and compared to that of an O/W emulsion. Furthermore, considering the different oxidation susceptibility of oils depending on their unsaturation degree, two different oils were evaluated. Thus, the emulsions were prepared with linseed oil or olive oil, and in each case, the incorporation of two antioxidants were assayed, a water *Melissa officinalis* extract and BHA, both added in the W_1 phase. In this sense, both the polarity of the antioxidant used, and the phase in which they are placed might be relevant factors that can affect the antioxidant effectiveness in different emulsion systems.

The mean value for total fat content for O/W and W/O/W emulsions was 52.6 g/100 g and 38.6 g/100 g, respectively. As expected, α-linolenic and linoleic acids were the predominant PUFA in the emulsions elaborated with linseed oil, accounting for 52.9 g/100 g and 14.8 g/100 g oil respectively. On the other hand, oleic acid (78.27 g/100 g oil), was the most relevant MUFA in olive oil containing emulsions. The lack of differences in the fatty acid profile of all emulsions during the storage

pointed out that the nutritional value of the oil was not affected by the heat treatment, regardless the type of emulsion considered.

Lipid oxidation assessment in both systems revealed that PV values in olive oil emulsions (including O/W and W/O/W) kept their values between 11.7-17.6 meq O_2/kg oil, without showing statistical differences at the end of the treatment between the use of *Melissa* or BHA ($p>0.05$). In linseed oil emulsions, gradual increases in PV were observed during the storage, with different behaviour depending on the antioxidant and the type of emulsion. In this sense, in W/O/W emulsions, *Melissa* extract showed significantly higher values at 48 h (10.61 meq O_2/kg oil) than those with BHA (8.57 meq O_2/kg oil) ($p<0.05$). When measuring secondary oxidation products, TBARS, very different results were noticed depending on the type of oil and emulsion. Whereas a great stability of olive oil emulsions was observed without differences between antioxidants or type of emulsion, in linseed oil emulsions TBARS gradually increased during the storage being higher in O/W than W/O/W emulsions. Moreover, in linseed oil emulsions, while the lipophilic antioxidant (BHA) seemed to be more efficient than the water *Melissa* extract, delaying the lipid oxidation in W/O/W emulsions, the opposite effect was found in the O/W emulsion.

Porter (1993) first described the "polar paradox" as a phenomenon where hydrophilic antioxidants were more effective in bulk oils, whereas lipophilic antioxidants were more effective in emulsified ones. In contrast to this theory, our study showed that water *Melissa* extract was far more effective to delay the increase of oxidation compounds than BHA, despite their polarities. As previously reported by García Íñiguez de Ciriano et al. (2010a), rosmarinic acid present in *Melissa* extract is a polar compound known by its antioxidant activity, which effectively delays the formation of oxidation products in O/W emulsions. Supporting these results, recent studies showed that not all antioxidants behave in a manner proposed by the "polar paradox", emphasizing that in O/W emulsions, not linear influence of the hydrophobicity on antioxidant capacity was found (Laguerre et al., 2015).

To deep in the effect of the incorporation of natural extracts in the aqueous phase of highly unsaturated O/W emulsions, two different *Fucus vesiculosus* extracts were evaluated, a water and an acetonic extract. The developed emulsions (non-food

grade) were stored in dark and room temperature, and were compared to those stored under accelerated conditions, (1) light and room temperature, (2) darkness and 40 °C, to assess the efficacy to inhibit lipid oxidation against photo- and thermoxidation, respectively.

Whereas both water and acetonic extracts, showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the water one showed antioxidant activity against photooxidation. Therefore, both the polyphenols content (radical scavenging activity) and in particular the presence of phlorotannins (ion-chelating capacity), contributed to decrease the lipid oxidation process slowing down fatty acid degradation. Moreover, the higher carotenoids content in the water extract could inhibit free radical chain reactions caused by photooxidation process.

These findings confirmed that natural antioxidant extracts containing polyphenols were effective in protecting the oily phase when the antioxidant was located in the aqueous phase. Consequently, the type of antioxidant remained a key factor in controlling oxidation in emulsion-based delivery systems prepared with highly unsaturated oils. As the interactions with other ingredients could modify these results, a further step would be necessary to incorporate the food-grade O/W and W/O/W emulsions into food products and evaluate their behaviour into a food matrix.

The second delivery system proposed in this work, as mentioned above, was an O/W emulsion in which the water phase was in gel state, named gelled emulsion. The use of carrageenan as biopolymer for the gel aimed to contribute to reach both desirable textural and sensory attributes. Furthermore, carrageenan could also contribute to prevent emulsions destabilization. In order to optimize the formulation of this new system, different amounts of linseed oil (40-70 %), carrageenan (0.5-1.5 %) and surfactant (polysorbate 80; surfactant-oil ratio: 0.003-0.005) were properly combined in a surface response design (RSM), to study the influence of these factors (oil and carrageenan percentage) on the hardness and the syneresis of the system. The model showed significant interaction between these two factors. Whereas at high carrageenan concentration (1.5 %) hardness decreased when the oil content increased, at lower carrageenan concentration (0.5 %) the hardness increased to a maximum point after which a decrease was again observed. Syneresis showed the opposite behaviour.

Considering that the gel was intended to be used as partial pork fat replacer in meat products, the objective was to obtain a gel with a maximum hardness (simulating the texture of pork back fat) and minimum syneresis (to avoid technological problems during the meat product elaboration). The resulting optimum formulation contained 40 % of oil and 1.5 % of carrageenan, keeping a surfactant-oil ratio of 0.003.

After the optimization of the gelled emulsion, a comparison of its chemical composition (protein, carbohydrates, fat, lipid profile, fibre, and cholesterol) and nutritional properties with those of pork back fat was done. Furthemore, to evaluate the influence of the lipid profile on the formulation of the gelled emulsions, linseed oil (LV), extra virgin olive oil (EVO) and high-oleic sunflower oil (HOSF) were used due to their highly unsaturated profile. The gelled emulsions showed a reduction of a 46.5 % of the energy supply, a 43.6 % lower amount of fat and no cholesterol content compared to animal fat. In addition, both the physicochemical and oxidative status of the gelled emulsions with HOSF was assessed during the storage (1 and 30 days, in products stored under vacuum, at 4 °C and at 25 °C). From the technological standpoint, storage of the gelled emulsions during 30 days at 4 °C ensured colour and texture stability and maintenance of low TBARS values (0.24 mg/100 g product). Storage at 25 °C might slightly increase oxidation susceptibility, without colour or texture modification. So, due to their nutritional advantages and physicochemical stability, it could be concluded that gelled emulsions could be good delivery systems to incorporate lipophilic bioactive compounds into food matrices.

It is well known that one of the most important causes of deterioration of oils and fats containing products is lipid oxidation. However, it is also important to highlight that one of the major factors that influences the evaluation and monitoring of lipid oxidation compounds rely on the availability of analytical techniques to detect them. A wide variety of analytical techniques have been developed to monitor lipid oxidation in bulk fats and oils, which can also be used to study emulsion systems, prior extraction of the oily phase (McClements & Decker, 2000). All the methods are based on measuring changes in the concentration of molecular species that are indicative of the progress of lipid oxidation. In this sense, lipids are oxidized into complex mixtures of primary and secondary oxidation products, which vary in nature and composition

according to the conditions (e. g. incidence of light, temperature) and the chemical composition of the FA. Thus, the usefulness of the results can be evaluated according to the chemical properties measured and the specificity, sensitivity and precision of the analyses (Frankel, 1998). For example, malondialdehyde (MDA) has been used as representative of non-volatile secondary oxidation products and its determination by the TBARS test measuring the absorbance at 532 nm has been widely used due to its simplicity (Barriuso, Astiasarán & Ansorena, 2013). However, this method exhibits some drawbacks, since it has been stated for instance, that the TBARS assay is nearly worthless with heat-treated oils (Guillén-Sans & Guzmán-Chozas, 1998) and that this test is reliable only when applied for the determination of MDA in unprocessed foods (Papastergiadis et al., 2012). On the other hand, significant amounts of TBARS are only formed when fatty acids with 3 or more double bonds are involved (Fennema, 1996; Waraho, Cardenia, Rodríguez-Estrada, McClements & Decker, 2009). This fact could lead to underestimation of oxidation in highly monounsaturated lipids (Waraho, Cardenia, Decker & McClements, 2010).

Therefore, due to the variety of oils used in both delivery systems formulations, and knowing that the lipid oxidation products can be substantially different in quantity and type depending on the composition of the lipid matrix, an optimization of the conditions to follow the oxidation rate of different edible oils was attempted.

Seven oils (virgin linseed oil, algae oil, sunflower oil, high-oleic sunflower oil, refined olive oil and two different extra virgin olive oils) with very different lipid profiles were chosen for the study, to evaluate the influence of the different composition on some lipid oxidation products formed during intense heating treatment. Oxidation at 0, 2 and 4 h at 180 °C was monitored by measuring the absorbance of thiobarbituric acid reactive substances (TBARS) along the absorption spectrum (300-600 nm), the volatile aldehydes (HS-SPME-GC-MS) and the fatty acid profile (FID-GC).

The results showed that TBARS absorption spectrum depended on the lipid composition of the heated oils, as we expected. The secondary oxidation compounds formed coloured compounds with TBA, whose absorbance was measured at different

wavelengths and correlated with the increments of the amounts of different families of volatile aldehydes. In the yellow area (350-450 nm), the highest correlation coefficients between the measured absorbances and the increments of the different families of volatile aldehydes were found at 390 nm. This wavelength was chosen as the best representative parameter for the assessment of the yellow pigment formed from the reaction between lipid oxidation products and TBA.

Moreover, higher absorbance increments during heating were noticed at 390 nm compared to those observed at the ordinary wavelength at which is usually measured the TBARS, 532 nm (from 2 to 21 fold higher depending on the oil). This finding pointed out the better sensitivity to detect oxidation at 390 nm as compared to 532 nm. Furthermore, a Principal Component Analysis (PCA) revealed a close relationship between the absorbance at 390 nm, the loss of unsaturated fatty acids and their corresponding volatile aldehydes. Moreover, a high association between the total volatile compounds formed and the loss of MUFA and PUFA was observed. In particular, high inverse correlations were especially found between the loss of ω-6 fatty acids and the presence of higher amounts of both alkadienals (R= -0.968) and total volatile aldehydes (R= -0.853). Aldehydes produced were substantially different in quantity and type (alkanal, alkenal or alkadienal) depending on the composition of the lipid matrix. These findings were in agreement with those obtained by Guillén and Uriarte (2012) after the analysis of different edible oils heated at high temperatures for long treatment periods. Therefore, oils rich in ω-3 FA (extra virgin linseed oil and algae oil) showed (*E,E*)-2,4-heptadienal as the main aldehyde formed. Sunflower oil, rich in ω-6 showed the highest value for (*E,E*)-2,4-decadienal, and oils rich in MUFA (high-oleic olive oil, extra virgin olive oils and refined olive oil) produced high amounts of 2-decenal and nonanal. One of the main outcomes of this work was the possibility of formulating multiparametric equations that allowed predicting the formation of volatile aldehydes of heated oils by measuring only two parameters: (1) the lipid profile in unheated oils (MUFA, ω-3 and ω-6) and (2) TBARS at 390 nm during their heating.

Thus, relationships between TBARS at 390 nm and the MUFA amount, ω-3 and ω-6 in the unheated oils were able to predict the amount of different families of

aldehydes (alkanals, alkenals and alkadienals) and also total volatile aldehydes and, in consequence, to estimate the intensity of the oxidation process. Our regression models were an easy, simple and good tool to predict the formation and semi-quantification of volatile aldehydes, under accelerated oxidation conditions (180 °C, 4 h), and found solid relationships among different parameters related to this process. So, these results pointed out the interest of choosing the absorbance at 390 when the oxidative evolution of vegetable oils under heating is assessed by the TBARS test.

Nutritional and technological implications of the use of emulsions in the meat industry

Meat and meat products are recognized in the Western diet as the main source of essential aminoacids, high biologic value proteins, bioavailable iron and vitamins of the B group (Hill, 2002; Biesalski, 2005; Williamson et al., 2005; McAfee et al., 2010). At the same time, processed meat products are not widely recommended in the dietary guidelines because they are also associated with other nutrients that are often considered negative, including high levels of fat, mainly SFA, cholesterol and sodium (Biesalski, 2002; Hill, 2002; Paik et al., 2005; Valsta et al., 2005; Ferguson, 2010). In fact, the excessive consumption of SFA has been linked to some chronic diseases such as cardiovascular diseases, obesity and some kinds of cancer (Lichtenstein et al., 1998; WHO, 2003; Rothstein, 2006; Dickinson & Havas, 2007; Havas et al., 2007).

Therefore, the development of nutritionally improved meat products using reformulation strategies has been accomplished with the aid of emulsion based systems, being the qualitative and quantitative improvement of the fat content one of the most important goals. Our research group previously assayed the incorporation of emulsified oils (using soya protein as emulsifier) with this purpouse (Barriuso, Ansorena, Calvo, Cavero & Astiasarán, 2015; Berasategi et al., 2014a; Berasategi, et al., 2014b; García-Íñiguez de Ciriano et al., 2009; García-Íñiguez de Ciriano et al., 2010a). Other researchers have tested also different conventional emulsions with the aim of reducing the SFA fraction and increasing the PUFA and MUFA fractions (Delgado-Pando, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2010b; Youssef, 2011; Rodríguez-Carpena, Morcuende & Estévez, 2012; Salcedo-Sandoval, Cofrades, Ruiz-Capillas, Solas & Jiménez-Colmenero, 2013; Salminen, Herrmann & Weiss, 2013; Triki, Herrero, Ruiz-Capillas & Jiménez-Colmenero, 2013; Ganesan, Brothersen & McMahon, 2014). In some cases, the application of these strategies has

caused the unacceptability of the developed products, due to technological or sensory problems.

In this sense, it is important to highlight that, when using fat replacers, a careful approach is needed in order to achieve the appearance and the technological, rheological and sensory properties required by the food industry in order to meet consumers' expectations (Tye, 1991; Salcedo-Sandoval et al., 2013; Salcedo-Sandoval et al., 2015). In fact, the use of low-fat replacers can cause, in some cases, technological problems due to the great impact on flavour, palatability and texture that fat provides to these products (Hort & Cook, 2007; Delgado-Pando, Cofrades, Ruiz-Capillas & Jiménez-Colmenero, 2010a; Horita, Morgano, Celeghini & Pollonio, 2011).

Consequently, after having evaluated the physicochemical properties of the delivery systems developed in this work, the gelled emulsion was selected to confirm its usefulness to be incorporated into meat products, due to its similar appearance with animal fat. Therefore, two different meat products were elaborated (Bologna-type sausages and burger patties) in order to evaluate the behaviour of the gelled emulsion in different processing conditions: in a cooked (80 °C) product, and in a fresh product intended to be cooked (180 °C) prior consumption.

Linseed oil was chosen as the oil to be incorporated into the gel in Bologna-type sausages, due to its high content in ω-3 FA (52.7 % of ω-3 FA). It is well known that ω-3 FA provides a wide range of benefits, including anti-trombotic and anti-inflamatory effects, immunomodulatory properties, lower insulin resistance, aids neural and brain development among others (Walker, Decker & McClements, 2015; Chen, McClements & Decker, 2013; Ganesan et al., 2014).

The gel was used for replacing a 50 % of the fat content of this Bologna products. These new product was compared with an O/W emulsion containing product and with a conventional formulation, with no modifications (control). The chemical composition (moisture, protein, fat content and lipid profile) of the different formulated Bologna-type sausages was determined, and the evolution of colour, texture (TPA) and TBARS were followed after 35 days of storage (4 °C). From the nutritional point of view, the gelled emulsions gave the same advantages as the conventional emulsion when both modified formulations were compared to control products. Regarding EU

Regulation (EFSA, 2009), both modified products supplied enough α-linolenic amounts to claim "high ω-3 FA". Moreover, whereas no differences were observed between O/W emulsion and gelled emulsions for colour and TBARS, TPA results revealed that gelled emulsions could be more efficient maintaining the hardness of the original product. To complete the study, a sensory analysis (triangle test) was performed to determine the existence of perceptible sensory differences in hardness, taste and appearance among final products. The sensory analysis did not show sensory problems related to odour, taste and juiciness, so it could be concluded that the gelled emulsion was an effective ingredient as partial pork back fat replacer (50 % of replacement) in cooked meat products. More studies related to the stability under different processing conditions of this ingredient, and its efficiency when used at different concentrations as fat replacer were needed, in order to highlight its potential use as animal fat replacer in a variety of products.

Therefore, a study focused on the use of a high-oleic sunflower oil gelled emulsion as total or partial fat replacer in a fresh meat product, and on the evaluation of the stability of the new product (burger patty) after usual cooking (180 °C, 15 min) was performed. The behaviour of the fat replacer on these samples was studied in raw and cooked conditions, by assessing nutritional (moisture, protein, fat content, lipid profile and cholesterol) and sensory properties of the resulting products, and by evaluating their stability against oxidation (TBARS and cholesterol oxidation products, COPs).

Increasing amounts (25, 50, 75 and 100 %) of the gelled emulsion were added into the product in order to reduce the fat content, while improving its fatty acid profile. Regarding the nutritional approach, a 41 % reduction of the total fat content with an increment of the 74.5 % of the UFA, and a significant decrease in cholesterol (47 %) and SFA (62 %) were achieved in the product with the highest level of substitution.

A number of potential nutrition claims could be applied depending on the amount of gelled emulsion incorporated in the developed burger patties referred to raw products according to the EU Regulation (Regulation 1924/2006). The highest substituted product (100 %) compared to a conventional formulation could be labelled

with five nutrition claims, (1) “energy reduced”, (2) “high protein”, (3) “reduced fat”, (4) “reduced saturated fat”, and (5) “high unsaturated fat”. Additionally, according to the Commission Regulation (EU) 432/2012, these nutritional modifications allowed to claim that “Replacing saturated fats in the diet with unsaturated fats contributes to the maintenance of normal blood cholesterol levels”, “Linoleic acid contributes to the maintenance of normal blood cholesterol levels; the beneficial effect is obtained with a daily intake of 10 g of LA” and “Reducing consumption of saturated fat contributes to the maintenance of normal blood cholesterol levels”. Moreover, the sensory test showed that the optimized gelled emulsion was an effective ingredient as partial or total pork back fat replacer in these products without negative influence on their sensory properties.

Aside from the fact that the gelled emulsion could improve the burger patties from the nutritional point of view, these products showed also significantly lower TBARS and COPs compared to control. However, when samples were subjected to thermal treatment (180 °C, 15 min, oven conditions) higher lipid oxidation rates were found when increasing amounts of the gelled emulsion were incorporated into the new formulations (without impairment of their final sensory properties), being advisable to control this process in future applications.

Therefore, the previously optimized gelled emulsion was demonstrated to be a suitable lipophilic delivery system for UFA compounds and applicable in meat formulations as partial or total fat replacer. So that, considered as a potential strategy, replacing animal fat with emulsified vegetable oils led to healthier meat products, towards enriching the fatty acid profile and resulting in low fat and/or lower cholesterol containing products.

Part VII
Conclusions

Conclusions

1. The use of hydrophilic polymers (carrageenan) allowed obtaining both multiple emulsions (W/O/W) and gelled emulsions for food use, physically stable and with a variable fat content, able to serve as delivery systems for bioactive compounds in the formulation of healthier foods.
2. The fatty acid profile of oils and the determination of the absorbance at 390 nm (resultant of the presence of thiobarbituric acid reactive substances) were suitable parameters, by using regression models, to estimate the amount of aldehydes formed during their oxidation at high temperatures.
3. The use of antioxidant plant extracts (*Melissa officinalis, Fucus vesiculosus* and *Lavandula latifolia*) of different polarity allowed to increase the oxidative stability of highly unsaturated emulsion systems (conventional, multiple and gelled emulsions).
4. A gelled emulsion (40 % oil, 1.5 % of carrageenan and a surfactant/oil ratio of 0.003) was found to be a physicochemically stable system, suitable for being used as animal fat replacer. The application of the gelled emulsion in fresh and cooked meat products was technologically feasible and sensory acceptable.
5. Meat products resulting from the partial or total replacement of animal fat for a highly unsaturated gelled emulsion, showed a healthier lipid profile, lower fat and cholesterol content and lower energy value. These nutritional characteristics allowed to establish, in some cases, nutrition and health claims according to the current European legislation.

Conclusiones

1. La utilización de polímeros hidrofílicos (carragenatos) permitió generar tanto emulsiones múltiples (W/O/W) como emulsiones gelificadas de uso alimentario, físicamente estables y con contenidos en grasa variables, capaces de vehiculizar compuestos bioactivos destinados a la formulación de alimentos más saludables.

2. La determinación del perfil de ácidos grasos en muestras de aceites y la medida de la absorbancia a 390 nm (resultante de la presencia de sustancias reactivas al ácido tiobarbitúrico) fueron suficientes para, a través de modelos de regresión, estimar la cantidad de aldehídos resultantes de la oxidación inducida en los aceites por altas temperaturas.

3. La utilización de extractos vegetales antioxidantes (*Melissa officinalis, Fucus vesiculosus* y *Lavandula latifolia*) de diversa polaridad permitió incrementar la estabilidad oxidativa de sistemas emulsión (emulsiones convencionales, múltiples y gelificadas) altamente insaturados.

4. Una emulsión gelificada (40 % aceite, 1,5 % de carragenato y un cociente surfactante/aceite de 0,003) resultó ser un sistema físico-químicamente estable, con una apariencia similar al tocino, adecuado para ser utilizado como sustituto de grasas animales. La aplicación de la emulsión gelificada en productos cárnicos frescos y cocidos fue tecnológicamente viable y sensorialmente aceptable.

5. Los productos cárnicos resultantes de la sustitución parcial o total del tocino por una emulsión gelificada altamente insaturada mostraron un perfil lipídico más saludable, menor contenido en grasa y colesterol, y menor valor energético. Estas características nutritivas permitieron establecer, en algunos casos, declaraciones nutricionales y saludables de acuerdo a la actual legislación europea.

Part VIII
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A

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Appendix

Appendix I

ANÁLISIS SENSORIAL TRIANGULAR

Pruebe las **tres muestras** presentadas, tantas veces como desee, y señale con una X si alguna de ellas es **diferente** respecto a las otras 2 para cada uno de los parámetros solicitados.

Los cuatro parámetros se deben juzgar de manera independiente sin que la valoración de cada uno ellos condicione los demás.

ENCIENDA LA LUZ ROJA

Parámetro 1: Diferencia en olor

CÓDIGO	IGUAL	DIFERENTE	¿Qué característica le permitió identificar la diferencia?
_ _ _			
_ _ _			
_ _ _			
¿Cuál prefiere?			

Parámetro 2: Diferencia en sabor

CÓDIGO	IGUAL	DIFERENTE	¿Qué característica le permitió identificar la diferencia?
_ _ _			
_ _ _			
_ _ _			
¿Cuál prefiere?			

Parámetro 3: Diferencia en jugosidad

CÓDIGO	IGUAL	DIFERENTE	¿Qué característica le permitió identificar la diferencia?
_ _ _			
_ _ _			
_ _ _			
¿Cuál prefiere?			

Parámetro 4: Diferencia en textura

CÓDIGO	IGUAL	DIFERENTE	¿Qué característica le permitió identificar la diferencia?
_ _ _			
_ _ _			
_ _ _			
¿Cuál prefiere?			

Observaciones: (¿nota alguna característica especial en alguna de ellas?; ¿las consumiría?; etc.)

Appendix II

SENSORIAL HAMBURGUESA COCINADA I

Enfrente de usted hay 5 muestras de hamburguesas. Una de las muestras está marcada con **R** y las otras tienen claves. Pruebe cada una de las muestras y compárelas con **R**, e indique su respuesta a continuación, marcando con una **X** donde corresponda.

Compare en cuanto a la intensidad del **COLOR.**

	601	279	481	529
Más COLOR que R	☐	☐	☐	☐
Igual que R	☐	☐	☐	☐
Menos COLOR que R	☐	☐	☐	☐

Indique cuál es la diferencia:

	601	279	481	529
Nada	☐	☐	☐	☐
Ligera	☐	☐	☐	☐
Moderada	☐	☐	☐	☐
Mucha	☐	☐	☐	☐
Muchísima	☐	☐	☐	☐

Comentarios:

Compare en cuanto a la intensidad del **OLOR**.

	601	279	481	529
Más OLOR que R	☐	☐	☐	☐
Igual que R	☐	☐	☐	☐
Menos OLOR que R	☐	☐	☐	☐

Indique cuál es la diferencia:

	601	279	481	529
Nada	□	□	□	□
Ligera	□	□	□	□
Moderada	□	□	□	□
Mucha	□	□	□	□
Muchísima	□	□	□	□

Comentarios:

Compare en cuanto a la intensidad del **SABOR**.

	601	279	481	529
Más SABOR que R	□	□	□	□
Igual que R	□	□	□	□
Menos SABOR que R	□	□	□	□

Indique cuál es la diferencia:

	601	279	481	529
Nada	□	□	□	□
Ligera	□	□	□	□
Moderada	□	□	□	□
Mucha	□	□	□	□
Muchísima	□	□	□	□

Comentarios:

Compare en cuanto a la **DUREZA**.

	601	279	481	529
Más DURA que R	□	□	□	□
Igual que R	□	□	□	□
Menos DURA que R	□	□	□	□

Indique cuál es la diferencia:

	601	279	481	529
Nada	□	□	□	□
Ligera	□	□	□	□
Moderada	□	□	□	□
Mucha	□	□	□	□
Muchísima	□	□	□	□

Comentarios:

Compare en cuanto a la **JUGOSIDAD**.

	601	279	481	529
Más JUGOSA que R	□	□	□	□
Igual que R	□	□	□	□
Menos JUGOSA que R	□	□	□	□

Indique cuál es la diferencia:

	601	279	481	529
Nada	□	□	□	□
Ligera	□	□	□	□
Moderada	□	□	□	□
Mucha	□	□	□	□
Muchísima	□	□	□	□

Comentarios:

Compare en cuanto a la **GRASOSIDAD**.

	601	279	481	529
Más GRASA que R	□	□	□	□
Igual que R	□	□	□	□
Menos GRASA que R	□	□	□	□

Indique cuál es la diferencia:

	601	279	481	529
Nada	□	□	□	□
Ligera	□	□	□	□
Moderada	□	□	□	□
Mucha	□	□	□	□
Muchísima	□	□	□	□

Comentarios:

SENSORIAL HAMBURGUESA COCINADA II

Tras haber probado las muestras de hamburguesas que se presentan indique, **según la escala,** su opinión sobre ellas.

Marque con una **X** la **valoración** que corresponda a la calificación para cada muestra.

ESCALA	R	601	279	481	529
Me gusta mucho	☐	☐	☐	☐	☐
Me gusta	☐	☐	☐	☐	☐
Me gusta ligeramente	☐	☐	☐	☐	☐
Ni me gusta ni me disgusta	☐	☐	☐	☐	☐
Me disgusta ligeramente	☐	☐	☐	☐	☐
Me disgusta	☐	☐	☐	☐	☐
Me disgusta mucho	☐	☐	☐	☐	☐

¿Cuál/es compraría?

Comentarios:

Appendix III

SENSORIAL HAMBURGUESA CRUDA

Observe cada una de las muestras de hamburguesas crudas marcadas con claves. Marque con una **X** la valoración que corresponda a la calificación para cada muestra.

ESCALA	801	167	588	343	753
Me gusta mucho	☐	☐	☐	☐	☐
Me gusta	☐	☐	☐	☐	☐
Me gusta ligeramente	☐	☐	☐	☐	☐
Ni me gusta ni me disgusta	☐	☐	☐	☐	☐
Me disgusta ligeramente	☐	☐	☐	☐	☐
Me disgusta	☐	☐	☐	☐	☐
Me disgusta mucho	☐	☐	☐	☐	☐

Observe las muestras de hamburguesas crudas y **compare** por grado de aceptación de cada muestra. Marque con una **X** sobre la escala.

Muestra **801**

MIN ..... MAX

Muestra **167**

MIN ..... MAX

Muestra **588**

MIN ..... MAX

Muestra **343**

MIN ..... MAX

Muestra **753**

MIN ..... MAX