UNIVERSIDAD DE BURGOS

DEPARTAMENTO DE BIOTECNOLOGÍA Y CIENCIA DE LOS ALIMENTOS

TESIS DOCTORAL

DEVELOPMENT OF MEAT PRODUCTS FORTIFIED WITH OMEGA-3 RICH OIL OBTAINED FROM FISH BY-PRODUCTS BY SUPERCRITICAL CARBON DIOXIDE EXTRACTION

SARA MARÍA DE DIEGO RUPÉREZ

BURGOS, 2015

DEVELOPMENT OF MEAT PRODUTS FORTIFIED WITH OMEGA-3 RICH OIL OBTAINED FROM FISH BY-PRODUCTS BY SUPERCRITICAL CARBON DIOXIDE EXTRACTION

Memoria presentada para optar al grado de Doctor Internacional en Ciencia y Tecnología de los Alimentos por la Universidad de Burgos

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Dña. ISABEL JAIME MORENO, Profesora Titular de Universidad en el área de Tecnología de los Alimentos del Departamento de Biotecnología y Ciencia de los Alimentos de la Universidad de Burgos.

Como Directora de la Tesis Doctoral **"Development of meat products fortified with omega-3 rich oil obtained from fish by-products by supercritical carbon dioxide extraction"/ "Desarrollo de productos cárnicos enriquecidos con aceites ricos en omega-3 procecentes de subproductos de pescado obtenidos mediante extracción supercrítica con dióxido de carbono"** desarrollada por **Dña. Sara María de Diego Rupérez.**

INFORMA:

Favorablemente la presentación de dicha tesis, ya que reúne las condiciones necesarias para su defensa en cuanto a la realización de la fase experimental y la elaboración de la memoria.

Y para que así conste y a los efectos oportunos, firma el presente informe,

En Burgos, a 3 de Noviembre de 2015

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CERTIFICA:

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Y para que conste, y a los efectos oportunos, firmo el presente certificado,

En Burgos, a 3 de Noviembre de 2015

Fdo. Pilar Muñiz Rodríguez

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Como no sabía que era imposible, lo hice

Albert Einstein

A mi familia

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A todos los que habéis formado parte de esta aventura ¡¡MUCHAS GRACIAS!!

Sara

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SUMMARY

SUMMARY

Fish oil is known to be an important source of omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, which play an important role in the prevention of different human diseases. Fortification of foods with such fatty acids is, thus, increasingly recommended. A growing public awareness about the benefits and the dietary sources of PUFA has created a substantial interest in the production of PUFA concentrates.

Fish by-products can be a valuable source for extraction of fish oil rich in polyunsaturated fatty acids, especially omega-3 PUFA; being a good opportunity for the use of these by-products and providing a huge income to the fish processing and related industries. In this study, four different fish by-products were evaluated (*Merluccius capensis – Merluccius paradoxus, Hoplostethus atlanticus, Genypterus capensis* and *Salmo salar).* Supercritical Fluid Extraction using carbon dioxide as the solvent ("green" technology) from salmon by-products provided higher oil yield and minimum degradation of long chain omega-3 polyunsaturated fatty acids including EPA and DHA. Then, this oil was an excellent source of nutrients for future incorporation in functional food fortified with omega-3 fatty acids.

Due to the presence of these highly unsaturated fatty acids, fish oil is very prone to oxidation, leading to the production of off-odours and off-flavours. Considerable attention has been focused in three different extracts (*Melissa officinalis*, *Salvia officinalis* and *Rosmarinus officinalis)* as natural antioxidants. Their antioxidant activity has been demonstrated in order to promote their use as natural food additives. The most suitable extract in salmon oil was sage at 5 % of concentration. Also the use of propolis as a natural antioxidant was studied, but for the moment not feasible due to the intense flavour that impart to the samples.

On the other hand, microencapsulation by spray drying technique protects salmon oil against oxidation in comparison to salmon oil non-encapsulated. In addition, sage effectively enhances protection against oxidation in salmon oil and its use in functional products.

Finally, reduced fat pork sausages fortified with omega-3 fatty acids can be manufactured using salmon oil obtained from fish by-products by supercritical fluid extraction stabilized with microencapsulation by spray-drying and natural antioxidants, getting more healthy products with sensory properties similar to the conventional sausages. They presented a low level of SFA and high level of PUFA, with a ratio n-3/n-6 of 1.6 and provided almost 250 mg of EPA + DHA per serving (125 g) that represent 50 % of the recommended daily intake.

OBJECTIVES

OBJECTIVES

Omega-3 fatty acids provide many health benefits, from reducing cardiovascular disease to improving mental health, and consumer interest in foods enriched with omega-3 fatty acids is increasing. Formulating a meat product enriched with these fatty acids that is stable and has an acceptable flavour is challenging. This study provides an overview of key topics in this area.

The objectives of the work, developed in five chapters, are summarised below:

Chapter 1: Identify motives that further or inhibit Spanish consumer's attempts to increase their intake of omega-3 and investigate whether certain products are more acceptable as vehicle for omega-3 than other. In addition, fortified products with omega-3 fatty acids that are commercially available in Spain and their evolution in the last five years were revised. Commercial products were analyzed for the actual content of omega-3 and then, to evaluate their contribution to omega-3 fatty acids intake.

Chapter 2: Potential use of offcuts from fish processing industry of different types of fish *(Merluccius capensis – Merluccius paradoxus, Hoplostethus atlanticus, Genypterus capensis* and *Salmo salar)* as source of omega-3 rich fish oil was investigated. Moreover, the viability of Supercritical CO_2 extraction to provide high quality omega-3 rich oils and its yield compared to other extraction methods were studied. The behavior of oils obtained by SFE was evaluated along storage in different conditions in order to know their stability since the oils than contain a high proportion of PUFAs are prone to oxidative deterioration. The final aim was a future incorporation of the oil in fortified functional foods.

Chapter 3: Investigates the efficacy of extracts from *Melissa officinalis*, *Salvia officinalis* and *Rosmarinus officinalis* to control the lipid oxidation in crude fish oil rich in omega-3 fatty acids.

Chapter 4 and **Chapter 5:** Focus on the fortification of meat products with omega-3 fatty acids by incorporation of fish oil, being the main targets:

- The study of the effectiveness of natural extracts (*Melissa officinalis*, *Salvia officinalis*, *Rosmarinus officinalis*), caffeic acid and propolis extracts, individually or combined with encapsulation, to extend the shelf life of salmon oil for a potential future incorporation in functional food fortified with omega-3 fatty acids.
- The evaluation of the effect of microencapsulation of fish oil by spray-drying to prevent oxidation of food-grade fish oil.
- The development of meat products enriched with omega-3 fatty acids by adding the salmon oil stabilised with the techniques previously selected, getting products that are stable, provide many health benefits and have an acceptable flavour.

INTRODUCTION

INTRODUCTION

1. WHAT ARE OMEGA-3´S?

Fatty acids are organic compounds formed by a hydrocarbonated chain and a carboxylic group that is normally bounded with glycerol forming acylglycerides (monoglycerides, diglycerides or triglycerides). Depending on the nature of the hydrocarbonated chain, fatty acids can be saturated or unsaturated, which in turn can be monounsaturated or polyunsaturated fatty acids (PUFA).

Many of the fatty acids can be synthesized by humans, but there is a group of PUFA, the essential fatty acids, than the human body cannot produce: Omega-3 and Omega-6 fatty acids. The most prominent omega-6 fatty acids in the human diet are arachidonic acid (found in animal meat) and linoleic acid (found in vegetable oils, seeds, and nuts), which can be converted into arachidonic acid by a desaturase enzyme (Figure 1) (Shaena et al. 2009).

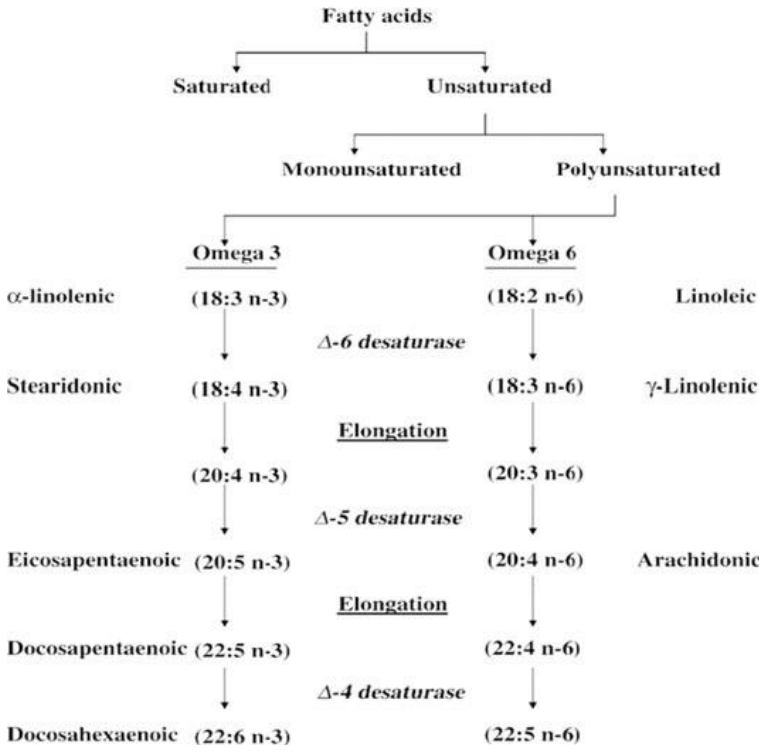
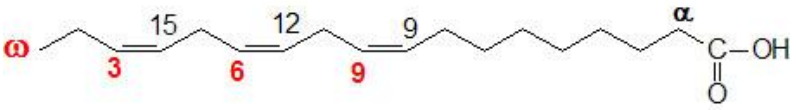
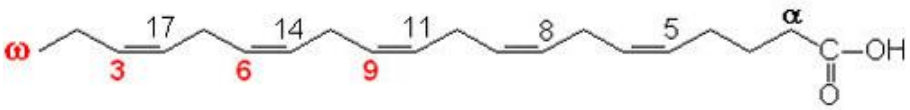


Figure 1. Metabolic pathway of omega-6 and omega-3 fatty acids synthesis

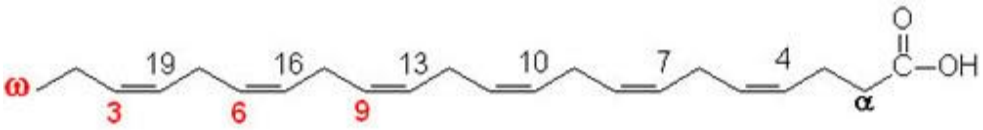
Omega-3 (n-3) PUFAs contain one of the double bonds located at three carbon atoms from the methyl end. The main n-3 PUFAs in the diet are α-linolenic acid (ALA, C18:3 n-3), eicosapentanoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) (Figure 2). The n-3 PUFAs are important structural components of cell membranes and contribute to various membrane functions such as fluidity, permeability, activity of membrane-bound enzymes and receptors, and signal transduction.



α- Linolenic C18:3 n-3



Eicosapentaenoic C20:5 n-3



Docosahexaenoic C22:6 n-3

Figure 2. Chemical structures of omega-3 polyunsaturated fatty acids

Major dietary sources of omega-3´s are fish containing EPA and DHA and nuts, seeds, and vegetable oils contained ALA, which can be converted to EPA and then DHA by the same desaturase enzyme (Figura 1). However, the conversion of ALA in EPA and DHA is low and these omega-3 are considered essential fatty acids for human.

During the last 30 years there has been increasing interest in n-3 polyunsaturated lipids in relation to food, nutritional and pharmaceutical applications. This is due to the increasing evidence that n-3 PUFA elicit a wide range of nutritional benefits in the human body.

2. OMEGA-3 FATTY ACIDS AND HUMAN HEALTH

Interest in Omega-3 fatty acids has steadily grown since the observation that Greenland's Eskimos have a low incidence of cardiovascular disease (CVD) in the setting of a diet rich in fatty fish. Importantly, both epidemiologic and experimental data have provided evidence for a beneficial effect of omega-3 fatty acids in the prevention of CVD (Dyerberg 1978).

Dietary intake of omega-3 and omega-6 fatty acids varies within and between different populations. The ratio of omega-6/omega-3 intake is estimated to be 20 to 1 in a modern Western diet, compared with that of our Paleolithic ancestor who ate a diet much richer in omega-3´s (Figure 3). A n-6/n-3 fatty acid ratio of 5:1 or less is desired, as suggested by nutrition experts (WHO/FAO 2010).

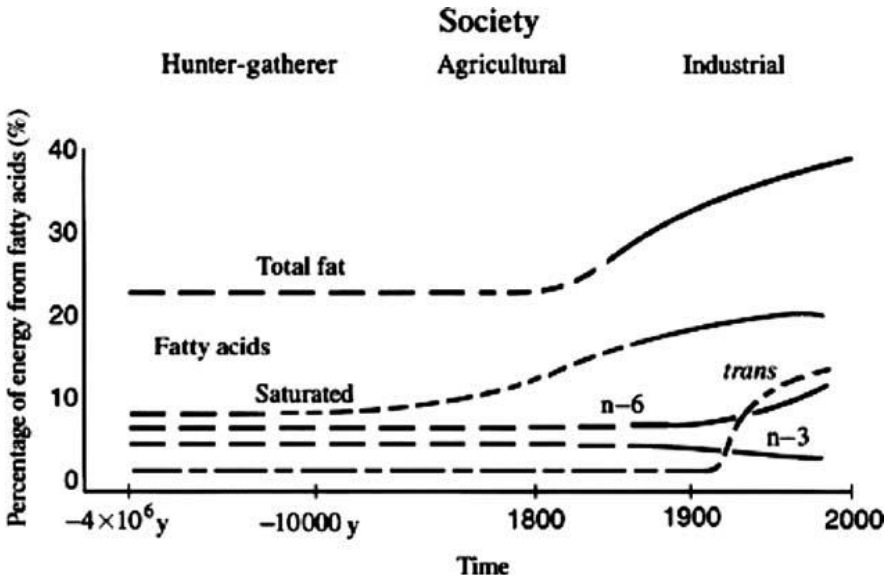


Figure 3. Historical schematic of relative percentages of fat and intake of different fatty acids in human beings (DeFilippis and Sperling 2006)

Many scientific articles and reviews about this subject have been published in the last years. One of the more complete compilations of scientific research can be found in the systematic review of the role of omega-3 fatty acids in the prevention and

treatment of diseases edited by Gil et al. (2012). The observations made lead to the conclusion that long chain polyunsaturated fatty acids (ARA, DHA and EPA) are conditionally essential nutrients for adequate growth, development and function in human. Despite the impressive documentation of EPA and DHA related health benefits, the cellular and molecular mechanisms for their actions are still insufficiently understood.

The evidence for the preventive effect of EPA and DHA on cardiovascular diseases is particularly strong (Maehre et al. 2015, Nestel et al. 2015, Saremi and Arora 2009, Shahidi and Miraliakbari 2004, Simopoulos 2008). Brain cell are especially rich in certain long chain PUFAs, this lead to the suggestion that dietary status of these long fatty acids might influence cognitive function and behavior (Bourre 2004, Denis et al. 2013, Denis et al. 2015, Innis 2008, Simopoulos 2011, Sinclair et al. 2007). It is well established that pregnant women must have an adequate supply of the long chain n-3 fatty acids before and throughout pregnancy and lactation to support normal growth, neurological development and cognitive function on the baby (Grieger and Clifton 2015, Klemens et al. 2012, Saccone et al. 2015, Simopoulos 1991). Unsaturated fatty acids have also been associated with a number of other diseases and although the evidence is by no means conclusive, it is an area that is attracting a huge amount of interest. Dietary fat affects a number of different metabolic pathways, including those involved with glycaemic control, so the types and amounts of dietary fats may have a role to play in the management of diabetes (Jeppesen et al. 2013, Li et al. 2015, Suresh and Das 2003). Unsaturated fatty acids may also be associated with a reduced risk of developing certain cancers, including cancers of colon, breast and prostate (Hardmand 2002, Laviano et al. 2013, Nabavi et al. 2015, Rose and Connolly 1999). There are a number of inflammatory conditions, such as asthma, Crohn´s disease and arthritis, which could potentially be alleviated by dietary modification. The fatty acids composition of cell membranes can be altered by consumption of both n-3 and n-6 PUFAs, and this can result in reduced inflammatory activity (Calder 2015, Hodge et al. 1998, Lev-Tizion et al. 2014). The effect of omega-3 fatty acids in the prevention of ocular diseases has been reviewed by Bretillon et al. (2011) and Rahman et al. (2013) have reviewed the works that examined the potential use of n-3 fatty acids in psoriasis.

However, whether this effect brings about a significant reduction in clinical symptoms is still unclear. It is also important to note that there are concerns that the beneficial effect on certain disease outcomes are observed with very high intakes of omega-3 fatty acids, either through the natural food products that contain them or as supplements in the diet for beneficial effect on health.

3. SOURCES OF OMEGA-3´S FATTY ACIDS

The most important natural sources of omega-3 PUFA include fish, algae, krill, and plants (Figure 4). It is largely cold water fish that serve as the major sources of EPA and DHA and even these shows marked variability in their EPA and DHA content. Seasonal variations of EPA and DHA in a single fish species can be substantial according to the season of catch and the geographic areas where harvested, with variations of as much as 1.5-fold (Racime and Deckelbaum 2007). The n-3 FA-rich oils are also obtained from sources other than fish. Marine microalgae that produce triglycerides very rich in DHA can be selectively cultivated and help solve the problem of seasonal variation and can provide a continuous supply of n-3 with consistent levels of quality. Still, obtaining DHA from microalgae presently involves high production costs. In addition, success in cultivating algae to produce high levels of EPA has been quite limited. Some species of krill, however, do have high levels of EPA and DHA, but production costs are also high (Herrero et al. 2015).

A few plants provide n-3 fatty acids. These include canola, soya, and flaxseed. Still, plant oils mainly provide ALA and contain no or only very low levels of EPA and DHA. With the inefficient conversion of ALA to EPA and DHA, overall there is weak evidence for higher dietary intakes of ALA on decreasing risk of heart disease and stroke compared to higher intakes of EPA and DHA. Obtaining n-3 FA from plants such as flaxseed is unlikely to have a major impact on improving cardiovascular disease outcomes as well as the other health benefits of the longer chain EPA and DHA.

Transgenic plants have been also proposed as an alternative source of omega-3 fatty acids (Napier et al. 2015), however nowadays the use of transgenic plants in agriculture is not well accepted in many part of the world, especially in Europe. Also due to the increased demand for EPA and DHA, new sources of the omega-3 fatty acid EPA have developed through fermentation using metabolically engineered strains of *Yarrowia lipolytica* (Xie et al. 2015).

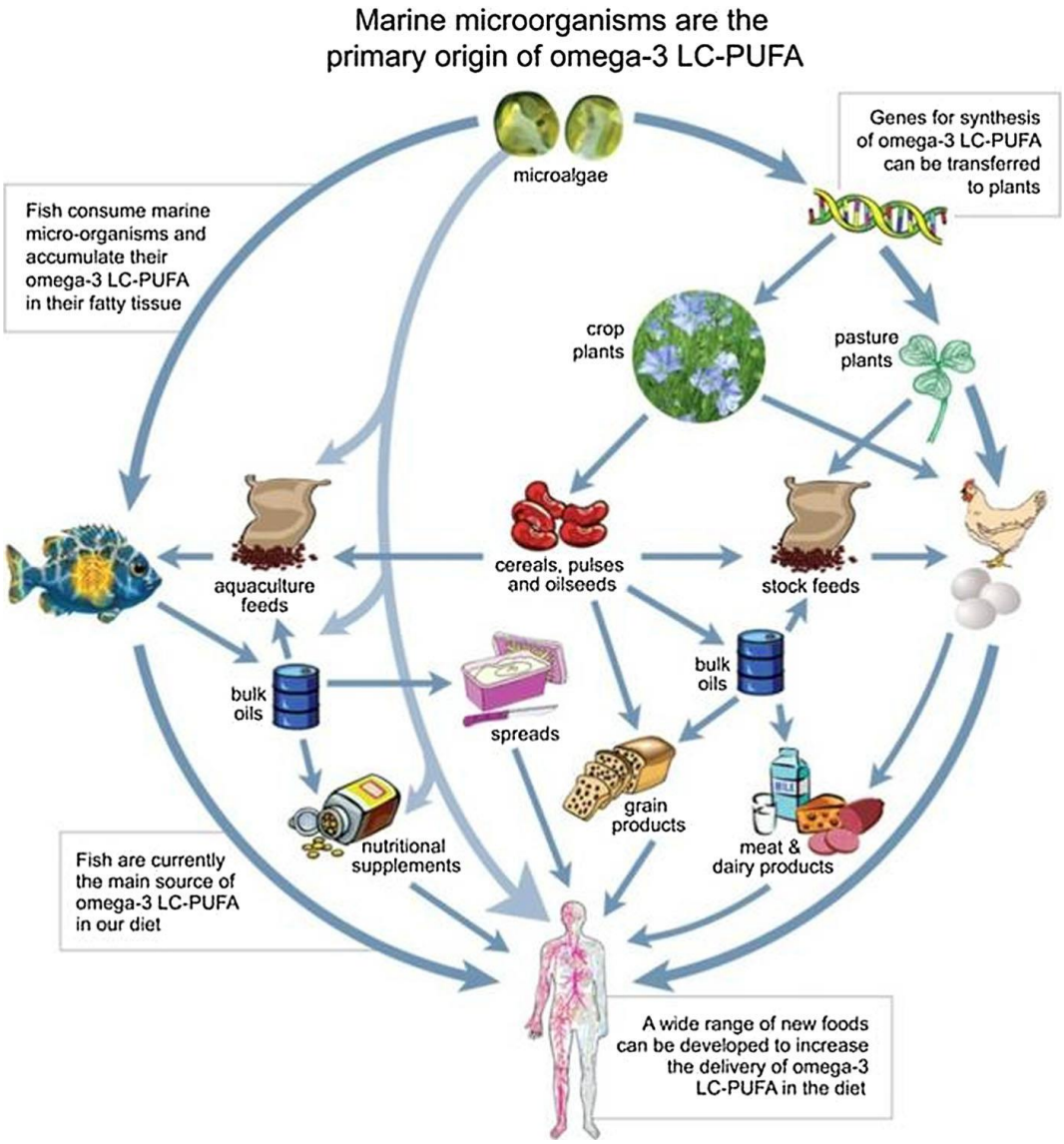


Figure 4. Exiting and possible new dietary sources of n-3 PUFAs. Source: Commonwealth Scientific and Research Organization (CSIRO) (Pottel et al. 2014)

Nowadays, fish is the most common source of omega-3 in human diet. The global fishery production, both from wild capture and aquaculture, is approximately 160 million tonnes, with more than 130 million tonnes destined for human consumption (FAO Fisheries and Aquaculture Department 2012). Fish currently represents about 16.6 % of animal protein supply with the value of exported products reaching US$136 billion (FAO Fisheries and Aquaculture Department 2012, Fisheries and Aquaculture Department 2014). As greater volumes of fish are caught, harvested and processed for export, more by-products such as backbones, heads, trimmings, fins, frames, roes and viscera are produced. In the past, fish by-products were considered low value and discarded.

The amount of fish discarded by fishing fleet or seafood processing plant varies between 50 and 75 % of the total weight of the catch, depending on species, size, season and fishing ground (Falch et al. 2006, Rustad et al. 2011). Fortunately, there has been a rise in awareness of economic, social and environmental aspects of optimal use of fishery by-products. With proper handling and processing, fish by-products may have a higher nutritional value than fillets, particularly in terms of essential polyunsaturated omega-3 fatty acids, proteins, vitamins and minerals, which can comprise an excellent means of fighting micronutrient deficiencies in developing countries (Fisheries and Aquaculture Department 2014). These solid wastes and by-products can be a valuable source for extraction of fish oil rich in polyunsaturated fatty acids, especially omega-3 fatty acids (Nurdiani et al. 2015, Wu and Bechtel 2008, Kim and Mendis 2006). The fish processing industry is focusing on the utilization of by-products from different types of fishes, such as tuna, herring, salmon and walleye (Ferdosh et al. 2015, Shaena et al. 2010). The lipids with are originally deposited in the viscera and beneath the skin were found to be a good source of omega-3 fatty acids than can be used as nutritional supplement or additive to human foods or animal feed (Aryee and Simpson 2009, Sathivel et al. 2003, Sahena et al. 2010, Ting et al. 2000). The amount of these fatty acids in fish oils depends on the raw material selected for oil production, i.e. fish species, age, and part of fish, time and place of the fish catch, and fish natural diet or fish feed (Usydus et al. 2007).

In this study, we use different types of fish by-products, specially the offcuts obtained from four particular species (orange roughy, hake, kingklip and salmon), in order to evaluate their suitability as sources of omega-3 rich fish oils (Figure 5-8).

Figure 5. Hake (*Merluccius capensis – Merluccius paradoxus)*

Figure 6. Salmon (*Salmo salar)*

Figure 7. Orange roughy **(***Hoplostethus atlanticus)*

Figue 8. Kingklip (*Genypterus capensis)*

4. OMEGA-3 FATTY ACIDS EXTRACTION

Several technologies involving solvents and high temperatures are currently used to extract fish oil (Rubio-Rodriguez et al. 2010, 2012, Hao et al. 2015), but these methods vary widely in terms of yield and quality of the extracted oils.

The production of fish oil from fish raw materials is usually achieved by wet extraction techniques consisting of steps of cooking, pressing, separation of the oily and watery phases, and finally the recovery of the oil and the protein-rich meal (FAO 1986).

The extracted fish oil is processed using conventional methods such as fractionation, vacuum distillation, urea crystallization, and conventional crystallization (Homayoomi et al. 2014, Sahena et al. 2010, Wayan Suriani et al. 2014). The method of rendering commonly used in extracting fish oils employs high temperatures, which increase the susceptibility of the extracted oil to thermal degradation. Non-heat treatment method for producing fish oil is being in practice since long. Solvent extraction with hexane and similar solvents, although applicable, is not a recommended option for extraction of food-grade fish oils (Rubio-Rodriguez et al. 2012).

Crude fish oils are inedible because they contains free fatty acids, primary oxidation products, minerals, pigments, moisture, phospholipids and insoluble impurities, which reduce their quality. Therefore, it is important to remove these impurities from the extracted oils to produce purified oils with acceptable shelf life and commercial value (Huang and Sathivel 2010). The oils are processed by chemical or alkaline refining steps including degumming to remove phospholipids and other gummy materials, neutralization with caustic soda to remove free fatty acids (FFA), washing with water to remove extra alkali, bleaching to remove soap and trace metals, and deodorization by vacuum distillation to remove residual FFA, aldehydes, ketones, alcohols and other compounds. Winterization is also used in fish oil refining in order to concentrate the valuable n-3 PUFAs, especially EPA and DHA (Crexi et al. 2010, Motalebi et al. 2014).

Other methods that are used to concentrate valuable n-3 PUFA include freezing, crystallization, urea complexation, molecular distillation, supercritical fluid extraction,

and lipase hydrolysis (Chakraborty and Joseph 2015, Rubio-Rodriguez et al. 2012, Wang et al. 2014).

However, a reliable alternative to obtain good quality fish oil from these by-products is Supercritical Fluid Extraction (Ferdosh et al. 2014, Rubio-Rodriguez et al. 2008, Sahena et al. 2014). The most widely used supercritical solvent is carbon dioxide, which is non-toxic, cheap and has mild critical conditions, which means it is suitable for processing thermo-degradable compounds such as PUFA.

In this study, oil extractions were carried out in a semi-pilot SFE-plant whose P&I diagram is presented in Figure 9 (Vaquero et al. 2006). The SFE-plant with solvent recycling is provided of an extractor (2L) and separator (1L) and other usual elements in this type of plants such as pumps, heating and cooling systems, pressure dampers; rupture disks installed for safety and instruments for measurement and control of the process parameters.

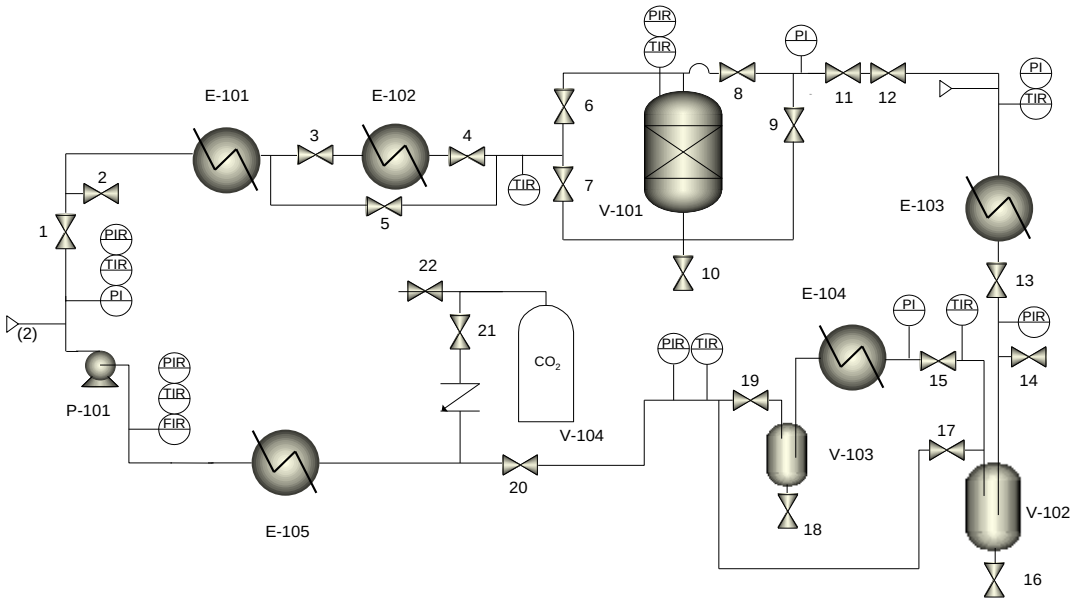


Figure 9. Flowsheet of the SFE semi-pilot plant used in this work: high pressure pump (1); rupture disks (2 and 11); shutoff valves (3, 6, 9, 14, 18, 21 and 25); purge valves (4, 15 and 20); thermostatic baths (5 and 13); discharge valves (7 and 17); extractor (volume=2L) (8); expansion valves (10); circulation pumps (12 and 23); separator (16); CO_2 storage (19); check valves (22); cooling bath (24).

5. STABILIZATION OF FISH OIL

Fish oils are highly susceptible to lipid oxidation due to their high degree of unsaturation. Lipid oxidation gives rise to the formation of undesirable off-flavours and unhealthy compounds such as free radicals and reactive aldehydes. The off-flavours formed from n-3 PUFA oxidation are particularly unpleasant. Furthermore, humans have a low threshold for volatile off-flavours resulting from oxidation of n-3 PUFA (Frankel 2005, Sullivan Ritter 2012).

Lipid oxidation is affected by numerous internal and external factors such as fatty acid composition, content and activity of pro- and antioxidants, temperature, oxygen presence, surface area in contact with oxygen, etc. Due to the complexity of the lipid oxidation mechanism in fish oil, more than one method are usually applied to determine oil quality (Aidos et al. 2002, Jacobsen et al. 2008, Kolanowski et al. 2007).

In order to prevent oxidation and undesirable off-flavours the industry has developed a wide variety of new antioxidants and encapsulation processes.

5.1. Natural antioxidants

Lipid oxidation is a highly deteriorative process in foods, as it leads to unacceptable properties for customers and a loss in nutritional value. Historically, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), were added PUFA containing food to stabilize them against autoxidation but toxicological effects of synthetic antioxidants and consumer preference for natural products have resulted in increased interest in the application of natural antioxidant (Arabshahi et al. 2007). Recently, interest in natural antioxidants found in plants has grown due to the increasing trend towards use of “natural” food additives (Arab-Tehrany et al. 2012, Maqsood et al. 2012, Oroian and Escribe 2015, O’Sullivan 2005, Pezeshk 2015, Yanishlieva 2006). Moreover, many new natural antioxidants for protecting omega-3 rich products appear every year (Anal et al 2014, Farvin and Jacobsen 2012, Raudoniute et al. 2011).

In this study, four different natural antioxidants were tested (Figure 10-12).

Figure 10. Lemon balm (*Melissa officinalis)*

Melissa officinalis, is an herb of long tradition and with a large variety of uses. Extract obtained from this plant exhibited antioxidant properties against lipid oxidation, due to phenolic acid components, such as rosmarinic and caffeic acids and their methyl esters (Dastmalchi et al. 2008, Marongiu et al. 2004, Mimika-Dukic et al. 2004, Pereira et al. 2009).

Figure 11. Rosemary (*Rosmarinus officinalis)*

The main compounds responsible for the high antioxidant effect of rosemary *(Rosmarinus officinalis)* are phenol diterpenes such as carnosic acid, carnosol and rosmarinic acid (Hernández-Hernández et al. 2009). According to Kolar and Urbancic (2007), rosemary extract possess antioxidant properties that make it useful in protection of polyunsaturated oils.

Figure 11. Sage (*Salvia officinalis)*

Salvia officinalis commonly called sage is widely used in traditional medicine and is a rich source of polyphenolic flavonoids and phenolic acids (Lu et al. 2002). The main antioxidant activity of sage was attributed to rosmarinic acid and the phenolic diterpenes carnosol and carnosic acid (Cuvelier et al. 1996, Farhat et al. 2009). Among the phenolic compounds, rosmarinic acid was the common phenolic compound in all the extracts.

On the other hand, other new antioxidant are arriving to the market. In recent years, many papers have provided antioxidant capacity of the propolis. Propolis is a resinous substance collected by honey bees from various plant sources. On account of its chemical composition, propolis possesses several biological and pharmacological properties. Polyphenols are one of the most important groups of natural antioxidants (Yasin and Abou-Taleb 2007) and propolis represents a widely available natural substance very rich in phenolic compounds (Pietta et al. 2002). Specifically, propolis is a bee product composed of more than 300 chemical compounds including the caffeic acid phenethyl ester that exhibits strong antioxidant properties (Sudina et al. 1993). In addition, several authors (Banskota et al. 2001, Burdock 1998, Marcucci 1995) have shown that the phenolic composition of propolis is responsible for other biological and therapeutic properties: antiseptic, antimycotic, antibacterial, anti-inflammatory and antitumor activities. Thus, propolis can be potentially used as a natural food additive and as a functional food ingredient (Mendiola et al. 2010) but its application to food

products is still limited because of its strong and unpleasant taste and odour that generally compromise food acceptability. The results of this study increase the possibility to embed the propolis as a microencapsulated natural ingredient in various foods to enhance antioxidant properties and phenolic contents. Microencapsulation may be an alternative for reducing this problem.

5.2. Encapsulation of omega-3 rich oil by spray drying

Due to strong clinical data supporting a variety of health benefits, omega-3 oils are being increasingly added to food and beverage products. However, these bioactive lipids need to be protected against autoxidation and degradation during food processing and storage. Microencapsulation technology is employed to stabilize omega-3 rich oils and enable their delivery into food and beverage products without impacting the taste and shelf life of the product.

There are a number of techniques used for fish oil microencapsulation, being spray-drying the method with the lowest costs; other techniques are fluidizing bed, freeze-drying and multilayer emulsions (Grigoriev and Miller 2009, Shaw et al. 2007). The process involves four stages: preparation of a dispersion or emulsion; homogenization of the dispersion; atomization of the feed emulsion; and dehydration of the atomized particles (Shahidi and Han 1993). Industry is defined as a technique in which tiny particles or droplets are surrounded by a coating wall to give small capsules. This technique increases stability of omega-3 fatty acids against oxidation, evaporation, reaction or migration during processing, heat treatments and storage and also controls the release of the incorporated compounds (Anwar and Kunz 2011, Calvo et al. 2012).

Although encapsulation itself prevents lipid oxidation, additional stabilization with antioxidants is required to ensure maximum protection during processing and subsequent storage of microencapsulated omega-3 fatty acids. The aim of this study was to investigate the impact of different antioxidant on the stability of an oil rich in long-chain polyunsaturated fatty acids in combination with microencapsulation process by spray-drying for their later incorporation in meat products.

6. MEAT PRODUCTS FORTIFIED WITH OMEGA-3 FATTY ACIDS

Among the vegetable oils with a high content of α-linolenic acid (ALA), are those obtained from linseed *(Linum usitatissimum* L.), chia (*Salvia hispanica* L.) and perilla (*Perilla frutescens*) seeds, and linseed oil has been the most frequently used to improve the lipid profile of different foods. According to the European Food Safety Authority (EFSA) set Dietary Reference Values (DRV) of 500 mg for EPA plus DHA for adults (EFSA, 2010). Algae oil has been described as one of the best natural sources of DHA, reaching in certain types of microalgae values up to 45 % of total fatty acids (Anderson and Ma 2009, Astiasarán and Ansorena 2009, Mozaffarian and Wu 2011). The use of algae oil from the micro-algaes (*Ulkenia* sp. and *Schizochytrium* sp.) as a novel food ingredient was recently regulated (European Commission 2009a, 2009b). Different works have studied the successful incorporation of algae oil in products such as eggs (Sedoski et al. 2012), surimi (Pietrowski et al. 2011), dry fermented-sausages (García-Íñiguez de Ciriano et al. 2010), yogurt and milk (Chee et al. 2005).

Lipids are the bioactive components that have received most attention in the development of healthier meat products (Delgado-Pando et al. 2010a, Delgado-Pando et al. 2010b, Delgado-Pando et al. 2011). Decreasing the amount of fat or changing the characteristics of the lipid fraction has been attempted. However, the production of reduced-fat meat products may cause technological problems due to the fact that fat affects the flavour, palatability and texture of foods (Delgado-Pando et al. 2010, Horita et al. 2011, Hort and Cook 2007). In order to solve this problem, different fat replacers have been tested, carrageenan providing good results for maintaining textural properties in different meat products (Ayadi et al. 2009, Candogan and Kolsarici 2003, Cierach et al. 2009, Kumar and Sharma 2004). In fact, it is possible to develop Bologna-type sausages with very low amounts of pork back-fat using different emulsifiers (Omana et al. 2012, Sanjeewa et al. 2010).

The lipid profile of cooked meat products can be modified by reformulation strategies (Jiménez-Colmenero et al. 2012, Trindade et al. 2011), including linseed oil, rich in α-linolenic acid (Berasategi et al. 2011, Delgado-Pando et al. 2012a, 2012b) in the formulations. However, to our knowledge, there are no studies where this enrichment is carried out with DHA and/or EPA. In addition, the increase in long chain unsaturated fatty acids could increase lipid oxidation, which might cause sensory problems. It has demonstrated that it is possible to develop new stable high fat meat products rich in n-3 PUFA by adding synthetic antioxidants such as butylhydroxyanisol (BHA) (Lee et al. 2006, Valencia et al. 2006a, 2006b, 2007). However, public attitude towards these substances is not as positive as that for natural ones, which are well appreciated (García-Íñiguez de Ciriano et al. 2009, Johnston et al. 2005). In this sense, different extracts obtained from *Melissa officinalis* have shown antioxidant properties in vitro (Lopez et al. 2007, Zandi and Ahmadi 2000), mainly due to the presence of flavonoids and hydroxycinnamic acid derivatives, known for their antioxidant capacity, with rosmarinic acid as the major component (Dastmalchi et al. 2008).

The objective of this study was to assess the effectiveness of a natural antioxidant in combination with microencapsulation in reduced-fat sausages enriched in DHA and EPA.

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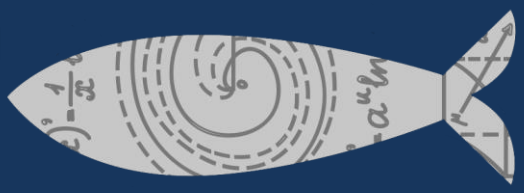

CHAPTER 1

Consumption of Omega-3 PUFAs Fortified Products in Spain: Exploratory Study of Consumer´Opinion and Products Availability

I. CONSUMPTION OF OMEGA-3 PUFA FORTIFIED PRODUCTS IN SPAIN: EXPLORATORY STUDY OF CONSUMERS' OPINION AND PRODUCTS AVAILABILITY

ABSTRACT

Growing consumer awareness of omega-3 enriched products and understanding of their positive nutritional effects have led to the need of specific studies and have captured more attention than ever before. The first aim was to identify motives that further or inhibit Spanish consumers´ attempts to increase their intake of omega-3 and investigate whether certain products are more acceptable as vehicle for omega-3 than others. And the second aim was to determine the fatty acid composition of 85 fortified products with omega-3 fatty acids that are commercially available in Spain and the feasibility of using manufactured food, enriched with eicosapentaenoic acids (EPA) and docosahexaenoic acid (DHA) as mean of increasing the intake of these n-3 polyunsaturated fatty acids (PUFA).

A survey with 112 participans was performed in order to know the consumers´ opinion about omega-3 fortified products. A 64 % of the participants did not know what are functional foods, despite they had mentioned several options and they know products enriched with omega-3 fatty acids (78 %). Most participants believed that omega-3 PUFAs could prevent certain diseases. All informants perceived fat spreads and canned products as "naturally" enriched products. Dairy products are by far found to be the most bought according to the informants. Eggs are perceived as unfit for enrichment by the majority of the informants and meat would be a potential target to

increase levels of omega-3 without drastic changes in the normal diet habits. Omega-3 fatty acids of eighty-five fortified products of these launches has been analyzed in order to define the best source to cover the demand for these nutrients.

In the second part, the last launches in omega-3 fatty acids were studied. In total, two hundred fifty new products with omega-3 have been launched to the Spanish market in the last five years.

1. INTRODUCTION

Beneficial health effects of omega-3 Polyunsaturated Fatty Acids (PUFA), especially eicosapentaenoic acid (EPA C20:5) and docosahexaenoic acid (DHA C22:6) are well demonstrated, mainly in the prevention of cardiovascular diseases, cancer, reduction in risk of Alzheimer disease and dementia, as well as the improvement of proper development and function of the brain and retina in infants (Lunn and Theobald 2006, Riediger et al. 2009). Although humans do not produce endogenous n-3 fatty acids, they can be obtained from the diet and through ingestion of fish oil capsules or fortified foods containing high levels of linolenic acid (ALA) or EPA and DHA.

It could be difficult to get high doses of n-3 fatty acids exclusively through the diet, but can be attained through ingestion of omega-3 fortified products without radical changes of eating habits.

A number of authorities have recently recommended increases in intakes of n-3 fatty acids by the general population (Tur et al. 2012). The European Food Safety Agency (EFSA 2010) proposed a recommended daily intake of 200-500 mg/day Eicosapentanoic acid and Docosahexaenoic acid for adults and The American Heart Association (AHA 2002) recommended for the general population a consumption of fish, a least twice a week (500 mg/day of EPA+DHA). To comply with this recommendation a variety of food products as eggs, milks, biscuits, breads, juices, spreads and meat products have been enriched with these fatty acids (Betoret et al. 2011, Ganesan et al. 2014, Tur et al. 2012).

Fortified/functional products saw the strongest growth over 2007-2012, as tough economic times led consumers to look to gain added value from products. Awareness of ingredients which offer added functionality is increasing and, as such, milk and dairy products have seen significant development (Euromonitor 2013).

The number of various food products enriched with omega-3 PUFA available on the market is maintaining the last five year in spite of the economic crisis in Spain, due to the bioavailability and health effects derived from regular consumption of those products still remain on the Spanish markets. In the last year, 2015, twenty-nine new

fortified products have been launched, exclude baby food products. Dairy category was the most active in launches after fish derivates.

Bioavailability and physiological effect of omega-3 fatty acids added to foods may differ depending on the type and quantity of omega-3; moreover they could be affected by the type and quantity of other nutrients present in the food product. Recently, the US Food and Drug Administration (FDA 2014) announced their final ruling that prohibits nutrient content claims for some omega-3 fatty acids in the labeling, including conventional foods and dietary supplements, that contain omega-3 fatty acids. This rule will be effective January 1, 2016.

Health behaviour theories aim to explain how and why individuals refrain from risk behaviours and adopt health behaviours (Weinstein 1993). A number of models have been proposed for this purpose and the most common ones are the Health Belief Model (Becker 1974), the Protection Motivation Theory (Rogers 1983), the Theory of Reasoned Action (Ajzen and Fishbein 1980) and its successor, the Theory of Planed Behaviour (Ajzen 1991), Transtheoretical Model/Stages of Change (Prochaska 1979), and the Health Action Process Approach (HAPA) (Schwarzer 1992). Several studies have made an attempt to compare these models (Baranowski et al. 2003, Garcia and Mann 2003).

The results of this study were approximated to the Health Action Process Approach as a theoretical framework to study how consumers can increase their intake of omega-3 polyunsaturated fatty acids. The purpose of the present study was two-fold. The first aim was to identify motives that further or inhibit consumers' attempts to increase their intake of omega-3, either in the form of dietary supplements, in the form of omega-3 enriched products, or through products naturally rich in omega-3 PUFAs (in particular, fatty fish). The second aim was to investigate the availability of rich omega-3 products and market evolution in recent years. This paper presents the results of a preliminary study that aimed to explore Spanish consumers' motives for choosing omega-3/fish oil enriched products and diversity of these products on the market.

2. MATERIAL AND METHODS

2.1. Participants and procedure

The purpose of the study was to identify motives that led participants to decide their diet, more specifically their intake of omega-3 PUFAs. There was a total of 112 participants, all recruited from Spain. The age of participants ranged from 20 to 79.

Semi-structured qualitative surveys were used in the study (Figure 1 and 2). They combined a structured agenda with the flexibility to ask subsequent questions. First, the participants were asked general questions about age, education and later the participants were asked about fish oil consumption in the form of dietary supplements or omega-3 enriched products.

Respondents were asked to reflect on the risk they perceived to be related to health problems (general risk perception). The following were some of the questions that were asked: Do you think that eating healthy foods is important? Do you think that low fat diet is good for your health? Do you eat foods that increase your cholesterol? Do you think that your risk of getting health problems would be smaller if you ate healthy food? As risk is considered as not being related to particular risk of not eating omega-3, but to more general risk of not eating healthily, the questions related to risk perception are not specified in relation to omega-3. Contrary to this, the questions related to the construct of ‘outcomes expectancy’ and ‘self-efficacy’ were specified in relation to omega-3 consumption. While asking about the outcomes respondents would expect from eating omega-3, the following questions were asked: Do you think that the omega-3 products are good for your health? What did you expect the omega-3 would do for you, if you started eating it?

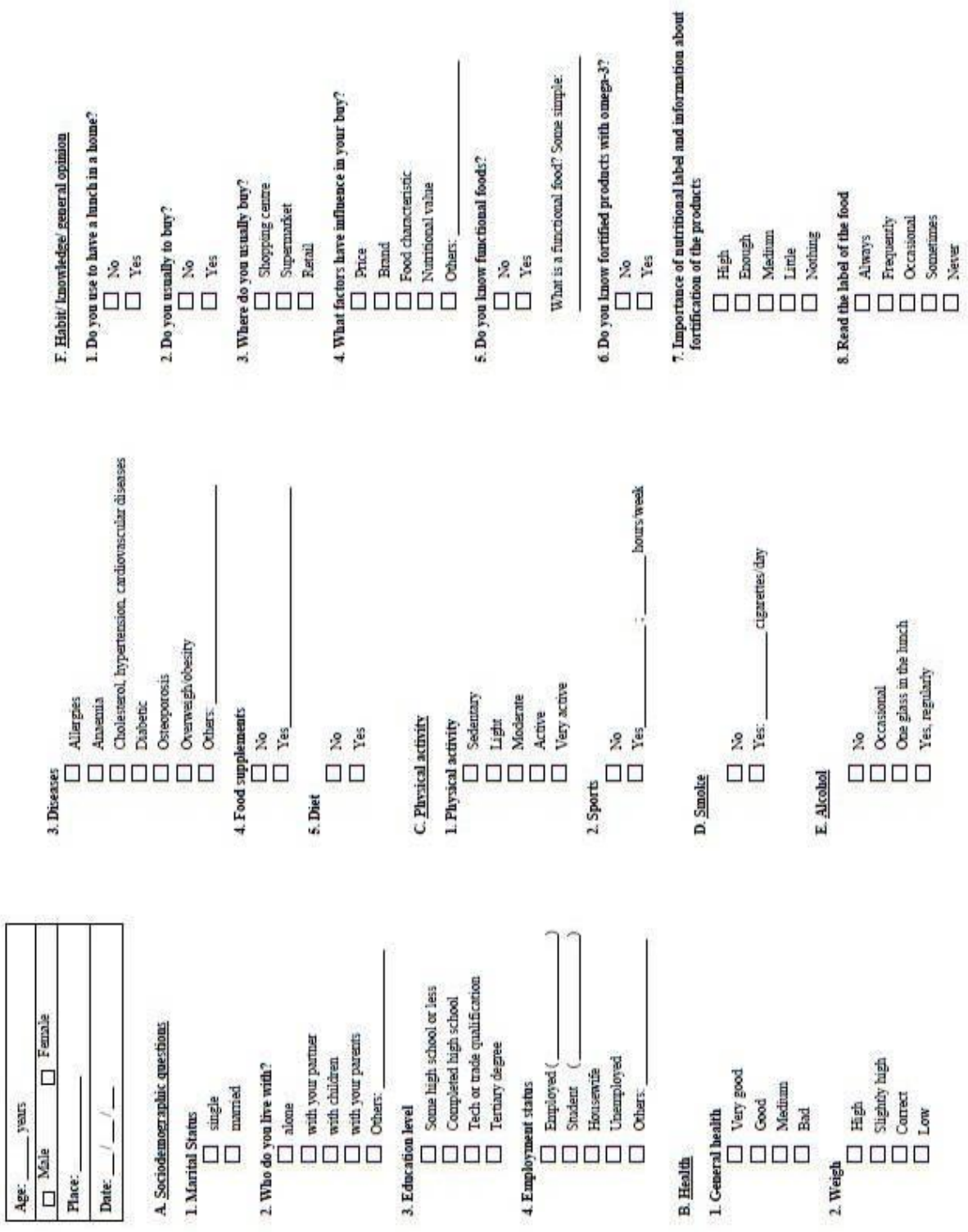

Figure 1. Model of questionary used in survey about fortified omega-3 products (part 1)

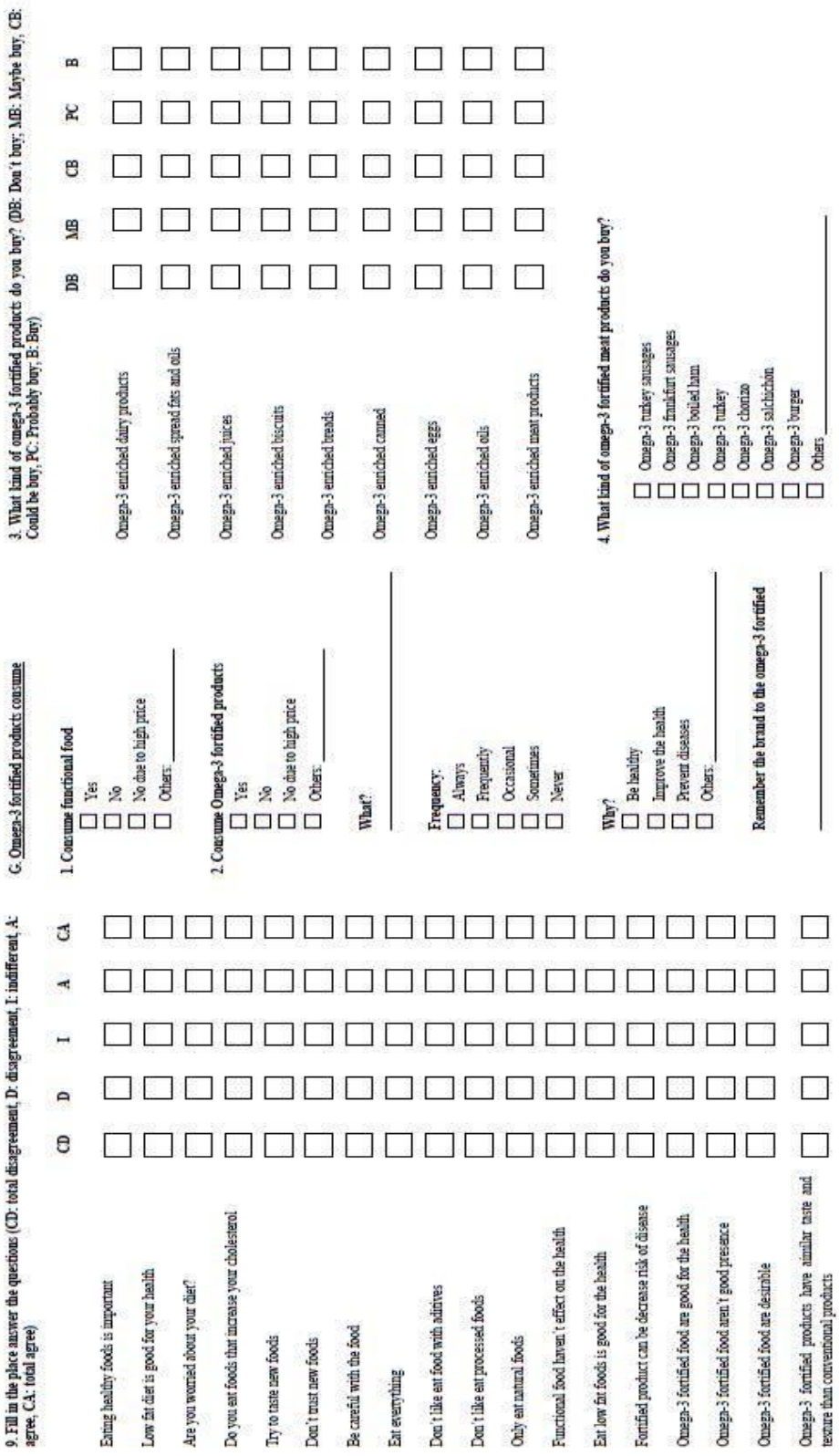

Figure 2. Model of questionary used in survey about fortified omega-3 products (part 2)

In the second part, respondents were asked to comment on nine prototype products (dairy products, butter or margarine, juices, cookies, bread, tinned food, eggs, oil and meat products) enriched with fish-oil, because we wanted to find out how consumers perceive omega-3 as an ingredient in selected foods. The following specific questions were asked: Do you think that products enriched with omega-3 have the same characteristic (taste and texture) than conventional products? Why do you buy products enriched with omega-3? We assumed that some products are more acceptable as being enriched with omega-3 than others.

The next question was: What kind of meat products do you buy? They were asked to comment on seven meat products (turkey sausages, pork sausages, boiled ham, turkey breast, "chorizo", "salchichón" spiced sausage similar to salami and hamburger) enriched with omega-3 fatty acids.

2.2. Fortified products analysis

Eighty-five fortified products were obtained from Spanish retail stores. Include: canned fish (tuna, sardine, mackerel), smoked salmon, fresh product (egg), fish products (surimi derivates, tuna sausages, tuna burger), dairy products (milks, kids milk), soya drink, spreading fats and oils (margarine, oils), biscuits, bread, juice, meat products (cooked sausages, "chorizo", "salchichón", cooked ham, turkey breast, turkey burger) and olives.

The fat content was determined by Soxhlet method using petroleum ether as solvent in an extraction system B-811 (Büchi, Flawil, Switzerland). The fatty acids profile was determined by the AOAC method (1995). The fatty acid methyl esters were firstly prepared and then analyzed by gas chromatography (GC) in a Hewlett Packard gas chromatograph (6890 N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector. The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 ºC during 20 min. A split

injection with a 50:1 ratio was used. Injector and FID were kept at 250 ºC during analysis. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co., St. Louis, Missouri, United States). Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (1995). Calibration curves were made for each pair internal standard + chromatographic standards in order to find the corresponding response factors.

3. RESULTS

3.1. Surveys on consumers´ perception omega-3 fortified products

A discussion on consumers' understanding of healthy eating is presented first, as it is assumed to be associated with consumers' willingness to increase their intake of omega-3. The likelihood buying of omega-3 enriched products will be discussed separately. Moreover, the results on how respondents perceived omega-3 as an ingredient in selected foods will be presented as well at the end of the section.

Participant characteristics

Of the 112 people who participated, 63 % were female, and their ages ranged from 20 to 79 years. 72.3 % of parcipants had a university degree, 13.4 % had technical or trade qualifications and the others had completed high school or less (Table 1).

The literature is conflicting with respect to the demographic profile of functional food consumers (Özen et al. 2014). Studies developed in different markets have shown results either highlighting stronger purchase behaviour by females or no gender differences. Whereas some studies indicate that women are more open to functional foods (Ares and Gámbaro 2007, Verbeke 2005). Both higher and lower levels of

education have been associated with positive attitudes toward functional foods (Herath et al. 2008, Urala and Lähteenmäki 2007).

Table 1. Demographic characteristics of the study population

Characteristics	All (n=112)		Males (n=41)		Females (n=71)	
	n	%	n	%	n	%
Age (years)						
18-29	72	64.3	21	51.2	51	71.8
30-39	19	7.0	11	26.8	8	11.3
40-49	8	7.1	4	9.8	4	5.6
50-59	8	7.1	3	7.3	5	7.0
>60	5	4.5	2	4.9	3	4.2
Education level						
No completed high school	5	4.5	1	2.4	4	5.6
Completed high school	11	9.8	7	17.1	4	5.6
Tech or trade qualification	15	13.4	9	22.0	6	8.5
University degree	81	72.3	24	58.5	57	80.3
Employment status						
Employed	66	58.9	27	65.9	39	54.9
Unemployed	7	6.3	1	2.4	6	8.5
Housewife	5	4.5	0	0.0	5	7.0
Student	34	30.4	13	31.7	21	29.6
Marital status						
Single	87	77.7	28	68.3	59	83.1
Married	25	22.3	13	31.7	12	16.9

Regarding age groups, it seems that older consumers perceive the use of functional foods as more beneficial than younger consumers (Herath et al. 2008, Landström et al. 2007). This relationship may be explained by the exposure of older consumers to more health problems.

Level of income may also play a role in functional food consumption, but research data lack consistency. Some studies show that consumption of functional foods is associated with lower income households (Herath et al. 2008). Others, however, highlight that it is a market dominated by well-off consumers, arguing that packaged functional foods tend to be available at a premium price and are perhaps beyond the reach of consumers with modest incomes, particularly retired consumers where the incidence of poverty is high (Petrovici and Ritson 2006). In general, the hypothesised effects of socio-demographic determinants are that acceptance of functional food increase with higher age, being female, with ill family members and low education level (Verbeke 2005).

The influence of gender, education and incomes were difficult to demonstrate due to high proportion of the interviewees presented similar demographic characteristics, maybe due to the surveys were fillings in environments close to the university. High proportion of the interviewees was females (63.0 %), between 18-29 years old (71.8 %), with tertiary degree (80.3 %), employed (54.9 %) or student (29.6 %) and singles (83.1 %). In the case of the males (37 %), they presented similar characteristics than the women, aged between 18-29 (51.2 %) with tertiary degree (58.5 %), employed (65.9 %) and singles (68.3 %). For this reason, the study did not demonstrate major gender effects on consumer attitude towards omega-3 fortified products.

Consumer understanding of healthy eating

In general, participants perceived their health as good and their weight as correct. When asked specifically about supplement, only 9.82 % consume food supplements. A 66.07 % of the participants did not know what functional foods are, despite they had mentioned several and they knew products enriched with omega-3 fatty acids, 78.43 % of participant knew this kind of products.

At the end, when asked about the diseases they thought taht healthy food could prevent, most respondents believed that healthy food can improve their general health

(46.15 %), well-being (3.84 %) and sometimes prevent cardiovascular diseases (30.76 %).

The level of nutritional knowledge about omega-3 fatty acids did not necessarily influence how much people liked omega-3 enriched products but was related to how much people consumed these products. In particular, consumers who were able to link attribute-related knowledge about omega-3 to consequence-related knowledge about consuming omega-3 fatty acids were much more likely to consume fortified products with omega-3 than were those who only had one type of knowledge.

Perceived risk as motivator for consuming omega-3

Overall, participants reported relatively little that could be categorized in terms of risk perception and vulnerability to chronic diseases. Perceived futures risks such as increased danger of life style diseases or increase of weight due to fat were mentioned with a high frequency by participants. All participants thought that a healthy diet could prevent cardiovascular disease. Almost all participants argued that they did not have any of the diseases they had referred to. Only a 3.57 % of the participants informed to have cholesterol and 16.07 % informed to have overweight. Belief in the health benefits of omega-3 fatty acids is the main positive determinant of acceptance.

Expected outcomes and circumstances of dietary change

Most participants believed that omega-3 PUFAs could prevent certain diseases. When asked in which way they expected omega-3 PUFAs to benefit them, participants referred to general health improvement and prevention of a number of chronic diseases. Some participants stated that they wanted to ‘‘live in good health’’ or ‘‘improve their well-being’’ and that they felt generally healthier.

There is evidence that consumers most likely to have a positive attitude toward functional foods are the ones who have faced illness among relatives or have experienced illness themselves (Verbeke 2005). Chronic diseases are strongly associated with the demand for functional foods—products targeted at preventing

widespread diseases such as heart diseases and cancer capture a great deal of attention from consumers.

Self-efficacy role in choosing omega-3

Self-efficacy refers to which nutrition practice people feel capable of undertaking, how big they expect the effort to be when changing their eating habits and how long they would persist in the face of obstacles. Most respondents reported support from reference groups, especially friends and sometimes parents, when sticking to consumption of omega-3 enriched products. Those that were not influenced by reference groups when choosing health foods were self-motivated due to willingness to improve their general health.

Previous experience with the product category (or familiarity) boosted all ratings: those who had used the products found fortified products more convincing, credible and attractive and also expressed higher intention to use them in the future.

Consumers had a greater belief in the importance of omega-3 fats and their associated health benefit and a belief that it is important to provide these novel foods on the market but insisted that it was important that they contain enough of the active ingredient to be useful. In this way, the US Food and Drug Administration (FDA 2014) recently announced their final ruling that prohibits nutrient content claims for some omega-3 fatty acids. Nutrients content claims are defined as labelling claims that characterize a level of the nutrient, such as “high in” or “a good source of”.

Consumer perception of omega-3/fish oil as an ingredient in selected foods

In this part of the survey, the participants were asked to choose the likelihood of purchase different kind of products enriched with omega-3 fatty acids. Dairy products are by far found to be the most bought according to the participants. Later cookies are chosen. All participants chose dairy products, cookies, juices, butter or margarine and canned products, meaning that the informants perceive these products as naturally enriched products (Figure 3).

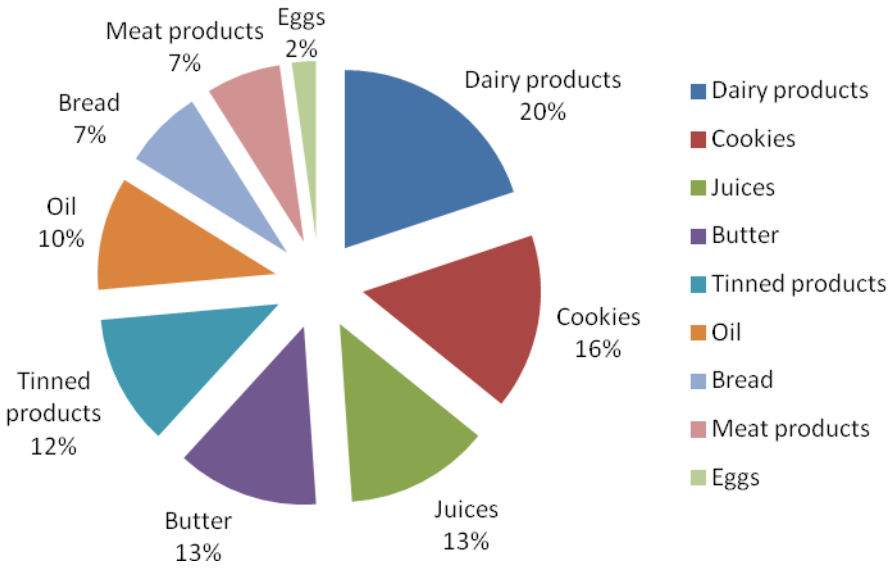

Figure 3. Likelihood of purchase of products fortified with Omega-3

The informants were also asked to choose which three products they found to be the least compatible with omega-3 enrichment. Three products stand out as being incompatible with being enriched: eggs, meat products and bread (Figure 3). They are perceived as unfit for enrichment by the majority of the informants. Some consumers feared that the functional ingredient may cause an off flavour in cases where there was no natural (subjective) link to the carrier product. Consumers expressed a concern that products enriched with fish oil would have an impact on the sensory properties. The combinations that seemed more natural, and where there was no fear of off-flavours, were regarded as more acceptable. In other hand, consumers thought that it is more acceptable to enrich carriers that are perceived as generally healthy (like milk) than unhealthy (like meat). They had also more negative perception for fresh products (eggs) than for processed products (meat products and bread).

Meat and meat products are important sources for protein, fat, essential amino acids, minerals and vitamin and other nutrients. In recent years, the consumer demands for healthier meat and meat products with reduced level of fat, cholesterol, decreased contents of sodium chloride and nitrite, improved composition of fatty acid profile and incorporated health enhancing ingredients are rapidly increasing worldwide.

Omega-3 fatty acids can be incorporated into meat products to improve the functional properties of meat products.

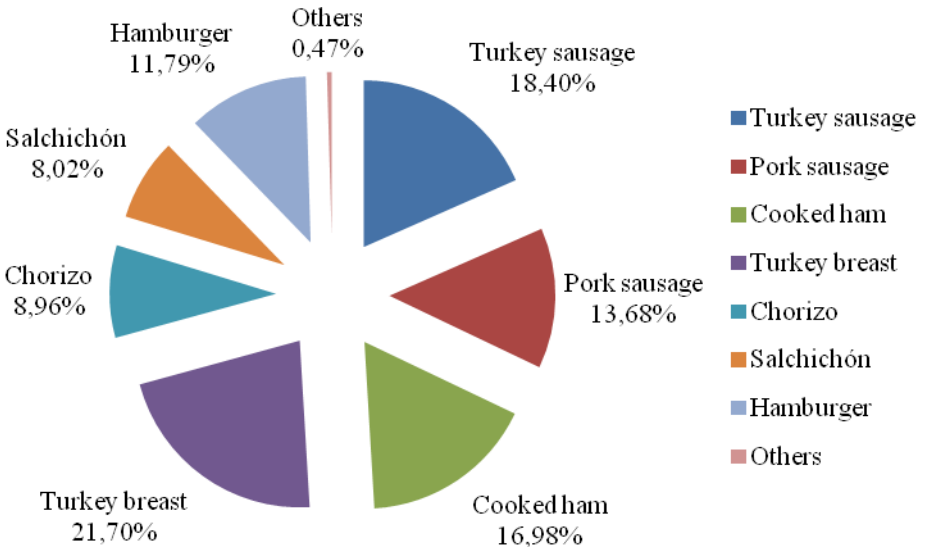

Figure 4. Likelihood of purchase of meat products fortified with Omega-3

A variety of meat products as turkey burgers, cooked pork sausages, turkey sausages, traditional Spanish dry fermented sausages as “chorizo” and “salchichón” have been enriched with omega-3 fatty acids. Cooked meat products like turkey breast, cooked ham and sausages are perceived as fit for enrichment by the majority of the informants whereas typical Spanish products, like “chorizo” and “salchichón” are perceived as unfit for enrichment with omega-3 fatty acids.

Caution should be taken when launching such products to the market. The interesting thing is that both products are perceived as sharing one particular attribute: being fermented cured sausages. However, some of the reluctance towards omega-3 in cured sausages is obvious from the above analysis, namely that the informants link omega-3 with spices and cured products, and thus feel repulsed when connecting the fish taste with cured taste.

Overall conclusion is that omega-3 fatty acids were seen as most appropriate when enriching turkey breast, sausages, cooked ham, and maybe hamburger, but not at all appropriate for enriching cured products as chorizo and salchichón.

3.2. Dietary intakes and recommendations for long-chain n-3 PUFA

Whilst a lot has been written on this topic there is still some confusion about Omega-3 health benefit claims, and nutrition claims in general.

The European Nutrition and Health Claims Regulation EC 1924/2006 came into force on 1 July 2007. It controls any nutrition, health and disease reduction claims made about a food. It does not just apply to labeling, but all types of commercial communication. However, this legislation is not clear regarding to omega-3 fatty acid. Then, on February 2010, the European Commission was assessing all health claims and published its full definitive list of approved health claims EC 116/2010. In this commission, concerning the claims 'Source of omega-3 fatty acids' and 'High in omega-3 fatty acids', the conditions of use should distinguish between the two types of omega-3 fatty acids, which have different physiological roles and for which different levels of consumption are recommended. A claim that a food is a source of omega-3 fatty acids may only be made where the product contains at least 0.3 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 40 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal. A claim that a food is high in omega-3 fatty acids may only be made where the product contains at least 0.6 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 80 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.

In other hand, a lot of health agencies and professional organizations worldwide have issued recommendations on the intake of long chain n-3 (Table 2), but dietary recommendations for long chain n-3 supplementation are still a matter of debate.

Table 2. Recommended Daily Intake of Long Chain n-3 Polyunsaturated Fatty Acids in adults from national and international organizations

Country	Organisation	EPA+DHA (mg/day)
Global	FAO/WHO (2010)	250 - 2000
	International Society for the Study of Fatty Acids and Lipids (ISSFAL 2004)	>500
	NATO Workshop on n-3 and n-6 fatty acids (Simopoulos 1998)	300 - 400
	World Gastroenterology Organization (2008)	3-5 fish per week
Europe	European Academy of Nutritional Sciences (EANS 1998)	200
	European Food Safety Authority (EFSA 2010)	200 - 500
	Eurodiet (2001)	200
	European Society of Cardiology and Societies CVD prevention (Perk 2012)	2 fish a week
	European Atherosclerosis Society (EAS) (Reiner 2011)	2-3 fish per week
Spain	Sociedad Española de Nutrición Comunitaria (SENC 2011)	500 - 1000
	Spanish Society of Intensive Care Medicine, Coronary Units (Jimenez 2011)	1000
Australia	National Heart Foundation of Australia (2008)	500
	Australian & New Zealand Health Authorities (NHMRC 2006)	Men 610 Women 430
	Defence Science and Technology Organisation (Forbes-Ewan 2009)	Men 610 Women 430
Austria	Austrian Society for Nutrition (DACH 2008)	250
Belgium	Belgian Superior Health Council (2005)	Two fish/week
Netherlands	Health Council of the Netherlands (2006)	450
UK	British Nutrition Foundation (1999)	1500 per week
	Scientific Advisory Committee on Nutrition (SACN 2004)	450
	Committee on the Medical Aspect of Foods, Nutrition Policy (COMA 1994)	200
France	Agence Francaise de Sécurité Sanitaire des Aliments (AFSSA 2010)	500
Germany	German Society for Nutrition (DACH 2008)	250
	Deutsche Geselischaft für Ernährung (DGE 2008)	250
Switzerland	Swiss Society fNutrition Research/Swiss Nutrition Association (DACH 2008)	250
Scandinavia	Nordic Council of Ministers (2004)	450
USA	American Heart Association (AHA) (Harris 2009)	500
	American Dietetic Association (ADA 2007)	500
Canada	Health and Welfare Canada (1990)	1100 - 1600
	Dieticians of Canada (1990)	500
India	Cardiology Society of India (Dalal 2012)	2000 – 4000
Japan	Ministry of Health, Labour and Welfare (2010)	>1000
Israel	Israel Heart Society (2011)	500 – 1000

A large variation in mean intake is apparent between studies and countries. Recommendations also vary depending on desire disease prevention. According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) for general population current recommendation ranges from 500 to 2000 mg EPA+DHA/day (FAO/WHO 2010). Specific recommendation for countries is detailed in Table 2. Medium values are 500 mg EPA+DHA/day. It is also recommended that the ratio of n-6 to n-3 should not exceed 4 to 1 (Simopoulos 2002).

3.3. Availability of omega-3 fortified products in Spanish market

2000s have seen growing use of omega-3 fatty acids in foods marketed as functional, providing heart or mental function benefits, mirroring the greater popularity of fish oil and omega-3 capsules in the supplements/vitamins markets. Fortified/functional products saw the strongest growth over 2007-2012, as tough economic times led consumers to look to gain added value from products. Awareness of ingredients which offer added functionality is increasing and, as such, milk and dairy products have seen significant development (Euromonitor 2013). From 2010 to 2015, two hundred fifty new products with omega-3 have been launched to the Spanish market (Figure 5).

Expansion on the existent range is the first reason of the launches, around 20 % is due to a new packaging and only a 4 % is a relaunch. Around 30 % of the launches are new products (Figure 6). Approximately, 1 in 4 new omega-3 products launches carry a private label (Mintel 2015).

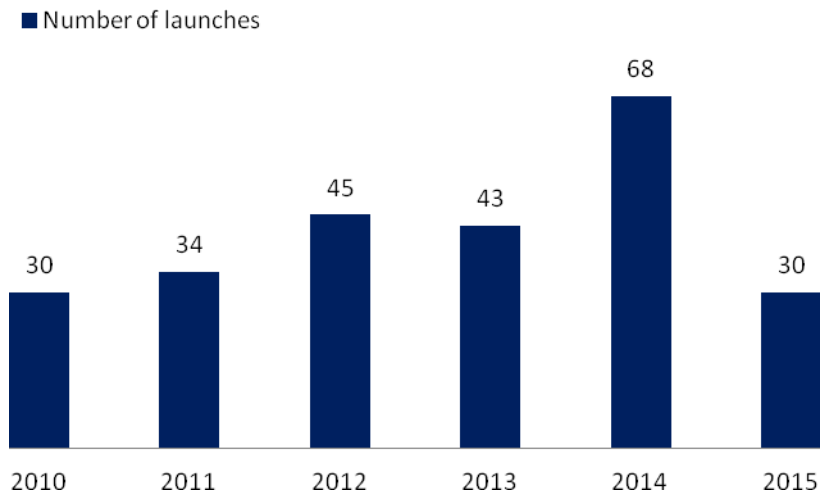

Figure 5. Functional food fortified with omega-3 fatty acids launched in Spanish market. (Mintel 2015)

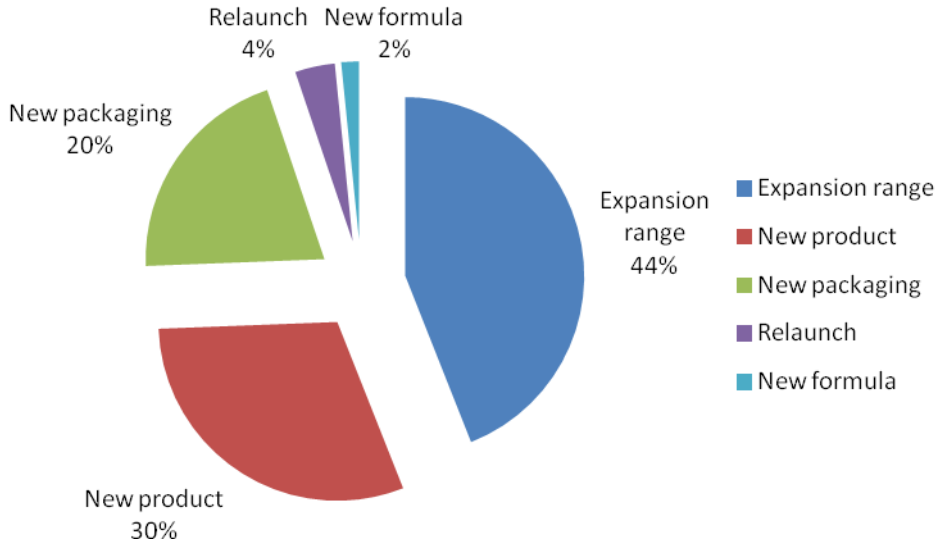

Figure 6. Main reason for the launches of omega-3 products. Source: Mintel, 2015

The range of foods benefiting from omega-3 fortification is discussed (Weylandt 2015). Omega-3 fatty acids as food makers around the world increase their efforts to provide the heart healthy benefits of fish without fishy odour and taste. Recent developments in food technology allow fortification of foods, such as bread, dairy products, egg, meat products, biscuits, juices, margarine, and other spreads, without the undesirable fish odour/taste and with reasonable shelf life (Kolanowski and Launfenberg 2006, Moghadasian 2008).

Therefore, there is a constantly growing range of food enriched in n-3 available for the consumer. The number of the fish derivates was increased on the Spanish market at the same time than the other products as meat products were decreased. Fish derivates, milks and soy drinks remain the main segments of omega-3 product development. New product developments in other sectors are nevertheless moving fast, with a notable trend towards margarines, oils and snacks (Table 3). Fish oil and soy remain at the forefront but a number of newer sources are being developed and sold on a health platform. These include flax, hemp, linseed, seaweed and chia seeds amongst others.

Eighty-five fortified products of these launches were obtained from Spanish retail stores and omega-3 content has been analyzed. Food sources of omega-3 are shown in Table 4. The products analysed included: natural canned fish (tuna, sardine, mackerel), smoked salmon, fish derivates (surimi derivates, tuna sausages, tuna burger), eggs, dairy products (milk, kids milk), soya milk, spread fats and oils (margarine, oils), olives, biscuits, bread, juice and meat products (pork sausages, chorizo, salchichón, cooked ham, turkey breast, turkey burger).

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Table 3. Summary of number of omega-3 fortified products launches in Spanish market

Category	2010	2011	2012	2013	2014	2015	Launches by category
Fish derivates	13	15	21	26	48	12	135
Milk	7	3	3	6	1	4	24
Soy drinks	4	2	7	2	4		19
Margarine		3	2		1	6	12
Snacks	1		2	2	4	1	10
Oils	1	1	1	1	2	1	7
Meat sucedaneous		2	2			2	6
Desserts	1	1	1		1		4
Mayonnaise		1		3			4
Nuts			1	1	1	1	4
Yogurths	1	1		1			3
Pasta	1	1	1				3
Meal sustitutes			1			2	3
Biscuits		2					2
Eggs	1				1		2
Cheese			1		1		2
Bread		1	1				2
Ready meals					2		2
Gum				1	1		2
Bakery		1					1
Breakfast cereal						1	1
Juice					1		1
Soup			1				1

Source: Mintel 2015

The purpose of this review was to discuss the various sources of omega-3 fatty acids within the context of the best source in order to cover the demand for these nutrients

Table 4. Amounts of omega-3 fatty acids in enriched products and natural sources of omega-3 in Spanish market and the amount of consumption required to provide 0.5 g of omega-3 fatty acids per day

Category	Type of product	n	Ration	Omega-3 FA per serving (g)*	Amount that provide 0.5 g of omega-3 FA (g)
Canned	Tuna	8	65g	0.17	194.81
	Sardine	8	60 g	1.72	17.42
	Mackerel	5	60 g	2.24	13.37
Smoked	Smoked salmon	1	100 g	2.30	21.74
Eggs	Eggs	4	50 g	0.18	140.35
Fish derivates	Surimi –based product	1	60 g	0.07	419.58
	"Gulas"	4	60 g	0.06	473.68
	Tuna sausages	1	125 g	0.23	277.78
	Tuna burger	1	125g	0.26	238.10
Dairy product	Milk	10	250 ml	0.13	983.28
	Kids milk	9	100 ml	0.18	284.58
Soya drink	Soy drink	2	250 ml	0.12	1041.67
Fat spread/oil	Margarine and butters	9	10 g	0.53	9.49
	Oils	1	10 g	0.35	14.29
Biscuits/Bread	Biscuits	3	30 g	0.06	263.16
	Bread toast	2	10 g	-	-
Juice	Juice	1	50 ml	0.13	1000.00
Meat product	Turkey cooked sausages	2	125 g	0.16	390.62
	Pork cooked sausages	2	125 g	0.15	416.67
	Chorizo	1	50 g	0.37	68.49
	Salchichón	1	50 g	0.33	75.76
	Boiled ham	3	50 g	0.21	117.51
	Turkey breast	2	50 g	0.01	1872.66
	Turkey burger	2	125 g	0.13	500.00
Others	Olives	2	25 g	0.004	3125.00

(*) Average values

The major food source contributing to omega-3 intake were fish products like smoked salmon or sardines. Meat fortified products also contributed to omega-3 fatty acid consumption in interesting proportion. Other products like biscuits, olives and

bread toasts had a very small contribution in the omega-3 intake. Products were also analyzed in order to evaluate the amount of consumption required to provide 500 mg of omega-3 fatty acid per serving (Tabla 4).

Canned, smoked fish and fish derivates

In the last 5 years more than 50 % of the total launches in products with omega-3 corresponding to canned, smoked fish and fish derivates (Table 3).

Figure 7. Some examples of launches in fish derivates fatty acids

The main source of long-chain omega-3 fatty acids in the diet came from fish intake. Fish contributed a different profile of long-chain omega-3 fatty acids than other fortified products, providing a higher proportion of EPA and DHA than ALA. Canned and smoked fish provided similar values than fresh fish. These amounts were enough to cover a normal average requirement per day of omega-3 fatty acids. Other products as fish derivates like “gulas” and other prepared fish products were far away to meet the dietary recommendation for a day with only one ration due to these products providing lower amounts of omega-3 fatty acids (63.3 and 71.5 mg omega-3 fatty acids/serving, respectively). However, these combinations, regarded as ‘natural’, were more attractive by survey respondents, as it previously described.

Dairy products

Awareness of ingredients which offer added functionality is increasing and, as such, milk and dairy products have seen significant development. Milk formula fortified

with omega-3 had estimated sales of US$24 billion in 2012, with growth predominantly from emerging markets (Euromonitor 2013).

Functional foods promise to deliver health and wellness to consumers in an environment where lifestyle diseases and an ageing population are threatening the wellness of society. Milk is a natural, multi-component, nutrient-rich beverage. Market trends indicate that milk-based beverages are ideal vehicles for newly discovered bioactive food ingredients targeting lifestyle diseases (Bechtold and Abdulai 2014). Low-fat milk is the most commonly used vehicle for the delivery of bioactive food ingredients and in particular is commonly used for delivery of omega-3 fatty acids. Drinks containing combinations of dairy and fruit juices with added bioactive components are also becoming common in the EU markets. Although a range of bioactive components is available for incorporation into dairy beverages, there are significant formulation challenges (García-Martínez and Márquez-Ruiz 2009). Therefore, functional foods need to be promoted as convenient, nutritious and tasty formulations with specific health benefits (Bhat and Bhat 2011, Shiby and Mishra 2013).

It is possible to increase levels of EPA and DHA in milk fat by including some fish oil in the diet of the cows (AbuGhazaleh et al. 2009, Toral et al. 2010); although the extent of enrichment in milk fat is very low. Another approach is to directly fortify milk with fish oil during processing to provide EPA and DHA (Kolanowski et al. 2007, Let et al. 2007, Sorensen et al. 2012) and in theory this process is much more efficient and controllable than inclusion in the diets of dairy cows.

In the last five year's twenty-nine new milks fortified with omega-3 fatty acids have been launched in the Spanish market (Table 5). Milk fortified in omega-3 found in Spanish market provides approximately 75 - 200 mg EPA+DHA/250 mL. Overall, fortified milk products are likely to generally provide small increases in EPA+DHA intake at normal levels of consumption, but many populations have substantially suboptimal intakes, and for them the availability of such milk products may be very valuable. Some examples of the last launches in milk products are shown in Figure 8.

Figure 8. Some examples of launches in milk with omega-3 fatty acids

The market has developed further by offering special milk catering for the need of particular sector of the population, such as children. Special target group for omega-3 PUFA rich food are infants. At the forefront of development in world market is the enrichment of infant formulae with essential omega-6 and omega-3 PUFA, especially EPA and DHA. Therefore the pediatric communities recommended use of omega-3 PUFA enriched formula, especially for pre-term infant nutrition (Dewey et al. 2009). Generally, every product can be enriched with omega-3 PUFA by fish oil addition. Currently good quality fish oils are the most commonly used sources of long chain omega-3 PUFA. The amount of EPA plus DHA in infant formulas ranged from 18 to 89 mg/100 mL of milk. These values are very lower than nutritional recommendations but it can be related with the nutritional requirement of omega-3 fatty acids in infants that in general are lower than in adult population.

Also, the milk is an everyday used drink which has been used as a carrier to try to increase the intake of certain nutrients (vitamins, minerals, protein, fats) and/or bioactives (phytosterols, probiotics, antioxidants, isoflavones, etc.). In the future, we will see more types of enriched or modified dairy drinks aimed to promote organ health and function and adapted to the different ages and physiology, an area of research that will certainly develop in the future. There is, however, a demand for new value-added soy-based drinks with improved “functional” (health benefiting) properties (Achouri et al. 2007). In this way, soya beverages are well appreciated for their nutritional and potential benefits. In the last 5 years, nineteen new soya products fortified with omega-3 have been launched to the Spanish market (Figure 9) due to soya beverages could be an

excellent carrier for functional or nutritive ingredients such omega-3 oils. A major driving force has been the increased publicity of the health benefits of soyabean products and the significant improvement in the taste (Achouri et al. 2007).

Figure 9. Some examples of soya product with omega-3 fatty acids

Similarly to the milk fortified with omega-3, fortified soya products covered approximately a ¼ of the daily recommendation for a day with only one serving due to these products provided around 120 mg of omega-3 fatty acids by serving (Table 4).

Yogurt also is included in dairy product category and there is supcetible to be fortified with omega-3 fatty acid. In 2014, Radulovic et al. demonstrated the viability of yogurt products fortified with PUFA omega-3 from fish oil as a new functional dairy product and nowdays this kind of products is present in the Spanish market (Figure 9).

Other commercial dairy products such as cheeses are also tried to introduce in the Spanish market (Figure 10). Some cheeses have been launched years ago, but nowadays fortified cheeses are not present in the Spanish market. Only one cheese was analyzed in order to evaluate the omega-3 concentration, but it was practically ineffective. In 2011, Bermúdez-Aguirre and Barbosa-Cánovas, demonstrated an easy way to incorporate omega-3 fatty acids from flaxseed oil and fish oil as an excellent nutrient into cheese. High retention of the nutrient omega-3 was achieved in three cheeses (fresh cheese, cheddar and mozarrella). Even though there were some differences in texture and physic-chemical changes, they obtained satisfactory results in a sensory evaluation for the fortified products. In 2015, Calligaris et al. tried to improve

of proportion of omega-3 fatty acids into cheese. In this study, it was stated the effect of formulation on the oxidative stability of omega-3 rich oil should be carefully taken into consideration. Probably, this is one of the reasons than fortified cheeses are not present at this moment in Spanish market.

Figure 10. Some examples of cheeses with omega-3 fatty acids

Spreadable fats and oils

The last years, fat spreads with reduced levels of total fat and increased level of unsaturated fatty acids have gained increasing market. Spreadable fats are a good example of food products suitable for enrichment with EPA and DHA by fish oil addition (Bower et al. 2003, Kolanoswki et al. 2006). Result in this study showed that ranged from 350 - 530 mg/serving (10 g). It can be concluded that daily portion of such enriched spreadable fat (10 g/per day) may provide approximately the recommended amount per day of EPA plus DHA.

Figure 11. Some examples of launches in spread with omega-3 fatty acids

Twelve spreadable fats have been launched in the last 5 years (Table 3). Some examples are shown in Figure 11. Omega-3 enriched spreads are perceived as more naturally fortified with omega-3 products than others. Omega-3 is also a non-natural enrichment per se, but being fatty acids they are a similar kind of compounds (fatty acids) that spreads already naturally possess, although combined with a less healthy product.

ALA occurs in plant foods such as nuts, particularly walnuts and flax, chia and hemp seeds, and vegetable oils, such as canola, linseed and soybean oils. In this way, seven new products have been launched to the Spanish market from 2010. These launches include walnut oil, linseed oil, seed oil (nut, seed and kernel oils) and soya oil (Figure 12).

The perception of the consumer for this kind of product is in line with fat spreads fortified with omega-3 fatty acids.

Figure 12. Some examples of launches in oils with omega-3 fatty acids

Eggs and egg derivates

Food products enriched with omega-3 PUFA are currently being marketed to increase omega-3 intake. Nowadays, commercial functional eggs are available, mainly enriched with n-3 PUFAs or with low cholesterol content.

Taking into account that eggs and egg-derived products are largely accepted by consumers, owing to their great culinary versatility and low economic cost, the development of functional eggs and egg-derived products could be an important way to

value the products and to gain profitability for egg producers and the food industry (Herron and Fernandez 2004). Nevertheless, as previously reported (Özen et al. 2014), functional eggs are still rarely consumed in Europe. Recent polls (Miranda et al. 2015) revealed that consumers mentioned the consumption of functional eggs in only two countries. In Sweden, only 3.8 % of those asked consumed eggs enriched with n-3 PUFAs, whereas in Spain, only 6.7 % of the respondents consumed eggs enriched with n-3 PUFAs or eggs that were low in cholesterol. Consumers in all other surveyed European countries reported using no eggs or functional eggs products. In the survey of this study we found that, in Spain, only 2% of the respondents consumed eggs enriched with omega-3. Some recently launches of fortified omega-3 egg products in Spanish market are seen in Figure 13.

Figure 13. Some examples of eggs product with omega-3 fatty acids

However, in most cases, these eggs are obtained through modification of laying hen's diet and management (Coorey et al. 2015), whereas much less attention has been paid to the development of functional eggs by means of technological methods (Fraeye et al. 2012, Miranda et al. 2015). One of the first marketed foods was n-3 fatty acid enriched eggs produced by hens fed diets rich in n-3 fatty acids (Arantes da Silva et al. 2009). However, some studies tried at creating such a product via processing (Kassis et al. 2010 a,b). The n-3 fatty acid fortified eggs that we can found in the market are typically developed through alteration of hen feed. When using fish oil as source of n-3 PUFA, it is highly recommended to include an antioxidant substance to prevent sensory hurdles that are mainly caused by oxidized n-3 PUFA in eggs (Fraeye et al. 2012). In this sense, recent research has shown that, when seaweed was used as source of n-3 PUFA, it could also act as an antioxidant, as seaweed naturally contains antioxidants such as carotenoids, polyphenols, and vitamins E and C. Therefore, one way to diversify the supply and to

possibly enlarge the market of egg products is to produce n-3 PUFA-enriched pasteurized liquid eggs and egg powders. Using technological methods, it is possible to fortify these products with n-3 PUFAs at the same time as reducing cholesterol content. Moreover, their use as ingredients in a wide range of processed foods could contribute to increased consumption of n-3 PUFA among the population (Meynier et al. 2014). Another important potential advantage of egg-derived products enriched with n-3 PUFA is that the lipid profile is better preserved at refrigeration temperatures, because storage at room temperature results in a loss of PUFAs.

Consequently, the development of functional egg products could be an interesting way to gain profitability for egg producers and the food industry, in addition to improving the general conditions of public health. Consumption of eggs enriched with omega-3 turned out to provide a mean value of 178.1 mg omega-3/serving (50 g). Thus, n-3 fortified eggs are a very interesting option for consumers.

Meat

Conventional meat products have an omega-6/omega-3 ratio higher than 15 while fortified meat products with omega-3 have been associated to ratios lower than four (Simopoulos 2002). The best strategy to improve the omega-6/omega-3 ratio in meat products is the addition of an appropriate amount of omega-3 PUFA (Olmedilla-Alonso et al. 2013). Meat products have great potential for delivering omega-3 into the diet. The nutritional composition of meat products can be altered by direct addition of omega-3 or by inclusion of omega-3 fatty acid into animal diets. Increasing unsaturated fatty acids is especially a problem in muscle foods. The fatty acid composition of ruminants is difficult to change because unsaturated fatty acids are biohydrogenated in the rumen (Buccione et al. 2012). There are also studies about improved fatty acid composition in grass fed beef but the nutritional importance of increased omega-3 is lower than the current recommendations (Scollan et al. 2006). In 2013, Zdunczyk and Jankowski demonstrated the addition of linseed oil to poultry diets has been found to increase n-3 PUFA concentration in chicken and turkey meat but this increases feed costs and may cause a deterioration in the sensory attributes and oxidative stability of meat.

Another way to fortify meat products with omega-3 fatty acids is direct addition of oils (Delgado-Pando et al. 2011, Reglero et al. 2008). This approach is more promising because it is only necessary to add low levels of omega-3 for significant nutritional effect. An advantage of this method is that the omega-3 fatty acids can be encapsulated in order to inhibit oxidation. A potential disadvantage of direct addition of omega-3 fatty acids into processed meats is that a portion of the bioactive lipid can be lost during cooking (Lee et al. 2006). Other problem is the high amounts of omega-3 promote lipid oxidation. To minimize the effect and to protect the added PUFA from autoxidation, the PUFA should be combined with antioxidants (Botsoglou et al. 2014, Cofrades et al. 2011). In other way, Wang et al. (2015) developed a novel semi-manufactured chicken surimi product with nutritional benefits by fortification with fish or flaxseed oil.

Functionalizing meat products has a great interest, considering their occurrence in the diet. Meat and meat products have a great nutritional value with high quality proteins, amino acids, vitamins, and minerals. Their fat content is the only deleterious aspect, and then replacing the fat excess by fish oil is a good option.

Figure 14. Some examples of meat products with omega-3 fatty acids

A variety of meat products as turkey burgers, cooked pork sausages, turkey sausages, traditional Spanish products as chorizo, salchichón, have been enriched with omega-3 fatty acids. Omega-3 fatty acids content varies between 13.4 mg to 370 mg per serving, an amount likely to stimulate commercial interests to fortify foods with omega-3 fatty acids.

According to Hathwar et al. (2012), development of healthier meat and meat products has health benefits of great importance at a population level, but marketing these products and convincing the consumers is an even more challenging task. Product development efforts have resulted in more failures than successes. The development and commerce of these products are rather complex, expensive and risky. The success of a product involves coordinated efforts between various disciplines especially nutritionists, epidemiologists, food technologists, natural product chemists and others. Also, the legislative aspects, sensory assessment and supermarket simulation studies play a vital role. Finally, the success or failure of the functional food is dependent on salient features of the product, its commercial viability and, on the nature, extent and management of collaboration between related disciplines.

Bakery products, cereals, nutritional bars and biscuits

Omega-3 acids are also effectively delivered through baked goods by means a variety of systems (Hernandez 2013). The lower water activity of baked foods, cereals and new products such as omega-3 nutritional bars now available in the market, makes them viable candidates for omega-3 fortification. Different types of fat are added during dough making and hence, flour can be easily fortified by addition of omega-3 fatty acids. Bakery products such as cakes, cookies, and pastries are manufactured from multiple ingredients wherein n-3 PUFA enriched ingredients such as eggs and dairy products can be specially used in combination to provide enchanced health benefits.

One of the main challenges for incorporation of omega-3 sources into baked goods and snacks is the extended shelf life required, sometimes over a year, for some products. In the case of bread, this is not as much of an issue since normally the shelf life required is only a few weeks. For products with long shelf life it is necessary to use more stable sources such as encapsulated or coacervated omega-3 oils. Torres et al. (2012) showed that the concentration of ω3 fatty acids as docosahexaenoic acid plus ecoisapentaenoic acid decreased approximately 6 % after 60 days of storage in cookies with omega-3 fatty acids.

Flax oil and flax meal, a good source of short chain omega-3 acids, are the more common ingredients in baked products. Flax meal also has the advantage that it can be used to substitute some of the wheat flour in several baked goods. The flax soluble fiber, gums and lignans, have been also reported as good stabilizers that can improve the general characteristics of bread. In 2015, Coelho and Salas-Mellado have demonstrated that it was possible to reduce the content of hydrogenated vegetable fat including chia (*Salvia hispanica* L.) seeds or flour in breads and to increase the levels of polyunsaturated fat, mainly n-3 fatty acids, in addition to increasing the level of fiber, yielding products with the features of functional foods. They have demonstrated by sensory evaluation that bread with chia flour or seeds added obtained high levels of acceptability and purchase plans, demonstrating the commercial viability of these products, with a higher index of purchase intention to the chia flour bread than the chia seed bread.

Figure 15. Some examples of cereal, biscuit and bread products with omega-3fatty acids

In 2014, Costantini et al. demonstrated that the combination of chia flour with tartary buckwheat *(Fagopyrum tataricum)* flour allow the creation of bread with enhanced functional properties without adversely affecting the technological parameters. Chia flour-supplemented breads contained significantly greater amounts of proteins, insoluble dietary fibres, ash and especially omega-3 fatty acids than the current whey breads. Moreover, tartary buckwheat flour may be suitable for preventing the oxidation of polyunsaturated fatty acids amount in bread fortified with 10 % of chia flour, providing a high amount of flavonoids and a 75 % increase in the total antioxidant

capacity. In addition, both chia flour and tartary buckwheat flour contain no gluten and can be consumed by patients with celiac disease.

These bakery products are also presented on the Spanish market are the omega-3, include biscuits, bread, breakfast cereals and cereals bars. These products provided very small amounts of omega-3 fatty acids. For example, biscuits only provided around 60 mg of omega-3 fatty acids. For this reason, this kind of products did not remain in the Spanish market long time. Moreover, these products are an example of products with a healthy image, but with an enrichment that were perceived as not natural since biscuits and breads are not a natural source of omega-3 fatty acid.

Snacks

Snack foods have become an integral part of the diet of majority of the world's population. Natural sources of omega-3 in snacks include some dried fruits as nuts, almonds and chestnuts. Also, blackberry and raspberry seed pomaces contain high quality and rich essential omega fatty acids as omega-3.

Figure 16. Some samples of snacks with omega-3 fatty acids

In other way, extrusion cooking is one of the major processes for producing expanded snacks. A number of studies have reported successful incorporation of fish flesh or fish powder into starch-based materials by extrusion processes to produce nutritious extruded products that were acceptable by consumers (Majumdar et al. 2011, Singh et al. 2012a,b). As extruded puffy snacks represent a frequently consumed diet

component, it can act as a potential medium for the delivery of health beneficial omega-3 PUFAs to the undernourished populations of the rural areas in the developing countries. However, only little efforts have been made so far on the fortification of extruded snack food with fish oil (Pansawat et al. 2008).

Meal substitutive and food supplements

There is now a large variety of medical foods, meal substitutes and food supplements targeted as general health promoting and also for persons suffering different types of diseases such as diabetes, cancer and renal disease. Examples of this kind of products in the Spanish market include a children supplement shake by Abbott Nutrition, containing 10.1 mg of DHA and 0.21 mg of the plant-based omega-3 fatty acid ALA per ration and which claims to support health. Also, in 2013, Nestlé launched a 200 mL nutrient drink called Resource SeniorActiv targeting the malnourished elderly. Besides containing EPA and DHA omega-3 fatty acids, it is fortified with vitamin D, calcium and protein. The main challenges for these types of products are prevention of oxidation and preservation of bioactivity of the omega-3 components (Mintel, 2015).

Figure 17. Some examples of meal substitutes and food supplements fatty acids

4. CONCLUSIONS

Three potential barriers for functional food acceptance are namely the price perception, the perception that there is no need for functional foods, and the perception that claims are mainly an advertising tool and thus the belief that functional foods are merely a marketing scam from the food industry.

But in the other hand, during the last decade, consumers' approach to healthy foods has changed dramatically, and today enhancing the health span of consumers through consumption of healthy food is more important than simply enhancing their life span. Rising medical costs are the prime factor forcing people to find cheaper and effective means of protecting their health. This fact has led to an increase in consumers' interest in functional foods. Omega-3 fortified products occupy a significant space in the functional foods market. This study reviews the perception of Spanish people of omega-3 fortified product and the situation of these products in the Spanish market.

The conflicting information found in the literature highlights the importance for companies to understand consumers in their global markets and define target audiences in a strategic manner. Our main hypothesis is that acceptance of functional foods depends on the combination of carrier and functional ingredient. Some consumers found that enrichments of food regarded as less healthy are perceived as more acceptable than enrichments of food that are perceived as healthy, like fat spreads, whereas some other consumers see products that are naturally healthy as more credible carriers for functional foods, like milk or eggs at the same time that these products are perceived as less credible carries due to be fresh products. It has also been shown that consumers are more likely to purchase combinations of carriers and ingredients where the functional ingredient also occurs naturally in that food, like canned or smoked fish or fish derivates. The study reporting that consumers see products that contain naturally omega-3 as credible carriers of fortified with omega-3. In other way the familiarity of the ingredient or of the ingredient/carrier combination has likewise been suggested to make functional foods more acceptable, and our own qualitative research on consumer

interest in products containing fish oil suggest that expected effects of the ingredient on the taste of the carrier may play a role as well.

With respect to influence of personal characteristics, our study can not confirm previous findings suggesting that demographic variables play a minor role in consumer acceptance of omega-3 enriched products.

The finding that perceived control over own health all ratings suggests that these consumers do not perceive a strong need for using functional foods. The finding that stronger perceptions of functional foods as a marketing scam decrease all ratings suggests that a too marketing- or advertising-driven strategy and positioning entail the risk of lower credibility and increased skepticism. Interestingly, the perception that functional foods are too expensive given their promised health benefits does not negatively influence consumer ratings in this study. These findings suggest that consumers' may express the price or cost argument to rationalize their eventual reserves against or rejection of omega-3 fortified products, whereas underlying reasons for rejection of these products are rather related to non-economic considerations.

Significant predictors of omega-3 products consumption are related to consumers'health motivation, perceived diet effectiveness of products, and knowledge about nutrition. There is evidence that consumers most likely to have a positive attitude toward enriched products are the ones who have faced illness among relatives or have experienced illness themselves. Cardiovascular diseases are strongly associated with the demand these products.

The most suitable to such enrichment seem to be food products that are frequently consumed, and which are not strongly heated, stored over long periods of time, or packed without access to light and oxygen. The presence of flavorings could allow higher levels of fish oil addition to the fortified products. Other way to avoid this problem is to add fish oil to short shelf life products or use microencapsulated fish oil that makes possible to convert fish oil to powder in order to increases shelf life of the fortified products.

A range of breads, milks, biscuits, juices, spread fats and fish and meat products fortified with omega-3 fatty acids was also launched onto the Spanish market. In the last 5 years two hundred fifty new products with omega-3 have been launched to the Spanish market. But the range has since been withdrawn and some products have disappeared in the last years from the market like fortified juices, breads and biscuits. This may reflect much lower levels of interest in this type of fortification but possible also reflects the problems in this kind of product in deodorizing the products to remove the fishy taste and smell or in the difficult to fortify in high content in omega-3 fatty acids in this kind of products. The study demonstrated that omega-3 enriched fruit juices; omega-3 enriched bread and omega-3 biscuits are the least accepted product concept. Although these products possess a healthy image, the less natural combination of bread, biscuits or bread with omega-3 apparently results in skepticism and lower acceptation of products.

An area of major interest in omega-3 fortification in recent years, however, has been EPA and DHA fortified milk. This kind of product has a medium contribution of omega-3 fatty acids but it had a very good acceptation between consumers. Furthermore, favorable perceptions may result from the specific association with omega-3, which has built a quite strong health reputation in previous years. Omega-3 contribution from egg is a little major than milk but eggs had a lower acceptation. Omega-3 spread was rated in between the other two products, despite the possible less healthy image of the carrier product combined with a non-natural combination. Existing marketing activities with several omega-3 enriched fat spread available and extensive investments in marketing and communication efforts may account for this product's positive evaluation. Moreover, taking into account that according to current dietary habits the meat consumption is high, meat products would be a potential target to increase levels of omega-3 without drastic changes in the normal diet habits.

In spite of the great offer of omega-3 fortified products, not all of them contribute in the same way in order to cover the average daily recommendation (500 mg), as we have observed in the study. The major food source contributing to omega-3 intake is fish products like canned or smoked fishs. Fat spreads, oils, milks and meat products also contribute to omega-3 fatty acids in interesting proportion due to their fat

content. Others products like biscuits, olives and bread toast have a very small contribution in the omega-3 intake.

In conclusion, this study indicates that whether one or another omega-3 fortified products appeals to consumers depends on the product type and the combined carrier product-omega-3 concept. It should be possible to produce functional foods that are regarded not only as healthy and convenient, but also natural and tasty.

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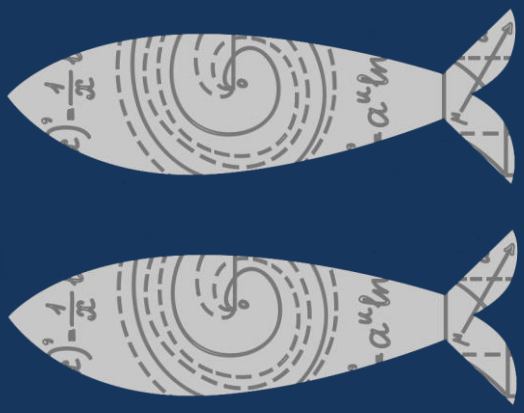

CHAPTER 2

Characterization of Omega-3 Rich Oils Obtained from Fish By-products by Supercritical Carbon Dioxide Extraction

II CHARACTERIZATION OF OMEGA-3 RICH OILS OBTAINED FROM FISH BY-PRODUCTS BY SUPERCRITICAL CARBON DIOXIDE EXTRACTION

ABSTRACT

The purpose of this study was to evaluate offcuts of hake (*Merluccius capensis – Merluccius paradoxus*), orange roughy (*Hoplostethus atlanticus*) and kingklip (*Genypterus capensis*) as sources of omega-3 rich fish oils obtained by means of supercritical CO_2 extraction. Supercritical CO_2 extraction is shown to provide high yields of omega-3 rich oil. The fish oils obtained in this way were stored for 75 days under pro-oxidative conditions (20 ºC, sunlight), and conventional conditions (4 ºC, without light). Peroxide and anisidine values, thiobarbituric acid reactive substances, free fatty acids and volatile compound profiles were all analysed. The hake offcuts were the most suitable for oil extraction, due to their medium fat content and high levels of n-3 PUFA. Orange roughy oil is shown to be much more stable against oxidation than hake oil. The influence of storage conditions on the stability of the oils was clearly demonstrated.

1. INTRODUCTION

Growing consumer interest in functional and healthy foods has led to a rise in demand for functional ingredients obtained using "natural" processes. Among the most interesting functional compounds are polyunsaturated fatty acids (PUFA), especially omega-3 (Jacobsen et al. 2013). Many scientific studies have examined the potentially positive effects of n-3 PUFA enriched functional food on health (Lane and Derbyshire 2013). Several reviews, such as that by Gil et al. (2012), Gogus and Smith (2010) and Riediger et al. (2009), refer to the role of PUFA in the reduction of cardiovascular disease risk, inflammation and autoimmune disorders, certain types of cancer, and the development of brain and nervous tissue in infants. Nutritionists have recommended increasing omega-3 consumption in order to maintain the omega-6/omega-3 ratio in a normal diet between 5:1 and 10:1 (Simopoulos 2002). Dietary habits in Western society are characterized by high consumption of saturated fatty acids and a higher-than-recommended omega-6/omega-3 ratio (Simopoulos 2008). The major dietary source of n-3 PUFA is fish, consumption of which often needs to be increased to reach the recommended average intake of omega-3 in a normal diet and to improve the omega-6/omega-3 ratio. Nevertheless, changing the dietary habits of the general public and encouraging greater consumption of fish is no easy task. Hence, the development over recent years of many n-3 PUFA enriched products. It is therefore important to find abundant, natural and economic sources of n-3 PUFA (Deckelbaum and Torrejon 2012, Kajla et al. 2015, Martins et al. 2013, Ward and Singh 2005).

An interesting source of n-3 rich fish oil is found in by-products (Kim and Mendis 2006, Nurdiani et al. 2015, Wu and Bechtel 2008) from the fish industry such as fish skin (Aryee and Simpson 2009, Shaena et al. 2010). Subcutaneous fish fat is by far the richest source of fat in some fishes and its recovery could be of economic interest. Several technologies involving solvents and high temperatures are currently used to extract fish oil (Hao et al. 2015, Rubio-Rodriguez et al. 2010, 2012). However, a reliable alternative to obtain good quality fish oil from these by-products is Supercritical Fluid Extraction (Ferdosh et al. 2014, Rubio-Rodriguez et al. 2008). The most widely

used supercritical solvent is carbon dioxide, which is non-toxic, cheap and has mild critical conditions, which means it is suitable for processing thermo-degradable compounds such as PUFA.

The purpose of this work was to characterize different types of fish by-products, specifically the offcuts obtained from three particular species (orange roughy, hake and kingklip), in order to evaluate their suitability as sources of omega-3 rich fish oil obtained by SFE. Whenever their extraction was presented as a viable economic alternative, the stability of the oils under different storage conditions was studied.

2. MATERIAL AND METHODS

2.1. Raw material

The raw material consisted of offcuts from three different species of fish: hake (*Merluccius capensis - Merluccius paradoxus*), orange roughy (*Hoplostethus atlanticus)* and kingklip *(Genypterus capensis*). The offcuts, provided by Pescanova, a Spanish food company located in Pontevedra (Spain), were obtained by peeling fishes with a TRIO™ peeler immediately after the catch while at sea, and they consisted mainly of skin, subcutaneous fat and some muscle. These by-products were frozen in the fishing boats at -20 ºC and remained so until the experiments were performed.

The frozen offcuts were cut into small pieces (1-10 mm equivalent diameter, D_e) with a cutter (CT25, Talleres Cato S.A. Spain), in order to decrease the internal mass transfer resistance during extraction. Then, the fish skin was freeze-dried (FreeZone 12 Liter Console Freeze Dry System with drying chamber, Labconco, Kansas City, USA) as described by Dunford et al. (1997), before extraction of the oil was performed.

2.2. Extraction of fish oil by solvents

Different solvent extraction methods were analysed in order to compare them with SFE. Bligh and Dyer (1959) proposed a method to extract total lipids using chloroform/methanol/water (1:2:0.8). Nilsson et al. (1994) proposed extractions that use n-hexane/isopropanol. Hara and Radin (1978) extracted the total lipids using n-hexane/isopropanol (3:2), followed by washing of the extract in aqueous sodium sulphate to remove nonlipid contaminants. Besides, the Soxhlet method, using petroleum ether and hexane as solvent in an extraction system model B-811 (Büchi, Flawil, Switzerland) was applied to extract fat from fish by-products.

2.3. Extraction of fish oil by supercritical carbon dioxide

Oil extraction was performed in a semi-pilot SFE-plant. Approximately 200 g of freeze-dried fish by-product were placed in the extractor, which was then pressured up to the extraction pressure of 25 MPa, with carbon dioxide (Carburos Metálicos, liquid $CO_2 \geq 99.9$ %). Then, the solvent was circulated at the extraction temperature of 40 ºC, at a flow rate of 10 kg CO_2/h for 3 h. It was continuously recycled to the extractor after removing the solute in one of the separators where the solvent power of CO_2 was reduced by lowering the pressure to 5 MPa (Rubio-Rodriguez et al. 2008).

2.4. Proximal composition

The fish skin was analyzed to determine its moisture, protein, and fat content. Moisture and protein content were determined by the AOAC International Official Methods (1990) 934.01 and 981.10, respectively, and total fat content was determined by Soxhlet method using petroleum ether as the solvent in a Büchi extraction system (model B-811, Flawil, Switzerland). The moisture content in the oils was determined by

weight loss after heating in an oven at 105 ºC for 30 min, according to the IUPAC (International Union of Pure and Applied Chemistry) Standard Method.

2.5. pH

10 g of product was blended with 10 mL of distilled water for 2 min to measure the pH in a pH meter 507 (Crison, Spain) equipped with a glass electrode.

2.6. Mineral composition

The samples (0.02 g of sample) underwent acid digestion in an ETHOS SEL (Milestone, Sorisole, Italy) microwave oven by using 10 mL of concentrated HNO_3 (65 %). The reactor was first held at 80 ºC for 4 min and then programmed to rise from 80 ºC to 130 ºC in 7 min and from 130 ºC to 170 ºC in 10 min. Mineral concentrations were determined by using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) (Agilent 7500i).

2.7. Fatty acid composition

The fatty acids profile was determined using the AOAC method (1995). This method requires the esterification of the the fatty acids to their methyl esters, which were then analyzed by Gas Chromatography (GC) in a Hewlett Packard gas chromatograph (6890 N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector. Separation was carried out with helium (1.8 mL/min) as the carrier gas in a fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.). The column temperature was programmed to start at a constant temperature of 180 ºC for 20 min, after which the heat was raised to 200 ºC at 1 ºC/min, held at 200 ºC for 1 minute, and then raised again to 220 ºC at 5 ºC/min and, finally, held steady at 220 ºC for 20 min. A split injection with a 50:1 ratio was used. Injector and FID were kept at 250 ºC during analysis. Most of the fatty acid methyl

esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co., St. Louis, Missouri, United Satates). Their quantification was performed by calculating the peak area and relating it to the area of an internal standard (methyl tricosanoate) as indicated in the AOAC method (1995). Calibration curves were obtained for each pair (internal standard + chromatographic standards) in order to find the corresponding response factors.

2.8. Oil oxidation

The effects of storage time and temperature on fish oil quality were all examined. Fish oil was analysed immediately after extraction (day 0) and throughout 75 days of storage under pro-oxidative conditions (20 ºC, sunlight), and conventional conditions (4 ºC, without light).

Oxidation process intensity was evaluated by means of various parameters, including Free Fatty Acids (FFA), Peroxide Value (PV), Thiobarbituric Acid Reactive Substances (TBARS), Anisidine Value (AV), colour, volatile compounds and odour fingerprint by electronic nose sensor responses.

Free fatty acids were determined by the official method Ca 5a-40 AOCS (American Oil Chemists´ Society) (1990).

Thiobarbituric acid reactive substances were analysed as described by the official AOCS method 19.90 (1990).

Peroxide value was determined according to the AOCS method Cd 8-53 (1990).

Anisidine value was analysed by the British Standard method BS 684-2.24 (1998).

Volatile compounds were analysed by Solid Phase Dynamic Extraction (SPDE) sampling and GC-MS. SPDE (Chromtech, Idstein, Germany) was performed after equilibration of the samples for 1 min at 70 ºC. The interior of the SPDE needle was coated with PDMS-AC. Gas chromatographic analyses were performed with an Agilent 6890N Series GC System coupled to an Agilent Technologies 5973i mass spectrometer

(Agilent, Palo Alto. CA.USA). The SPDE syringe was then removed from the sample vial and immediately inserted into the "gas station" port where 50 µL of carrier gas (helium) were pulled into the SPDE-syringe for VOC desorption during 30 s and pumped into the GC-inlet (heated to 250 ºC, splitless mode) at 15 µL/s. Postdesorption bake-out of the neddle at 250 ºC during 10 min ensured full desorption of all analyses from the needle sorbent, thus avoiding carry-over between injections (González 2008). The compounds were separated in a 007-5MS capillary column (50 m x 0.32 mm I.D fused silica capillary column coated with 1.05 µm film thickness (Quadrex Corporation. New Haven. USA)). The column temperature was increased at a rate of 3 ºC/min from 40 to 240 ºC. Effluent from the capillary column went directly into the mass spectrometer and as the volatile compounds were identified their mass spectra were compared with those in the NIST and the Wiley spectrum libraries.

Odour fingerprints were determined by an electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors. The vials with samples were incubated under agitation (cycles 5 s on and 2 s off at 500 rpm) in an oven at 50 ºC for generating the equilibrated headspace. The injection temperature was 60 ºC. Nitrogen was used as the carrier gas through the injection chamber at a flow rate of 150 mL/min, and synthetic air was used to maintain the thermal stability of the sensors.

2.9. Statistical analysis

All data were evaluated with STATGRAPHICS PLUS using ANOVA to compare the data of each oil sample for significant change (95% confidence interval). In all the tables, appropriate letters indicate statistical significance.

3. RESULTS

3.1. Comparison of different extraction methods

Six different solvent extraction techniques were applied to the hake, orange roughy and kingklip skin samples. The main objective consisted in studying the effect of the extraction of oil from fish skin with different solvents including SFE on extraction yield and oil composition: EPA, DHA and FFA content. The final objective was to determine the influence of supercritical CO_2 extraction on the quality and yields of omega-3 enriched oil obtained from various by-products (Table 1).

The Bligh and Dyer method (1959) is taken as the standard method for lipid extraction in marine samples (Smedes and Thomasen 1996, Smedes and Askland 1998). It gave higher oil yields, and EPA and DHA yields in comparison with the other extraction methods, but as shown in Table 1 these were very similar to those obtained by SFE. Nilson (1994) and Hara and Radin (1978) proposed a relatively safer organic solvent in response to stricter solvent disposal programs and issues relating to chloroform toxicity. The Hara and Radin extraction method produced higher oil yields but lower EPA and DHA yields in comparison with SFE, except for klinglip fish skin. Nilson extractions have resulted in poor oil, and DHA and EPA yields. The low yield of the Nilson extraction method has been attributed to the low polarity of isopropanol in comparison to methanol and to the limited efficiency of hexane in extracting polar lipid in comparison to chloroform (Undeland et al. 1997). Moreover, lipid composition must be studied in order to evaluate the effects of different solvent types on lipid composition.

Soxhlet-petroleum ether compared favourably with Soxhlet-hexane (Table 1) and it presented similar yields in comparison with SFE, although SFE presented higher EPA and DHA recovery rates.

FFA content is an important quality index in oils, thus the effect of each extraction method on FFA content in the oil was determined. Data on the FFA content in the extracted oils are presented in Table 1. Permissible levels of FFA in food-grade

crude fish oil are set at 1-7 % (Boran et al. 2006). As shown in Table 1, the oils obtained by SFE had appreciably less FFA than the oils extracted by other solvents. Although higher amounts of FFAs were obtained in the solvent extractions, in no case exceeded the proposed limits. Generally, oils prepared at higher temperature had higher FFA values (Chantachum et al. 2000). Aryee and Simpson (2009) studied the effects of extraction methods on the FFA content of the oils. A relatively lower FFA content of SFE oil in this study can be attributed to the low temperature and short extraction time. Fish oils are highly susceptivle to oxidative damage and the rate of oxidation increases with increasing unsaturated fatty acid content in oil.

Supercritical CO_2 extraction yields of oil from fish skin are comparable to those obtained with other methods and the quality of the omega-3 rich oil is better with respect to acidity, since it presented lower levels of FFA in comparison with other extraction methods. In this way, the quality of SFE extracted fish by-products oil was superior to that others solvent extracted oils. Similar conclusion obtained Ferdosh et al. (2014) about the quality of tuna fish oils extracted from processing the by-products of three species of tuna using SC-CO_2 in comparison with Soxhlet extraction.

Table 1. Total lipid yield (%), Free Fatty Acids (% Oleic acid) and Eicosapentaenoic acid (EPA) (%) and Docosahexaenoic acid (DHA) (%) content corresponding to different extraction methods

Extraction Method	Hake				Orange roughy				Kingklip			
	Yield	FFA	EPA*	DHA*	Yield	FFA	EPA	DHA	Yield	FFA	EPA	DHA
SFE	4.0	4.7	7.40	18.99	31.9	0.4	1.02	2.29	0.07	-	6.42	30.68
Bligh and Dyer	4.9	5.3	7.44	21.16	44.8	0.5	1.03	2.07	0.06	-	7.62	36.01
Nilsson et al.	3.7	5.3	6.79	18.67	32.5	0.8	1.09	1.95	0.05	-	6.42	30.74
Hara and Radin	4.1	6.6	4.64	11.66	35.3	0.5	0.93	2.00	0.09	-	7.31	34.04
Soxhlet (ether)	4.0	5.7	7.23	17.88	33.7	0.8	1.19	2.45	0.01	-	7.60	32.01
Soxhlet (hexane)	3.7	5.2	7.07	17.61	25.6	0.7	0.90	1.80	0.01	-	7.17	31.80

(*) % Fatty acid in comparison with total fatty acids.

3.2. Proximal composition

It is known that the fat content of different fish species is extremely variable, depending on several factors (Kucukgulmez et al. 2008). Thus, it is necessary to determine the proximal compositions of the fish by-products used as raw material in this work. These are presented in Table 2 for the three fish species under study: hake, orange roughy and kingklip. Orange roughy offcuts contained higher amounts of fat, hake offcuts were considerably less fatty, and kingklip by-products contained the lowest amount of fat. The very low fat content in kingklip offcuts made this species unsuitable for oil extraction. Moisture content usually varies inversely with fat content in fish flesh; thus, higher moisture levels usually imply less fat in the raw material. There was little or no difference in protein content between the three species and they shared similar pH levels; being slightly lower the pH of orange roughy than that of hake and kingklip.

The proximal composition determined for hake offcuts coincided with those reported for fillets of other species of hake (*Merluccius merluccius, Merluccius hubbsi*) by Kucukgulmez et al. (2008) and Méndez and Gonzalez (1997), except for the higher fat content found in the offcuts analysed in this study, demonstrating the greater presence of fat in fish skin than in the filleted fish. This confirms the suitability of the selected fish by-products as a source of fish oil.

Table 2. Proximate composition in raw offcuts from three species of fish (means ± standard deviation)

Composition	Hake	Orange roughy	Kingklip
Moisture[a]	79.71 ±1.09	54.53 ± 1.84	83.79 ± 0.79
Protein	16.60 ± 0.80	18.08 ± 0.79	12.37 ± 0.67
Fat	3.97 ± 0.13	31.90 ± 1.41	0.07 ± 0.01
pH	6.89 ± 0.02	6.55 ± 0.04	6.80 ± 0.02

[a] expressed as percentage of wet weight.

3.3. Mineral composition

The content of essential elements (Cu, Fe, Zn) and undesirable heavy metals (As, Cd, Hg, Pb) in raw offcuts of hake and orange roughy and in the fish oils obtained from them are shown in Table 3. These heavy metals occur naturally in the marine food chain and contaminate fish and fishery products (Liaset et al. 2003). Cadmium and lead were not present in any of the fish. Slightly higher contents of the other minerals were found in the hake offcuts than in the orange roughy offcuts, with the exception of zinc. The fish oils obtained both from hake and orange roughy had very low or even undetectable mineral levels. SFE is therefore shown to be an effective technique for the production of fish oil that is free of heavy metals, such as As, Hg and Pb, and which is therefore suitable for human consumption. This is a remarkable fact as maximum permissible levels of these compounds in fish and fish products have gradually been reduced (EU 2008). According to Hajeb et al. (2015) the fish oil extracted using conventional methods contained toxic elements at levels much higher than the accepted limits. Hajeb et al. (2015) showed that toxic element in fish oil could be reduced using supercritical CO_2 at moderate temperature and pressure.

Table 3. Mineral concentration in raw offcuts and oils from three species of fish (means ± standard deviation)

	Hake		Orange roughy	
	Offcuts	Oil	Offcuts	Oil
Fe[a]	3.34 ± 2.86	n.d.	1.73 ± 0.09	n.d.
Cu	0.46 ± 0.16	0.07 ± 0.05	0.27 ± 0.08	n.d.
Zn	4.59 ± 1.05	n.d.	10.30 ± 2.61	1.51 ± 0.64
As	1.35 ± 0.03	0.05 ± 0.02	0.85 ±0.08	0.26 ± 0.03
Cd	n.d.	n.d.	n.d.	n.d.
Hg	0.12 ± 0.12	0.0 2± 0.00	n.d.	n.d.
Pb	n.d.	n.d.	n.d.	n.d.

[a] expressed as ppm. n.d.: not detected

3.4. Fatty acid composition

The fatty acid composition of the three fish oils studied in this work differed considerably. According to Sahena et al. (2010) the greastest recoveries of PUFA from by-products were in the skin, followed by flesh, viscera and heads of Indian mackerel. Classified by their respective percentages of unsaturated fatty acids, the distribution was shown to be monounsaturated (46 %) > saturated (28 %) ≈ polyunsaturated (26 %) fatty acids in hake oil; monounsaturated (92 %) >> saturated (3 %) ≈ polyunsaturated (5 %) fatty acids in orange roughy oil; and, polyunsaturated (50 %) > monounsaturated (34 %) ≈ saturated (28 %) fatty acids in kingklip oil. Table 4 shows the detailed fatty acid composition of the three fish oils. Orange roughy oil had low concentrations of polyunsaturated and saturated fatty acids, whereas it had higher concentrations of monounsaturated acids. The principal difference with the other two oils is found in the ratio of monounsatured and polyunsatured fatty acids, which was higher in orange roughy oil than in the other two oils. Oleic acid, the main monounsaturated fatty acid in orange roughy oil, is responsible for the high value of this fraction. Hake oil presented high concentrations of monounsaturated fatty acids and similar concentrations of saturated and polyunsaturated fatty acids; the proportion between monounsatured and polyunsatured fatty acids in this oil was under two. The major PUFA content was found in kingklip oil. Values for C20:5 n-3 (EPA) and C22:6 n-3 (DHA) varied widely between the three species. EPA and DHA levels in kingklip oil were similar to EPA and DHA levels found in hake oil and about 10 times higher than concentrations of these fatty acids in orange roughy oil. The predominance of n-3 PUFA might suggest the use of kingklip offcuts for extracting fatty acids oil rich in n-3, but its low fat content makes this fish unsuitable for commercial exploitation. An inverse correlation between lipid content and the relative proportions of n-3 to n-6 PUFA has already been shown in other fish species (Sikorski and Kolakowska 2003). On the contrary, orange roughy oil presented a high fat content, but a low content of n-3 PUFA. In comparison, the hake offcuts are the most suitable for oil extraction, because they have a medium fat content and high n-3 PUFA levels. Due to its low fat content, kingklip was excluded from further oxidation studies.

Table 4. Fatty acid composition (mg/g oil) in fish oil

Fatty acid	Kingklip	Hake	Orange roughy
C14:0	4.00	18.79	3.72
C16:0	70.10	129.12	6.58
C18:0	31.80	20.51	1.99
SFA	105.90	168.42	12.29
C16:1	8.70	33.85	38.05
C18:1	103.90	164.13	209.16
C20:1	14.40	36.87	31.45
C22:1	1.90	32.22	25.33
C24:1	4.60	7.80	9.68
MUFA	133.50	274.87	313.67
C18:2	10.10	7.05	3.84
C18:3 n-3	-	4.40	3.35
C18:4	-	3.18	0.36
C20:3	-	0.82	0.22
C20:4	23.50	5.52	1.77
C20:5 n-3	24.50	36.32	2.72
C22:4	-	4.03	0.20
C22:5	11.50	8.03	0.61
C22:6 n-3	117.10	82.07	4.06
PUFA	186.70	151.42	17.13
Total n-3	141.60	122.79	10.13

SFA: saturated fatty acids. MUFA: monounsaturated fatty acids

PUFA: Polyunsaturated fatty acids

3.5. Oil oxidation

The effects of storage time and temperature on the quality of hake and orange roughy oils were examined. According to Hao et al. (2015), the oxidative stability of oils extracted by SFE is higher than that obtained by the other extraction methods. The fish oils were analysed immediately after supercritical fluid extraction (day 0) and throughout 75 days of storage under pro-oxidative conditions (20 ºC, sunlight), and conventional conditions (4 ºC, without light). Several parameters such as FFA, PV, AV and TBARS were analysed to follow the evolution of oil oxidation (See Tables 5 and 6, hake oil and orange roughy oil, respectively). These parameters have been extensively used to estimate the progress of lipid oxidation in oils (Laguerre et al. 2007).

The increment of PV, AV and TBARS values, observed in Tables 5-6, reflect increasing levels of primary and secondary products of lipid oxidation, such that, higher values of these parameters indicate that the oil is more susceptible to degradation due to lipid oxidation. The results showed that orange roughy oil was more stable than hake oil under storage at 20 ºC. Depending on their storage conditions, fish oils with high levels of PUFA are liable to oxidative deterioration (Boran et al. 2006). The oils under study differed greatly in the relative amounts of saturated and unsaturated fatty acid (Table 4), which probably influences their stability to lipid oxidation. In this study, hake oil was found to be less stable than orange roughy oil and it presented high initial oxidation.

FFA content is an important quality index in oils (Aidos et al. 2002a), and it is therefore a suitable parameter to evaluate the effect of storage conditions on fish oil. The FFA content of fish oil obtained was high; nevertheless a high FFA content is usual in this type of fish oil irrespective of the extraction method. FFA levels in fish oil obtained by SFE were lower than the FFA content of the oils obtained using other solvent extraction systems (Table 1). FFA values in hake oil increased with storage time and temperature (Table 5). Hake oil stored at 20 ºC had significant ($p < 0.05$) higher FFA values than the samples stored at 4 ºC. Hake oil at initial conditions presented fairly high FFA concentrations, even higher than values found in orange roughy oil after 75 days of storage. In orange roughy oil, FFA remained almost constant throughout storage at the two above-mentioned storage temperatures. The maximum FFA value (8.62 %) for hake oil was found after 75 days of storage at 20 ºC, which was higher than the recommended value of 1-7 % for food-grade fish oil (Boran et al. 2006). The increase observed in the FFA content of hake oil may well be related to its moisture content (1.31 %). Lower moisture content in orange roughy oil (0.58 %) coincides with a lower FFA content and less variation over time. The reported FFA values for frozen fish and fish oils and their evolution over time were extremely variable. Aubourg et al. (2007) observed a gradual increase in FFA during frozen storage of hake (*Merluccius merluccius*), reaching values between 10-20 % after 2 months; however, Aidos et al. (2002a) noted variations below 1 % or hardly any changes in the FFA of herring oil stored under different temperatures for 160 days.

Peroxide value, which measures hydroperoxide concentrations, is generally used to determine the extent of primary oil oxidation. A much lower peroxide value was evident in orange roughy oil than in hake oil; this value significantly increased in the two fish oils during storage, as shown in Tables 5-6. The acceptability limit for PV is 5 meq O_2/kg fish oil (GOED 2008). Orange roughy oil remained within the acceptability limit throughout 75 days of storage at 4 ºC and at 20 ºC, whereas hake oil exceeded this limit after only 6 days of storage.

Determination of anisidine value is one of the oldest methods of evaluating secondary lipid oxidation; it is related to the content of secondary oxidation products such as α and β-alkenals (Laguerre et al. 2007). The AV limit set by GOED (2008) is 20. In hake oil, the generation of secondary oxidation products begins almost simultaneously with the generation of hydroperoxides, as may be observed from Table 5. AV gradually increased in the hake oil during storage at both temperatures. The increase during storage was considerably lower at 4 ºC than at 20 ºC. Hake oil exceeded the limit after only 6 days of storage at 20 ºC and after 14 days of storage at 4 ºC. Different rates for PV and AV modification have been reported in other fish oils (O´Sullivan et al. 2005). Aidos et al. (2002a) also demonstrated that temperature had a clear influence on peroxide value and anisidine values in crude herring oil, the formation of these products being more intense at 20 ºC than at 0 ºC. The most significant effect, however, was observed at temperatures above those studied in this work. At 50 ºC, the peroxides no longer accumulated over time as they decomposed into secondary oxidation products quantified by anisidine, which increased considerably over time. On the contrary, in our study it was found that the AV and PV in both the hake and the orange roughy oils increased during storage (Tables 5 and 6).

The TBARS test is based on the formation of coloured products when ThioBarbituric Acid (TBA) reacts with malonaldehyde or other TBA reactive substances which are presumed to be produced from oxidized lipids. The intensity of the reaction increases with the degree to which unsaturated fatty acids are present

(Kolanowski et al. 2007); therefore, a parameter such as AV will allow us to track the evolution of secondary oxidation. TBARS values were notably higher in hake oil than in orange roughy oil, which is in line with the high EPA and DHA contents of the first one (Table 4). The TBARS and the AV and PV values increased in both types of oils. The TBARS level underwent a greater increase in hake oil stored at 20 ºC. Quality limits imposed by regulatory agencies for the acceptability of oils for human consumption is 7-8 mg malonaldehyde/kg of oil (Boran et al. 2006). This limit was exceeded after 6 days of storage in the case of hake oil, but orange roughy oil did not reach this value even after 75 days of storage.

Table 5. Changes in the quality of hake oil extracted by Supercritical CO_2 along storage at two different temperatures

Days of storage	FFA (% oleic)	PV (meq O_2/kg oil)	AV	TBARS (mg MA/kg)
at 4ºC				
0	4.70 ^{a}	4.11 ^{a}	7.75 ^{a}	7.83 ^{a}
6	4.89 ^{a}	7.64 ^{b}	16.27 ^{b}	17.74 ^{a}
14	4.66 ^{a}	11.43 ^{c}	26.43 ^{c}	30.30 ^{b}
32	4.90 ^{a}	20.95 ^{d}	32.58 ^{d}	61.48 ^{c}
45	5.46 ^{b}	19.59 ^{d}	61.42 ^{e}	121.93 ^{d}
60	5.80 ^{b}	26.10 ^{e}	81.42 ^{f}	121.43 ^{d}
75	6.49 ^{c}	28.47 ^{e}	203.73 ^{g}	146.60 ^{e}
at 20ºC				
0	4.70 ^{a}	4.11 ^{a}	7.75 ^{a}	7.83 ^{a}
6	4.87 ^{a}	13.10 ^{b}	29.31 ^{b}	55.80 ^{b}
14	5.08 ^{a}	14.31 ^{b}	43.21 ^{c}	39.33 ^{ab}
32	5.23 ^{b}	28.12 ^{d}	62.78 ^{d}	72.13 ^{b}
45	5.73 ^{c}	21.77 ^{c}	98.03 ^{e}	123.43 ^{c}
60	7.49 ^{d}	27.67 ^{d}	130.38 ^{f}	163.33 ^{d}
75	8.62 ^{e}	29.26 ^{d}	299.50 ^{g}	366.43 ^{e}

Values in the same column with different superscript letters are significantly different ($p < 0.05$). FFA: Free Fatty Acids, PV: Peroxide Value, AV: Anisidine Value; TBARS: thiobarbituric acid reactive substances

Table 6. Changes in the quality of orange roughy oil extracted by Supercritical CO_2 along storage at two different temperatures

Days of storage	FFA (% oleic)	PV (meq O_2/kg oil)	AV	TBARS (mg MA/kg)
at 4ºC				
0	1.55 ^{ab}	0.20 ^{a}	0.00 ^{a}	0.12 ^{a}
6	1.49 ^{ab}	0.28 ^{b}	1.20 ^{c}	0.20 ^{a}
14	1.67 ^{bc}	0.29 ^{b}	1.46 ^{cd}	1.07 ^{b}
32	2.30 ^{d}	0.49 ^{c}	1.90 ^{e}	4.30 ^{c}
45	1.88 ^{c}	0.55 ^{d}	0.12 ^{a}	1.24 ^{b}
60	1.66 ^{bc}	0.86 ^{e}	1.59 ^{d}	1.12 ^{b}
75	1.30 ^{a}	0.89 ^{e}	0.61 ^{b}	1.27 ^{b}
at 20ºC				
0	1.55 ^{ab}	0.20 ^{a}	0.00 ^{a}	0.12 ^{a}
6	1.55 ^{b}	0.47 ^{a}	1.69 ^{c}	0.61 ^{ab}
14	2.04 ^{c}	0.58 ^{a}	1.10 ^{b}	3.28 ^{e}
32	1.67 ^{b}	1.10 ^{b}	3.14 ^{e}	8.22 ^{f}
45	1.91 ^{c}	1.17 ^{b}	1.02 ^{b}	2.85 ^{e}
60	1.63 ^{b}	2.30 ^{c}	3.25 ^{e}	2.30 ^{cd}
75	1.30 ^{a}	2.57 ^{c}	2.37 ^{d}	1.49 ^{bc}

Values in the same column with different superscript letters are significantly different ($P < 0.05$). FFA: Free Fatty Acids, PV: Peroxide Value, AV: Anisidine Value; TBARS: thiobarbituric acid reactive substances

Large differences have been observed when comparing PV, AV and TBARS found in different types of oils throughout storage, which in some cases, could be due to the different storage conditions used in these studies. In general, AV and TBARS were lower (Aidos et al. 2002a, Boran et al. 2006, Hao et al. 2015, O´Sullivan et al. 2005, Zuta et al. 2007) than the values found for hake oil in this work, and peroxide values were either lower (Aidos et al. 2002a, Zuta et al. 2007) or comparable (O´Sullivan et al. 2005). Orange roughy oil showed lower values for all the parameters related to lipid oxidation than those found in the literature for several other species. This stability against oxidation could be related to its fatty acid profile, since it is well established that oil is particularly susceptible to lipid oxidation and rancidity development because of its high PUFA content (Kolanowski et al. 2007). Another factor to be taken into account is the content of wax-esters, which is very high in orange roughy oil (Buisson et al. 1982).

This type of lipid could be less prone to undergo oxidative changes, but it is indigestible and potencially problematic for sensitive consumers.

3.6. Volatile compounds

The oxidation of PUFA-containing lipids causes the development of off-flavours and aromas, often referred to as 'rancidity' in fish. According to Sullivan Ritter (2012), by monitoring these volatiles, it may be possible to create a simple method to asses oxidation in fish oils that correlates well with sensory properties of the oil without the use of a sensory panel. Compounds that have rancid flavours and aromas are volatile secondary oxidation products derived from the breakdown of lipid hydroperoxides (Kolanowski et al. 2007). The main intrinsic factors that determine the rate and extent of rancidity development in fish are lipid levels, the fatty acid composition of the lipids and levels of endogenous antioxidants and endogenous oxidative catalysts. External influencing factors include oxygen concentration, surface area exposed to atmospheric oxygen, storage temperature and processing procedures that lead to tissue damage (Baigrie 2003). As the fatty acid profiles of hake and orange roughy oils were very different, it was also expected that the volatile profile and rancidity development would differ considerably in both oils. Studies on the evolution of volatile compounds in oil oxidation are scarce, but the volatile profile in fish oil enriched products and fresh and frozen fish throughout storage have been more extensively studied. According to Roh et al. (2006), supercritical carbon dioxide can successfully eliminate off-flavours from tuna fish oil by removing volatile compounds and can also decrease the oxidation of PUFAs. After this, Wang et al. (2015) indicated that the odour wich may affects fish oil were removed up to 100 % by using supercritical CO_2 extraction technology, besides, the acids were removed efficiently. In this study, the main volatile components of fish oil can be grouped into several types of compounds; the most important families being alcohols, aldehydes, alkanes, ketones, furans and amides. The hake oil presented a larger number and much higher concentrations of volatile compounds than the orange roughy oil (Table 7). At initial conditions, furans, alcohols, ketones and aldehydes were only present in hake oil. Amides and alkanes were identified in both oils; the amides

were more abundant in hake oil and alkanes in orange roughy oil. In general, volatile compounds in hake oil underwent an increase during storage. At the end of storage, the predominant groups were aldehydes, alcohols and furans. Orange rough oil hardly showed any modifications, as only alkanes and aldehydes increased, but their concentrations remained fairly low.

Thirty-five volatile compounds were identified in the fish oils studied throughout storage, 1 alcohol, 13 aldehydes, 9 alkenes, 6 ketones, 3 furans and 3 amides (Table 7). Lipid-derived volatile compounds contribute to the characteristic and desired flavour attributes of foods, but can also cause off-flavours. The formation of secondary oxidation products such as volatile aldehydes and ketones is responsible for flavour and sensory changes. Some of these volatiles, such as hexanal and pentanal have been commonly used to measure the extent of lipid oxidation since these off-flavour components have very low threshold values (Karahadian and Lindsay 1989). The evolution of the six aforementioned groups of compounds (alcohols, aldehydes, alkanes, ketones, furans and amides) was followed in this work over time.

Alcohols: One alcohol found in hake oil, 1-penten-3-ol, was clearly identified and characterized as a lipid oxidation product in fish oil (Horiuchi et al. 1998). However, no alcohols were identified in orange roughy oil, due probably to its different fatty acid composition, as alcohols mainly result from the degradation of unsaturated fatty acids through autoxidation of lipids. The level of alcohols in hake oil increased with higher temperatures and longer storage times.

Aldehydes: Only two aldehydes were identified in orange roughy oil throughout storage. Hexanal was present in hake oil from the start, but it was initially absent in orange roughy oil, only appearing after a storage period of 60 days at 4 ºC and 14 days at 20 ºC. The other aldehyde found in orange roughy oil after 75 days of storage at 20 ºC was 2,4-heptadienal. In contrast, in addition to hexanal, other twelve different aldehydes were found in hake oil. According to Baigrie (2003), the main aldehydes arising from oleic acid are octanal and nonanal, and the main ones from linoleic acid are hexanal, (E)-2-heptenal and (E,E)-2,4-decadienal**,** whereas linolenic acid yield a

complex mixture that is very rich in (E,Z)-2,4-heptadienal; all of these except nonanal were identified in hake oil.

It has been reported that volatile compounds such as Z-4-heptenal, (E,Z)-2,4-heptadienal and (E,Z)-3,5-octadien-2-one, are specifically derived from lipid oxidation of n-3 PUFA (Aidos et al. 2002b, Iglesias et al. 2007, Venkateshwarlu et al. 2004). These compounds were identified in hake oil, but not in orange roughy oil, probably due to its higher content of n-3 PUFA, the precursor of these aldehydes. Iglesias et al. (2007) also reported that propanal resulted from the oxidation of n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), although no such volatile compound was found in hake oil.

The concentration of aldehydes increased in hake oil throughout storage (Table 7), undergoing greater changes at 20 ºC than at 4 ºC. This agreed with the evolutionary pattern of different aldehydes throughout storage reported by several authors (Aidos et al. 2002b, Hartvigsen et al. 2000, Venkateshwarlu et al. 2004). This tendency has been noted in several studies, although Karahadian and Lindsay (1989) observed a fall in certain individual aldehydes in fish oil at the end of a long period of storage. Among the aldehydes associated with marine oil oxidation, Z-4-heptenal, (E,E)-2,4-heptadienal and (E,Z)-2,6-nonadienal have been characterized as very powerful odorants, contributing to an unpleasant rancid, fishy off-flavour (Hartvigsen et al. 2000). Other aldehydes present in hake oil have also been associated with sensory odour attributes that could explain off-flavours that arise in the oil during storage (Table 7). There was a large amount of these odorants in hake oil, which is probably related to its intense odour.

Alkanes: A large number and quantity of alkanes were found in orange roughy oil, whereas they were hardly detected in hake oil at all (Table 7). Hydrocarbons are usually regarded as flavourless; n-alkanes contribute little to overall food flavour in general due to their high aroma thresholds (Hartvigsen et al. 2000). However, 2,6,10,14-tetramethylpentadecane, a branched alkane, that was found in both hake and orange roughy oil, is asociated with a waxy, oily and slight fishy odour.

Furans: Furans can be formed by cyclization of oxygen containing radicals or peroxides (Horiuchi et al. 1998). Three furans, 2-ethylfuran, 5-ethyl-2(5H)furanone and cis-2-(1-pentenyl)furan, have been detected in hake oil, their quantity being influenced by temperature and storage time, but they were not identified in orange roughy oil. The compound 2-ethylfuran has been reported to have a strong sweet-ethereal odour (Table 7). The furans most usually identified in fish oils or enriched fish oil emulsions are ethylfuran and pentylfuran (Horiuchi et al. 1998, Iglesias et al. 2007, Karahadian and Lindsay 1989, Sullivan Ritter and Suzanne 2012, Venkateshwarlu et al. 2004). In fish oil enriched mayonnaise, seven furans were identified by Hartvigsen et al. (2000), two of them, 2-ethylfuran and 2-(1-pentenyl)furan, were also found in hake oil. They were associated with flowery and "green" odours, but are unlikely to have an important sensory impact.

Ketones: Ketones may be produced by thermal oxidation of PUFA and amino acid degradation (Iglesias et al. 2007). They have been reported as lipid oxidation products in fish (Alasalvar et al. 2005, Giogios et al. 2009). In this work, six different ketones were identified in hake oil, and none was identified in orange roughy oil after 75 days of storage at 20 ºC (Table 7). Higher storage temperatures enhanced ketone production. The unsaturated ketone, 3,5-octadien-2-one was present in hake oil throughout storage. Two further important ketones 1-penten-3-one and 2-nonanone that, according to Hartvigsen et al. (2000) caused unpleasant pungent odours, were also found; 1-Penten-3-one, originating from n-3 PUFA, is associated with fishy off-flavours and a pungent and rancid green odour. 2-Undecanone was also found in hake oil, at the end of storage, at day 60 in oil stored at 20 ºC, and at day 75 in oil stored at 4 ºC, but it was not detected in orange roughy oil.

Amides: One amide N,N-dimethylformamide was identified in orange roughy oil and two N,N-dimethylacetamide and N,N-propanamide in hake oil at the end of the storage.

Table 7. Volatile compounds (AAU x 10^6) in fish oils before and after 75 days of storage

	Volatile compounds [a]	RI [b]	MI [c]	Characteristic odour [d]	Hake oil (AAU x 10^6)			Orange roughy oil (AAU x 10^6)		
					Initial	After 75 days of storage		Initial	After 75 days of storage	
						4ºC	20ºC		4ºC	20ºC
	Alcohols									
1	1-Penten-3-ol	<700	MS	Pungent, grassy	291.94	564.82	927.20	-	-	-
	Aldehydes									
2	2-Butenal	<700	MS	Sharp, warm, spicy	51.29	51.47	92.82	-	-	-
3	(E)-2-Pentenal	784	MS+LRI	Powerful, fruity	110.82	139.50	343.61	-	-	-
4	Hexanal	828	MS+LRI	Fatty green, penetrating	77.22	149.05	304.63	-	28.70	33.69
5	(E)-2-Hexenal	885	MS+LRI	Strong, leafy-green	18.52	9.78	17.60	-	-	-
6	(Z)-4-Heptenal	928	MS+LRI	Creamy and fried-like odour like cream and	25.58	14.95	43.61	-	-	-
7	2,4-Hexadienal	951	MS	Fresh, green and spicy	7.74	6.72	11.75	-	-	-
8	(E)-2-Heptenal	987	MS+LRI	Green-fatty-vegetable	-	8.16	14.08	-	-	-
9	Benzaldehyde	1013	MS+ LRI	Odour of bitter almond oil	5.93	4.11	5.29	-	-	-
10	Octanal	1031	MS+LRI	Fatty-fruity	4.57	5.88	15.99	-	-	-
11	(E,E)-2,4-Heptadienal	1034	MS+LRI	Pungent, spicy	22.39	32.98	59.05	-	-	12.79
12	(E,Z)-2,6-Nonadienal	1188	MS+LRI	Cucumber	1.86	1.44	1.62	-	-	-
13	(E)-2-Nonenal	1190	MS+LRI	Strong, fatty-orris odour	-	2.69	3.81	-	-	-
14	(E,E)-2,4-Decadienal	1360	MS+LRI	Strong, deep fatty odour	-	2.26	2.88	-	-	-
	Alkanes									
15	Nonane	900	MS+LRI	Hydrocarbon odour	3.60	2.74	6.18	-	-	-
16	Decane	1000	MS+LRI	Hydrocarbon odour	6.63	-	-	16.00	26.80	17.90
17	2-Methyldecane	1064	MS+LRI		-	-	-	4.00	6.83	2.79
18	3-Methyldecane	1070	MS+LRI		-	-	-	6.27	4.00	2.13
19	Undecane	1100	MS+LRI	Gassy, hydrocarbon odour	-	-	-	26.97	34.91	34.40
20	Dodecane	1200	MS+LRI	Sweet hydrocarbon like	3.21	3.10	2.31	9.47	11.15	5.91
21	Tridecane	1300	MS+LRI	Hydrocarbon odour	-	2.13	1.85	3.84	6.43	2.84
22	Pentadecane	1500	MS+LRI	Mild hydrocarbon odour	5.89	4.42	3.38	12.89	16.00	19.66
23	2,6,10,14-Tetramethylpentadecane	1698	MS+LRI	Waxy, oily, slightly fishy	49.46	23.28	23.68	182.67	295.04	354.41
	Ketones									
24	1-Penten-3-one	<700	MS	Strong, penetrating, pungent	93.38	33.07	70.12	-	-	-
25	(E)-3-Penten-2-one	762	MS+LRI	Pungent, spicy, fruity, fishy	-	4.94	15.12	-	-	-
26	3-Hexen-2-one	868	MS+LRI		-	-	1.02	-	-	-
27	2-Nonanone	1112	MS+LRI	Fruity, fatty, cheesy	-	3.94	14.64	-	-	-
28	3,5-Octadiene-2-one	1132	MS	Woody	29.85	14.69	31.94	-	-	-
29	2-Undecanone	1314	MS+LRI	Oily-fruity	-	1.53	3.40	-	-	-
	Furans									
30	2-Ethylfuran	<700	MS	Strong, sweet-ethereal	195.50	184.93	366.46	-	-	-
31	5-Ethyl-2(5H)-furanone	1005	MS+LRI	Caramel	-	38.53	48.28	-	-	-
32	(Z)-2-(2-Pentenyl)furan	1021	MS+LRI		8.35	21.83	30.59	-	-	-
	Amides									
33	N,N-dimethyl-Formamide	802	MS		-	-	-	5.80	35.22	43.65
34	N,N-dimethyl-Acetamide	911	MS		4.02	31.69	30.14	-	-	-
35	N,N-dimethyl-Propanamide	986	MS		-	5.18	5.33	-	-	-

[a] Volatile compounds (Z: cis, E: trans)

[b] Retention indices relative to C_8-C_{20} n-alkanes on 007-05NS column

[c] Method of identification: MS, mass spectrum comparison using Wiley and NIST libraries; LRI, Kovats index in agreement with literature values

[d] Characteristic odour according to Flavour Base 2007 by John C. Leffingwell (Leffingwell & Associates).

[e] AAU: Arbitrary Area Unit. Values are means (area units x 10^6) of three analyses

3.7. Odour fingerprint by electronic nose analysis

Due to the great diversity and quantity of volatile compounds found in hake oil, significant implications in oil odour were expected. In this work, odour fingerprints of the oils were analysed during storage with an electronic nose. In hake oil, the PCA model of the first two latent variables accounted for 98.2 % of the total explained variance (Figure 1).

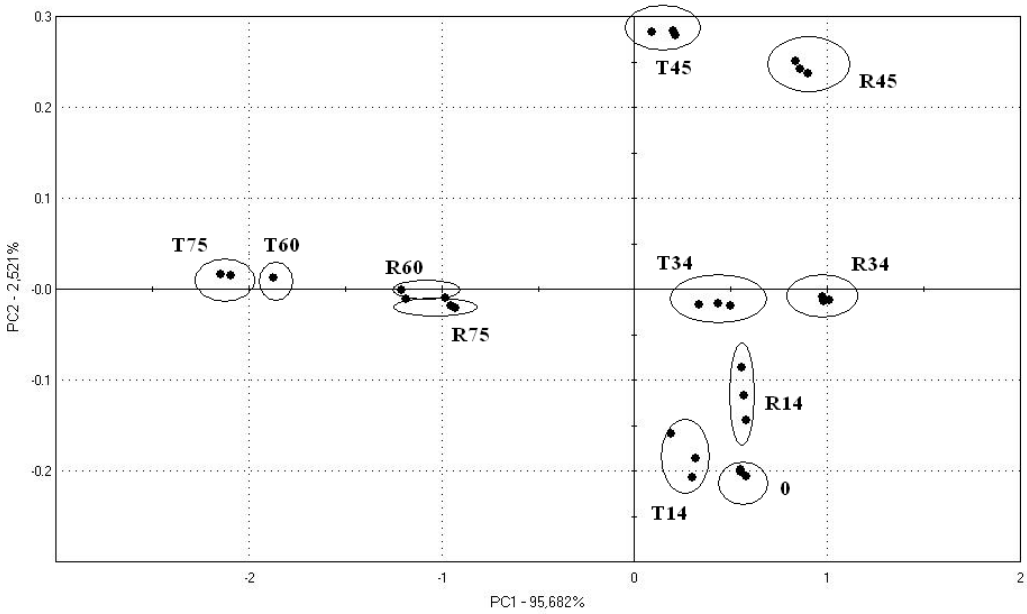

Figure 1. PCA of sensor reponses to hake oil headspace throughout storage (0, 14, 34, 45, 60 and 75 days) at two different temperatures: 4 ºC (R) and 20 ºC (T).

The relative score plot shows a clear discrimination of the samples according to the storage conditions under the first component. The samples stored at room temperature are clearly separate from the samples stored at 4 ºC. In the PCA score plot of the electronic nose sensor responses to hake oil headspace, samples at day 60 and at day 75 differed from earlier storage times under PC1, which explained 95.7 % of the variance. From the start date until day 45, samples were mainly separated by PC2, which only explains 2.5 % of the variance and they showed similar PC1 coordinates. This result agrees with the evolution of volatile compounds (Table 7), since the amount

of several families such as aldehydes and ketones underwent a remarkable increase, and some individual compounds, such as (E)-2-nonenal, (E,E)-2,4-decadienal, (E)-3-penten-2-one, 3-hexen-2-one and 2-undecanone, only appeared after 60 or 75 days of storage. Orange roughy oil fingerprints (not shown) hardly experienced any changes throughout storage; although samples showed a gradual evolution from the initial oil, differences between the two first principal components were small.

4. CONCLUSIONS

It has been demonstrated that supercritical carbon dioxide extraction is an efficient technique by which to obtain fish oil free of heavy metals. Supercritical CO_2 extraction of omega-3 rich oil from fish by-products gives similar yields than other conventional solvent extraction systems. Solvent extraction with hexane and similar solvents, although applicable, is not a recoomended option for extractions for food-grade fish oil.

The fatty acids profile of oils obtained by SFE is comparable to those oils extracted by conventional methods and the FFA content of fish oil obtained by SFE is lower than the FFA content of the oil obtained by conventional solvent extraction. The fat content and fatty acid profiles of three fish by-products studied in this work (orange roughy, hake and kingklip offcuts) differed greatly; hake being the most suitable raw material for SFE of omega-3 rich oil. The behaviour of oils obtained by SFE agrees with the well established poor stability of the oils that contain a high proportion of PUFA against oxidation; hake oil undergoing high oxidative deterioration. Moreover, the clear influence of storage conditions on oil stability has been demonstrated. Therefore, techniques to stabilize SFE fish oils with a high PUFA content must be developed and used in order to avoid their oxidation over time.

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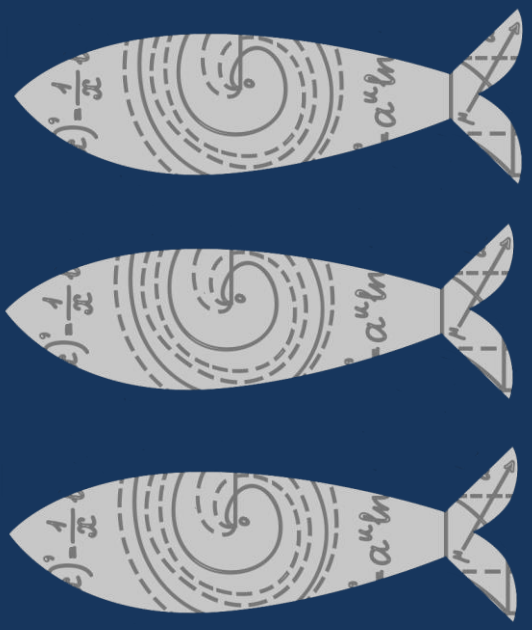

CHAPTER 3

Use of Natural Antioxidants (*Melissa officinalis, Salvia officinalis* and *Rosmarinus officinalis*) to Protect Fish Oil against Oxidation

III USE OF NATURAL ANTIOXIDANTS (*MELISSA OFFICINALIS, SALVIA OFFICINALIS* AND *ROSMARINUS OFFICINALIS*) TO PROTECT FISH OIL AGAINST OXIDATION

ABSTRACT

The ability of natural extracts from three different species of plants: lemon balm (*Melissa officinalis*), sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) to protect crude fish oil obtained from by-products of fishery industry was investigated. The oxidative stability of fish oil was evaluated by chemical and sensory analyses during 42 days of storage at 20 ºC. A control stored at light was compared with the fish oil added with three different extracts (lemon balm, rosemary and sage) in two different proportions (2 % and 5 %). Oxidation increased along storage at room temperature as it showed the evolution of various parameters such as fatty acids (FA) composition, free fatty acids (FFA), peroxide values (PV), p-anisidine values (AV), olfative profile, antioxidant activity, volatile compounds and sensory analysis. Fish oil with natural antioxidants was more stable than fish oil without antioxidants. Fish oil containing 5 % natural antioxidant had lower levels of lipid oxidation in comparison to the samples containing 2 % of antioxidant. Lemon balm showed high content of rosmarinic acid and fish oil with lemon balm presented the greatest stability against oxidation. On the other hand, results suggest that the electronic nose is capable of measuring changes in volatile compounds associated with oil oxidation and could be used to supplement data obtained from sensory evaluations.

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1. INTRODUCTION

Fish oil contains high levels of long chain polyunsaturated fatty acids (PUFA) that are considered to have a number of health benefits (Gil et al. 2012, Lunn and Theobald 2006, Riediger et al. 2009, Ruxton et al. 2004). There are reports on production of good quality fish oil according to the extraction method (Sahena et al. 2009, Shahidi and Wanasundara 1998). In the last decade, several studies have been published about oil obtain from different types of fish by-products as a viable economic alternative in order to obtain of omega-3 rich fish oil (Aryee and Simpson 2009, Nurdiani et al. 2015, Sahena et al. 2010, Rubio-Rodriguez el al. 2010, Sathivel et al. 2009). However, the n-3 fatty acids are highly vulnerable to oxidative deterioration. Lipid oxidation in fish oil is a detrimental process. It deteriorates the quality, results in the production of off-flavour and odours. Lipid oxidation is affected by numerous internal and external factors such as fatty acid composition, content and activity of pro- and antioxidants, temperature, oxygen presence, surface area in contact with oxygen, etc. Due to the complexity of the lipid oxidation mechanism in fish oil, more than one method is usually applied to determine oil quality (Aidos et al. 2002, Kolanowski et al. 2007a).

Oxidation may be inhibited by various methods (Arab–Tehrany et al. 2012, Jacobsen et al. 2008, Kamal-Eldin and Yanishlieva 2002, Kolanowski et al. 2007b, Maqsood et al. 2012, O'Sullivan et al. 2005). Usually, to delay lipid oxidation antioxidants are added. In general, there are two basic categories of antioxidants, natural and synthetic. Carocho and Ferreira (2013) also studied the prooxidant effect of some antioxidants. Recently, interest in natural antioxidants found in plants has grown due to the increasing trend towards use of "natural" food additives (Oroian and Escribe 2015, Pezeshk et al. 2015, Yanishlieva et al. 2006). Moreover, many natural antioxidants are shown to have health benefits (Valenzuela et al. 2003). New natural antioxidants for protecting omega-3 rich products appear every year (Anal et al. 2014, Farvin and Jacobsen 2012, Raudoniute et al. 2011). Lemon balm, *Melissa officinalis*, is an herb of long tradition and with a large variety of uses. Extract obtained from this plant exhibited antioxidant properties against lipid oxidation, due to phenolic acid components, such as

rosmarinic and caffeic acids and their methyl esters (Dastmalchi et al. 2008, Marongiu et al. 2004, Mimika-Dukic et al. 2004, Pereira et al. 2009). The main compounds responsible for the high antioxidant effects of rosemary extract *(Rosmarinus officinalis)* are also phenol diterpenes such as carnosic acid, carnosol and rosmarinic acid (Hernández-Hernández et al. 2009). According to Kolar and Urbancic (2007), rosemary extract possess antioxidant properties that make it useful in protection of polyunsaturated oils.

Salvia officinalis commonly called sage is widely used in traditional medicine and is a rich source of polyphenolic flavonoids and phenolic acids (Lu and Yeap Foo 2002). The main antioxidant activity of sage was attributed to rosmarinic acid and the phenolic diterpenes carnosol and carnosic acid (Cuvelier et al. 1996, Farhat et al. 2009). Among the phenolic compounds, rosmarinic acid was the common phenolic compound in all the extracts. According to Lindberg Madsen and Bertelsen (1995) rosemary and sage are two of the most potent spices as natural antioxidants, isolating from both of them the same compounds, indicating a close botanical relation between these two spices.

The present study was undertaken to further evaluate the efficacy of *Melissa officinalis*, *Salvia officinalis* and *Rosmarinus officinalis* extracts to control the lipid oxidation in crude fish oil rich in omega-3 fatty acid from fish by-products. Therefore, this study also aimed to investigate the potential of using a natural preservative to extend the shelf life of fish oil for future incorporation in functional food fortified with omega-3 fatty acids.

2. MATERIALS AND METHODS

2.1. Fish oil preparation and storage

The crude fish oil was provided by AFAMSA, a Spanish food company located in Vigo (Spain). The antioxidant extract came from three different species of plants: lemon balm (*Melissa officinalis*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*). They were provided by Soria Natural (Soria, Spain). The lemon balm, sage

and rosemary extracts were homogenized with fish oil at 0 % (control), 2 % and 5 % (w/w). The samples were storage with light at 20 ºC along 42 days. Each experiment was conducted in triplicate.

2.2. Determination of Antioxidant Capacity by free radical scavenging by the Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed essentially as described by Huang et al. (2002). Briefly, AAPH (0.414 g) was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM and made fresh daily. A fluorescein stock solution (4 x 10^{-3} mM) was made in 75 mM phosphate buffer (pH 7.4) and stored wrapped in foil at 5 °C. Immediately prior to use, the stock solution was diluted 1:1000 with 75 mM phosphate buffer (pH 7.4). The diluted sodium fluorescein solution was made fresh daily. A Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) was used with a 485 nm, 20 nm bandpass, excitation filter and a 528 nm, 20 nm bandpass, emission filter. The plate reader was controlled by KC4™ version 3.4. In regards to the plate usage, the exterior wells were not used for experimental determinations. These wells were filled with 300 µL of water, while the interior wells were used for experimental determinations. To experimental wells, 150 µL of working sodium fluorescein solution was added. In addition blank wells received 25 µL of 75 mM phosphate buffer (ph 7.4), while standards received 25 µL of Trolox dilution and samples received 25 µL of sample. The plate was then allowed to equilibrate by incubating for a minimum of 30 minutes in a Synergy HT Multi-Detection Microplate at 37 °C. Reactions were initiated by the addition of 25 µL of AAPH solution (153 mM) followed by shaking at maximum intensity for 10 seconds. The fluorescence of each well was then measured from the bottom every 60 seconds at a sensitivity setting of 60. ORAC values were calculated as described by Prior and Cao (1999). The AUC and the Net AUC of the standards and samples were determined using KC4™ Data Reduction

Software (BioTek Instruments, Winooski, VT, USA) using equations 1 and 2 respectively.

AUC = 0.5+(R2/R1)+(R3/R1)+(R4/R1)+…….+ 0.5(Rn/R1) (Eq. 1)

Where R1 is the fluorescence reading at the initiation of the reaction and Rn is the last measurement.

Net AUC = AUCsample – AUCblank (Eq. 2.)

The standard curve was obtained by plotting the Net AUC of different Trolox concentrations against their concentration. ORAC values of samples were then calculated automatically using the KC4 software to interpolate the sample's Net AUC values against the Trolox standard curve.

2.3. Determination of rosmarinic acid

Natural extracts were analysed by reverse-phase HPLC-MS using a ThermoQuest Finningan instrument operating in electrospray positive mode. A linear gradient elution was performed using water:trifluoroacetic acid (97:3) as solvent A and acetonitrile:methanol (80:20) as solvent B in a C18 Symmetry column. Peak was identified by comparing the relative retention time with this of standard and by its mass spectra.

2.4. Fatty acid analysis

The fatty acids profile was determined by the AOAC method (1995). The fatty acid methyl esters were firstly prepared and then analyzed by gas chromatography (GC) in a Hewlett Packard gas chromatograph (6890 N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector. The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 ºC during 20 min, heated to 200 ºC at 1 ºC/min, held at 200 ºC during 1 minute, heated again to 220 ºC at 5 ºC/min and finally held at 220 ºC for 20 min. A split injector (50:1) at 250 ºC was used. The FID was

also heated at 250 ºC. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co.). Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (AOAC, 1995). Calibration curves were made for each pair internal standard + chromatographic standards in order to find the corresponding response factors.

2.5. Oil oxidation

The effects of antioxidants extracts were examined in fish oil. Fish oil was analysed immediately after reception (day 0) and along 42 days of storage under pro-oxidative conditions (20 ºC, sunlight) without antioxidant and with two different concentrations of antioxidant extract (2 % and 5 %).

The intensity of the oxidative process was evaluated by means of various parameters, including free fatty acids (FFA), fatty acids profile, peroxide value (PV), anisidine value (AV), totox value, volatile compounds and odour fingerprint by electronic nose sensor responses.

Free fatty acids were determined by the official method Ca 5a-40 (AOCS 1990).

Peroxide value was determined according to the AOCS method Cd 8-53 (AOCS 1990).

Anisidine value was analysed according to the British Standard method BS 684-2.24 (BSI 1998).

Totox value was calculated as 2PV+AV according to Shahidi and Wanasundra (2002).

Volatile compounds were analysed by Solid Phase Dynamic Extraction (SPDE) sampling and GC-MS. SPDE (Chromtech, Idstein, Germany) was performed after equilibration of the samples for 1 min at 70 ºC. The interior of the SPDE needle was coated with PDMS-AC. Gas chromatographic analyses were performed with an Agilent 6890N Series GC System coupled to an Agilent Technologies 5973i mass spectrometer (Agilent, Palo Alto. CA.USA). The SPDE syringe was then removed from the sample vial and immediately inserted into the “gas station” port where 50 µL of carrier gas

(helium) were pulled into the SPDE-syringe for VOC desorption during 30 s and pumped into the GC-inlet (heated to 250 ºC, splitless mode) at 15 µL/s. Postdesorption bake-out of the neddle at 250 ºC during 10 min ensured full desorption of all analyses from the needle sorbent, thus avoiding carry-over between injections (González, 2008). The compounds were separated in a 007-5MS capillary column (50 m x 0.32 mm I.D fused silica capillary column coated with 1.05 µm film thickness (Quadrex Corporation. New Haven. USA)). The column temperature was increased at a rate of 3 ºC/min from 40 to 240 ºC. Effluent from the capillary column went directly into the mass spectrometer and as the volatile compounds were identified their mass spectra were compared with those in the NIST and the Wiley spectrum libraries.

Odour fingerprint was determined by an electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors. The vials with samples were incubated under agitation (cycles 5 s on and 2 s off and 500 rpm) in an oven at 50 ºC for generating the equilibrated headspace. The injection temperature was 60 ºC. Nitrogen was used as the carrier gas through the injection chamber at a flow rate of 150 mL/min, and synthetic air was used to maintain the thermal stability of the sensors.

2.6. Sensory analysis

Method development

Ten trained panellists of the internal panel of the University of Burgos participated in the sensory study. They were selected based on their experience in detecting odours and on their availability to participate in testing sessions over the period of this study. Training sessions were performed with different commercial samples to familiarize the judges with the products, to develop a preliminary list of sensory attributes and to establish a testing procedure. In order to obtain a vocabulary including terms that describe possible off-flavours, one part of the samples were previously artificially oxidized.

This preliminary work led to a standardized sensory language for the description of oxidative changes in fish oil during storage. Ten attributes were defined: fish, rancid,

boiled, acid, sweet, herb, fruit, lemon balm, rosemary and sage. Each attribute was provided with a quantitative reference for concept alignment.

Sensory sample description during storage

The fish oil was described using a descriptive analysis technique. During training sessions, the standardized language, developed in the preliminary phase, was checked for completeness. Moreover, the panel was further familiarized with the testing procedure. For formal testing, samples were presented simultaneously in randomized order in 3 digit coded vials at room temperature. The intensity of attributes was assessed on 5 point intensity scales, where 1 corresponding to low intensity and 5 to high intensity. The testing sessions were conducted in separate booths under standardised light conditions. All the panellists evaluated each sample, corresponding to 7, 14, 28 and 42 days of storage.

2.7. Statistical analysis

All data were evaluated with STATGRAPHICS PLUS using ANOVA to compare the data for each oil sample for significant change (95% confidence interval). In all the tables, appropriate letters indicate statistical significance.

3. Results and Discussion

3.1. Antioxidant characterization

Radical Scavenging Capacity and rosmarinic acid content

ORAC values varied from 656.45 to 812.74 µmol Trolox equivalent per mL of sample. The plant extract that showed the highest antioxidant capacity was rosemary (812.74 µmol Trolox equivalent/mL), followed by the sage extracts (681.21 µmol Trolox equivalent/mL). Lemon balm extract showed the lowest antioxidant potential (656.45 µmol Trolox equivalent/mL). The ORAC assay requires expensive equipment and is long time consuming, but is to date the only method that takes free radical action to

completion and uses the AUC technique for quantisation. It thus combines inhibition percentage and the length of inhibition time of the free radical action by antioxidants in a single quantity. Moreover, this assay is considered to be more significant as it uses a biologically relevant radical source. High correlation was found between the ORAC assays and the prevention against oxidation of the different extracts (Huang et al. 2005, Prior et al. 2005).

The difference in antioxidant activity between lemon balm, sage and rosemary extracts may be largely due to the different types of phenolic compounds present in the extracts (Cuvelier et al. 1996, Farhat 2009, Maqsood 2010). It was reported that antioxidant capability of rosemary extract was due to rosmarinic acid, which is a flavonoid with high antioxidant activity (Erkan et al. 2008, Frankel et al. 1996). Then, only rosmarinic acid was determined in the extracts. Lemon balm extracts had significantly higher rosmarinic acid concentrations (3.10 %) as compared to rosemary extracts (1.85 %) and sage extract (0.85 %). According to Cuvelier et al. (1994) rosmarinic acid is the most abundant caffeic dimer in *Salvia* species. Several studies have reported on the detection and quantification of rosmarinic acid in various sage preparations (Janicsák et al. 1999). Shahidi et al. (1992) suggested that antioxidant capacity of substances may not be solely characterized by the total phenolic content without consideration of the presence and abundance of specific phenolic components and their particular structural characteristics.

Volatile compounds of natural extracts

The volatile compounds of the natural antioxidant (lemon balm, rosemary and sage) are summarized in Table 1. Twenty-seven constituents were identified. The principal volatile compounds in lemon balm extract were linalool, benzaldehyde and benzene acetaldehyde. As it is known from the literature the major components in *Melissa officinalis* are aldehydes such as citronellal and neral (Basta et al. 2005). Other aldehydes have also been detected in the lemon balm extract, except citral or citronella that were not detected.

The main compounds in sage extract were the following: linalool, γ-terpinene and α-terpineol. Dzamic et al. (2008) reported the chemical composition of wild *Salvia*

sclarea. According to this study the main constituents of the extracts were linalyl acetate, linalool and α-terpineol.

Table 1. Volatile Compounds (AAU x 10^6) of natural extracts (*Melissa officinalis*, *Rosmarinus officinalis*, *Salvia officinalis*) by SPDE GC-MS.

Volatile compounds	RIa	Characteristic odour	Lemon balm	Rosemary	Sage	Identification
Camphene	976	Oily, sweet	-	0.88	1.97	GC-MS + LRI
β-Myrcene	999	Herbaceous citrus taste	8.62	0.93	6.00	GC-MS
Benzaldehyde	1011	Floral, sweet, vainilla, herbaceous	15.20	5.08	4.52	GC-MS
3-Octanol	1013	Herbaceous, oily-nutty mushroom odour	-	-	12.77	GC-MS + LRI
α-Phellandrene	1031	Fresh, spicy, citrus, woody	1.28	-	9.52	GC-MS + LRI
α-Terpinene	1040	Refreshing, lemos-citrus, terpene odour	1.37	-	14.50	GC-MS + LRI
(Z)-β-Ocimene	1049	Herbaceous	2.54	-	1.56	GC-MS + LRI
Limonene	1064	Sweet minty	9.08	-	-	GC-MS + LRI
(E)-β-Ocimene	1050		7.96	-	-	GC-MS
1,8-Cineole	1064	Woody, fresh, fruity note	-	27.13	1.94	GC-MS + LRI
γ-Terpinene	1081	Herbaceous citrus like terpene odour	-	-	17.70	GC-MS + LRI
Benzene acetaldehyde	1093		23.78	9.31	4.57	GC-MS
(Z)-Linalool oxide	1095		10.56	-	-	GC-MS + LRI
Terpinolene	1108		3.45	2.30	6.16	GC-MS + LRI
(E)-Linalool oxide	1113		3.97	-	-	GC-MS
Linalool	1119		26.26	2.60	30.54	GC-MS + LRI
(Z)-Rose oxide	1134		25.70	1.50	-	GC-MS + LRI
α-Thujone	1145		-	-	3.52	GC-MS + LRI
(E)-Rose oxide	1153		9.96	-	-	CG-MS + LRI
β-Thujone	1157	Spicy, cinnamon, green, sweet	-	-	1.15	GC-MS + LRI
Camphor	1200		-	36.42	8.23	GC-MS + LRI
Borneol	1221		-	20.88	-	GC-MS + LRI
α-Terpineol	1236		8.56	14.81	15.30	GC-MS + LRI
α-Copaene	1409		-	0.20	-	GC-MS + LRI
Caryophyllene	1465		-	2.77	2.77	GC-MS + LRI
δ- Cadinene	1554		-	-	1.33	GC-MS + LRI
α-Calacorene	1593		-	4.99	-	GC-MS

[a] Retention indices relative to C_8-C_{20} n-alkanes on 007-05NS column. [b] Characteristic odour according to Flavour Base 2007 by John C. Leffingwell (Leffingwell & Associates). [c] Method of identification: MS, mass spectrum comparison using Wiley and Nist libraries; LRI, Kovats index in agreement with literature values. AAU: Arbitrary Area Unit. Values are means (area units x 10^6) of three analyses

The principal volatile compounds in rosemary extract were camphor and 1,8-cineole, followed by borneol and α-terpineol. Other components such as camphor, 1,8-cineole and borneol were also reported to be present in *R. officinalis* (Papageourgiou

et al. 2008, Pino et al 1998). According to Diaz-Maroto et al. (2007) in rosemary some attributes as fresh, vegetable, pine and herbaceous decrease, but others as hay-like, earthy, woody and infusion increase. In this study, samples with rosemary decreased their characteristic herb attribute along time.

3.2. Fatty acid composition

Table 2 shows the fatty acid composition of the fish oil at day 0 without antioxidant and fish oil with and without antioxidant after 42 days of storage. PUFAs constitute the majority of fatty acids, followed by the saturated and MUFAs in the fish oil before storage. After 42 days of storage, saturated constitute the majority of fatty acids, followed by monounsaturated and polyunsaturated due to the oxidation of polyunsaturated fatty acid along storage. After 42 days, a decrease of the n-3 polyunsaturated fatty acids content was recorded (Table 2). This fact could be related to the oxidation of these compounds. Saturated and monounsaturated acids presented a relative increase due to the decrease of the n-3 acids previously mentioned. Changes in the fatty acids composition were also analyzed in oil with extracts.

The effect of storage time and protective effect of different natural antioxidant on the concentration and lipid and fatty acid compositions of fish oil were studied. Fatty acids were analyzed at 0, 7, 14, 28 and 42 days of storage at room temperature. At day 0, the major fatty acids present in fish oil were DHA (C22:6 n-3), palmitic (C16:0) and oleic (C18:1) acids. DHA was the main polyunsaturated fatty acid in fish oil. DHA content showed a significant decrease ($p < 0.05$) along time of storage, decreasing in 42 days of storage from 22.64 % up to 6.61 %. Palmitic acid, the main saturated fatty acid was present in high concentration (20.74 %) and its concentration was significantly increased after 42 days of storage.

Oleic acid was the dominant monounsaturated fatty acid in fish oil. Oleic acid content significantly increased ($p < 0.05$) after 42 days of storage from 17.06 % to 22.58 % in fish oil sample.

Then 20:5 n-3 and 22:5 n-3 were also reduced, suggesting that all these fatty acids could be involved in the oxidation process. The n-3/n-6 ratio significantly decreased from 7.68 at 0 days to 3.07 at 42 days ($p < 0.05$).

Table 2. Fatty acid composition (g/100 g oil) of fish oil with different antioxidant extracts

Fatty acid	Control Day 0		Fish oil after 42 days of storage													
			Control		Lemon balm				Rosemary				Sage			
					2 %		5 %		2 %		5 %		2 %		5 %	
14:0	3.39	a	4.83	d	3.99	bc	3.92	b	4.04	bc	3.96	bc	4.14	c	3.92	b
16:0	20.74	a	29.01	d	24.13	bc	23.52	b	24.21	bc	23.92	bc	24.89	c	23.68	bc
18:0	7.14	a	9.80	c	8.21	b	7.96	b	8.19		8.14	b	8.44	b	8.04	b
Saturated	31.27	a	43.64	d	36.32	bc	35.40	b	36.44	bc	36.02	bc	37.47	c	35.63	bc
16:1	5.66	a	7.70	d	6.56	bc	6.45	b	6.63	bc	6.51	bc	6.79	c	6.45	b
18:1 n7	2.94	a	4.00	c	3.38	b	3.31	b	3.40	b	3.35	b	3.49	b	3.33	b
18:1 n9	17.06	a	22.58	c	19.24	b	18.91	b	19.46	b	19.28	b	20.22	b	19.15	b
20:1 n9	2.49	a	3.42	d	2.86	bc	2.77	b	2.87	bc	2.84	bc	2.98	c	2.81	bc
22:1 n11	1.43		2.02		1.65		1.73		1.67		1.64		1.08		1.63	
22:1 n9	0.39		0.54		0.44		0.42		0.44		0.43		0.29		0.44	
24:1	0.89	a	1.10	d	0.99	bc	0.94	ab	0.97	abc	0.92	ab	1.03	cd	0.99	bc
Monounsaturated	30.84	a	41.34	e	35.12	bcd	34.52	b	35.41	cd	34.99	bc	35.86	d	34.79	bc
18:2 n6	2.30	a	2.27	a	2.40	ab	2.39	ab	2.41	ab	2.40	ab	2.47	b	2.39	ab
18:3 n3	0.77	c	0.56	a	0.72	b	0.74	bc	0.73	bc	0.72	b	0.74	bc	0.73	bc
18:4 n3	1.00	c	0.60	a	0.85	b	0.88	b	0.84	b	0.84	b	0.86	b	0.85	b
20:4 n6	1.82	c	0.96	a	1.53	b	1.57	b	1.51	b	1.54	b	1.55	b	1.56	b
20:5 n3	7.38	d	2.91	a	5.61	b	5.90	c	5.54	b	5.69	bc	5.64	bc	5.77	bc
22:4 n6	0.25	a	0.48	b	0.92	cd	0.96	d	0.90	cd	0.93	cd	0.92	cd	0.94	cd
22:5 n3	1.74	e	0.66	a	1.31	b	1.37	d	1.28	b	1.32	bcd	1.31	bc	1.35	cd
22:6 n3	22.64	e	6.61	a	15.25	b	16.32	d	14.95	b	15.55	bc	15.21	b	16.00	cd
Polyunsaturated	37.9	d	15.03	a	28.56	bc	30.09	c	28.15	bc	28.99	bc	26.67	b	29.58	c
EPA+DHA	30.02	d	9.51	a	20.86	bc	22.21	c	20.49	bc	21.24	bc	18.84	b	21.76	bc
n3	33.53	d	11.33	a	23.72	bc	25.18	c	23.34	bc	24.12	bc	21.74	b	24.69	bc
n6	4.37	b	3.70	a	4.83	c	4.91	c	4.81	c	4.87	c	4.93	c	4.89	c
n3/n6	7.67	c	3.06	a	4.91	b	5.135	b	4.855	b	4.95	b	4.41	b	5.05	b

Values in the same row with different letters are significantly different ($p < 0.05$)

The present study showed that the n-3 concentration decreased along time of storage due to its high susceptibility to oxidation. Results showed that all the natural antioxidants were able to inhibit oxidation of polyunsaturated fatty acids. The capability to inhibit oxidation was higher at 5 % concentration for the extract.

3.3. Oxidation

Chemical analyses, such as FFA, PV and AV were carried out to monitor oil oxidation under defined storage conditions with or without natural antioxidant. These results are shown in Figures 1-2. In general, it was observed, in all oil samples, that FFA, PV and AV gradually increased.

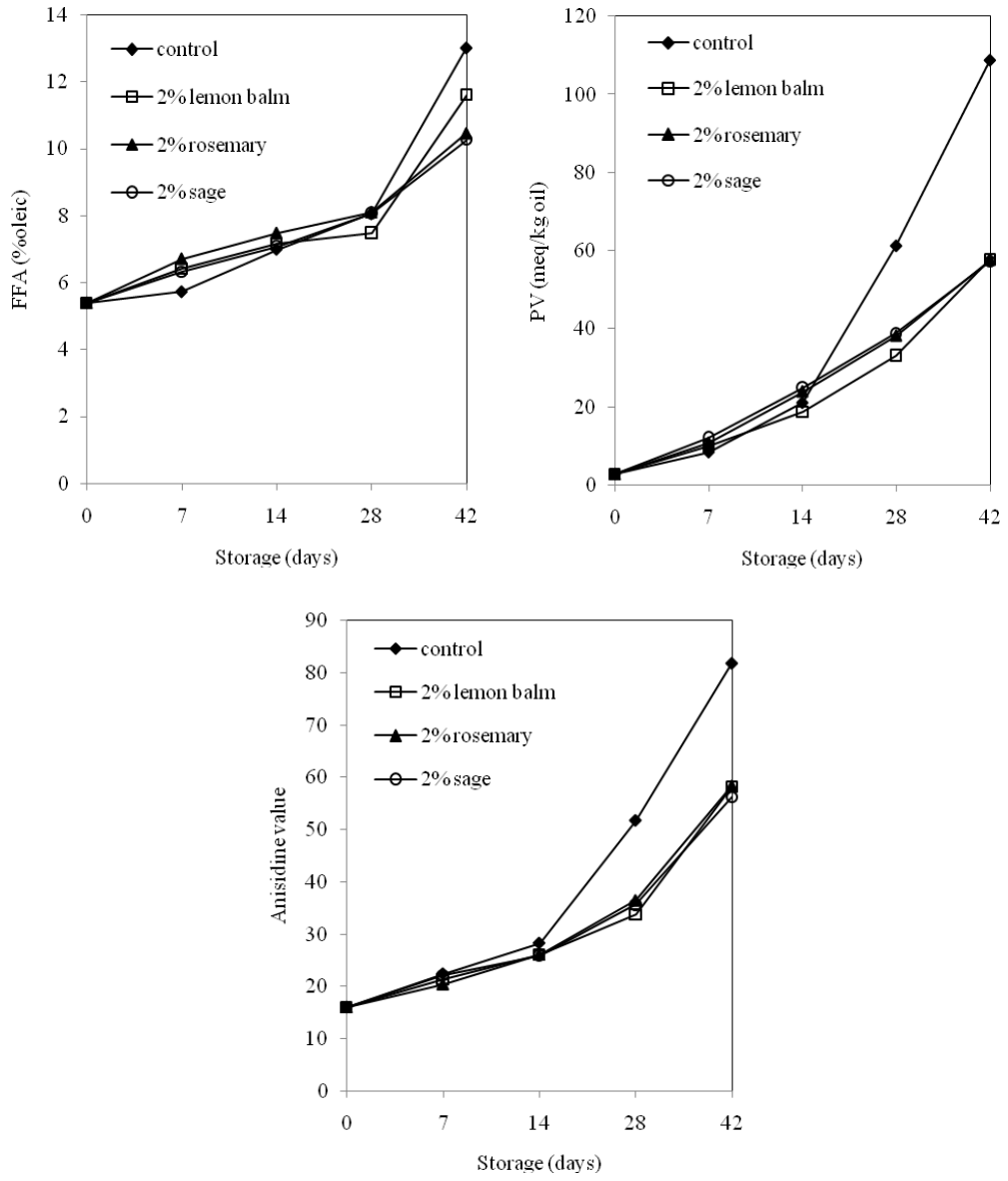

Figure 1. Effect of different antioxidant extract at 2 % concentration on the formation of lipid oxidation products in crude fish oil stored at 20 ºC for a period of 42 days. Free fatty acid (FFA), peroxide value (PV) and anisidine value (AV)

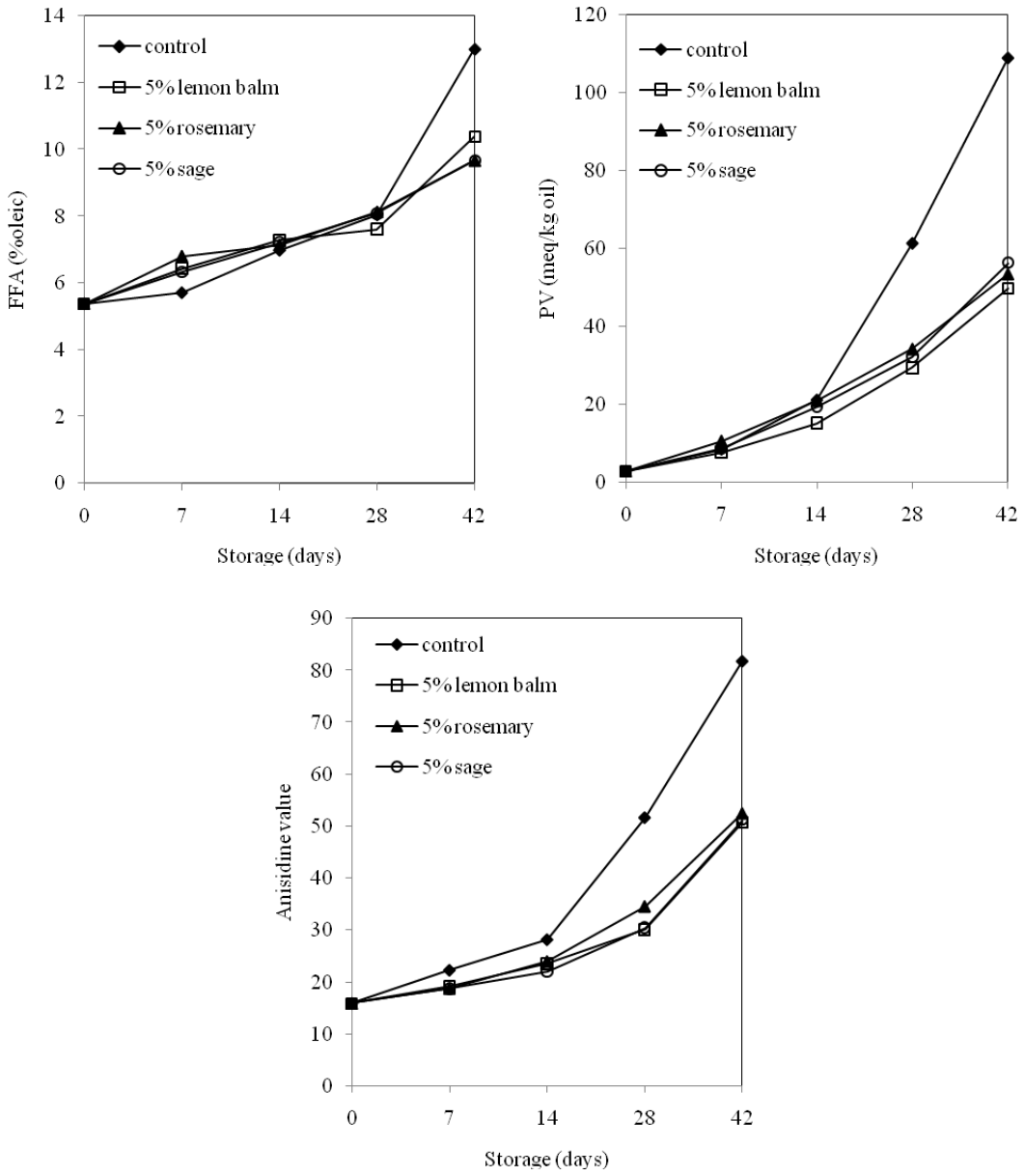

Figure 2. Effect of different antioxidant extract at 5 % concentration on the formation of lipid oxidation products in crude fish oil stored at 20 ºC for a period of 42 days. Free fatty acid (FFA), peroxide value (PV) and anisidine value (AV)

The Free Fatty Acid (FFA) proved to be very sensitive to the time of storage of the fish oil, increasing significantly over time. The amount of FFA in the fish oil with antioxidants was low (Figures 1-2). Increaments in FFA are generally associated with lipase activity originating from microorganism or biological tissue (Boran et al. 2006).

Free fatty acid (FFA) concentration is considered to be an important quality parameter. Quality specifications for crude fish oil state that the FFA content should were lower than the 7-8 % for food-grade fish oil (Bimbo 1998). The initial values of FFA were 5.39 %. Our results showed that this limit was exceeded beyond 28 days of storage, except in lemon balm samples where the limit was exceeded after 42 days of storage.

The Peroxide Value (PV) of fish oil is presented in Figures 1-2. A gradual increase in PV was observed in all samples during 42 days of storage. When comparing PV of all samples, it was found that the control sample contained higher PV than the samples added with natural antioxidant throughout the storage, particularly after 28 days of storage. The acceptability limit for crude oil is 5 meq O_2/kg of oil (GOED 2008), which, in the present study, was reached at days 7 for control and after 14 days in the fish oil with rosemary and sage. Only lemon balm at 5 % was not reached after 14 days. Lemon balm at 5 % was more effective in lowering the increase in PV in fish oil than the other two antioxidant extracts used in the present study ($p < 0.05$). The higher efficiency of lemon balm in prevention of hydroperoxide formation correlated well with the higher rosmarinic acid concentrations. Rosemary and sage extracts showed a similar effect on retarding the formation of PV in fish oil ($p < 0.05$) (Figure 2) but their efficiency was lower than that of lemon balm extract ($p < 0.05$). The highest inhibition of hydroperoxides development was observed in the samples stabilized with 5 % lemon balm.

The impact of natural antioxidant on anisidine value (AV) formation in fish oil during the storage is shown in Figures 1 and 2. The increase in AV indicated the formation of secondary oxidation products. These were more presented in the control samples and was retarded in the samples added with natural antioxidant, particularly lemon balm and sage at 5 % ($p < 0.05$). AV of samples added with rosemary, lemon balm and sage at 2 % concentration was lower than that of the control samples ($p < 0.05$), but was higher than that of samples added with natural antioxidant al 5 %. Sage and lemon balm showed similar efficiency in preventing the lipid oxidation in fish oil, but lemon balm was more efficiency in preventing the hidroperoxide formation. The inhibitory effect of rosemary extract on AV was slightly lower than that of other extracts tested. According to the GOED (2008) the limit of AV is 20. All samples with 2 % of

extract presented high values after 7 days of storage. In the case of 5 % of concentration, all extracts presented high values after 14 days storage except fish oil with 5 % of sage. According to Sullivan Ritter (2012) the PV and AV typically used to indicate fish oil quality have little relationship with sensory properties.

3.4. Odour fingerprint by electronic nose analysis

Using an "electronic nose" to monitor the formation of volatile compounds associated with off-flavours could help to interpret oil oxidation studies as a complement to the sensory panels. The e-nose showed greater sensitivity than the sensory pannel. The first principal component and the second principal component were enough to display the data structure, since they explained 99 % of the total variance. Examining the score plot (Figure 3) in the area defined by the first two principal components, a separation of the samples along PC1 was found, according to the days of storage. Control sample increase the gradient after 7 days of storage. Figure 3 shows that fish oil samples with increasing oil oxidation appear ordered from right to left along the first component.

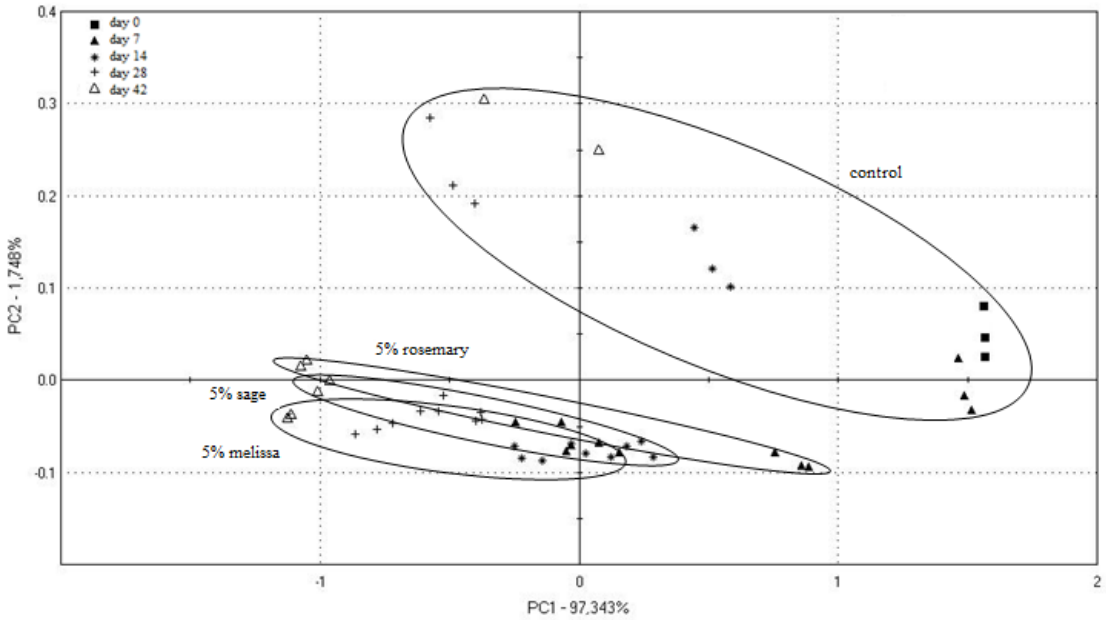

Figure 3. PCA of sensor reponses to fish oil throughout storage at 20 ºC with natural antioxidant

3.5. Sensory analysis

Figure 4 shows the descriptive sensory analysis (DSA) profiles of fish oil with different antioxidant extracts. The study includes four groups of samples: control, lemon balm, rosemary and sage. Control sample (fish oil without antioxidant) was characterized by intense fish and rancid odour. According to Sullivan (2012), numerous factors, including lipid class composition, concentrations of oxygen present, presence of antioxidants and light can also influence hydroperoxide formation and degradation. Being primary oxidation products, hydroperoxides have very little impact on flavour of oils and are primarily measured because they are precursors to compounds that negatively affect sensory characteristics. Measurement of secondary oxidation products is also used to assess oxidation. For instance, AV measure aldehydes with α- and β- unsaturations, however, this is not a sensitive method and there is some uncertainty whether those specific components are linked to oil flavour. It is well documented that sensory properties of fish oil are highly affected by the presence of volatile ketones and alcohols, in addition to aldehydes, that are also breakdown products of hydroperoxides (Frankel 2005). In some cases aldehyde and ketone compounds have a taste and/or odour threshold of less than 1 ppm, well below the detection limits of AV testing.

On other hand, odour notes of the natural extract have impact on flavour of oils. Lemon balm extract was characterized by intense sweet, vegetable, herbaceous and toast odour notes. Rosemary extract was characterized by intense sweet odour, honey notes and spices odour notes and lightly acid. Sage extract was characterized by honey, fruit, aromatic herbs notes and sweet and boiled odour. These descriptors were previously reported in dry rosemary (Díaz-Maroto et al. 2007). The samples stored at light without antioxidant were significantly more easily oxidized than the samples with antioxidant. In this study, differences in rancid odour between samples stored with antioxidant and samples stored without antioxidant were observed in Figure 4 and were explained due to the protective action of natural antioxidant against oxidation of unsaturated fatty acids present. Sweet odour significantly increased after 42 days of storage in samples with rosemary extract. Fish oil with sage extract showed more acid odour than the other

samples. Control and samples with rosemary extract presented higher level of boiled odour than the other samples, but this odour decreased after 42 days of storage.

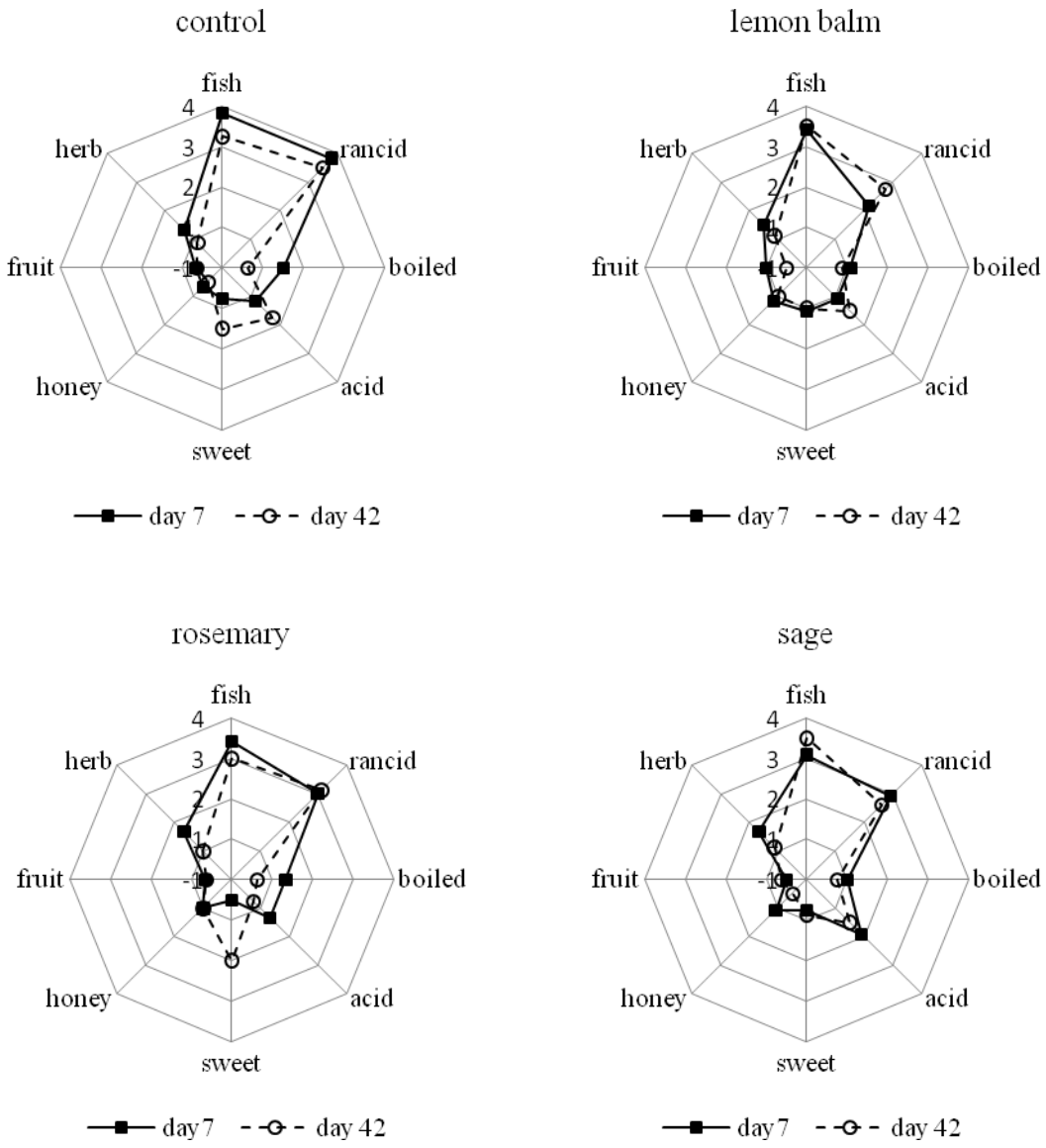

Figure 4. Descriptive Sensory Analysis of odour attributes in crude fish oil with different antioxidants (5% lemon balm, 5% rosemary and 5% sage)

The poor relationship between conventional oxidation testing and sensory parameters draws attention to the need for an alternative method to monitor oil quality. Taste panels are the most accurate method of evaluating sensory qualities of oils, as

humans can detect lower levels of volatile components that traditional tests of oxidation cannot. Unfortunately, the high costs associated with establishing and maintaining a panel makes their use unattractive. In many cases it is difficult to recruit panelists as people are reluctant to participate if the product being tasted has poor sensory properties. An alternative to sensory panels are methods that monitor amounts of volatile secondary oxidation products in the headspace of samples, as these are the compounds most responsible for oil flavours, using techniques such as solid phase microextraction (SPME). While over 200 volatiles have been detected in fish oils, so far only about 20 have been shown to have a relationship with sensory characteristics (Frankel 2005). These volatiles are present at such low levels that they are impossible to detect using conventional quality testing.

3.6. Volatile compounds

The oxidation of PUFA-containing lipids causes the development of off-flavours and odours, often referred to as 'rancidity' in fish. Compounds that have rancid flavours are volatile secondary oxidation products derived from the breakdown of lipid hydroperoxides (Kolanowski et al. 2007a). The main volatile components of fish oil protected with antioxidants can be grouped into several types of compounds; the most important families being alcohols, aldehydes, alkanes, furans and ketones. The results were shown in Table 4.

All the families of volatiles compounds increased during storage time. Eighty-one volatile compounds were identified in the fish oils studied throughout storage, 6 alcohols, 26 aldehydes, 23 alkanes, 12 ketones and 14 furans. The formation of secondary oxidation products such as volatile aldehydes and ketones is responsible for flavour changes. Iglesias et al. (2007) also reported that propanal resulted from the oxidation of n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), although no such volatile compound was found in this fish oil. According to Baigrie (2003), the main aldehyde arising from linoleic acid is hexanal. Hexanal concentration increased in fish oil during storage, undergoing minor changes at fish oil with 5 % of

lemon balm than 5 % of sage was added. There was a large increase of total aldehydes in fish oil, which is probably related to its intense odour throughout storage.

Table 4. Volatile compounds (AAU x 10^6) in fish oil with antioxidants (lemon balm, rosemary and sage) per family groups

	Alcohols		Aldehydes		Alkanes		Furans		Ketones	
Antioxidant										
2% lemon balm	294.66	b	516.81	a	202.23	a	148.50	ab	39.19	ab
2% rosemary	294.86	b	544.52	ab	203.16	a	149.18	ab	41.70	ab
2% sage	321.00	b	586.44	b	217.23	a	167.56	ab	48.21	b
5% lemon balm	234.11	a	547.91	ab	201.51	a	144.87	a	36.91	a
5% rosemary	282.95	ab	574.94	ab	209.84	a	180.68	b	43.86	ab
5% sage	279.44	ab	576.57	ab	212.19	a	157.22	ab	38.92	ab
Days of storage										
0	146.47	a	376.81	a	195.00	a	128.91	ab	11.84	a
7	175.47	a	360.42	a	194.14	a	108.81	a	28.25	b
14	292.90	b	534.98	b	207.69	a	153.15	b	45.13	c
28	523.17	c	959.25	c	233.94	b	241.14	c	80.64	d

Values in the same column with different letters are significantly different ($p < 0.05$)

4. CONCLUSION

The potential use of natural extracts as natural preservative to extent the shelf life of fish oil for future incorporation in functional foods was demonstrated. All samples with natural extracts presented lower oxidation values than fish oil without antioxidant but this antioxidant activity of the natural extracts did not vary considerably with the different types. Lemon balm at 5 % exhibited a little higher antioxidative activity and could prevent lipid oxidation effectively in fish oil. The use of lemon balm at 5 % as an antioxidant in fish oil was superior in reducing the production of primary oxidation products and the use of lemon balm or sage at 5 % was more effective in delaying the production of secondary products and in the reduction on n-3 content than the other antioxidant systems analyzed (rosemary at 5 % or lemon balm, rosemary and sage at 2 %). However, sage extracts also contain flavonoids and others phenolic that may

contribute to the total antioxidant activity. Study of phenolic compounds must be developed and used in order to evaluate the effect of their use as natural food additives. Future work should focus on determining the optimum concentration of antioxidant and optimal condition in fish oil in order to maximize its antioxidizing potential.

A similar evolution along storage was shown in odour fingerprint scores and headspace volatile compounds, these fact could advise the use of electronic nose to monitoring odour modification during storage. The goal was to identify the key oxidation products that are important in distinguishing between acceptable and poor quality oils in order to create a method to monitor fish oil oxidation that correlates well with the sensory characteristics of the oil.

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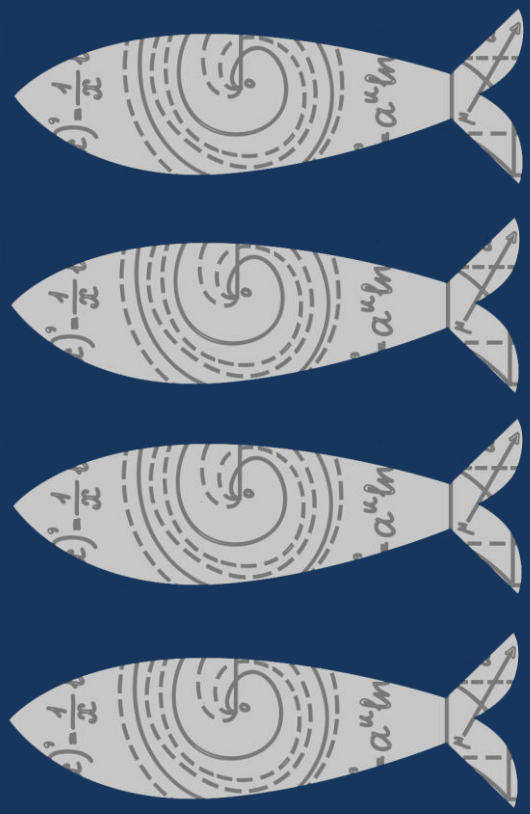

CHAPTER 4

Development of Reduced Fat Pork Sausages Fortified with Fish and Olive Oils: Sensory and Technological Considerations

IV DEVELOPMENT OF REDUCED FAT PORK SAUSAGES FORTIFIED WITH FISH OIL AND OLIVE OILS: SENSORY AND TECHNOLOGICAL CONSIDERATIONS

Abstract

Low fat cooked sausages were manufactured with the substitution of pork fat by olive and fish oils. Oils were added to sausage emulsion in two ways: directly or previously microencapsulated by spray-drying. Five different batches were tested: control, olive oil, microencapsulated olive oil, salmon (*Salmo salar*) oil extracted by supercritical CO_2 and microencapsulated salmon oil. The n-6/n-3 ratio decreased from more than 15 in the control to less than 2.5 in the sausages with salmon oil and with microencapsulated salmon oil. From a sensory point of view is possible to formulate sausages with reducted fat content with fish and olive oils with suitable sensory flavour quality, irrespective of the microencapsulation during one month of storage. Storage determined differences in flavour profile of sausages with salmon oil that could be avoided if oil was added after microencapsulation. Encapsulation increased the stability of fish oil during 60 days. Incorporation of effective antioxidant in combination with microencapsulation could be necessary in order to ensure oxidative shelf stability of fish oil sausages during long storage times.

1. INTRODUCTION

Meat industry is continuously undergoing transformations, driven among other factors by changes in consumer demands. One of the main trends shaping developments in the consumption of meat derivatives is consumer interest in the possibilities of improving health and wellbeing through diet. Low-fat sausages are desirable from a diet/health standpoint as they contain less calories and less saturated fatty acids. Potential positive effects of low-fat sausages could be further enhanced by substituting animal fat with healthier lipids. Olive oil (Delgado-Pando et al. 2010, Jiménez-Colmenero et al. 2010, Moon et al. 2008, Paneras and Bloukas 1994, Paneras et al. 1998, Park et al. 1990) and fish oils (Caceres et al. 2008, Delgado-Pando et al. 2011, López-López et al. 2009a, 2009b, Marchetti et al. 2014, Oliveira et al. 2014, Park et al. 1989) have been used to supply substantial amounts of oleic acid and omega-3 fatty acids in order to produce enriched sausages. In the last years, others new fat replacements have been studied, like linseed oil (Berasategi et al. 2014, Ferreira et al. 2015), tomato peel powder (Wang et al. 2015), inulin and pectin (Méndez-Zamora et al. 2015), inulin and citrus fiber (Tomaschumas et al. 2013), pig skin and wheat fiber (Choe et al. 2013).

Olive oil is one of the most monounsaturated vegetable oils and has a high biological value attributed to a high ratio of vitamin E. Partial substitution of pork fat by olive oil can be used in order to increase the monounsaturated fatty acid content in sausages. Olive oil intake is associated with a decreased risk of cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes and hypertension. Furthermore, it is associated with a reduction in cancer risk (mainly breast, colorectal and prostate cancer) and it may be protective against age-related cognitive decline and Alzheimer´s disease (López-Miranda et al. 2010).

Fish oil is considered an important natural source of omega-3 fatty acids whose benefits on human health has been extensively reported in the literature. Lunn and Teobald (2006) reviewed the role of PUFA in the reduction of cardiovascular disease risk, inflammation and autoimmune disorders, certain types of cancer, and the development of brain and nervous tissue in infants. In 2012, Gil deeply analysed the role of omega-3 fatty acids in the prevention and treatment of disease. On the other hand, Tur

et al. (2012) reviewed the dietary sources of omega-3 and analyzed the public health risks and benefits. Nutritionists have recommended an increment of omega-3 consumption in order to maintain the omega-6/omega-3 ratio in a normal diet between 5:1 and 10:1 (Simopoulos 2002). It is important to find abundant, natural and economic sources of n-3 PUFA. The by-products from the fish industry could be an interesting option. Several technologies involving solvents and high temperatures are currently used to extract fish oil (Rubio-Rodriguez et al. 2010). However, a reliable alternative to obtain good quality fish oil from these by-products is Supercritical Fluid Extraction (Rubio-Rodriguez et al. 2008). The most widely used supercritical solvent is carbon dioxide, which is non-toxic, cheap and has mild critical conditions, being suitable for processing thermo-degradable compounds such as PUFA. As it has been described in chapter two, there is a high variability in content and characteristics of oil extracted by supercritical CO_2. Hake by-products could be a good source of rich omega-3 oil taking into account their composition; however the extraction yield was not so good. Salmon oil by-products could be a more suitable source of omega-3 fatty acid due to their higher content of fat. The problem of n-3 PUFA is to be prone to oxidation because of the high number of unsaturated double bonds. This factor limits the shelf life of these products.

Microencapsulation has emerged as a potential technology to offset the ingress of oxygen that could promote lipid oxidation (Kolanowski et al. 2006). There are a number of techniques used for fish oil microencapsulation, being spray-drying the method with the lowest costs; other techniques are fluidizing bed, freeze-drying and multilayer emulsions (Grigoriev and Miller 2009, Shaw et al. 2007). During the microencapsulation process, fish oil microdroplets are coated with various materials creating the wall of the microcapsules. Various hydrocolloids are used as coating materials, including combinations of gelatin, maltodextrins or other carbohydrates, starch, etc. as well as plant gums, skimmed milk powder and milk proteins. Spray drying has been shown to be an effective technique for the protection of vitamins, minerals, flavours, preservatives and leavening agents (Arshady 1993). Microencapsulation of fish oil produces a dry powder from liquid fish oil, which enables its use for the enrichment of instant foods, infant formulas, bread, etc. in order to deliver the omega-3 fatty acids to the diet. One of the challenges facing the incorporation of fish oil in non-fish containing meat products is the

undesirable presence of residual fishy odours and taste. This may be offset by the encapsulating material which creates a physical barrier between the fish oil and the inside of the mouth (Keenan et al. 2015).

The aim of this study was to evaluate the effect of addition of encapsulated and non-encapsulated fish and olive oils on the physico-chemical and sensory characteristics of low-fat sausages.

2. MATERIAL AND METHODS

2.1. Extraction of salmon oil by CO_2 supercritical fluid

Salmon oil was extracted in a semi-pilot supercritical fluid plant. Salmon by-products were provided by Pescanova S.A. The by-products were cut into small pieces (1-10 mm equivalent diameter, D_e), with a cutter (CATO, Spain), in order to improve the extraction rate. The water of by-products was removed before extracting the oil by freeze-drying (FreeZone 12 L Console Freeze Dry System with drying chamber, Labconco). Approximately 200 g of freeze-dried salmon by-product were placed in the extractor that was later pressurized up to the extraction pressure of 25 MPa, with carbon dioxide (Carburos Metálicos, liquid $CO_2 \geq 99.9$ %). Then, the solvent was circulated at the desired extraction temperature of 40 ºC, with a flow rate of 10 kg CO_2/h during 3 hours. The solvent was continuously recycled to the extractor after removing the solute in the separator where the solvent power of CO_2 was reduced by reducing pressure down to approximately 5 MPa according to Rubio-Rodriguez et al. (2008).

2.2. Production of fish oil and olive oil emulsion for microencapsulation

Two powders were prepared on the basis of formulation containing 20 % oil in powder. Olive oil was obtained from a Spanish retail store (FFA < 1º, PV < 15 meq O_2, wax ester < 300 mg/kg). The ratios of maltodextrin/sodium caseinate/oil in emulsion were 2:2:1. To obtain the emulsions, initially sodium caseinate were dissolved in water. Subsequently, maltodextrines were added to the solution very slowly, while stirring vigorously using a homogenizer (Ultraturrax T25). The oil and 0.4 % of emulsifier (tween 80) were then added to the mixture and the mixture was stirred for 5 min.

2.3. Spray drying

A spray-dryer (Mini Spray-dryer B-290, Buchi, Flavil, Switzerland) was used to convert infeed emulsions into encapsulated powders. The operational conditions of the spray drying were air inlet temperature of 180 ºC, nitrogen outlet temperature of 110 ºC. The dried powder was collected and stored in glass bottles at 20 ºC in prooxidant conditions during 120 days in order to evaluate their oxidation.

2.4. Microencapsulation parameters

Extraction of total oil

The procedure was based on the Rose-Gottlieb method (Richardon, 1985), widely accepted for quantitative determination on fat in milk and milk powders. Briefly, 1 g of dried ingredient was weighed and dispersed in 10 mL of water at 65 ºC. After shaking, 2 mL NH_4OH 25 % were added and the solution was heated at 65 ºC for 15 min in a stirring water bath. Then, the solution was cooled, transferred to a separator funnel and the flask rinsed with 10 mL ethanol. Oil was extracted three times with, first, 25 mL diethyl ether and 25 mL petroleum ether, second, 5 mL ethanol, 15 mL diethyl ether and 15 mL petroleum ether and third, the same solvents without adding ethanol. After

filtration through anhydrous Na_2SO_4, solvents were evaporated under reduced pressure and sample dried to constant weight using a stream of nitrogen.

Extraction of non-encapsulated oil

The determination of non-encapsulated oil was performed using the method described by Westergaard (2004). Briefly, 10 g of sample were weighed into a screw capped flask. Petroleum ether (50 mL) was added and the sample was mixed slowly for 15 min. The dispersion was filtered and 25 mL of the filtrate were evaporated to dryness. The residual oil was weighed and the percentage of extractable oil was calculated.

Encapsulation efficiency (MEE %)

Microencapsulation efficiency (MEE %) was determined as a function of the encapsulated oil related to the total oil of the microcapsules. For that, the external and total oil of the microcapsules were quantified, and the MEE % was calculated with the equation provided by Velasco et al. (2006):

MEE (%) = (total oil – external oil) / total olive/fish oil) x 100

2.5. Oxidative stability of salmon oil microencapsulated

Rancimat method

The oxidation stability test was conducted according to the Rancimat method according to the AOCS method Cd 12b-92 (AOCS 1992) with Rancimat 743 (Metrohm, Herisau, Schwizerland). A stream of air (20 L/h) was blown through the sample (3 g of oil) at a temperature of 100 °C. Easily volatile oxidation products, which were formed, were transferred by the stream of air into a measuring vessel containing deionized water whose conductivity was continuously measured. Plotting the conductivity as a function of time produces the oxidation curve, whose point of inflection is known as the induction time of oil oxidation. Longer induction time indicates higher stability of oil. The

difference between the induction time of salmon oil and microencapsulated salmon oil is the basis for evaluation of the effectivity of the microencapsulation process. The effect of microencapsulation is expressed as a protection factor and is calculated as a ratio of the induction time of the oil stabilized by microencapsulation and the induction time of the non-microencapsulated salmon oil:

Protection factor = Induction time of stabilized sample / Induction time of control sample

A protection factor greater than 1 indicates inhibition of lipid oxidation. The higher the value, the better the antioxidant activity.

Peroxide value

The stability of salmon microcapsules in comparison with non-encapsulated salmon oil stability was evaluated for 120 days at prooxidant temperature 20 ºC. Lipid oxidation was monitored through the analysis of peroxide value. Peroxide value was determined according to the AOCS method Cd 8-53 (AOCS 1992).

2.6. Sausages formulation and processing

Five batches of low fat sausages were manufactured: control, olive oil, microencapsulated olive oil, salmon oil and microencapsulated salmon oil (Table 1). Fish oil sausages were formulated in order to cover a quarter of the average consumption per day of omega-3 fatty acids according to the nutritional advice of 500 mg/day (FAO/WHO 2000), considering a serving of 125 g of sausages. Pork meat and fat obtained from a local slaughterhouse were frozen, separately, at -20 ºC one week prior to use. The lean meat was thawed slowly at 0 ºC and the fat was tempered (-2 ºC). Both materials were chopped in a 25 L cutter (Cato, Sabadell, Spain) to particles of about 3 mm diameter. The lean meat (40.0 %) was mixed with the soya protein (2.0 %), the phosphate (0.3 %) and the salt (1.6 %) and about 1/3 of the water was added in the form of ice (38.0 %). The batter was chopped at high speed. Then, tempered fat and 1/3 of the ice were added and

mixed at high speed. Finally, the remaining ice and ingredients (0.1 % carboximetilcelulose, 1.0 % milk powder, 0.8 % flavour, 3.5 % potato starch, 0.1 % sodium ascorbate, 1.0 % lactate, 0.3 % dextrose) were added in the cutter and mixed to obtain a suitable emulsion. The maximum temperature reached during the process was 10 ºC. The mixture was stuffed into 22 mm cellulose casings and the sausages were placed in an oven (Eller, Meran, Italy).

Table 1. Formulation (g/kg) of the different low fat sausages with fish and olive oils

Sausage formulation	Control	Olive oil	Microencapsulated olive oil	Salmon oil	Microencapsulated salmon oil
Lean pork meat	400.0	400.0	400.0	400.0	400.0
Fat meat	102.0	95.3	68.7	95.3	88.7
Olive oil	-	6.7	-	-	-
Salmon oil	-	-	-	6.7	-
Microcapsules oilve oil	-	-	33.2	-	-
Microcapsules salmon oil	-	-	-	-	33.2
Ice	380.0	380.0	380.0	380.0	380.0
Phosphate	3.0	3.0	3.0	3.0	3.0
Salt	16.0	16.0	16.0	16.0	16.0
Carrageenan	5.0	5.0	5.0	5.0	5.0
Carboximetilcelulose	1.0	1.0	1.0	1.0	1.0
Milk powder	10.0	10.0	10.0	10.0	10.0
Caseinate	8.0	8.0	-	-	-
Potato starch	35.0	35.0	10.0	10.0	10.0
Soya protein	20.0	20.0	-	-	-
Flavour	7.0	7.0	7.0	7.0	7.0
Sodium ascorbate	0.5	0.5	0.5	0.5	0.5
Lactate	10.0	10.0	10.0	10.0	10.0
Dextrose	2.5	2.5	2.5	2.5	2.5

A four-step process was performed: drying at 55 ºC for 15 min, smoking at 60 ºC with a HR of 75 % for 15 min, cooking at 75 ºC until the internal temperature was 72 ºC, and finally cooling by spraying with cool water until an oven temperature of 15-20 ºC was reached. The casings were removed and the sausages were vacuum packed,

pasteurised in hot water at 75 ºC for 45 min, and then chilled in water at 10 ºC. The products were kept under refrigeration at 4-5 ºC, and were analysed in the same week as they were manufactured.

2.7. Microbiological analyses

The sausages were subjected to microbiological analysis to monitor the dynamic changes of the spoilage populations during storage. A 25 g sausage portion from each treatment was homogenized in a Stomacher (Stomacher 400, Lab. Blender, London, UK) for 120 s with 225 mL sterilized saline peptone water: NaCl 8 g/L, bacteriological peptone 1g/L, (Oxoid, Basingstoke, UK), prior to the preparation of 1/10 serial dilutions for microbiological analysis. The following microorganisms were determined: total viable count (TVC) plated on PCA agar (Pronadisa, Madrid, Spain) and incubated at 30 ºC for 48h; Lactic Acid Bacteria (LAB), grown in MRS agar (Man, Rogosa and Shaper agar, Oxoid) and incubated anaerobically in 6 % CO_2 at 30 °C for 48 h; *Clostridium perfringens*, tested by inoculating in TSN agar (Tryptone Sulfite Neomycin, Biokar Diagnostics, Beauvais, France) and incubated at 45 ºC for 24-48 h in anaerobic conditions; *Bacillus cereus*, grown in MYP agar (Mannitol Egg Yolk Polymyxin, Oxoid) and incubated at 30 ºC for 24-48 h; and finally *Brochothrix thermosphacta* tested in STAA agar (Streptomycin Sulphate Thallous Acetate Actidione, Oxoid) and incubated at 22 ºC for 48-72 h. After counting, means and standard deviations were calculated.

2.8. Chemical analysis

Water and protein content were determined by the AOAC Official Methods 934.01 and 981.10, respectively, and total fat content was determined by Soxhlet method using petroleum ether as solvent in a Büchi extraction system (model B-811, Flawil, Switzerland).

pH was measured by blending 10 g of products with 10 mL of distilled water for 2 min. A pH meter 507 (Crison, Barcelona, Spain) equipped with a glass electrode was

used for this measurement. Water activity (a_w) was measured by CX2 AQUA LAB equipment (Decagon, Washington, USA).

Fatty acids were determined on the lipid extract from sausages. The Bligh and Dyer (1959) method was used for the lipid extraction. The fatty acids profile was determined by the AOAC method (AOAC 1995). This method required the esterification of the fatty acids to their methyl esters, which were analyzed by Gas Chromatography (GC) in a Hewlett Packard gas chromatograph (6890 N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector. The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 ºC during 20 min, heated to 200 ºC at 1 ºC/min, held at 200 ºC during 1 minute, heated again to 220 ºC at 5 ºC/min and finally held at 220 ºC for 20 min. A split injection 50:1 was used. Injector and FID were kept at 250 ºC during analysis. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co.). Their quantification was made by relating the peaks area to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (AOAC 1995). Calibration curves were obtained for each pair internal standard + chromatographic standards in order to find the corresponding response factors.

2.9. Colour

Surface colour measurements in sausages were performed using a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japan). CIE *L*a*b** colour parameters (considering illuminant D65 and standard observer 10º) were then calculated. Colour changes were measured by the lightness (*L**) and the coordinates greenness–redness (*a**) and blueness–yellowness (*b**). Colour measurements were made at days 0, 30 and 90 in sausage samples (n=3).

2.10. Instrumental texture measurement

Instrumental texture profile analysis (TPA) (Breene 1975) was performed with a texture analyzer TA-XT2 (Stable Micro Systems, Haslemere, UK). The Texture Expert, version 1.20, computer program by Stable Micro Systems was used for data collection and calculations. Ten cubes of sausages (1x1x1 cm) were compressed twice with a cylindrical probe of 4.5 cm diameter, at 1 mm/s speed and the level of compression was 60 % of the thickness of the sample. The test was always accomplished in a conditioned room at 21±1 ºC. The parameters determined from the force-time curves were hardness, springiness, cohesiveness and chewiness. Hardness was defined by peak force during first compression cycle and expressed in grams. Springiness was defined as a ratio of time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve. Chewiness was obtained by multiplying hardness, springiness and cohesiveness and expressed in grams.

2.11. Electronic nose

Odour fingerprint was determined by an electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors. 1.0 g of sample was transferred into 10 mL glass vial. The vials with samples were incubated under agitation (cycles 5 s on and 2 s off at 500 rpm) in an oven at 50 ºC for generating the equilibrated headspace. The injection temperature was 60 ºC. Nitrogen was used as the carrier gas through the injection chamber at a flow rate of 150 mL/min, and synthetic air was used to maintain the thermal stability of the sensors.

2.12. Sensory evaluation

The sausages were sensory evaluated by a 15 member sensory panel.

The evaluations were performed in individual booths under standardised white light. The assessors were given toasts without salt and water to clean the palate and neutralize the taste between samples. The samples, initially vacuum packed and held under refrigeration, were heated to 70 ºC internal temperature in a microwave oven. Samples were divided in pieces of 10 mm thickness, cutting off the ends to get homogeneous pieces. Assessors were served hot slices of sausage from each treatment. The following attributes were tested: colour, odour (smoked, vegetal and fishy or others), texture (hardness and chewiness), and taste (salty, smoked, vegetal and fishy). A seven point structured scale was used for scoring the intensity; 1 corresponded to absence for each attribute and 7 to the highest intensity.

2.13. Statistical analysis

Data were analysed using Statgraphics Plus 5.1 (STSC Inc. Rockville, MD, USA). The effect of each formulation was tested by ANOVA one-way. Tukey test was used to identify significant differences ($p < 0.05$) among means.

3. RESULTS AND DISCUSSION

3.1. Salmon oil obtained by Supercritical CO_2 extraction

It is known that the fat content of different fish species by-products is extremely variable, depending on several factors. In a previous experiment (Chapter 2), rich omega-3 fatty acids oil was extracted from hake by-products with a extraction yield fairly low (3.9 % oil). Salmon by-products could be a more suitable source of omega-3 fatty acids since that present high extraction yield (57.0 % oil) and similar amount of these fatty acids than the hake oil (Table 2).

Table 2. Fatty acids profile of salmon oil obtained by SFE from salmon by-products

Fatty acid	mg fatty acids/g oil
C14:0	40.4 ± 0.1
C16:0	143.0 ± 0.4
C16:1	59.0 ± 1.0
C18:0	46.4 ± 0.1
C18:1 n9	146.0 ± 2.0
C18:1 n7c	28.9 ± 0.1
C18:2 n6	93.0 ± 1.0
C18:3 n6	5.5 ± 0.1
C18:3 n3	14.0 ± 0.2
C18:4 n3	5.9 ± 0.1
C20:1 n9	13.1 ± 0.4
C20:3 n6	3.2 ± 0.2
C20:4 n6	6.7 ± 0.1
C20:5 n3	79.0 ± 1.0
C22:5 n3	38.4 ± 0.7
C22:6 n3	63.0 ± 1.0
C24:1	2.5 ± 0.1
ΣSFA	230.0 ± 1.0
ΣMUFA	250.0 ± 3.0
ΣPUFA	309.0 ± 5.0
Σn3	200.3 ± 3.0
Σn6	108.4 ± 2.0

3.2. Microencapsulation effectiveness

The values of encapsulation efficiency are shown in Table 3. The total oil incorporated to the microcapsules was slightly higher than 20 % in the two types of oil microcapsules. The non-encapsulated oil was lower than 2 % in both samples, and the values of encapsulation efficiency also presented similar values.

Table 3. Total oil, non-encapsulated oil, and microencapsulation efficiency (MEE) of microcapsules of olive and salmon oils

Microcapsules	Total oil (%)	Non-encapsulated oil (%)	MEE (%)
Olive oil	22.46	1.93	91.4
Salmon oil	21.38	1.87	91.2

3.3. Stability of salmon oil microcapsules

Due to the degree of unsaturation of fish oils, salmon oil microcapsules stability was studied by Rancimat method and peroxide value, before sausages formulation.

Induction times of salmon oil encapsulated and non-encapsulated and protection factor were obtained, in order to evaluate the influence of microencapsulation on the stability of the fish oil (Table 4).

Table 4. Induction time and protection factor by Rancimat analysis of non-encapsulated and microencapsulated salmon oil

Sample	Induction time (h)	Protection factor
Non-microencapsulated oil	0.81	-
Microencapsulated oil	10.12	12.49

Concerning to Rancimat results, a protection factor greater than 1 indicates inhibition of lipid oxidation. When the induction time of non-microencapsulated salmon oil was compared with the microencapsulated oil, the effect of microencapsulation on fish oil protection was verified. Induction time increased around 10 times when the salmon oil was protected by spray-drying microencapsulation.

Also, peroxide values were determined in order to evaluate the effect of microencapsulation. It is evident from Figure 1 that microencapsulation had some stabilizing effects on the oil. Encapsulated salmon oils samples presented lower PV than the control batch.

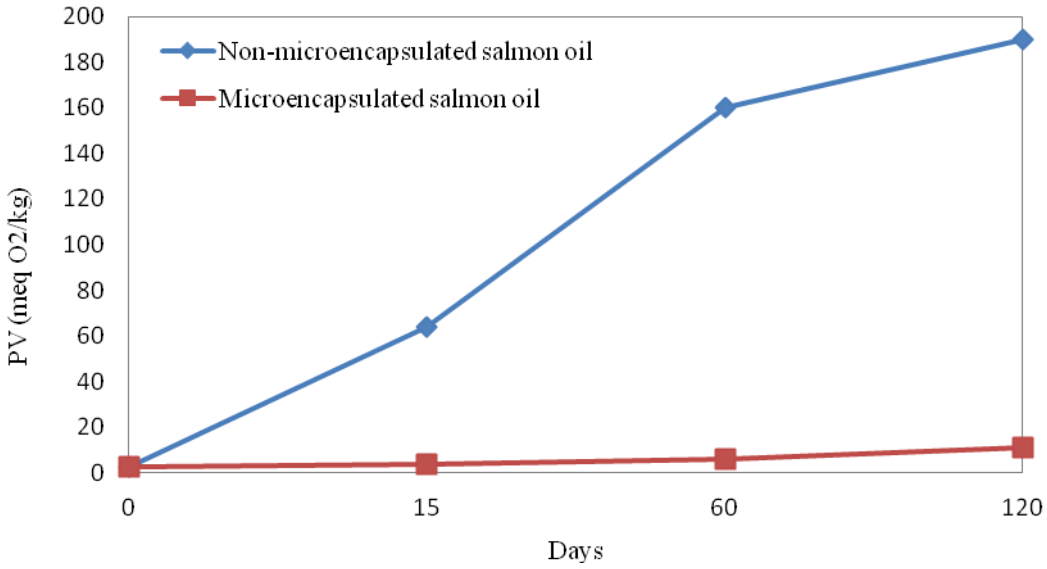

Figure 1. Peroxide value along 120 days of storage of non-encapsulated and microencapsulated salmon oil

The acceptability limit of PV for crude oil of 5 meq O_2/kg of oil (GOED 2008) was reached by non-microencapsulated salmon oil after the first days of storage. Salmon oil microcapsules presented values higher than 5 meq after 60 days of storage (5.57 meq O_2/kg). These results agreed with the results obtained in Rancimat test. This means that it is possible to use microencapsulation as barrier for fish oil but a combination with other antioxidant strategies could be necessary in order to improve the shelf life of the fish oil.

3.4. Proximate composition of sausages

The effect of a partial substitution of pork fat by salmon oil or olive oils (direct addition or microencapsulated) on the quality of sausages was studied in this chapter. Proximate analysis of fortified sausages showed some significant differences between

formulations (Table 5). Protein and fat contents of fortified sausages were close to the target levels of 13 % and 10 %, respectively, and small differences between treatments were found. No significant differences were observed in the protein content of the fortified sausages irrespective of the formulation.

pH of sausages ranged between 6.02 and 6.07 in control and fortified sausages. Similar values have been reported by Bloukas and Paneras (1993), Cáceres et al. (2004), Grigelmo et al. (1999) in different types of sausages and are considered within the normal range for these products. An increase in pH was observed with the incorporation of oils, and even a higher pH occurred when the added oil was microencapsulated. This increase has been described by other authors (Cofrades et al. 1995) in low-fat meat products emulsions and could be related to some of the components of pre-emulsion, mainly caseinates, used for the microencapsulation of fish and olive oils (Cáceres et al. 2008).

A_w presented the same levels in all sausages, ranging from 0.979 to 0.982. Neither pH nor a_w changed after 90 days under refrigeration.

Table 5. Proximate analysis (% on wet basis), pH and a_w of sausages with different fat formulation

Sample	Moisture	Fat	Protein	pH	A_w
Control	66.12 b	9.78 bc	13.72 a	6.02 a	0.980 ab
Olive	66.47 b	9.99 bc	13.19 a	6.04 b	0.982 b
Microencapsulated olive	66.60 b	7.88 a	13.57 a	6.06 c	0.979 a
Salmon	66.22 b	8.52 ab	13.38 a	6.06 c	0.979 a
Microencapsulated salmon	63.72 a	10.04 c	13.21 a	6.07 c	0.979 a

Means with different letters in the same column are significantly different ($p < 0.05$).

3.5. Fatty acid composition

As expected, the fatty acid composition of the sausages was affected by the type of formulation (Table 6). In all the samples, the principal SFA in quantitative terms was palmitic acid, followed by stearic acid, which together accounted for 50 % of total fatty

acids in control sausages and 45 % in fortified sausages. The concentration of these fatty acids decreased 5 % when pork fat was replaced by olive oil and salmon oil. MUFA content was higher ($p < 0.05$) in fortified sausages compared with control. Oleic acid was the most abundant fatty acid in all sausages, and sausages fortified with olive oil presented higher concentration of this fatty acid. No effect of the technique of incorporation, direct or microencapsulated, was observed. The most abundant PUFA was linoleic acid. EPA and DHA fatty acids only were present in sausages fortified with salmon oil.

Table 6. Fatty acid profile (% of total fatty acids) of sausages with different fat formulation.

Fatty acid	Control		Olive		Micro-olive		Salmon		Micro-salmon	
C14:0	1.63 ± 0.01	c	1.44 ± 0.00	a	1.56 ± 0.01	b	2.10 ± 0.01	e	1.97 ± 0.01	d
C16:0	31.92 ± 0.03	e	28.92 ± 0.01	a	29.83 ± 0.01	c	30.54 ± 0.03	d	29.51 ± 0.01	b
C18:0	19.42 ± 0.06	c	17.34 ± 0.16	b	16.85 ± 0.00	a	16.89 ± 0.08	a	16.75 ± 0.05	a
ΣSFA	**52.97 ± 0.09**	**d**	**47.70 ± 0.16**	**a**	**48.24 ± 0.01**	**b**	**49.52 ± 0.12**	**c**	**48.23 ± 0.07**	**b**
C16:1	2.37 ± 0.00	a	2.45 ± 0.01	b	2.46 ± 0.01	b	3.24 ± 0.01	d	3.14 ± 0.01	c
C18:1n9	38.07 ± 0.09	a	43.57 ± 0.22	e	43.18 ± 0.01	d	39.14 ± 0.02	b	39.74 ± 0.11	c
C18:1n7c	3.44 ± 0.00	b	3.44 ± 0.01	b	3.34 ± 0.01	a	3.76 ± 0.02	c	3.82 ± 0.05	c
ΣMUFA	**43.88 ± 0.09**	**a**	**49.46 ± 0.24**	**e**	**48.98 ±0.01**	**d**	**46.20 ± 0.03**	**b**	**47.24 ± 0.13**	**c**
C18:2n6	2.95 ± 0.01	a	2.84 ± 0.09	b	2.60 ± 0.02	b	2.97 ± 0.01	c	3.14 ± 0.06	d
C18:3n6	-		-		-		0.07 ± 0.03	a	0.06 ± 0.00	a
C18:3n3	0.19 ± 0.00	c	0.17 ± 0.00	a	0.18 ± 0.00	b	0.21 ± 0.01	d	0.22 ± 0.00	d
C20:5n3	-		-		-		0.26 ± 0.00	a	0.31 ± 0.00	b
C22:5n3	-		-		-		0.12 ± 0.00	a	0.13 ±0.02	a
C22:6n3	-		-		-		0.64 ± 0.01	a	0.67 ± 0.00	b
ΣPUFA	**3.15 ± 0.01**	**a**	**2.84 ± 0.08**	**b**	**2.78 ± 0.02**	**b**	**4.28 ± 0.07**	**c**	**4.54 ± 0.09**	**d**
Σn3	0.19 ± 0.00	a	0.18 ± 0.00	a	0.18 ± 0.00	a	1.23 ± 0.01	b	1.33 ± 0.02	c
Σn6	2.95 ± 0.01	b	2.66 ± 0.08	a	2.60 ± 0.02	a	3.05 ±0.04	b	3.21 ± 0.05	c
n6/n3	15.26 ± 0.26	c	15.03 ± 0.13	c	14.44 ± 0.02	b	2.47 ± 0.06	a	2.41 ± 0.12	a

Means ± SD. Different letters in the same row indicate significant differences ($p < 0.05$)

Scientific evidences suggests that a high n-6/n-3 PUFA ratio promotes many diseases, including CVD, cancer, etc., whereas increase of n-3 PUFA have a suppressive

effect (Simopoulos 2002). According to the results, one serving of 125 g of sausages fortified with microencapsulated salmon oil provides a 110 mg of DHA+EPA. The dietary recommendation for prevention of CVD is to reduce the ratio n-6/n-3 PUFA to less than 4. Enrichment allowed to get sausages with a ratio n-6/n-3 close to 2 in the case of salmon oil fortified sausages, which is much lower than the ratio of control sausages, which was higher than 15. The addition of olive oil increased the proportions of MUFA and decreased the proportions of SFA. The ratio n-6/n-3 of the sausages containing olive oil was similar to the ratio of the control sausages. In the case of salmon oil fortified sausages no significant differences were found between sausages formulated by oil direct addition and with microencapsulated salmon oil.

3.6. Colour

The colour parameters are shown in Table 7. In relation to the lightness, the *L** parameter increased in fortified sausages. This increase was evident in all fortified sausages and the values obtained significantly differed ($p < 0.05$) between control and fortified sausages. In our case, the olive and fish oils were incorporated as a pre-emulsion component giving a bright aspect to the sausages and the microencapsulated oils led to a whitish appearance of sausages due to caseinates. Then, the incorporation of oils or microencapsulated oils caused these colour changes.

The values of *a** parameter (redness) were very similar for all sausages. Only in sausages with microencapsulated fish oil, higher redness was observed but the differences were not significant ($p > 0.05$) respect to control sausages. Yellowness (*b**) was quite similar in all sausages and it was not possible to establish a clear effect of the fish or olive oil fortification in this colour parameter.

After 90 days of storage, the values of colour components hardly changed demonstrating that the sausage colour was stable during this time. No significant differences were found for *L** and *a** values, but *b** value increases were observed with longer storage. Other studies reported that storage time had no effect on the colour characteristics of low sausages with added oils (Bloukas et al. 1993).

Table 7. Effect of oil addition in sausages on the CIE *L**, *a**, *b** values

	L*	a*	b*
Type of sausage			
Control	72.95 a	4.63 ab	19.30 ab
Olive oil	73.39 ab	4.36 a	19.85 c
Microencapsulated olive oil	73.87 b	4.30 a	19.08 a
Salmon oil	74.10 b	4.39 a	19.52 bc
Microencapsulated fish oil	73.86 b	4.86 b	19.57 bc
Days of storage			
0	73.46 a	4.44 a	19.00 a
30	73.76 a	4.56 a	19.68 b
90	73.68 a	4.53 a	19.71 b

Values in column with common letters are not different ($p < 0.05$)

3.7. Texture

Hardness values were lower in sausages fortified with microencapsulated olive and fish oils (Table 8). Different results have been reported about the effect of vegetable oils on the texture of sausages. Substitution of pork fat by olive oil in reduced-fat sausages has been reported to produce a harder product, or to have no influence on texture or even that olive oil addition combined with fat reduction caused a decrease in hardness and chewiness of frankfurters (Bloukas and Paneras 1993, Paneras et al. 1998). Other authors like Lureña-Martínez et al. (2004) reported a decrease the firmness of low-fat sausages due to olive oil addition in agreement with this study. Apparent discrepancies regarding the effect of incorporation of olive oil may be related with some of the other components of the sausages.

Table 8. Effect of fortification of sausages with olive and fish oil on textural properties

	Hardness	Chewiness	Cohesiveness	Springiness
Type of sausage				
Control	6494.88 bc	4776.77 b	0.774 c	0.954 a
Olive	6835.52 c	4964.17 b	0.768 bc	0.949 a
Micro-olive	5269.86 a	3680.26 a	0.749 ab	0.940 a
Salmon oil	7265.75 c	5318.97 b	0.768 bc	0.955 a
Micro-salmon	5615.40 ab	3890.29 a	0.739 a	0.942 a
Days of storage				
0	6727.05 b	4806.66 b	0.755 a	0.945 a
30	5826.90 a	4167.02 a	0.755 a	0.950 a
90	6334.89 ab	4604.59 b	0.769 b	0.950 a

Means in the same column with different letters are significantly different ($p < 0.05$)

Although the caseinates usually increase the consistency and, as consequence, the hardness of the product (Muguerza et al. 2001), in this case probably the lower values could be related with a larger amount of trapped air in sausage emulsion when microencapsulated oil was used.

Springiness values were quite similar in all sausages independently of the technique of fortification assayed and the type of oil incorporated. No significant differences were observed ($p < 0.05$).

No differences in texture parameters due to storage were found after 90 days, indicating a stable texture over the time of storage.

3.8. Microbiological analysis

The results of the microbiological analysis are presented in table 9. Formulation and storage time affected the total viable counts of mesophilic bacteria, lactic acid bacteria and *Brochothrix thermosphacta*.

Table 9. Microbiological parameters during storage of sausages (log CFU/g)

	Mesophilic bacteria	Lactic acid bacteria	*Brochothrix thermosphacta*	*Clostridium perfringens*	*Bacillus cereus*
Type of sausage					
Control	3.02 a	1.24 a	2.45 a	nd	nd
Oliva	2.83 a	1.49 a	nd	nd	nd
Micro-oliva	4.07 c	2.93 c	2.32 a	nd	nd
Salmon	4.05 c	3.46 d	2.38 a	nd	nd
Micro-salmon	3.55 b	2.32 b	2.55 a	nd	nd
Days					
0	2.00 a	nd	nd	nd	nd
30	2.64 a	0.00 a	1.48 a	nd	nd
90	4.37 b	4.58 b	2.40 b	nd	nd

Means in the same column with different letters are significantly different ($p < 0.05$)
nd: not detected

Similar values were described for total viable counts and lactic acid bacteria by López-López et al. (2009b) in low-fat sausages enriched with n-3 PUFA, edible seaweed and olive oil. Whereas no significant differences were found between control sausages and sausages fortified by direct addition of olive oil, significant differences with the other sausages were detected, probably due to the greater manipulation during salmon oil extraction and encapsulation of both types of oils. Even with all, numbers after processing were very low and were maintained throughout the storage below 5 log CFU/g. *Clostridum perfringens* and *Bacillus cereus* were not detected.

3.9. Sensory properties

Figure 2 shows the results of sensory evaluation of sausages. Each sample was evaluated in terms of colour, odour, flavour and texture. No significant differences were found in colour.

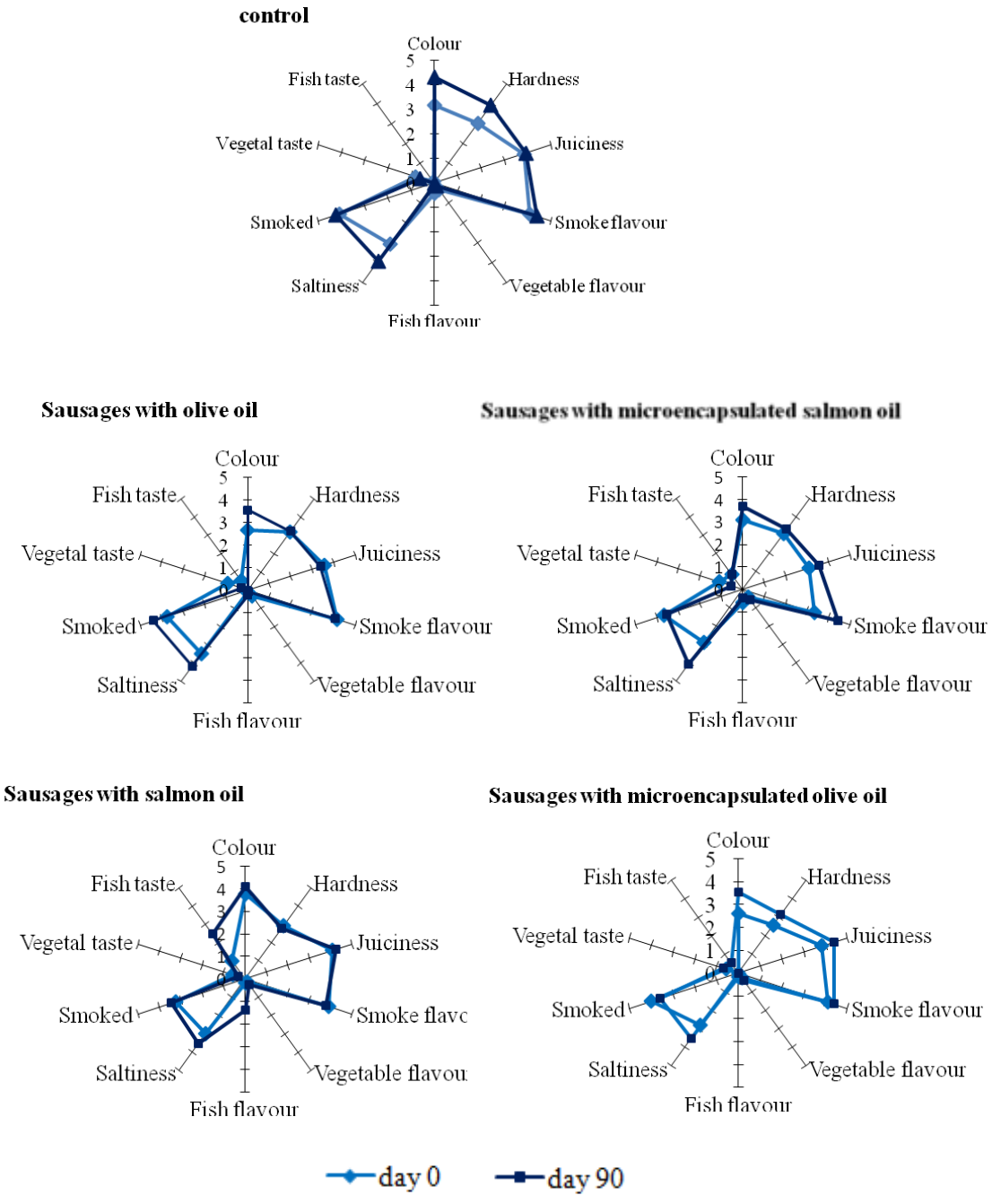

Figure 2. Sensory evaluation scores of sausages with olive and fish oil

The hardness and juiciness scores were higher in the samples fortified by direct addition of oils, but differences between the control and the other treatments were no significant ($p > 0.05$). No vegetal or fishy flavour attributes were detected at day 0, irrespective of the technique used to incorporate the oil. This may have been due to the sausages spices used, which could provide a predominant aroma and result in flavour masking in the salmon or olive oil sausages. However, at day 90 fishy flavour was significantly higher in sausages with salmon oil directly added.

3.1. Odour fingerprint by electronic nose analysis

Odour fingerprints of the sausages were analysed with an electronic nose. In the PCA model the first two latent variables accounted for 99.95 % of the total explained variance (Figure 3).

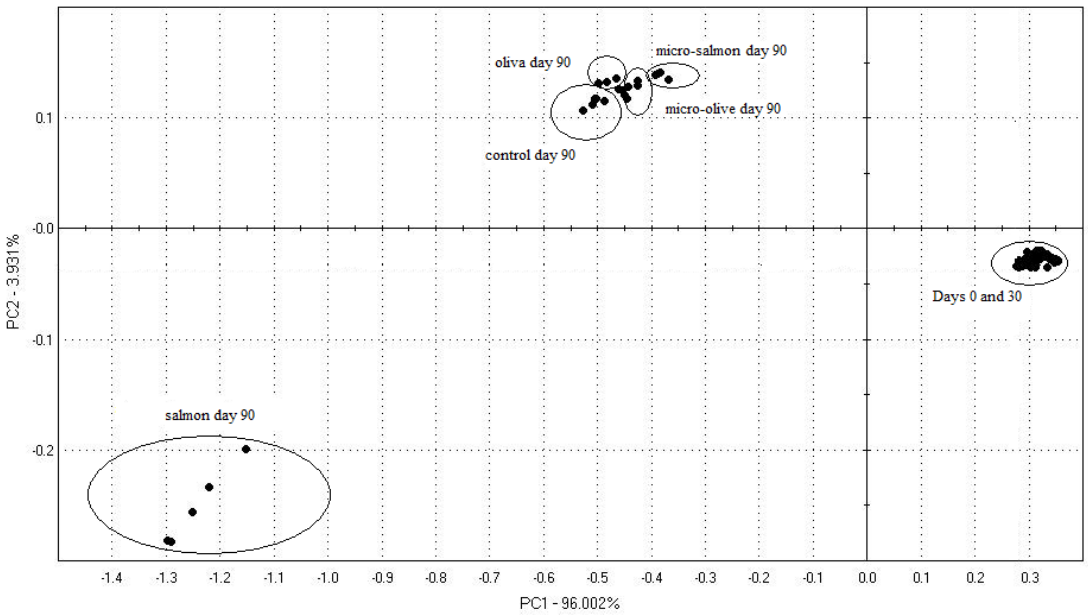

Figure 3. PCA of maximum response of electronic nose sensors for sausages with olive and fish oil along storage at 4 ºC

The relative score plot shows a clear discrimination of the sausages in the first 30 days of storage and sausages stored during 3 months according to the first and second

component. The samples from day 0 are not clearly separate from the samples from day 30. These results demonstrated that microencapsulation could be used as vehicle of salmon oil to fortified sausages without differences in the odour fingerprints respect to the control samples. In other hand, the addition of salmon oil directly to formulation demonstrated clear differences with the other samples.

4. CONCLUSION

Sensory, chemical and microbiological parameters showed that low fat pork sausages fortified with microencapsulated olive and salmon oils and the formulation of these products by direct addition of the oils were comparable shortly after processing. The inclusion of microencapsulated oils improved the fatty acids profile of the pork sausages. In the case of sausages fortified with salmon oil the ratio n-6/n-3 was reduced to less than 4. The sensory quality, after a storage period of 90 days, was improved by microencapsulation of salmon oil due to the prevention of fishy off-flavours related to polyunsaturated fatty acids oxidation. This means than, it is possible to use microencapsulation as barrier for fish oil but encapsulated oil oxidized on storage of more than 90 day. Then, a combination with other antioxidant strategies could be necessary in order to improve the shelf life and increase the omega-3 fatty acids concentration in fortified products with salmon oil.

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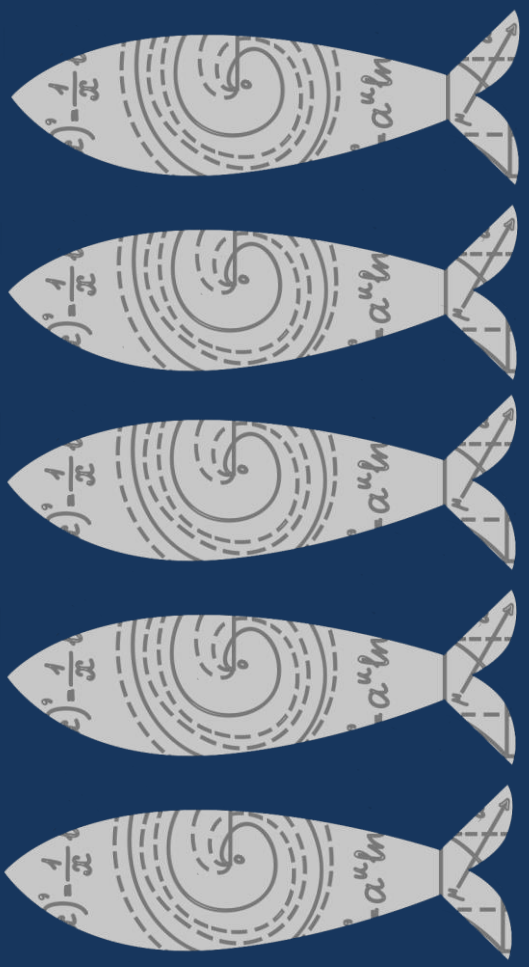

CHAPTER 5

Influence of Fortification with Omega-3 Rich Fish Oils Stabilized by Microencapsulation and Natural Antioxidants on the Characteristic of Reduced Fat Pork Sausages

V INFLUENCE OF FORTIFICATION WITH OMEGA-3 RICH FISH OIL STABILIZED BY MICROENCAPSULATION AND NATURAL ANTIOXIDANT ON THE CHARACTERISTIC OF REDUCED FAT PORK SAUSAGES

ABSTRACT

Ease of use and oxidative stability of fish oil were improved by microencapsulation and natural antioxidants for the development of reduced fat pork sausages fortified with omega-3 rich oil extracted by Supercritical Fluid Extraction (SFE) from fish by-products. The effect of microencapsulation of salmon oil, and addition of natural antioxidants as sage and propolis on pH, water activity, lipid oxidation, colour stability, texture and sensory properties of cooked pork sausages vacuum packaged stored at 4 ºC was investigated. Five types of cooked pork sausages enriched in n-3 PUFA were developed. One of them was supplemented only with salmon oil by direct addition, other with microencapsulated salmon oil without antioxidants, the third one was fortified with microencapsulated salmon oil with 5 % sage (*Salvia officinalis*) as antioxidant, and the last one was supplement with microencapsulated salmon oil with 5 % propolis of honey bee. Furthermore, a traditional formulation of this type of cooked pork sausage was also manufactured. Microencapsulation of salmon oil was effective to delay lipid oxidation and addition of sage and propolis extracts improved the protection against oxidative deterioration in cooked pork sausages fortified with salmon oil. Antioxidant effectiveness followed the order: propolis > sage, but propolis application is limited because it has strong flavour. Microencapsulation of salmon oil in combination with sage extract, as natural antioxidant ingredient, could be useful in the production of functional meat

products fortified with omega-3 fatty acids to extend their shelf life delaying deterioration due to lipid oxidation.

1. INTRODUCTION

According to joint statements by the WHO and FAO, the recommended ratio of polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs) in diets should be between 0.4 and 1.0 while n-6/n-3 PUFA ratio should be between 1 and 4 (FAO/WHO 2010). Unfortunately, it is a characteristic trait of current western diets, that they are not only deficient in n-3 PUFAs (especially long chain fatty acids) but also contain excessive amounts of n-6 PUFAs, with a n-6/n-3 PUFA ratio of 15–20 as opposed to the recommended range of 1–4 (Simopoulos 2002). For this reason, a dietary supplementation of food products with n-3 PUFAs, and especially long chain n-3 PUFAs such as eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; C22:6) has been suggested as a potential way to compensate and/or replace saturated, monounsaturated and n-6 polyunsaturated fatty acids in foods (Jimenez-Colmenero et al. 2010).

Fish and algae oils are one of the dietary sources that are rich in long chain n-3 PUFAs. In particular cold water fish such as salmon, herring, mackerel, anchovies and sardines are major sources of n-3 PUFAs. Oils extracted from salmon by-products are excellent sources of long chain omega-3 polyunsaturated fatty acids including EPA and DHA (Rubio-Rodriguez et al. 2012, Skara et al. 2004, Wu and Bechtel 2008) and Supercritical Fluid Extraction using carbon dioxide as solvent (“green” technology) provided very good yields in comparison with other extraction methods (Rubio-Rodriguez et al. 2012).

Despite the dietary benefits of consuming n-3 PUFAs, fish oils are difficult to include in foods. The unsaturated lipids have an increased sensitivity for lipid oxidation and the generated lipid oxidation products results in a rapid development of the characteristic “fishy” flavour of these oils. In addition to using a delivery system such as

emulsion (Delgado-Pando et al. 2010) or microencapsulation (Anwar and Kunz 2011) for the n-3 fatty acids, a variety of different processing operations such as deodorizing or refining of the oil, and applying chelators and antioxidants could be used (Jimenez-Colmenero et al. 2010, Rubio-Rodriguez et al. 2010).

Microencapsulation is believed to protect fish oil against oxidation by limiting undesirable influences of the environment, i.e., oxygen, light, humidity, etc. Microencapsulation of fish oil produces a dry powder from liquid fish oil, which enables its use for the enrichment of instant foods, infant formulas, bread, etc. in order to deliver the omega-3 to the diet. During the microencapsulation process, fish oil microdroplets are coated with different materials creating the wall of the microcapsules. There are a number of techniques used for fish oil microencapsulation, the method with the lowest costs being spray-drying; other techniques are fluidizing bed, freeze-drying and multilayer emulsions (Grigoriev and Miller 2009, Shaw et al. 2007). Various hydrocolloids are used as coating materials, including combinations of gelatin, maltodextrins, starch or other carbohydrates, as well as plant gums, skimmed milk powder and milk proteins.

Microencapsulated fish oil powder is usually considered as an easy delivery system of omega-3 to the human diet. In industrial practice, in order to diminish off-flavour detection, antioxidants are added to fish oil enriched foods. Synthetic antioxidants such as BHT or BHA as well as natural plant extracts were used to prevent PUFAs from rapidly oxidizing (Shah et al. 2014). For this reason, the industry's quest for natural plant extracts with a high antioxidant potential and low impact on taste and flavour continues. In contrast to synthetic antioxidants, the use of natural antioxidants from spices is increasing since their application is less stringently regulated in most countries around the world.

Recently, interest in natural antioxidants found in plants has grown due to the increasing trend towards use of “natural” food additives (Oroian and Escribe 2015). Lemon balm (*Melissa officinalis*), rosemary *(Rosmarinus officinalis)* and sage (*Salvia officinalis)* are usually used as antioxidant extracts. Lemon balm is an herb of long tradition and with a large variety of uses. Extract obtained from this plant exhibited antioxidant properties against lipid oxidation, due to phenolic acid components, such as

rosmarinic and caffeic acids and their methyl esters (Pereira et al. 2009). The main compounds responsible for the high antioxidant effects of rosemary extract are also phenol diterpenes such as carnosic acid, carnosol and rosmarinic acid (Hernández-Hernández et al. 2009). Sage is widely used in traditional medicine and is a rich source of polyphenolic flavonoids and phenolic acids (Lu et al. 2002). The main antioxidant activity of sage was attributed to rosmarinic acid and the phenolic diterpenes carnosol and carnosic acid (Farhat et al. 2009). Among the phenolic compounds, rosmarinic acid was the common phenolic compound in all the extracts. According to Lindberg Madsen and Bertelsen (1995) rosemary and sage are two of the most potent spices as natural antioxidants.

Also propolis can be potentially used as natural food additive and functional ingredient, but its application to food products is still limited because of its strong and unpleasant taste and odour that generally compromise food acceptability (Spinelli et al. 2014). It is well known that propolis presents a strong and characteristic smell because of its volatile phenolic acid fraction (Thomson 1990, Banskota et al. 2010, Da Silva et al. 2011, Spinelli et al. 2014) and this is the main discriminating attribute that affects the overall quality and limits the use of propolis. Microencapsulation may be an alternative for reducing this problem. The aim of this study was to encapsulate propolis extract by spray-drying using sodium caseinate and maltodextrin. In order to obtain it in the form of powder, stable, with antioxidant property and with the possibility of fortified functional foods. According to Espinosa et al. (2015), propolis represents a potential alternative as natural antioxidant to be applied in functional emulsions containing n-3 FA due to their phenolic composition. Other compound that has been studied as natural antioxidant is caffeic acid, which is present in the composition of lemon balm, rosemary and sage and also propolis. The antioxidative effect of caffeic acid was demonstrated in different fish oil enriched food products such as mayonnaise and milk (Aleman et al. 2015, Sorensen et al. 2012).

The objective of this study was to investigate the viability of developing low fat pork sausages fortified with salmon oil rich in omega-3 fatty acids obtained by SFE from fish industry by-products, using microencapsulation in combination different natural antioxidants to stabilize it against oxidation.

2. MATERIAL AND METHODS

2.1. Extraction of salmon oil by supercritical CO_2

Salmon oil was extracted in a semi-pilot supercritical fluid plant. Salmon by-products were provided by Pescanova S.A. The by-products were cut into small pieces (1-10 mm equivalent diameter, D_e), with a cutter (CATO, Spain), in order to improve the extraction rate. The water of by-products was removed before extracting the oil by freeze-drying (FreeZone 12 L Console Freeze Dry System with drying chamber, Labconco) in order to reduce the volume of the samples. Approximately 200 g of freeze-dried salmon by-product were placed in the extractor that was later pressurized up to the extraction pressure of 25 MPa, with carbon dioxide (Carburos Metálicos, liquid $CO_2 \geq$ 99.9 %). Then, the solvent was circulated at the desired extraction temperature of 40 ºC, with a flow rate of 10 kg CO_2/h during 3 hours. The solvent was continuously recycled to the extractor after removing the solute in the separator where the solvent power of CO_2 was reduced by reducing pressure down to approximately 5 MPa according to Rubio-Rodriguez et al. (2008).

2.2. Antioxidant selection

Antioxidant effectiveness of extracts from different aromatic herbs: lemon balm (*Melissa officinalis*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) on crude oil was previously analysed in Chapter 3. They showed a protective effect on fish oil; however the concentration should be higher for effectively delaying oil oxidation. In this chapter the effect of higher concentrations of the three extracts on salmon oil was studied in order to select the most effective. The extracts of lemon balm, rosemary and sage were provided by Soria Natural (Soria, Spain). The extracts were homogenized with salmon oil by mixing. Fish oil without antioxidant (control) and with antioxidant extracts at two different concentrations (5 % and 7 % w/w) were analysed immediately after extraction (day 0) and along 45 days of storage under pro-oxidative conditions (20 ºC, sunlight).

The extract and the suitable concentration were defined according the results of Rancimat test and oxidation parameters various parameters, including free fatty acids (FFA), peroxide value (PV), anisidine value (AV) and volatile compounds: 1-penten-ol, 1-penten-3-one and hexanal).

Two new antioxidants were added to salmon oil before microencapsulation. Caffeic acid was selected due to the effectiveness demonstrated in a parallel study on the stability of fish oil enriched milk (Sorensen et al. 2012) and because it is one of the compounds present in the aromatic herb extracts of which has shown its antioxidant activity (Zheng and Wang 2001). Also, propolis was included in the study; due to its high content of polyphenols and literature information (Mendonça et al. 2015, Machado et al. 2015) that demonstrated the high antioxidant activity of propolis, similar to the synthetic antioxidant TBHQ, representing a potential alternative as natural antioxidant to be applied in a functional emulsion containing n-3 FA (Spinelli et al. 2014, Espinosa et al. 2015). Propolis extract was provided by IDOKI SCF Technologies (Derio, Spain) and caffeic acid (purity > 98 %) was purchased from Sigma Aldrich, Steinheim, Germany.

2.3. Parameters to evaluate the stability against oxidation

2.3.1. Rancimat method

The oxidation stability test was conducted according to the AOCS method Cd 12b-92 (AOCS 1992) with Rancimat 743 (Metrohm, Herisau, Schwizerland). A stream of air (20 L/h) was blown through the sample (3 g of oil) at a temperature of 100 °C. Easily volatile oxidation products, which were formed, were transferred by the stream of air into a measuring vessel containing deionized water whose conductivity was continuously measured. Plotting the conductivity as a function of time produces the oxidation curve, whose point of inflection is known as the induction time of oil oxidation. Longer induction time indicates higher stability of oil. The difference between the induction time of oil and oil containing antioxidants or microencapsulated is the basis for evaluation of the effectiveness against oil oxidation. It is expressed as a protection factor and is

calculated as a ratio of the induction time of the oil stabilized and the induction time of the control sample. A protection factor greater than 1 indicates inhibition of lipid oxidation. The higher the value, the better the effect against oxidation.

2.3.2. Evaluation of oil oxidation

The intensity of the oxidative process was evaluated by means of various parameters, including free fatty acids (FFA), peroxide value (PV), anisidine value (AV) and volatile compounds.

Free fatty acids

Free fatty acids values were determined by the official method Ca 5a-40 (AOCS 1992).

Peroxide value

Peroxide value was determined according to the AOCS method Cd 8-53 (AOCS 1992).

Anisidine value

Anisidine value was analysed according to the British Standard method BS 684-2.24 (BSI 1998).

Volatile compounds

Volatile compounds were analysed by Solid Phase Dynamic Extraction (SPDE) sampling and GC-MS. SPDE (Chromtech, Idstein, Germany) was performed after equilibration of the samples for 1 min at 70 ºC. The interior of the SPDE needle was coated with PDMS-AC. Gas chromatographic analyses were performed with an Agilent 6890N Series GC System coupled to an Agilent Technologies 5973i mass spectrometer (Agilent, Palo Alto. CA. USA). The SPDE syringe was then removed from the sample vial and immediately inserted into the “gas station” port where 50 µL of carrier gas (helium) were pulled into the SPDE-syringe for VOC desorption during 30 s and pumped into the GC-inlet (heated to 250 ºC, splitless mode) at 15 µL/s. Postdesorption bake-out of the needle at 250 ºC during 10 min ensured full desorption of all analyses from the

needle sorbent, thus avoiding carry-over between injections (González, 2008). The compounds were separated in a 007-5MS capillary column: 50 m x 0.32 mm I.D fused silica capillary column coated with 1.05 µm thickness film (Quadrex Corporation. New Haven. USA). The column temperature was increased at a rate of 3 ºC/min from 40 to 240 ºC. Effluent from the capillary column went directly into the mass spectrometer and as the volatile compounds were identified their mass spectra were compared with those in the NIST and the Wiley spectrum libraries. 1-penten-ol, 1-penten-3-one and hexanal were the volatiles compounds studied during 45 days of storage.

2.4. Microencapsulation of salmon oil with different antioxidants

2.4.1. Preparation of fish oil emulsions for encapsulation

Formulations of the emulsions for spray drying were established after several preliminary tests with different materials and proportions of the components. Four different emulsion formulations for encapsulation were made. Four powders were prepared on the basis of formulation containing 20 % oil in powder. The ratios of maltodextrin/sodium caseinate/oil were 2:2:1. To obtain the emulsions, initially sodium caseinate was dissolved in water. Subsequently, maltodextrins were added to the solution very slowly, while stirring vigorously using a homogenizer (Ultraturrax T25). The oil, the antioxidants and 0.4 % of emulsifier (tween 80) were then added to the mixture and the mixture was stirred for 5 min.

The antioxidants previously selected (as described in 2.2), added in the different formulations were 5 % extract of sage (*Salvia officinalis*), 5 % propolis extract and 100 ppm of caffeic acid.

2.4.2. Spray-dying

A spray-dryer (Mini Spray-dryer B-290, Buchi, Flavil, Switzerland) was used to convert infeed emulsions into encapsulated powders. The operational conditions of the

spray drying were air inlet temperature of 180 ºC, nitrogen outlet temperature of 110 ºC. The dried powder was collected and stored in glass bottles at 20 ºC in prooxidant conditions during 120 days in order to evaluate their oxidation.

2.4.3. Microcapsules characteristics

A_w

Water activity (a_w) was measured by CX2 AQUA LAB equipment (Decagon, Washington, USA).

Morphological analysis by scanning electron microscopy (SEM)

The surface morphology of the microencapsulated powders was evaluated using a scanning electron microscopy (SEM, Quanta-200, FEI, Brmo, Czech Republic). The microencapsulated powders were coated with a 10 nm gold-palladium film under vacuum by a fine coat iron sputter (E-1011, Hitachi, Japan). Scanning electron microscopy was carried out at an accelerating voltage of 10 kV and 20 kV.

Extraction of total oil

The procedure was based on the Rose-Gottlieb method (Richardon, 1985), widely accepted for quantitative determination on fat in milk and milk powders. Briefly, 1 g of dried ingredient was weighed and dispersed in 10 mL of water at 65 ºC. After shaking, 2 mL NH_4OH 25 % were added and the solution was heated at 65 ºC for 15 min in a stirring water bath. Then, the solution was cooled, transferred to a separator funnel and the flask rinsed with 10 mL ethanol. Oil was extracted three times with, first, 25 mL diethyl ether and 25 mL petroleum ether, second, 5 mL ethanol, 15 mL diethyl ether and 15 mL petroleum ether and third, the same solvents without adding ethanol. After filtration through anhydrous Na_2SO_4, solvents were evaporated under reduced pressure and sample dried to constant weight using a stream of nitrogen.

Extraction of non-encapsulated oil

The determination of non-encapsulated oil was performed using the method described by Westergaard (2004). Briefly, 10 g of sample were weighed into a screw capped flask. Petroleum ether (50 mL) was added and the sample was mixed slowly for 15 min. The dispersion was filtered and 25 mL of the filtrate were evaporated to dryness. The residual oil was weighed and the percentage of extractable oil was calculated.

Encapsulation efficiency (MEE %)

Microencapsulation efficiency (MEE %) was determined as a function of the encapsulated oil related to the total oil of the microcapsules. For that, the external and total oil of the microcapsules were quantified, and the MEE % was calculated with the equation provided by Velasco et al. (2006):

MEE (%) = (total oil – external oil) / total olive/fish oil) x 100

2.5. Sausages fortified with rich omega-3 salmon oil

2.5.1. Sausages development

Five batches of low fat sausages were manufactured: control, salmon oil, salmon oil microencapsulated, salmon oil + 5 % sage microencapsulated, salmon oil + 5 % propolis microencapsulated (Table 1).

All sausages were formulated in order to cover almost 50 % of the recommended consumption per day (CDR) of omega-3 fatty acids according to the nutritional advice of 500 mg/day (FAO/WHO 2000), considering a serving of 125 g of sausages.

Table 1. Formulation (g/kg) of the different sausages manufactured

Sausage formulation	Control	Salmon oil	Microcapsules		
			Salmon	Sage	Propolis
Lean pork meat	400.0	400.0	400.0	400.0	400.0
Fat meat	102.0	88.7	88.7	88.7	88.7
Salmon oil	-	13.3	-	-	-
Microcapsules of salmon oil	-	-	66.3	66.3	66.3
Ice	380.0	380.0	380.0	380.0	380.0
Phosphate	3.0	3.0	3.0	3.0	3.0
Salt	16.0	16.0	16.0	16.0	16.0
Carrageenan	5.0	5.0	5.0	5.0	5.0
Carboximetilcelulose	1.0	1.0	1.0	1.0	1.0
Milk powder	10.0	10.0	10.0	10.0	10.0
Caseinate	8.0	8.0	-	-	-
Potato starch	35.0	35.0	10.0	10.0	10.0
Soya protein	20.0	20.0	-	-	-
Flavour	7.0	7.0	7.0	7.0	7.0
Sodium ascorbate	0.5	0.5	0.5	0.5	0.5
Lactate	10.0	10.0	10.0	10.0	10.0
Dextrose	2.5	2.5	2.5	2.5	2.5

Pork meat and fat obtained from a local slaughterhouse were frozen, separately, at -20 ºC one week prior to use. The lean meat was thawed slowly at 0 ºC and the fat was tempered (-2 ºC). Both materials were chopped in a 25 L cutter (Cato, Sabadell, Spain) to particles of about 3 mm diameter. The lean meat (40.0 %) was mixed with the soya protein (2.0 %), phosphate (0.3 %) and salt (1.6 %) and about 1/3 of water was added in the form of ice (38.0 %). The batter was chopped at high speed. Then, tempered fat and 1/3 of the ice were added and mixed at high speed. Finally, the remaining ice and ingredients (0.1 % carboximetilcelulose, 1.0 % milk powder, 0.8 % flavour, 3.5 % potato starch, 0.1 % sodium ascorbate, 1.0 % lactate, 0.3 % dextrose and microencapsulated powders) were added in the cutter and mixed to obtain a suitable emulsion. The maximum temperature reached during the process was 10 ºC. The mixture was stuffed into 22 mm cellulose casings and the sausages were placed in an oven (Eller, Meran,

Italy). A four-step process was performed: drying at 55 ºC for 15 min, smoking at 60 ºC with a HR of 75 % for 15 min, cooking at 75 ºC until the internal temperature was 72 ºC, and finally cooling by spraying with cool water until an oven temperature of 15-20 ºC was reached. The casings were removed and the sausages were vacuum packed, pasteurised in hot water at 75 ºC for 45 min, and then chilled in water at 10 ºC. The products were kept under refrigeration at 4-5 ºC, and were analysed in the same week as they were manufactured and during 90 days of storage.

2.5.2. Chemical analysis

Water and **protein content** were determined by the AOAC Official Methods 934.01 and 981.10, respectively, and total **fat content** was determined by Soxhlet method using petroleum ether as solvent in a Büchi extraction system (model B-811, Flawil, Switzerland).

pH was measured by blending 10 g of products with 10 mL of distilled water for 2 min. A pH meter 507 (Crison, Barcelona, Spain) equipped with a glass electrode was used for this measurement.

Water activity (aw) was measured as described in section **2.4.3.**

Fatty acids were determined on the lipid extract from sausages. The Bligh and Dyer (1959) method was used for the lipid extraction. The fatty acids profile was determined by the AOAC method (AOAC, 1995). This method required the esterification of the fatty acids to their methyl esters, which were analyzed by Gas Chromatography (GC) in a Hewlett Packard gas chromatograph (6890 N Network GC System) equipped with an auto-sampler (7683B series) and a flame ionization (FID) detector. The separation was carried out with helium (1.8 mL/min) as carrier gas. A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. The column temperature was programmed starting at a constant temperature of 180 ºC during 20 min, heated to 200 ºC at 1 ºC/min, held at 200 ºC during 1 minute, heated again to 220 ºC at 5 ºC/min and finally held at 220 ºC for 20 min. A split injection 50:1 was used. Injector and FID were kept at 250 ºC during analysis. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co.). Their quantification was made by relating the peaks area to the

area of an internal standard (methyl tricosanoate) as indicated by the AOAC method (AOAC, 1995). Calibration curves were obtained for each pair internal standard + chromatographic standards in order to find the corresponding response factors.

Peroxide value (PV) measurements were carried out according to the previously describes in section **2.3.2.**

2.5.3. Colour

Surface colour measurements in sausages were performed using a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japón). CIE L*a*b* colour parameters (considering illuminant D65 and standard observer 10º) were calculated. Colour was defined by the lightness (L*) (L*: 0 = black and 100 = white) and the coordinates a* (-a*: green and +a*: red) and b* (-b*: blue and +b*: yellow). Colour measurements were made at days 0, 30 and 90 in sausage samples (n=3).

2.5.4. Instrumental texture measurement

Instrumental texture profile analysis (TPA) (Breene, 1975) was performed with a texture analyzer TA-XT2 (Stable Micro Systems, Haslemere, UK). The Texture Expert, version 1.20, computer program by Stable Micro Systems was used for data collection and calculations. Ten cubes of sausages (1x1x1 cm) were compressed twice with a cylindrical probe of 4.5 cm diameter, at 1 mm/s speed and the level of compression was 60 % of the thickness of the sample. The test was always accomplished in a conditioned room at 21±1 ºC. The parameters determined from the force-time curves were hardness, springiness, cohesiveness and chewiness. Hardness was defined by peak force during first compression cycle and expressed in grams. Springiness was defined as a ratio of time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve. Chewiness was obtained by multiplying hardness, springiness and cohesiveness and expressed in grams.

2.5.5. Electronic nose

Odour fingerprint was determined by an electronic nose αFOX 4000 (AlfaMOS, Toulouse, France) with a sensor array of 18 metal oxide sensors. 1.0 g of sample was transferred into 10 mL glass vial. The vials with samples were incubated under agitation (cycles 5 s on and 2 s off at 500 rpm) in an oven at 50 ºC for generating the equilibrated headspace. The injection temperature was 60 ºC. Nitrogen was used as the carrier gas through the injection chamber at a flow rate of 150 mL/min, and synthetic air was used to maintain the thermal stability of the sensors.

2.5.6. Volatile compounds

Volatile compounds were analysed as described previously in section **2.3.2.**

2.5.7. Sensory evaluation

The sausages were sensory evaluated by a 15 member sensory panel. The evaluations were performed in individual booths under standardised white light. The assessors were given toasts without salt and water to clean the palate and neutralize the taste between samples. The samples, initially vacuum packed and held under refrigeration, were heated to 70 ºC internal temperature in a microwave oven. Samples were divided in pieces of 10 mm thickness, cutting off the ends to get homogeneous pieces. Assessors were served hot pieces of sausage from each treatment. The following attributes were tested: colour, odour (smoked, vegetal and fishy or others), texture (hardness and chewiness), and taste (salty, smoked, vegetal and fishy). A seven point structured scale was used for scoring the intensity; 1 corresponded to absence for each attribute and 7 to the highest intensity.

2.6. Statistical analysis

Data were analysed using Statgraphics Plus 5.1 (STSC Inc. Rockville, MD, USA). The effect of each formulation was tested by one-way ANOVA. Tukey test was used to identify significant differences ($P<0.05$) among means.

3. RESULTS AND DISCUSSION

3.1. Antioxidant selection

The first objective of the study was undertaken to further evaluate the efficacy and the optimum concentration of lemon balm (*Melissa officinalis*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) extracts to control the lipid oxidation in salmon oil rich in omega-3 fatty acids extracted by supercritical CO_2 from fish industry by-products. The aim was to investigate the potential of using one of the antioxidant extracts as natural preservative to extend the shelf life of fish oil for incorporation in meat products fortified with omega-3 fatty acids.

According to Rancimat results, a protection factor greater than 1 indicates inhibition of lipid oxidation. The highest value indicates the best antioxidant activity. Sage extract prolonged the induction period and protected fish oil appreciably, whereas rosemary and especially lemon balm seemed to be much less effective. In this case, 5 % sage had the greatest protecting factor following of rosemary 7 %. High concentration of sage probably had a prooxidant activity since the protecting factor of 7 % sage was lower than 5 % of sage concentration. The relative efficiency of these antioxidants is given in the Table 2.

Table 2. Induction time and protection factor (Rancimat analysis) of salmon oil with different vegetal extracts

Sample	Induction time (h)	Protection factor
Control	0.21	1
Lemon balm 5 %	0.37	1.8
Lemon balm 7 %	0.51	2.4
Rosemary 5 %	0.46	2.2
Rosemary 7 %	0.59	2.8
Sage 5 %	0.69	3.3
Sage 7 %	0.63	3.0

These results corresponded with those of parameters analysed to follow oil oxidation (Figure1). All fish oils with antioxidants presented higher FFA values than the control. According Bimbo (1998), the FFA values should were lower than 7-8 % for food grade fish oil. All samples presented FFA values lower than 5 % after to 45 days of storage.

Peroxide values and anisidine values of all samples were determined. It is evident from Figure 1 that all extracts showed some effect in stabilizing the oil. All the fish oils with extracts showed lower PV and AV than the control batch. Sage with 5 % concentration was more effective reducing the increase in PV in salmon oil than the other two natural antioxidants.

The acceptability limit of PV for crude oil is 5 meq O_2/kg of oil (GOED 2008). Salmon oil without antioxidant reached this level after the first days of storage. In oil with lemon balm and rosemary the limit was also reached after 15 days of storage. In oil with 7 % of sage extract it was reached after 30 days of storage. Perhaps the prooxidant effects of high concentration of sage begin to arise. Only oils with 5 % of sage presented values below the acceptability limit after 45 of storage. Other protection techniques, like microencapsulation, could be necessary in order to increase the shelf life of salmon oils.

Regarding anisidine values the acceptability limit for crude oil is 20 according to GOED (2008). Only fish oils without antioxidant presented higher values after 45 days of storage (Figure 1).

Antioxidant activity of vegetal extracts depends on the containing types of phenolic compounds. According to Wojdylo et al. (2007) total phenolic contents of six natural antioxidants decreased in the following order: lemon balm > sage > horehound > rosemary > thymus > oregano. The discrepancies with our results were likely due to genotypic and environmental differences (namely, climate, location, temperature and fertility) within spices, choice of parts tested, time of taking samples and determination methods.

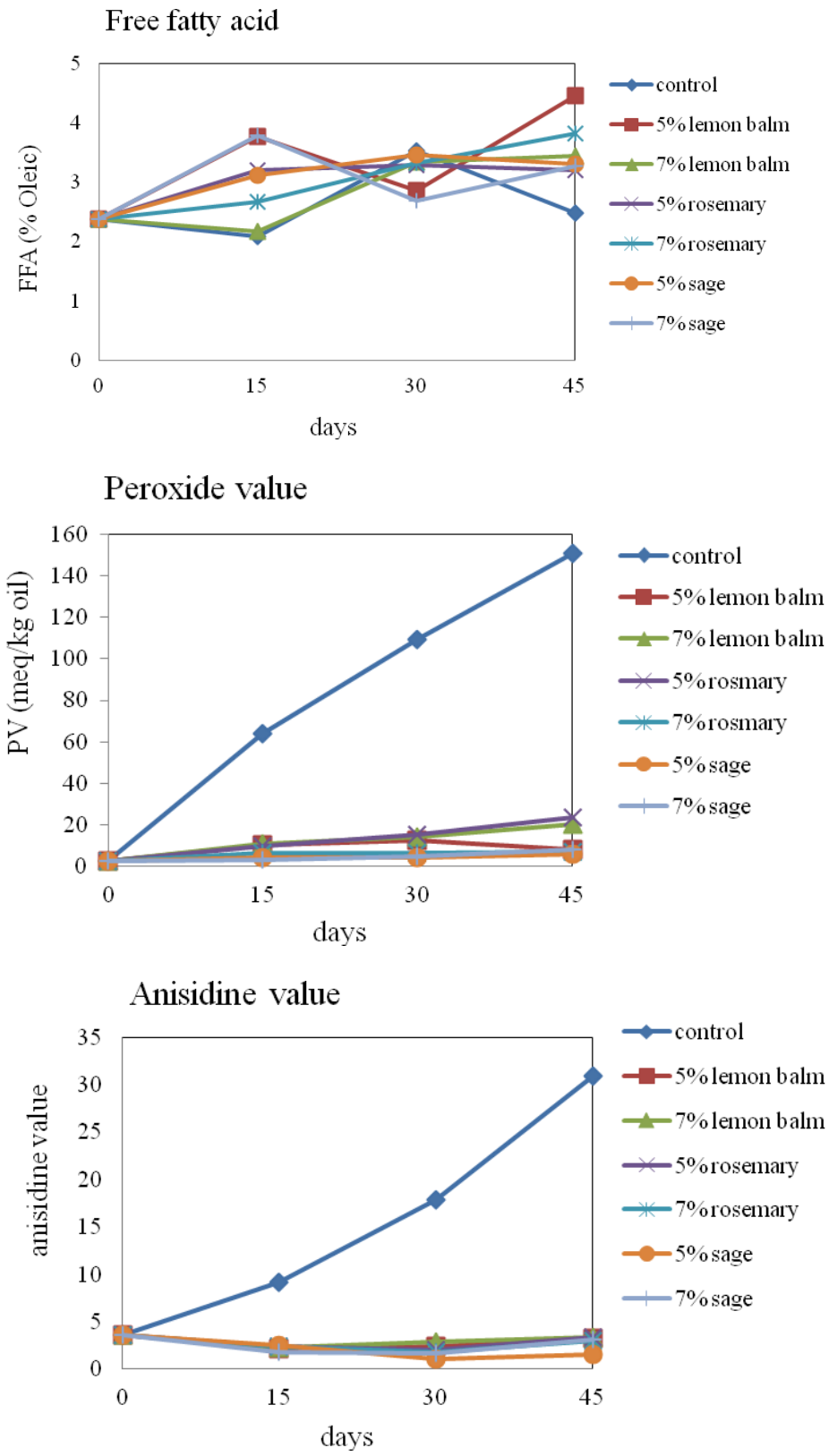

Figure 1. Effect of lemon balm, rosemary and sage extract in two different concentration in salmon oil on free fatty acids, peroxide value and anisidine value

The development of three secondary lipid oxidation products: 1-penten-3-one, 1-penten-3-ol and hexanal were monitored via headspace gas chromatography after solid-phase microextraction. The three different volatile compounds were analysed along 45 days of storage.

1-penten-one increased in salmon oil without antioxidant along of storage (Figure 2). This compound was usually associated to strong plastic and leatherlike odours. It has been reported earlier by Badings (1970) a sharp and fishy odour due to 1-penten-one in cold stored butter. It has also been indicated as the most noticeable volatile, with a very intense odour in rancid sardine oil (Nakumura et al. 1980). According to Aidos et al. (2002) in a study on oxidative stability of crude herring oil, the concentration of 1-penten-3-one showed a good correlation with peroxide value and anisidine value.

Other two oxidative derived compounds (1-penten-3-ol and hexanal) contributing to rancidity according to Jónsdóttir et al. (2008). Both of them were detected in the studied samples and contributed to the overall fish-like sweet odour. Hexanal, generally, appears to contribute with green-fishy notes according to Olafsdottir et al. (2005). The three compounds considerably increased along the storage in control batch. It is evident from Figure 2 that all extracts showed some effect in stabilizing the oil except 7 % of rosemary. In this case, volatiles compounds showed a strange evolution for which there is not an easy explanation.

Assessing jointly the results of Rancimat method and oxidation analyses, sage at 5 % of concentration could be considered the most suitable natural antioxidant for controlling oxidation of salmon oil extracted by SFE.

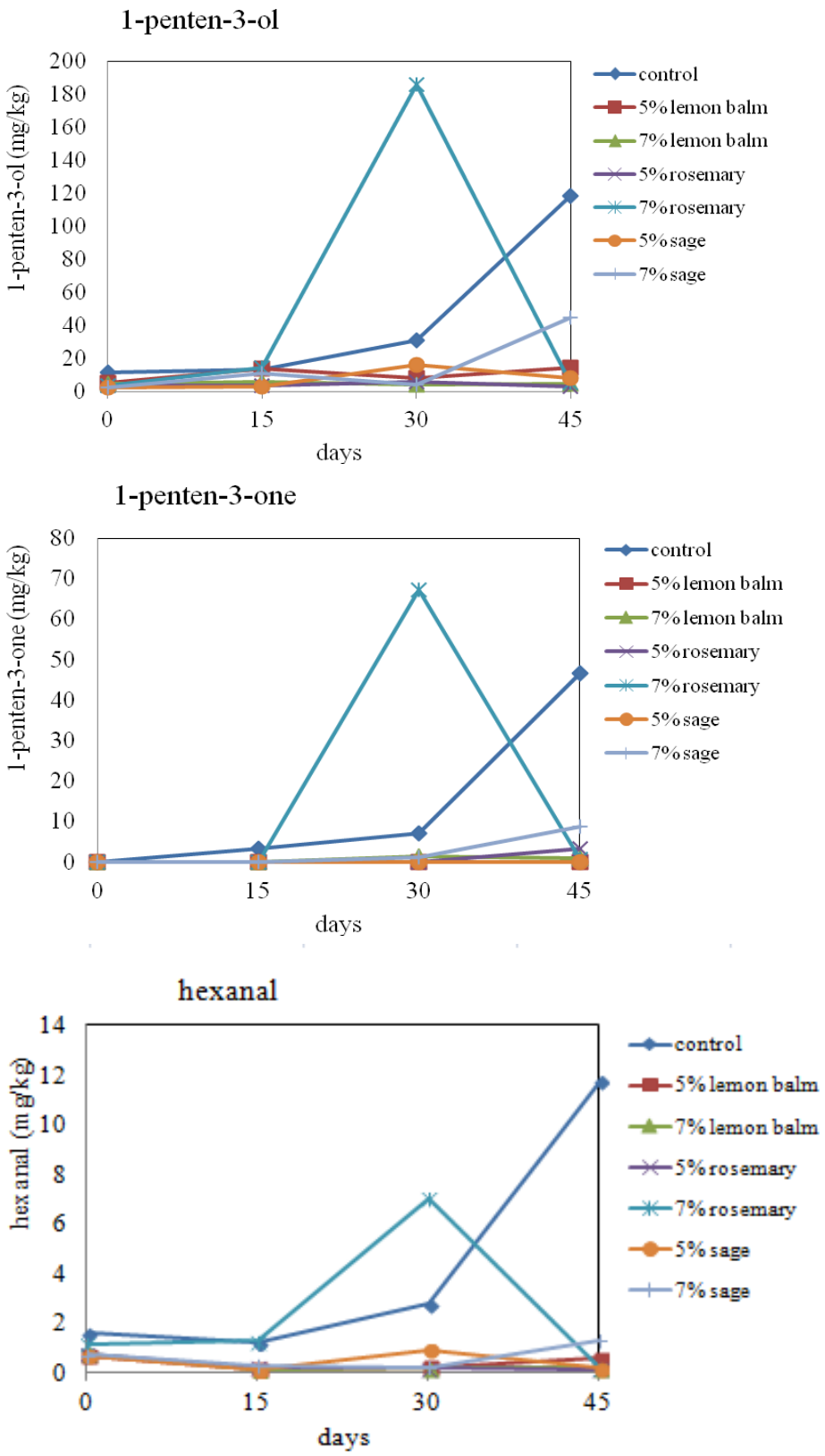

Figure 2. Effect of lemon balm, rosemary and sage extract in two different concentrations in salmon oil on 1-penten-3-ol, 1-penten-3-one and hexanal development

3.2. Microencapsulation and natural antioxidants as tools to enhance stability of salmon oil

3.2.1. Visual Observation

The four types of salmon oil microcapsules differed substantially in odour, appearance, and particles size. Visual observations showed that colour of microcapsules with only salmon oil were yellowish, microcapsules with 5 % of sage were a little more brownish, microcapsules with 100 ppm of caffeic acid were orange coloured and microcapsules with 5 % propolis were white-caramel coloured (Figure 3).

1 2 3 4

Figure 3. Different types of microcapsules: 1. Salmon oil without antioxidants, 2. Salmon oil + 5 % sage extract, 3. Salmon oil + 100 ppm caffeic acid, 4. Salmon oil + 5 % propolis extract.

Propolis can be potentially used as natural food additive and functional ingredient, but its application to food products is still limited because of its strong and unpleasant taste and odour that generally compromise food acceptability (Spinelli et al. 2014).

3.2.2. Morphological analysis by scanning electron microscopy

Spray-dried microcapsules were examined by scanning electron microscopy (SEM). The results are shown in Figure 4. Particles formed spherical structures and some unbound wall material occurred around the microcapsules. The microcapsules had no cracks and holes on the surface, showing smooth shape, and reduced indentation, as can be seen in the image for sample B.

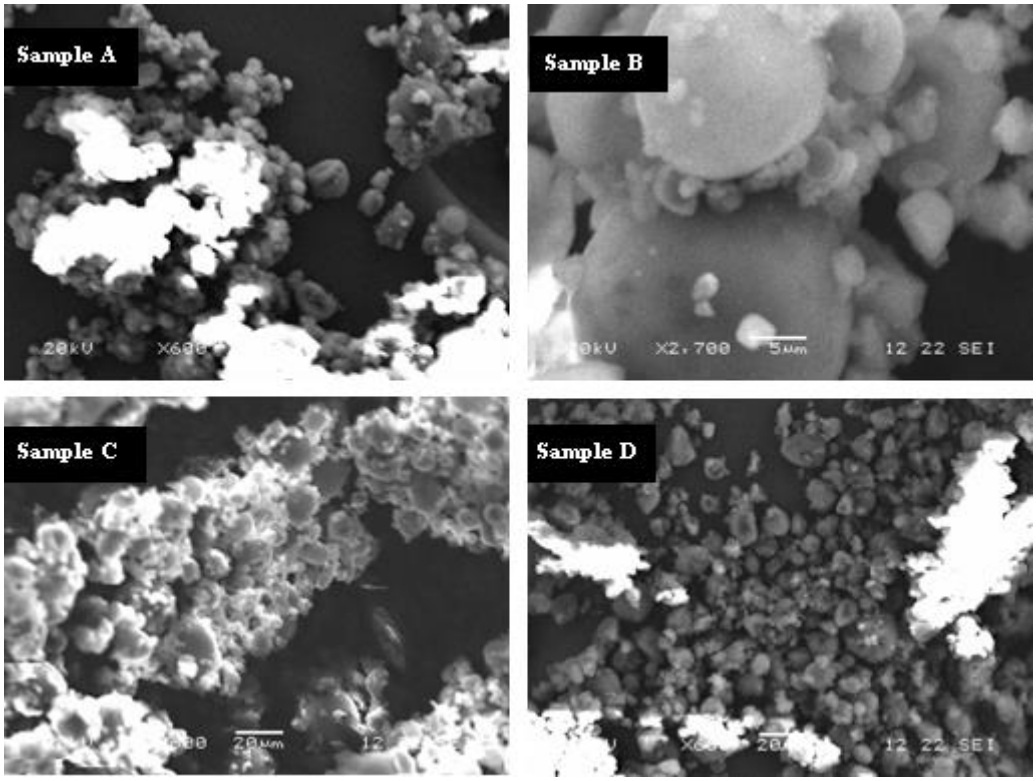

Figure 4. Scanning electron microscopy images of salmon oil microencapsulated. Sample A: Salmon oil microcapsules (600x), Sample B: Salmon oil microcapsules (2700x), Sample C: Salmon oil + caffeic acid microcapsules (600x), Sample D: Salmon oil + 5 % sage extract microcapsules (600x).

3.2.3. Water activity

It is well known that the water activity of the samples as well as porosity has a significant effect on the lipid stability. Initial water activity (a_w) of tested microcapsules were estimated to be in the range of 0.181 – 0.195 (Table 3). According to Rocklant et al. (1987) the ideal range for storage stability against lipid oxidation is 0.2 – 0.4. All samples presented very similar a_w and there were no significant differences ($p < 0.05$) among a_w of microcapsules.

Table 3. Water activity (a_w) of different types of microcapsules of salmon oil

Microcapsules	a_w
Without antioxidants	0.181
With 5 % sage extract	0.195
With 100 ppm caffeic acid	0.183
With 5 % propolis extract	0.193

3.2.4. Encapsulation efficiency

The values of encapsulation efficiency ranged from 81.8 % for salmon oil with propolis to 91.4 % for salmon oil with sage. The total oil ranged from 16.69 to 23.22 % for microcapsules with propolis and microcapsules with caffeic acid, respectively. Microcapsules without antioxidant (21.38 %) and microcapsules with sage (22.45 %) and caffeic acid (23.22 %) presented similar results. The non-encapsulated oil was always below than 3 % being slightly higher in microcapsules with propolis.

Table 4. Total oil, non-encapsulated oil, and microencapsulation efficiency (MEE) of different types of microcapsules of salmon oil

Salmon oil microcapsules	Total oil (%)	Non-encapsulated oil (%)	MEE (%)
Without antioxidant	21.38	1.87	91.2
With 5 % sage extract	22.45	1.93	91.4
With 100 ppm caffeic acid	23.22	2.05	91.2
With 5 % propolis extract	16.69	2.93	81.8

3.2.5. Rancimat method

Protection factor test was conducted according the Rancimat method by comparing induction time and stabilization factor of salmon oil microcapsules in the presence and absence of antioxidants (Table 5).

Table 5. Induction time and protection factor by Rancimat analysis of different types of microcapsules of salmon oil

Sample	Induction time (h)	Protection factor
Non microencapsulated oil	0.81	-
Microencapsulated oil		
Without antioxidant	10.12	12.49
With 5 % sage extract	13.17	16.26
With 100 ppm caffeic acid	4.60	5.68
With 5 % propolis extract	33.8	41.73

Concerning to Rancimat results, a protection factor greater than 1 indicates inhibition of lipid oxidation. The highest value indicates the best antioxidant activity. Although all microencapsulated samples tested showed effective protection of microencapsulation against oxidation, the combination of natural antioxidants with microencapsulation resulted in higher antioxidant effect than only microencapsulation. When the induction time of non-encapsulated salmon oil is compared with microencapsulated oil without antioxidant, the effect of microencapsulation on fish oil protection can be verified. Induction time increase around 10 times when the salmon oil is protected by spray-drying microencapsulation. When microencapsulation was combined with different antioxidant the induction time and protection factor increased, indicating an antioxidant effect on salmon oil. Maximum antioxidant effect was observed in microcapsules with 5 % of propolis followed by 5 % sage samples. In microcapsules of salmon oil with caffeic acid a prooxidant action can be appreciated, since this sample showed lower protection factor than the remaining microcapsules.

3.2.6. Oxidative stability of fish oil microencapsulated

Peroxide values of all samples were determined. It is evident from Figure 5 that all microencapsules showed some effects in stabilizing the oil. All the encapsulated salmon oil samples presented lower PV than the control batch. Microencapsules with 5 % of sage extract were more effective reducing the increase in PV in salmon oil than the others microcapsules.

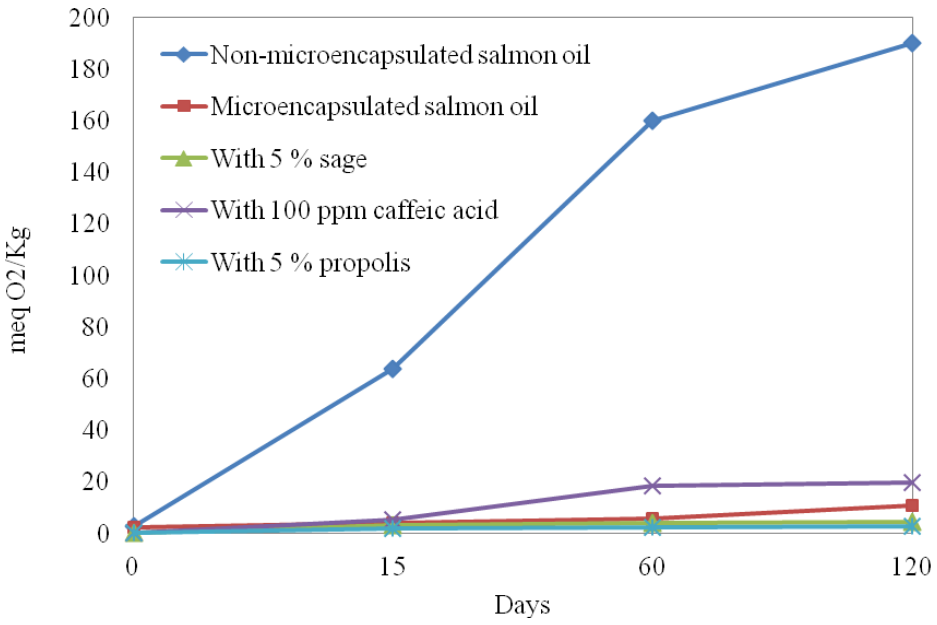

Figure 5. Peroxide values of salmon oil from different types of microcapsules along 120 days of storage

The acceptability limit of PV for crude oil is 5 meq O_2/kg of oil, in non-microencapsulated salmon oil was reached after the first days of storage. Microcapsules without antioxidant presented values higher than 5 meq O_2/kg of oil after 60 days of storage, this mean than microencapsulation is a technique very effective to protect fish oil from the oxidation but it is necessary to combine it with other barriers like antioxidants in order to improve the shelf life of the salmon oil. Only microcapsules with 100 ppm of caffeic acid presented values higher than 5 meq O_2/kg after 15 days of storage. These results agreed with the results obtained in Rancimat test, and probable prooxidant action can be appreciated in microcapsules with caffeic acid. This means that, in the case that

use caffeic acid as antioxidant, the effect of concentration should be previously studied to define the most suitable to avoid oil oxidation. Microcapsules with sage and propolis extracts presented values lower than the acceptability limit even after 120 days of storage.

3.3. Development of reduced fat pork sausages rich on omega-3 fatty acids by addition of microencapsulated salmon oil with antioxidants

3.3.1. Proximate composition

Similar proximate composition was observed in the sausages with different formulations (Table 6).

Table 6. Proximate analysis (% on wet basis), pH and a_w of sausages with salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis)

Type of sausage	Moisture	Fat	Protein	pH	A_w
Control	68.46 ± 0.28 ^{bc}	8.16 ± 0.02 ^{a}	13.72 ± 0.31 ^{a}	5.95 ±0.01 ^{a}	0.980 ±0.001 ^{a}
Salmon oil	68.23 ± 0.34 ^{abc}	8.05 ± 0.32 ^{a}	13.38 ± 0.52 ^{a}	5.97 ±0.00 ^{a}	0.978 ±0.001 ^{a}
Microsalmon	68.71 ± 0.25 ^{cd}	9.54 ± 0.32 ^{a}	13.21± 0.16 ^{a}	5.97 ±0.01 ^{a}	0.979 ±0.001 ^{a}
Sage	69.19 ± 0.18 ^{d}	8.42 ± 1.10 ^{a}	13.57 ± 0.45 ^{a}	5.97 ±0.02 ^{a}	0.979 ±0.001 ^{a}
Propolis	67.67 ± 0.39 ^{a}	9.26 ± 0.31 ^{a}	13.21± 0.16 ^{a}	5.99 ±0.02 ^{a}	0.976 ±0.002 ^{a}

Means with different letters in the same column are significantly different ($p < 0.05$)

Similar fat and protein contents for cooked pork sausages were found in different types of sausages, and then microencapsulation had no effect on these parameters ($p > 0.05$). Moisture content not just presented differences between different types of

sausages, although they can be seen among cooked pork sausages containing sage and propolis extracts. Neither the pH and water activity differed between the different types of sausages (Table 6).

3.3.2. Oxidation

The peroxide values (PV) of sausages with salmon oil added without and with different antioxidant extracts during the storage are presented in Figure 6. An increment of peroxide values was observed in all samples after 90 days of storage. When comparing PV of all samples after storage, it was found that the control sample showed higher PV than the samples added with encapsulated salmon oil, and the difference was even greater when added natural antioxidants. This means that microencapsulation had a protective effect against fish oil oxidation, and this effect was enhanced with the addition of antioxidants. Propolis microcapsules at 5 % were more effective in lowering the increase in PV than microcapsules with sage extract in the same proportion.

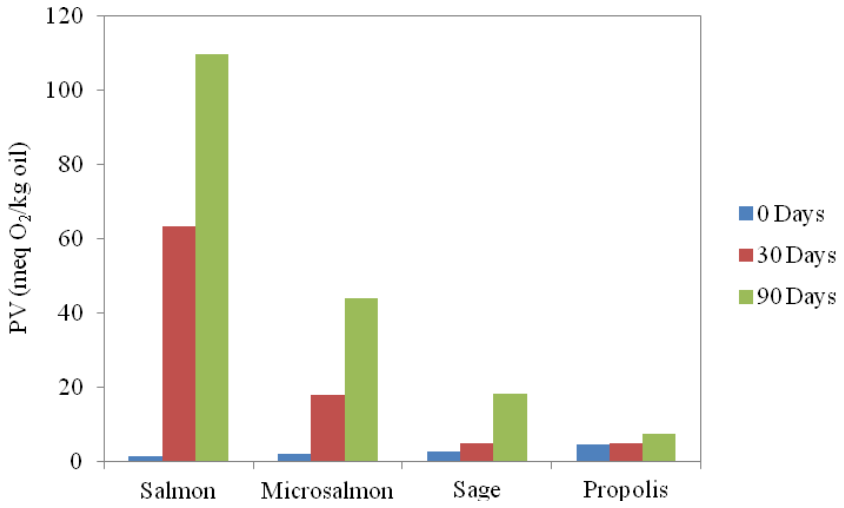

Figure 6. PV values of sausages with salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis).

3.3.3. Colour

Colour parameters were affected by formulation and storage time (Table 8). Generally, lightness, redness and yellowness values were higher ($p < 0.05$) in samples where pork back fat had been replaced by microcapsules of salmon oil. There were also some differences in colour parameters depending on the extract used for fish oil microencapsulation.

Table 8. Effect of salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis) on the colour CIE *L**, *a**, *b** values of low fat sausages

	L*		a*		b*	
Type of sausages						
Control	67.32	a	4.94	a	21.00	c
Salmon	71.58	c	5.06	a	20.60	b
Microsalmon	73.53	d	4.97	a	19.61	a
Sage	69.12	b	5.63	b	22.42	d
Propolis	67.74	a	5.42	b	23.42	e
Days of storage						
0	70.44	b	4.91	a	20.57	a
30	70.29	b	5.06	a	21.43	b
90	69.72	a	5.35	b	21.53	b

Means with different letters in the same column are significantly different ($p < 0.05$)

For instance, the increase in the parameter L* (as compared with the control) produced by addition of fish oil microencapsulated was smaller when the microencapsulated fish oil contained natural antioxidants (sage and propolis). This parameter decreased along the storage period in all different sausages and at the same time redness increased. Different opinions have been reported on the differences in the colour of sausages due to the type of fat added. According to Delgado-Pando et al.

(2010), depending on the protein system used for oil-in-water emulsion stabilization some differencies in colour parameters could appear. Keenan et al. (2015) observed that lightness values were higher in samples containing vitamin E and non-encapsulated fish oil in beef burgers with partial fat replacement for fish oil than in the control samples.

3.3.4. Texture

Texture parameters of sausages were affected ($p < 0.05$) by the presence of microencapsulated fish oil and natural antioxidants (Table 9).

Table 9. Effect of salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis) on textural properties of low fat sausages

Type of sausages	Hardness	Springiness	Cohesiveness	Chewiness
Samples				
Control	8333.31 ^{c}	0.93 ^{ab}	0.70 ^{b}	5780.92 ^{d}
Salmon	7524.07 ^{c}	0.92 ^{ab}	0.74 ^{b}	5527.32 ^{d}
Microsalmon	5615.40 ^{b}	0.94 ^{b}	0.73 ^{b}	4126.41 ^{c}
Sage	3854.47 ^{a}	0.89 ^{a}	0.54 ^{a}	2040.34 ^{b}
Propolis	3210.30 ^{a}	0.89 ^{a}	0.50 ^{a}	1554.54 ^{a}
Days of storage				
0	5365.86 ^{a}	0.91 ^{a}	0.64 ^{a}	3538.18 ^{a}
30	5928.25 ^{b}	0.91 ^{a}	0.67 ^{a}	4066.81 ^{b}
90	6187.93 ^{b}	0.91 ^{a}	0.65 ^{a}	4114.05 ^{b}

Means in the same column with different letters are significantly different ($p < 0.05$).

The sausages with microencapsulated fish oil presented lower hardness than the control sample (all pork fat), especially the sausages with natural antioxidants (sage and propolis). The sausage with emulsified fish oil added presented similar values than the control samples. This mean than the incorporation of fish oil by microencapsulation have

an effect on the texture of the final meat products. Contrasting with our observations in the present experiment, an increment of hardness by the presence of fish oil-in-water emulsions in the meat matrix have been reported by Delgado-Pando et al. (2010) due to that oils achieved a better distribution than animal fat in meat emulsion matrixes, thus producing firmer sausages due to improved association with the protein. However, conflicting results have been found for the effect of natural antioxidants. The sausages with propolis and sage presented significantly lower values of hardness, springiness, cohesiveness and chewiness than the other sausages. In general, samples which include microcapsules in their formulation presented lower values in all TPA parameters than the control sample. Along 90 days, hardness and chewiness increased but storage time had no effect on the springiness and cohesiveness characteristics of low fat sausages with microencapsulated salmon oil.

3.3.5. Volatile compounds

Aldehydes are well known to be important indicators of the oxidation of fish oil. Some aldehydes were detected in all the samples during storage period.

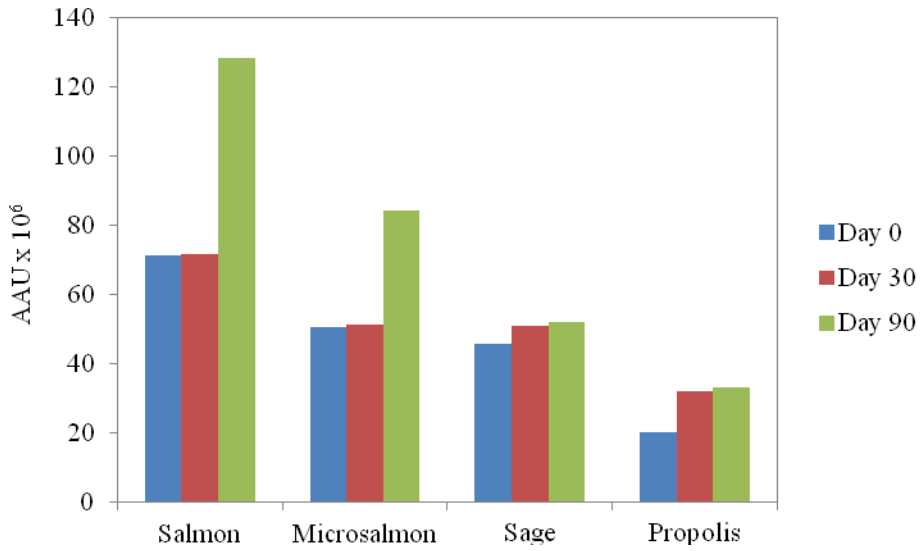

Figura 7. Hexanal content (AAU x 10^6) in low fat sausages with salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis)

Hexanal is often used as a marker compound for C18:2*n*−6 oxidation in meats (Olsen et al. 2005). Hexanal strongly increased during storage in sausages with emulsified salmon oil (Figure 7). In sausages with microencapsulated oil and microencapsulated with antioxidant extracts the increment of hexanal was relatively smaller. Sausages with propolis presented less hexanal concentration than the other sausages.

Figura 8. Nonanal content (AAU x 10^6) in low fat sausages with salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis)

Nonanal is a very typical compound derived of fish oil oxidation (Giogios et al. 2009). It was detected in the sausages of this study (Figure 8). In this case, sausages with sage extract presented less nonanal concentration. In both volatile compounds, the protection of microencapsulation in relation with the control was observed and the combination with natural antioxidants improved the stabilization of the salmon oil.

3.3.6. Sensory properties

Colour, odour and flavour descriptors were designated as predictors of sensory deterioration and acceptability of the sausages fortified with n-3 fatty acids. The effect of the incorporation of salmon oil microencapsulated is shown in Figure 9.

The colour of a product is an important factor in its consumer acceptance and thus ultimately for the sale of the product. The results indicated that sage had less colour than the other sausages. The control sample had a major colour intensity compared with the other samples.

Flavour is also an important parameter in the sensory properties of a food product. The flavour arises from the combined perceptions of odour, taste and mouth feel. This makes a food attractive to consumers. It was obvious from the data that the n-3 enrichment of sausages had significant effects on the evaluated descriptors of the flavour and odour. The balance of the volatile compounds produced from the oxidative breakdown of the n-3 fatty acid produces a fishy odour and flavour. In this study the panelists perceived more pronounced fishy flavour in the sausages with emulsified salmon oil than in the samples where fish oil was protected with microencapsulation and natural antioxidants. Fishy odours were not detected in microencapsulated samples and microencapsulated samples with antioxidants.

Propolis sausages presented a characteristic odour with a score under the acceptability of the sensory panel, even though several authors assessed that the spray drying is generally used to mask unpleasant feelings, bitterness and astringency of polyphenols and other compounds (Da Silva et al. 2013, Favaro-Trinidade et al. 2010). In this case, the results are according to Spinelli et al. (2014), who found that only a mass ratio of core to wall material equal to 1:20 as carrier during the spray drying technique could retain great amounts of propolis and mask the characteristic smell. It is likely that the concentration of the encapsulating material in our study was unable to hide the pungent smell of propolis. Panelists evaluated also texture parameters; sensory scores agreed with the result obtained by instrumental measurement.

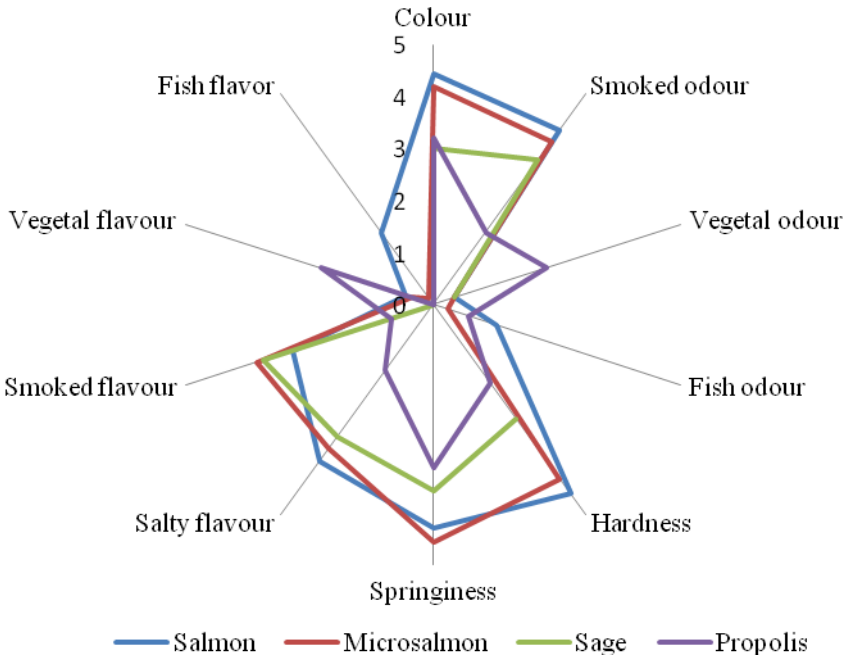

Figure 9. Sensory evaluation scores of sausages with salmon oil incorporated in different ways: emulsified (salmon), encapsulated (microsalmon), encapsuled with 5 % antioxidant extracts (sage and propolis)

4. CONCLUSIONS

In the first part of the study, antioxidant activity of some natural extracts (lemon balm, rosemary and sage) has been demonstrated in order to promote their use as natural food additives. Taking into account the concentration tested of 5 % and 7 %, the most suitable extract was 5 % sage. Further studies of phenolic compounds of these natural antioxidants must be developed to define an optimal concentration for each use.

On the other hand, microencapsulation by spray drying technique protects salmon oil against oxidation in comparison to salmon oil non-encapsulated. In addition, natural extracts such as sage and propolis can effectively enhance protection against oxidation in salmon oil. However, the use of propolis as a natural antioxidant was not feasible due to the intense flavour that impart to sausages.

The sausages designed presented a low level of SFA and high level of PUFA, with a ratio n-3/n-6 of 1.6 and provided almost 250 mg of EPA + DHA per serving (125 g) that represent 50% of the recommended daily intake.

In conclusion, low fat sausages fortified with omega-3 fatty acids can be manufactured using salmon oil obtained from fish by-products by supercritical fluid extraction stabilized with microencapsulation by spray-drying and natural antioxidants, getting more healthy products with sensory properties similar to the conventional sausages.

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CONCLUSIONS

CONCLUSIONS

In this work, at different stages, challenges for the incorporation of fish oil into meat products to fortify them with omega-3 fatty acids have been gradually overcome, and the following general conclusions were drawn:

1. Omega-3 fortified products occupy a significant space in the functional foods market. Two hundred fifty new products with omega-3 have been launched to the Spanish market in the last five years. The acceptance of functional foods depends on the combination of carrier and functional ingredient. With respect to influence of personal characteristics, our study confirms previous findings suggesting that demographic variables play a minor role in consumer acceptance of omega-3 enriched products.

2. In spite of the great offer of omega 3 fortified products, not all of them contribute to the same degree to cover the average daily recommendation (500 mg). As expected, the major food source contributing to omega-3 intake are fish products like canned or smoked fish. Fat spreads, oils, milks and meat products also contribute to omega-3 fatty acids intake in interesting proportions. Other products like biscuits, olives and bread toasts have a very small contribution in the omega-3 intake.

3. Supercritical carbon dioxide extraction is an efficient technique to obtain fish oils rich in omega-3 fatty acids from fish by-products giving similar yields than other conventional solvent extraction systems, but a better quality oil free of heavy metals and lower FFA content.

4. Fish by-products are suitable as sources of omega-3 rich fish oil obtained by SFE. *Salmo salar* is the most appropriate species due to their fat proportion and omega-3 fatty acid composition. Due to the high proportion of omega-3 in the fish oil it needs to be protected from the oxidation.

5. The antioxidant activity of sage, lemon balm and rosemary extracts have argued to promote their use as natural food additives in the protection of rich omega-3 fish oil oxidation. The best option to protect salmon oil is 5% sage extract. The use of propolis as a natural antioxidant should be optimized due to the intense flavour that impart to final fortified products. Further studies of phenolic compounds of these natural antioxidants must be developed to define an optimal concentration for each use.

6. Microencapsulation of salmon oil by spray drying is a good tool as barrier for fish oil oxidation but a combination with other strategies such as antioxidant extracts could be necessary in order to extend the shelf life and to increase the omega-3 fatty acids concentration in fortified products with salmon oil.

Finally, reduced fat pork sausages fortified with omega-3 fatty acids have been developed using salmon oil obtained from fish by-products by supercritical fluid extraction, stabilized with microencapsulation by spray-drying and natural antioxidants, getting more healthy products with sensory properties similar to the conventional sausages.

PUBLICATIONS

LIST OF PUBLICATIONS

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