UNIVERSIDAD DE BURGOS

Departamento de Biotecnología y Ciencia de los Alimentos

NEW TECHNOLOGIES AND BY-PRODUCT VALORISATION TO IMPROVE THE QUALITY AND SHELF LIFE OF MINIMALLY PROCESSED FISH-PRODUCTS.

PhD Thesis

Irene Albertos Muñoz

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(Con Mención de Doctor Internacional)

TESIS DOCTORAL

NEW TECHNOLOGIES AND BY-PRODUCT VALORIZATION TO IMPROVE THE QUALITY AND SHELF LIFE OF MINIMALLY PROCESSED FISH-PRODUCTS.

NUEVAS TECNOLOGÍAS Y VALORIZACIÓN DE SUBPRODUCTOS PARA MEJORAR LA CALIDAD Y LA VIDA ÚTIL DE PRODUCTOS DE PESCA MÍNIMAMENTE PROCESADOS.

UNIVERSIDAD DE BURGOS
DEPARTAMENTO DE BIOTECNOLOGÍA Y
CIENCIA DE LOS ALIMENTOS

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Como Directores de la Tesis Doctoral **"New technologies and by-product valorisation to improve the quality and shelf life of minimally processed fish products"**, desarrollada por **Dña. Irene Albertos Muñoz**

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Favorablemente sobre 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.

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En Burgos, a 11 de Marzo de 2016

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“Mientras los hombres sean libres para preguntar lo que deben, para decir lo que piensan, para pensar lo que quieran, la libertad nunca se perderá y la ciencia nunca retrocederá”

Robert Oppenheimer

Si al franquear una montaña en la dirección de una estrella, el viajero se deja absorber demasiado por los problemas de la escalada, se arriesga a olvidar cual es la estrella que lo guía.

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Me enervan los que no tienen dudas
y aquellos que se aferran a sus ideales
sobre los de cualquiera

Extremoduro basado en Ideario (Francisco M. Ortega Palomares)

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GENERAL INDEX

SUMMARY

Consumer demand for more healthy, natural and minimally processed food is pushing the processing industry to constantly investigate novel treatments which provide these characteristics while ensure food safety. Fish has a high nutritional interest, with special emphasis on omega-3-polyunsaturated fatty acids, but which however make it highly perishable. In this work, the use of natural additives and novel technologies is proposed to increase fish shelf life.

This thesis presents the application of previously underutilised agroindustrial by-products as natural antioxidants and antimicrobials. These wastes can be turned into valuable resources. The use of brine (by-product from herring marinating industry) can be successfully employed as coating/glazing and natural additive to prevent oxidation in frozen herring fillets and chilled herring mince respectively. Seed peel from carob fruit, which holds a high antioxidant activity, is also proposed for fish preservation. This by-product showed interesting antioxidant properties, preventing lipid oxidation in minced oily fish. On the other hand, olive leaf powder was successfully employed in chilled horse mackerel as antioxidant. As a further method of application, fish gelatin films containing olive leaf extract were effective controlling the growth of *L. monocytogenes* on smoked salmon, showing the potential application of these antimicrobial films on ready-to-eat fishery products to improve their quality and safety .

The effect of a combination of selected hurdle technologies (high pressure processing, chitosan edible films and chitosan films with clove oil) on the self-life of rainbow trout fillets was studied. First, the most appropriate antimicrobial concentration of clove oil for its formulation into chitosan films was obtained based on *in vitro* tests. The use of edible films showed as a suitable strategy to improve the physicochemical parameters

and extend the shelf life of refrigerated trout fillets compared to high pressure processing and cooking.

Cold atmospheric plasma was shown to be a suitable treatment for reducing the spoilage bacteria in fish, increasing its shelf-life. Treatment conditions, voltage and time, were evaluated at different processing levels, and shown to influence lipid oxidation.

Finally, the effect of vacuum frying as a novel processing technology on nutritional, oxidative, and sensory aspects of fish patties was compared with that of conventional (atmospheric) deep frying. The results obtained support the applicability of vacuum frying technology on fish products, reducing oxidation and improving sensorial properties. Vacuum frying could be a promising way of obtaining attractive yet highly nutritional products, and contribute to increase fish consumption, which remains below that recommended by health authorities, especially in infant populations.

INTRODUCTION

Seafood consumption around the world is on the rise. The global demand for seafood into the future will be increased due to the growth of human population and increasing income in developing countries (Swartz, Rashid Sumaila, Watson, & Pauly, 2010). Besides its protein content, seafood is also rich in vitamins and antioxidants (tocopherols, carotenoids) which have associated health benefits. Seafood contain high levels of long-chain omega-3 polyunsaturated fatty acids (ω3 PUFAs), especially in fatty fish species. These fatty acids have a structural role as main components of cell membranes and contribute to various membrane functions (Lee & Lip, 2003). It is widely accepted that the high content of PUFAs in seafood reduced the risk of cardiovascular diseases. In general, dietary recommendations to consumers advise consumption of two-three fish servings per week (Maehre, Jesen, Elvevoll, & Eilertsen, 2015). However, marine lipids are relatively more susceptible to oxidation, compared to other food lipids, because of their high content of PUFAs (Kolakowska, Olley, & Dunstan, 2003). Lipid deterioration in seafood is directly related to the production of off-flavours and odours (Harris & Tall, 1994), as well as a number of other reactions that reduce the shelf life and nutritive value of seafood. Nevertheless, the major cause of seafood spoilage is microbial growth (Olafsdottir et al., 1997; Gram & Dalgaard, 2002). Also, incidences of seafood-associated foodborne pathogen outbreaks are a major concern. Seafood is commonly contaminated with several pathogenic microorganisms, including *Listeria monocytogenes*, *Escherichia coli* and *Vibrio parahaemolyticus* (Vogel, 2009).

Minimally processed food has gained interest in industrialised countries. This concept describes approaches to food safety and preservation that are designated to retain the natural and fresh-like properties of foods. A hurdle technology is a minimal processing

technology that exploits synergistic interactions between traditional preservation treatments. According to the hurdle concept, preservation treatments combined at lower individual intensities have additive or synergistic antimicrobial effects, while their impact on sensory and nutritive properties of the food is minimised (Ross, Griffiths, Mittal, & Deeth, 2003).

Consumers are increasingly demanding more natural, minimally processed products. To satisfy these requirements, one of the major challenges in the food industry consists of replacing synthetic additives by natural alternatives (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011).

This research is aimed to develop minimally processed seafood with acceptable quality to meet new trends in lifestyles. These strategies used include the application of novel technologies and use of natural antioxidant and antimicrobial ingredients. Among novel technologies, high pressure processing, cold atmospheric plasma and vacuum frying have been tested to that end. On the other hand, bioactive ingredients, either added directly to the seafood and/or through the use of edible films and coatings, included clove essential oils and environmentally-friendly alternatives from by-products valorisation (marinated herring brines, carob seed peel and olive leaves).

New technologies

High pressure processing

The inactivation of microorganisms through high pressure processing (HPP) is attributed to changes in cell membranes, with consequent damage and alterations of cell permeability, transport systems, loss of osmotic responsiveness, organelle disruption and inability to maintain intracellular pH (Campus, 2010). HPP enabled shelf life extension of ready-to-eat salmon loins, previously submitted to sous-vide cooking (Picouet, Cofan-Carbo, Vilaseca, Carboné-Balbe & Castells, 2011). HPP has also been successfully applied to smoked-salmon without affecting sensory properties (Lakshmanan & Dalgaard, 2004). Nevertheless, it has been shown that the application of HPP on fresh fish is limited and changes in colour and a final cooked appearance can be expected (Yagiz, Kristinsson, Balaban & Marshall, 2007; Matser, Stegeman, Kals & Bartels, 2000). The mechanism behind these changes is thought to be a pressure-induced denaturation of haem proteins (Matser et al., 2006). Another possible disadvantage of HPP would be an enhanced lipid oxidation (Cheftel and Culioli, 1997; Medina-Meza et al., 2014) which extends to different degrees, depending on factors such as pressure and time of the HPP treatment, and also on the fish species used.

The combination of other hurdles along with HPP for preservation of raw fish would allow for adjusting the HPP pressure and time levels, and in this sense it could be useful for reducing the changes on the final quality of the product. Timing of antimicrobial application is important, and its presence is required during pressurisation for an additive or synergistic effect to occur with HPP (Ross et al., 2003). Raouche, Mauricio-Iglesias, Peyron, Guillard, and Gontard (2011) proposed a mechanism of a pressure triggered uptake of antimicrobial compounds by HPP sublethally injured microorganisms. Available evidence suggests the existence of synergistic antimicrobial

effects of essential oils and HPP, although the synergistic effect between essential oils and HPP would be less evident in foodstuff than that observed in buffered solutions (Rauche et al., 2011). The incorporation of these natural compounds to edible films seems to be a good strategy to reduce the amount of essential oil needed for a certain effect, through a gradual release of the additive to the food over its shelf life (Campos, Gerschenson, & Flores, 2011). In addition, the efficiency is improved due to the localisation of the antimicrobial activity on the surface of foods (Marcos, Aymerich, Garriga, & Arnau, 2013).

The combination proposed in this work of HPP and edible films with antioxidant and/or antimicrobial properties as hurdle strategy pretended to reduce the treatment time and high pressure intensity, avoiding or minimising the potential negative effects of HPP. Nuñez-Flores, Castro, López-Caballero, Montero and Gómez-Guillén (2013) found that the combined use of gelatin-lignosulphonate films and HPP prevented more efficiently the lipid oxidation and microbial spoilage in sardine fillets during chilled storage. Similar results were found by Günlu, Sipahioğlu, and Alpas (2014). Rainbow trout fillets wrapped with chitosan films and submitted to HPP extended the refrigerated shelf life of the product.

Cold atmospheric plasma

Plasma is an emerging technology in food field. However, it is successfully used industrially in medial engineering, material manufacturing and illumination technology (Schlüter et al., 2013). Plasma consisted on a gas containing free electrons, ions, and photons particles. The plasma possesses a net neutral charge because the number of positive charge carriers is equal to the number of negative ones. Electrons and photos are designated as “light” species. Plasma is often referred to as the fourth state of matter

due to its energy properties. An increase of energy occurred from solid to liquid to gas and ultimately to plasma (Misra, Tiwari, Raghavarao, & Cullen, 2011). It is difficult to classify the different kinds of plasma due to the existence of a wide range of varying parameters: pressure, gas flow, gas type…

Two kinds of plasma can be distinguished on the basis of temperature at which they are generated (Schlüter et al., 2013; Misra et al., 2011; Surowsky, Schlüter, & Knorr, 2014):

- ✓ *Thermal plasma*
    - Generated at relatively high pressure, requiring high power.
    - Thermodynamic equilibrium between electrons, ions and neutral particles exists. The former are in a thermodynamic equilibrium state so that electrons and ions occurring in the plasma have approximately the same temperature and energy ($T_{ion} = T_{overall} = T_{electron}$).
    - Ways of application: Plasma cannot be directly applied on food. The distance between the food and plasma source is required to ensure that the temperature remains within the desired range.
        - Indirect: No interaction with plasma particles. Plasma is used to treat gases or liquids.
            - UV lamps.
            - Ozone generator.
        - Semi-direct: Distance between plasma and substrate is much larger than the mean free particle path. No interactions with charged particles exist.
            - Sterrad process with plasma-activated hydrogen peroxide.

✓ *Non thermal plasma (Cold plasma).*

- Gas temperature can be close to ambient temperature (30-60 ºC).
- It is obtained at atmospheric or reduced pressure (vacuum) and requires less power.
- Non-equilibrium plasma: Non thermal plasma is characterised by an electron temperature much above that of the gas (macroscopic temperature) and consequently it does not present a local thermodynamic equilibrium.
- Sources:
    - Plasma Jet. This source produces small "plasma flames" through a single-electrode configuration with a needle electrode, as shown in **Figure** 1.

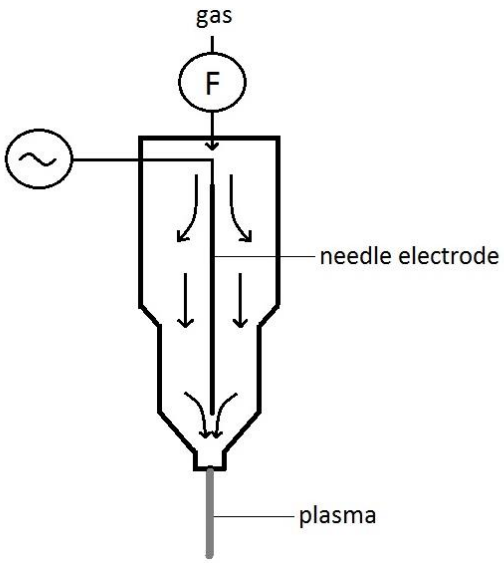

Figure 1. Schematic diagram of atmospheric pressure plasma jet.

    - Dielectric barrier discharges (DBD). Using this type of source plasma is generated through two electrodes (**Figure 2**). DBD has great advantages such as great variety of gases used, low gas flow

needed and good adaptability due to different electrode geometries among others.

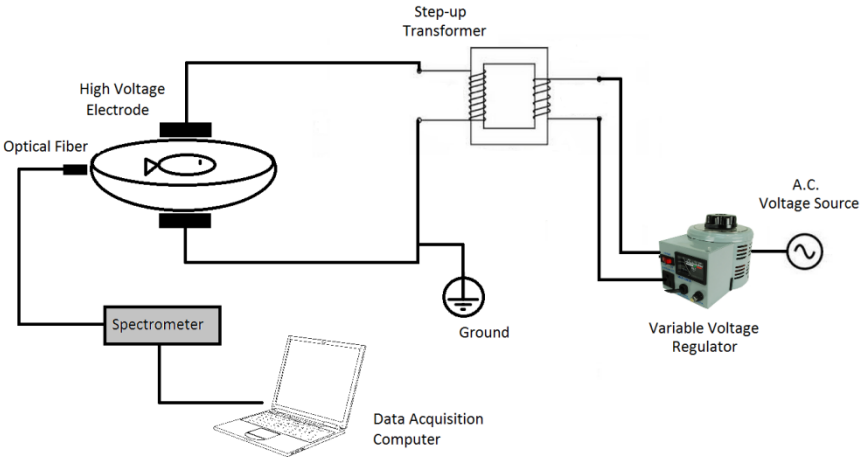

Figure 2. Schematic diagram of Dielectric Barrier Discharge (DBD).

- Corona discharges. The use of this type of source results in corona discharges appearing near sharp electrode geometries where the electric field is sufficiently large to accelerate randomly produced electrons up to the ionization energy level of surrounding gas atoms or molecules (**Figure 3**).

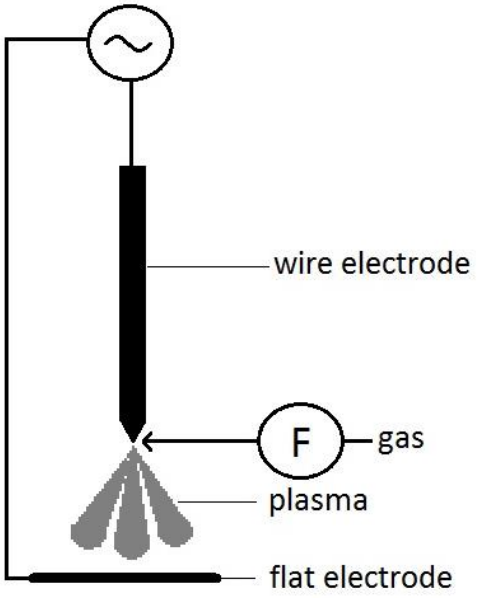

Figure 3. Schematic streamer corona discharge

- Microwave discharges. The main difference that presents this kind of source compared with other sources of plasma is that the discharges are generated without electrons. The microwaves are generated by a magnetron and are guided to the process chamber by a wave guide or a coaxial cable. The electrons present in the process gas absorb the microwaves, leading to an increase in kinetic energy and thus ionization reactions by inelastic collisions (**Figure 4**).

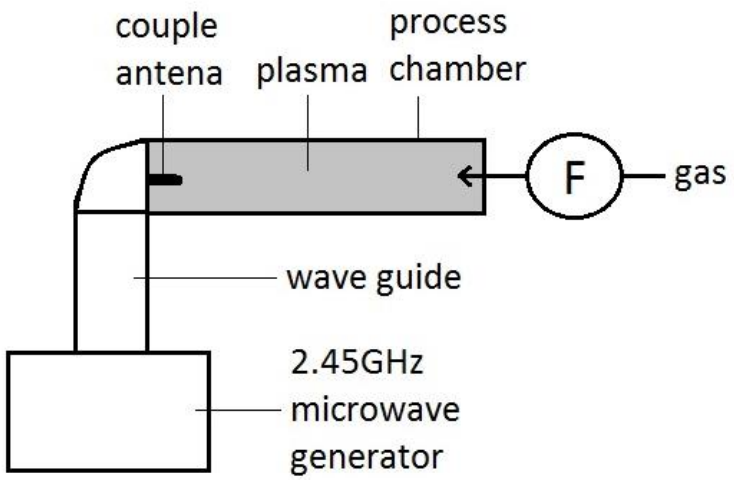

Figure 4. Schematic microwave discharge.

Atmospheric pressure cold plasma (ACP) has recently drawn considerable attention for food decontamination. Species such as hydroxyl radicals, hydrogen peroxide, ozone, singlet oxygen, superperoxide or nitrogen oxide are produced during treatment with nonthermal plasma. These molecules act on the microorganisms, affecting macromolecules like DNA, proteins and lipopolysaccharides. The effectiveness of ACP in meat inoculated with pathogenic bacteria has been demonstrated (Noriega, Shama, Laca, Díaz, & Kong, 2011; Kim, Yun, Jung, Jung, Choe, & Jo, 2011; Rød, Hansen, Leipold, & Knøchel, 2012; Kim, Yong, Park, Choe, & Jo, 2013).

Main research is this area of knowledge is aimed to achieve the highest possible reduction on microbe count with the lowest possible effect on food quality. Investigations have not revealed significant changes in dry products such as wheat flour (Misra et al., 2015) or vegetables such as tomatoes (Pankaj, Misra, & Cullen, 2013; Misra, Keener, Bourke, Mosnier, & Cullen, 2014) and strawberries (Misra et al., 2014). However, the suitability of ACP for high lipid content-food is doubted. These products

have susceptibility to oxidation due to the formation of hydroxyl acids, keto acids, short-chain acids and aldehydes (Misra, Tiwari, Raghavarao, & Cullen, 2011). The effect of ACP on food quality in meat products has not been studied extensively, and no studies on fish products exist. Further studies should be conducted to clarify these results (**Table 1**).

Table 1: Impact of atmospheric pressure cold plasma (ACP) on food properties of meat products.

Food properties	**Matrix**	**Plasma Source**	**Process gas**	**Treatment Conditions**	**Results**	**Reference**
pH Colour TBARS	Bacon	DBD*	Helium Helium and O_2	Input power (P) (0, 75, 100 & 125W) Time (T) (60, 90 sec)	= pH Colour <L* with >power & T in DBD =TBARS	(Kim et al., 2011)
Colour TBARS	Bresaola (Ready to eat meat product)	DBD	Ozone	Input power (P) (0, 15.5, 31 & 62W) Time (T) (0,2,5,10,20 &60 sec)	=Colour > TBARS with DBD; being P&T dependent	(Rød, Hansen, Leipold, & Knøchel, 2012)
pH Colour TBARS	Pork loin	DBD	Helium Helium and O_2	Input power (P) (30kHz) Time (T) (5 &10min)	<pH with DBD <L* with >power & T in DBD < TBARS with DBD at day 0 > TBARS with DBD during storage	(Kim, Yong, Park, Choe, & Jo, 2013)

*DBD: Dielectric Barrier Discharge

Vacuum frying

Vacuum frying is an alternative process to conventional frying, which is carried out under pressures below atmospheric level, preferably below 70 mbar (Shyu, Hau & Hwang, 1998). This pressure reduction allows diminishing the boiling point of the oil. The common temperatures for frying at atmospheric pressure are 160-180 °C, while the optimum temperature for vacuum frying are between 100-110 °C.

Basically, a vacuum fryer consists of three components: the frying vessel, vacuum pump and condenser, as it shown in **Figure 5**.

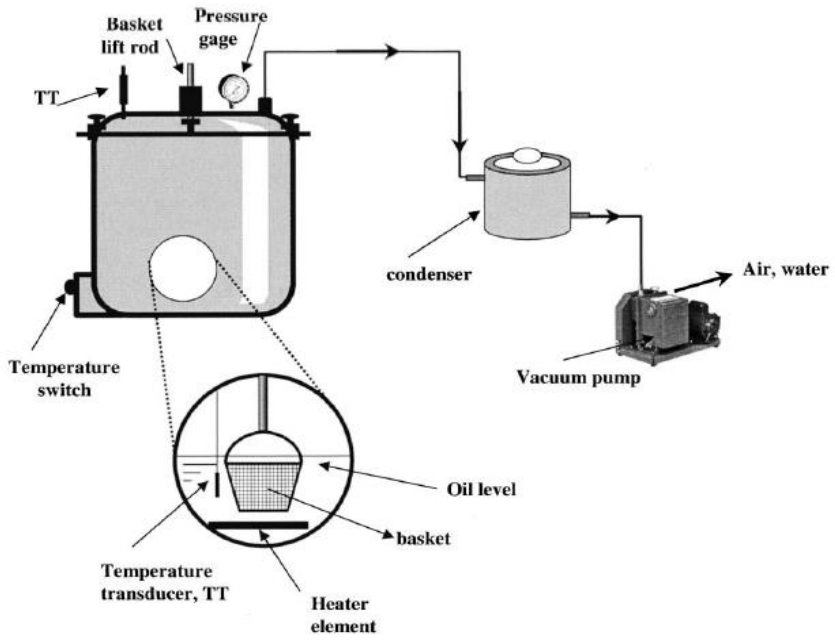

Figure 5. Schematic of the vacuum frying system adapted from Garayo and Moreira (2002).

The vessel is perfectly sealed and the fryer´s basket held by a shaft-seal. This shaft-seal allows manipulation of the basket within the vacuum side from the atmosphere side through the chamber wall. A heating element and a thermostat are also contained in the

vessel element. A condenser is required to protect the pump from the water vapour, which would damage its mechanism and cause cavitation (Moreira, 2014).

Normal procedure is as follows. Firstly, the vessel is set to the target temperature; then, oil is heated approximately one hour prior to vacuum frying. The product is loaded in the basket, the vessel´s lid is closed and depressurised. When the pressure in the vessel achieved vacuum, the basket is submerged into the hot oil and frying begin for the desired frying time. Once the product is fried, the basket is lifted from the oil and the vessel pressurised (Garayo & Moreira, 2002). Then, the lid of the vessel is opened and the product is removed from the basket. In this stage, several methods have been developed to eliminate the surface fat such as centrifugation (Tarmizi & Niranjan, 2010) or just drying with towel paper (Garayo & Moreira, 2002). According to Garayo and Moreira (2002) the oil absorption occurred mainly in the cooling stage during vacuum frying. In fact, this stage exerted a crucial role in the observed reduction in oil content due to the vacuum frying (Tarmizi & Niranjan, 2010).

Some advantages of vacuum frying as a result of low temperatures employed and minimal exposure to oxygen are:

- ✓ Preservation of natural colour and flavour. Vacuum frying allows retaining better the original colour of food due to the lower temperatures used. Nonenzymatic browning reactions and subsequent darkness are minimised, resulting in products with higher lightness (L*) values, which is a critical parameter in fried food because it is usually the first quality characteristic evaluated by consumers when determining product acceptance (Dueik, Robert & Bouchon, 2010).

- ✓ Preservation of nutritional compounds. Dueik et al. (2010) demonstrated that vacuum frying maintained carotenoid compounds in carrot different from traditional thermal methods. In fact, heating caused major degradation due to the isomerisation of trans-carotenoids, which represent almost 100 % of total carotenoid content in raw carrots. This isomerization affects pro-vitamin A activity, bioavailability and antioxidant capacity of carotenoids (Dueik et al., 2010).
- ✓ Reduction of adverse effects on oil quality. The oil is preserved better during vacuum frying due to lower temperatures used. Oil submitted to vacuum frying has lower values (peroxide value, carbonyl value, total polar components, and dielectric constant) than atmospheric frying oil (Shyu et al., 1998).
- ✓ Decrease of negative compounds for health. Granda and Moreira (2005) obtained a reduction of 94 % in acrylamide content when potatoes were fried under vacuum compared to those atmospheric fried. Furthermore, *trans* fatty acids were formed during atmospheric frying because of the high oil temperature. It is widely accepted that *trans* fatty acids increase blood levels of low density lipoproteins (LDL) and decrease high density lipoproteins (HDL),among other negative effects on health (Dueik & Bouchon, 2011).
- ✓ Reduction of oil content in vacuum fried products. Da Silva and Moreira (2008) showed that an oil content decrease is not observed in all types of products, and the mechanism for this phenomenon has not been completely clarified. Sahin and Gülum Sumnu (2009) explained that the oil content reduction can be due to faster air diffusion at low pressure, hindering the oil access to the inner parts of the product pores, and consequently reducing the oil absorption when compared to atmospheric frying.

In Europe, vacuum frying was developed to produce potato chips, achieving the necessary moisture content without severe darkening of these products (Moreira, Castell-Perez, & Barrufet). Another possible reason to introduce this technology in the potato chip industry was the necessity of reducing the acrylamide content (Granda & Moreira, 2005). In recent years, vacuum frying has focused on fruit and vegetable snack development (Moreira, 2014). Nevertheless, very little research has been carried out on fish (Andrés-Bello, García-Segovia, & Martínez-Monzó, 2010; Chen, Zhang, & Fang, 2014; Pan, Ji, Liu, & He, 2015). A few studies have focused on the effect of vacuum frying on proximate composition, colour, texture and/or sensory properties of fish, as shown in **Table 2.** To best our knowledge, no research to date has been reported on the effect of the use of this frying technology (under vacuum conditions) on the fatty acid composition and lipid oxidation. Vacuum frying could be an alternative to atmospheric one. Traditional frying process exerts significant changes on the fat content, lipid fraction and fatty acids profile of fish, with special emphasis on DHA and EPA. High long-chain omega-3 polyunsaturated fatty acids (ω3 PUFAs) are found especially in fatty fish species (blue fish), and their health benefits are widely accepted. However, these high levels of PUFAs make oily fish highly susceptible to oxidation, which is directly related to the production of off-flavours and off-odours (Harris & Tall, 1994).

Table 2. Effect of Vacuum Frying treatment in fish and seafood products.

Fish Product	Processing conditions	Main effects/benefits	Reference
Gilthead sea bream (*Sparus aurata*) fillets.	VF*: 90 ºC at 15 kPa, 100 ºC at 20 kPa, 110 ºC at 25 kPa 1, 2, 3, 4, 5, 6, 8 & 10 min AF*: 165 ºC 1, 2, 3, 4, 5, 6, 8 & 10 min	AF produced a greater decrease in the mass of the fillets and oil content than VF. A reduction in colour attribute changes was observed for VF (>L*, < a* b*).	Andrés-Bello et al., 2010.
Desalted grass carp (*Ctenopharyngodon idellus*) fillets.	VF: 90 ºC, 100 ºC, 110 ºC & 120 ºC at 0.08 MPa 10, 15 & 20 min AF: 170 ºC 4 min	Optimal conditions (<oil content, better colour and texture conditions) in VF carps fillets at 0.08 MPa, 100-110 ºC for 15 min).	Chen et al., 2014.
Bread Shrimps.	VF: 80 ºC, 100 ºC & 120 ºC at 0.090 MPa 1, 2, 3, 4, 5, 6, 8 & 10 min AF: 170 ºC 1, 2, 3, 4, 5, 6, 8 & 10 min	VF had lower moisture loss and oil absorption, compared to AF. VF maintained better colour (>L*, < a* b*), had lower hardness and acrylamide content than AF.	Pan et al., 2015.

*VF: Vacuum frying, AF: Atmospheric frying.

Natural ingredients

Essential oils

Essential oils (EOs) have been widely used as flavouring agents in food since the earliest recorded history. However, nowadays they attract increasing interest due to their antimicrobial and antioxidant properties (Holley & Patel, 2005), interest that is enhanced by the fact that consumers demand less use of chemicals on processed food products. The antimicrobial and the antioxidant activity of plant EOs is assigned to a number of small terpenoid and phenolic compounds (Du, Avena-Bustillos, & McHugh, 2009). The antimicrobial mechanism may relate to the ability of phenolic compounds to

alter microbial cell permeability, damage cytoplasmic membranes, interfere with cellular energy and disrupt the proton motive force (Burt, 2004). Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén and Montero (2010) tested the antimicrobial activity of different essential oils (clove, fennel, cypress, lavender, thyme, herb-of-the-cross, pine and rosemary), finding that clove oil had the highest inhibitory effect over a range of microorganisms. Similar results were found by Alboofetileh, Rezaei, Hosseini, and Abdollahi (2014). In this work, the authors previously tested the qualitative antimicrobial activity of six selected essential oil against three common pathogenic foodborne bacteria. After that, the most effective essential oils were incorporated in alginate/clay nanocomposite films with the aim of evaluating their antibacterial activity against *L. monocytogenes, E. coli* and *S. aureus*. In both assays clove essential oil was the second more effective, only behind marjoram. The highest concentration tested (15 g kg^{-1}) inhibited the three pathogens growth. Burt (2004) proposed that eugenol is the main compound responsible for the antimicrobial properties of clove EOs. With respect to the antioxidant properties, the inhibitory activity of clove EOs on lipid peroxidation indicated a higher antioxidant activity than a common used synthetic antioxidant such as BHT (Jirovetz et al., 2006).

Essential oils and edible films

The application of EOs through their formulation into film pretends to lessen their levels needed for obtaining desired results. In this way, disadvantages derived of their use such as intense aroma and even their high cost would be reduced (Sánchez-González et al., 2011). On the other hand, edible films with essential oils result in a gradual release of this additive to the food along its shelf life (Campos, Gerchenson, & Flores, 2011). Furthermore, the use of edible films as carriers of EOs is efficient due to the localisation of antimicrobial activity on the surface of foods (Aasen et al., 2003).

These films can also enhance oxygen-barrier, reduce the need for synthetic packaging materials and improve their recyclability by simplifying their structure (Du, Avena-Bustillos, & McHugh, 2009).

These combined techniques can be useful in the preservation of fish, a very perishable product with a limited shelf life. The main objectives of using edible films in seafood are to prevent contamination by spoilage microbiota, to avoid oxidative deterioration and to decrease the humidity loss in the products (Campos et al., 2011). Andevari and Rezaei (2011) used gelatin coatings with cinnamon EOs, efficiently preserving the quality of refrigerated rainbow trout fillets over a period of 15 days. Other EOs contained in edible films such as laurel (Alparslan, Baygar, Baygar, Hasanhocaoglu, & Metin, 2014) and clove (Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2013; Gómez-Estaca et al., 2010) have also been applied on fish with the aim of increasing the shelf life.

A synergistic effect between EOs and HPP can be found in literature. HPP induces damage in the bacterial cell membrane, facilitating the uptake of the antimicrobial agents into the cells (Wouters, Glaasker, & Smelt, 1998). Raouche, Mauricio-Iglesias, Peyron, Guillard and Gontard (2011) proposed a mechanism involving a pressure-triggered uptake of antimicrobial compounds by HPP sublethally injured microorganisms. Nevertheless, the synergistic effect of EOs and HPP decreased in foods when compared to results in buffered solutions.

The combination of HPP and edible films with antioxidant and/or antimicrobial properties as hurdle strategy for food products has recently been proposed, and there are very few reports concerning its application in fish. Gómez-Estaca, Montero, Giménez and Gómez-Guillén (2007) showed that the combination of gelatin films with oregano

and rosemary extract and HPP yielded the best results in terms of both preventing oxidation and inhibiting microbial growth. Ojagh, Núñez-Flores, López-Caballero, Montero and Gómez-Guillén (2011) found a quality improvement in salmon fillets using HPP and gelatin-lignin, whereas the effect on microbial growth was negligible. These results differed from Nuñez-Flores, Castro, López-Caballero, Montero, and Gómez-Guillén (2013), who found the combination of gelatin-lignosulphonate films and HPP was feasibly to increase the shelf life of sardine fillets during chilled-storage. Similarly, Günlu, Sipahioğlu, and Alpas (2014) found a rise in the shelf life of chilled trout submitted to HPP by the use of chitosan films.

By-products revalorization

Agri-food processing industries generate large quantities of by-products that would cause an environmental problem. Nowadays, efforts are focussed on reducing waste through the use of more efficient processes and upgrading the waste into added-value by-products. For a long time, these by-products have only been used for animal feed. Nevertheless, most of them have antioxidant and antimicrobial activities, and some of them include bioactive compounds. Thus, these by-products would be likely to be used as additives or for developing functional foods. Nevertheless, before used, it is necessary, according to Pérez-Jiménez and Viuda-Martos (2015), to previously evaluate whether their profile in bioactive compounds have indeed a beneficial *in vivo* effect and determine whether they can really be incorporated into food matrixes, properly addressing technological and sensory aspects.

In this work, solutions of by-products valorisation have been proposed, taking into account the possible limitations aforementioned when they are incorporated into food products.

Marinated herring brines

In Northern of Europe there is a long tradition of producing and consuming herring as marinated product. Marinated process consisted on two stages: salting and ripening. The salting stage includes an initial soaking of the fish in salt, which initiates the extraction of fluids from the fish and creates natural brine (Vokresensky, 1965). Afterwards, the herring fish or fillet is placed in brine. This stage is called ripening and its duration changed depending on the the type of marinated herring brines. The ripening is produced by enzymes, causing the degradation of proteins due to both digestive and muscle proteases (Nielsen, 1995). During this long ripening period, biomolecules such as proteins, lipids and peptides leach out from the fish to the brine (Svensson, Nielsen, & Bro, 2004), leading to a brine rich in organic matter. After the ripening period, the maturating brine is removed and discarded and, before barred-salted herring´s commercialisation, the fish is packed with fresh brine containing spices and flavourings. Therefore, during the production, very large volumes of marinade with high organic load are discarded. Specifically, this waste can reach more than 700 L per 100 kg of marinated herring produced (Gringer, Osman, Nielsen, Undeland, & Baron, 2014).

Carob seed peel

Carob peel seed is a by-product from carob bean gum extraction, which is the main exploitation of this commodity nowadays. This gum is added to a variety of products as thickener, stabilizer or flavouring (Bouzouita et al., 2014). A novel process allows the peel or cuticle, accounting for the 30-33 % of the seeds, to be separated intact as a by-product (Gharnit, El Mtili, Ennabili & Sayah, 2006). This by-product has not yet received attention. Although, carob peel seed which are a food grade waste and it could represent a good source of natural additive due to high polyphenol and fiber content.

Olive leaves

Olives leaves are abundant by-products worldwide. Many works have reported the antioxidant properties of olive leaves (Pereira et al., 2007; Mylonaki, Kiassos, Makris, & Kefalas, 2008; Lee & Lee, 2010; Peralbo-Molina & Luque Castro, 2013; Apostolakis, Grigorakis, & Makris, 2014). The four predominant constituents of olive leaves are secoiridoids (oleuropein), hydroxycinnamic acid derivatives (verbascoisde), flavonoids (luteolin, apigenin) and tyrosols (hydroxytyrosol) (Mylonaki et al., 2008; Pereira et al., 2007; Apostolakis et al., 2014; Benavente-García, Castillo, Lorente, Ortuño, & Del Río, 2000). Oleuropein is the most abundant phenolic compounds in olive leaf. It has shown potent antioxidant (Benavente-García et al., 2000) and antimicrobial activities (Lee & Lee, 2010). Furthermore, the antioxidant and antimicrobial effect of combined phenolics was significantly higher than those of the individual phenolics (Benavente-García et al., 2000; Lee & Lee, 2010). Markin, Duek and Berdicevsky (2003) and Pereira et al. (2007) reported antimicrobial activity of olive leaves against a range of bacterial species. The antimicrobial activity has been related to their terpenes content and involved cell membrane disruption, although the mechanism of action was not fully understood.

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OBJECTIVES

Seafood contain high levels of long-chain omega-3 polyunsaturated fatty acids (ω3 PUFAs), which are associated to health benefits. However, high PUFAs content increases seafood susceptibility to oxidation. This fact, along with easiness to microbial spoilage, limit seafood stability.

This research proposes the use of natural ingredients and novel technologies to extend seafood shelf life while maintaining their natural properties. Natural antioxidants and antimicrobials, including essential oils and bioactive ingredients obtained from by-product valorisation, are proposed for direct inclusion on seafood or through the use of edible films or coatings. Among novel technologies, high pressure processing, atmospheric cold plasma and vacuum frying have been used in this study.

The main objective of this work has been broken down in particular objectives, covered in following chapters. Chapters 1, 2 and 3 explore the use of valorised bioactive by-products directly applied on different seafood products or through their formulation in edible films or coatings. Chapter 4 relates to the use of a natural bioactive essential oil formulated in edible films in combination with a novel treatment technology as High Pressure Processing (HPP). Finally, chapters 5 and 6 are dedicated to the exploration of the application of other novel processing technologies on seafood preservation, such as atmospheric cold plasma and vacuum frying.

Chapter 1: Effluent by-products (brines) of marinated herring processing are rich in low molecular weight peptides with possible antioxidant properties. This chapter explores the applicability of three types of such brines either as coating agents in frozen herring fillets or directly formulated into chilled minced herring, and their ability to improve product shelf life.

Chapter 2: The efficacy of carob seed peel, a by-product from carob gum production, to control oxidation in chilled minced horse mackerel is investigated. Physicochemical characterisation of this novel by-product is also carried out.

Chapter 3: This chapter is focused on novel strategies for olive leaf valorisation:

-Antioxidant and antimicrobial properties of olive leaves are previously determined.

-Olive leaf powder is evaluated as a means to prevent oxidation of minced mackerel over chilled storage.

-Gelatin films are formulated including olive leaf by-products and characterised.

-The effectiveness of gelatin films with olive leaf extract in controlling *Listeria monocytogenes* in inoculated cold smoked salmon is investigated.

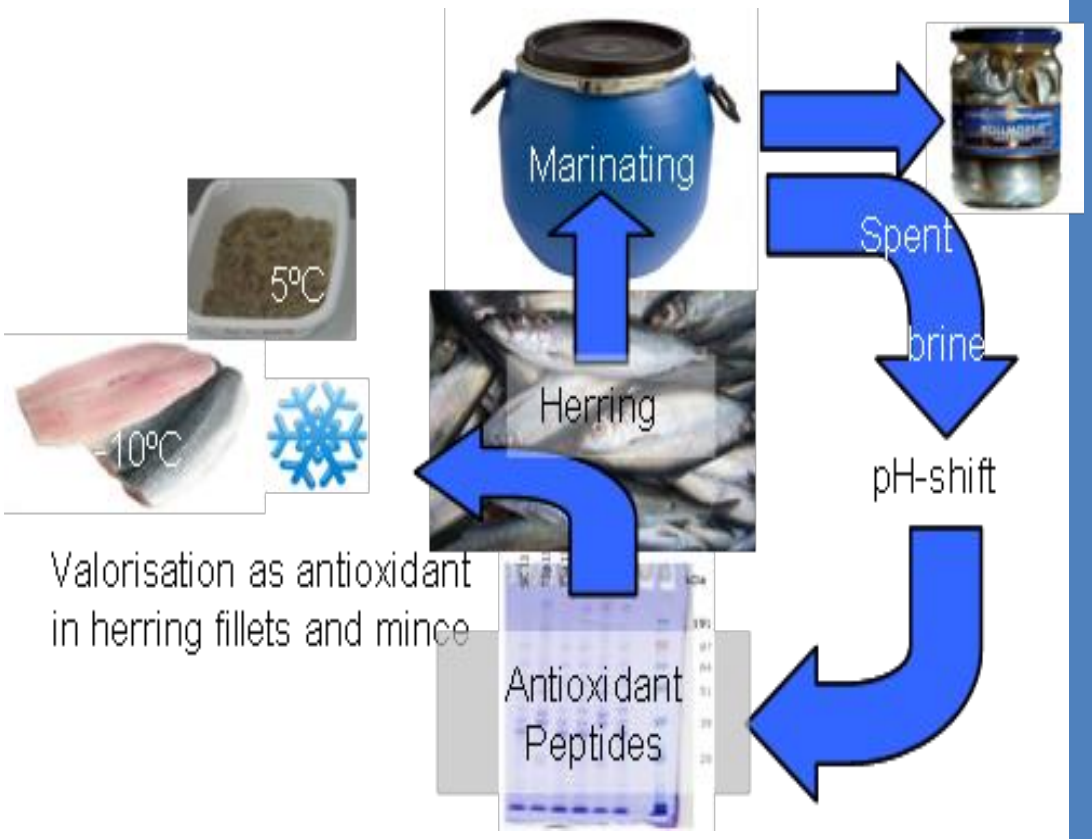
Chapter 4: The use of chitosan films with clove essential oil combined with high pressure processing (HPP) is proposed; this treatment is compared to fresh and treated samples (HPP and Cook) and/or films (chitosan with or without clove essential oil) in trout fillets over chilled storage. Clove essential oil amount in films is previously optimised through in vitro antimicrobial and antioxidant assays.

Chapter 5: In this chapter, the application of atmospheric cold plasma on microbiological and quality markers of oily fish (Atlantic mackerel and herring) is evaluated. Different voltages and time conditions of plasma treatment are used, and changes in fish monitored immediately after treatment and over chilled shelf life storage.

Chapter 6: Vacuum frying as alternative to atmospheric frying in mackerel patties is explored in this chapter. Specifically, proximate composition and fatty acids composition, oxidative markers and organoleptic parameters are evaluated at different frying times of vacuum and atmospheric (conventional) frying processes with same thermal driving force (temperature differential over water boiling point).

Chapter 1

Salted herring brine as a coating or additive for herring products – A source of natural antioxidants?



This chapter has been partially published as poster communication (Albertos et al, 2014) in WEFTA meeting (9-11 of June of 2014, Bilbao, Spain), as well as it has been submitted to Innovative Food Science and Emerging Technologies.

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Salted herring brine as a coating or additive for herring products

– A source of natural antioxidants?

ABSTRACT

The objective of this study was to characterize herring brine and assess its use as natural antioxidant. Herring brine from different marinated herring products (brine from fillet-ripened spice-cured herring: SC, traditional barrel-salted spice-cured herring: TSp and brine from traditional barrel-salted herring: TSa) were used without any pre-treatment or with a pH-shift method isolation and were tested as coating agents for frozen herring or as additives in fresh mince herring in order to prevent oxidation.

TSa and TSp were the most effective glazing, retarding lipid oxidation as confirmed by both the measurement of peroxide and volatiles oxidation products and were better than water. Brines tested as additive retarded lipid and protein oxidation in a similar trend than herring mince containing salt and/or protein. SC brine was a better as additive against lipid and protein oxidation compared to the other tested brines TSa and TSp. Using protein fractions isolated from herring marinating brines as glazing and/or additive seems feasible for preventing oxidation of both frozen and fresh herring.

Keywords: Herring, brine, pH-shift method, glazing, oxidation, natural antioxidant.

1. INTRODUCTION

Barrel-salted herring is an important fish product in the Nordic fishery industry whilst in the South of Europe; anchovies are more common as salted product. During the salting process a long maturation period takes place, where degradation of proteins occurs due to both digestive and muscle proteases (Nielsen, 1995). During this long period of ripening biomolecules such as proteins, lipids and peptides leach out from the fish to the brine (Svensson, Nielsen, & Bro, 2004), leading to a brine rich in organic matter. After the ripening period, the maturating brine is removed and discarded and before barred-salted herring´s commercialisation the fish is packed with fresh brine containing spices and flavourings. Therefore, during the production very large volumes of brine with high organic load are discarded. There is thus a need to prove if these liquid wastes, which contain high-value marine biomolecules such as protein, lipids and peptides, could be reused and valorised. Furthermore, marinating brines, which are a food grade waste, could represent a good source of natural additives such as antioxidants. Herring brines have been characterised and contain proteins and peptides (Gringer, Osman, Nielsen, Undeland, & Baron, 2014) which may be able to protect the lipids from oxidative damage under herring ripening (Andersen, Andersen, & Baron, 2007). A recent study characterising brine from different Scandinavian products demonstrated, using *in vitro* tests, that brine possesses radical scavenging activity, iron chelating activity and reducing properties and therefore has the potential to be used as a source of antioxidants (Gringer et al., 2014). Similarly, a study that tested herring press juice, in a model fish system and a simulated gastrointestinal digestion, showed that it had good antioxidant capacity, attributed to low molecular weight compounds (Sannaveerappa, Sandberg, & Undeland, 2007a; Sannaveerappa, Carlsson, Sandberg, & Undeland, 2007b). Recently, Taheri, Farvin, Jacobsen, & Baron (2014) isolated proteins fractions from barrel-salted herring brines and reported that they exhibit good antioxidant properties *in vitro* and in simple emulsion systems. However, despite their potentially interesting

antioxidant properties, no studies have showed how these protein-rich wastes could be directly valorised using minimal and simple procedures such as one-step fractionation and centrifugation.

Glazing/coating is a common practice in the seafood industry to preserve frozen fish from oxidation and dehydration and as alternative to this procedure, there is increasing research on edible coating based on proteins (e.g. soy, albumin and whey), fish skin hydrolysates and chitosan, (Sathivel, 2005, Sathivel, Liu, Huang, & Prinyawiwatkul, 2007, Gómez-Estaca, Montero, Giménez, & Gómez-Guillén, 2007, Rodríguez-Turienzo et al., 2011). Kakatkar, Sherekar, & Venugopal (2004) reported that acidic fish protein dispersion applied as a glaze to frozen fish blocks or fillets had a positive impact on quality, reducing oxidation and dehydration. Another investigation also showed that acidic dispersion of fish protein applied to seer fish improved its microbiological quality compared to water glazing (Phadke, Pagarker, Kumar Reddy, & Kumar Meena, 2012). Herring brines are dispersions of fish proteins and no investigation has been performed demonstrating their ability to be used as edible coating to protect frozen fish.

One of the processing technologies that have received a great deal of attention in the marine sectors to obtain functional proteins is the pH shift method to prepare protein isolate. In brief, in a first step muscle proteins are homogenised with water and solubilised at low pH (pH ≤ 3) or at high pH (pH ≥10.5). Centrifugation allows separation of insoluble material and in a second step the solubilised proteins are re-precipitated at their isoelectric point and a protein isolate is obtained (Undeland, Kelleher, & Hultin, 2002). This technique has been used to obtain protein isolate from several fish species including fish solid waste (Chitsomboon, Yongsawadigul, & Wiriyaphan, 2012, Park, 2012). Application of the soluble proteins fraction (before precipitation) has been investigated as coating agent and several patents exist demonstrating its use as moisture retention and coating agent preserving fish products quality (Kelleher, 2006, 2011). However, the pH shift has been investigated for sardine stick water as a way to recover proteins for its potential use as food/feed compositional

ingredient (García-Sifuentes et al., 2009), but until now there has been no research on liquid effluents from the fatty fish processing industry such as herring marinades.

OBJETIVES

Therefore, in this study brine protein was solubilised at extreme (2 or 11) pH, freeze-dried and applied to herring. Herring brine was tested either as coating agent for frozen fish or as an additive in fresh herring mince in order to investigate possible routes for valorisation of this liquid waste rich in proteins and peptides as a source of natural antioxidants.

2.MATERIALS AND METHODS

2.1. Brines

2.1.1. Initial brine

Brine from fillet-ripened spice-cured herring (*Clupea harengus*) (SC), traditional barrel-salted spice-cured herring (TSp) and brine from traditional barrel-salted herring (TSa) were obtained from the local fish herring processing industry (Lykkeberg A/S, Hørve, Denmark) and selected for valorisation. The pH of the raw brines was measured directly using a 780 pH meter (Metrohnm, Switzerland). All analyses on the brines were performed in triplicate and in two different sampling days.

2.1.2. Acidic and alkaline brine solutions

Fifty-millilitre samples of brines were adjusted to pH 2 or pH 11 (acidic or alkaline methods, respectively). All brines were centrifuged (Sorvall RC 5B Plus, Dupont, Norwalk, CT, USA) at 11,403 xg for 20 minutes at 10 ºC. Samples were filtrated through cotton and the collected

permeates were freeze dried (Heto DryWinner 8, Thermo Fisher Scientific, Loughborough, UK) or refrigerated at 4 ºC until further analysis.

2.2. Characterisation of brines

2.2.1. Mass balance

The mass balance is expressed as volume yield (final volume of permeate/sample volume after adjusting pH) in percentage (%).

2.2.2. Protein and salt content

Soluble protein content (mg/mL) of the initial brine and each acidic and basic permeate was assayed using a BCA kit (Thermo Scientific Pierce®, Rockford, USA), with bovine serum albumin (BSA) as standard.

The salt content in percentage (w:w) of the initial brine and the acidic and basic permeates was determined using the AOAC standard method (AOAC, 2000).

2.2.3. Protein profile: SDS-PAGE

Brine samples were diluted to 1 mg/mL in 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA, and further mixed 1:1 (v:v) with Laemmli buffer with 10% DDT. Subsequently the mixture was boiled for 3 minutes and centrifuged for 3 minutes at 13,684 xg. Samples and standard See-blue (10 µL) were loaded onto a 10% NuPAGE® Bis-Tris Gels (Novex®, 1.0 mm, 12 well, Life Technologies™, Denmark) and run with MOPS (4-morpholinepropanesulfonic acid) running buffer at 200V for approximately 50 minutes). After the run, gels were stained with Coomassie Brilliant blue G-250 overnight. Finally, the gels were washed with a destaining solution of 15% ethanol and 5% acetic acid until protein bands became clearly visible in a colourless gel matrix.

2.3. Storage experiments

2.3.1. Frozen herring coating

Ten kilograms of fresh herring (*Clupea harengus*) were obtained from local fishmongers, filleted with skin on and subsequently vacuum packed and stored at -80 °C until further experiment.

Coating was performed at 4 °C and frozen herring fillets were randomly allocated into three batches: initial brine SC, TSp and TSa adjusted to protein concentration of 1 mg/mL. Briefly dipping the fish fillet in fish protein solution for 10 seconds three consecutive times at 15 second intervals resulted in a thin coating layer onto the fillet surface. Controls consisted of non-coated fillets, fillets coated with water and vacuum packed fillets. For each treatment a minimum of 6 randomised fillets were used. The fish were placed in aluminium foil and stored for 4 and 10 weeks at -10 °C before they were stored at -80 °C until further analysis.

2.3.2. Fresh herring mince

Ten kilograms of fresh herring (*Clupea harengus*) were obtained from the local fishmongers, filleted, skinned and minced using a kitchen blender (Robot Coupe Ra A7S, France). The mince was stored in plastic bags under vacuum at -80 ºC until further experiment. Samples of mince (250 g) were randomly allocated into the following batches containing 1 g per kg of SC; TSp; TSa powder or their corresponding alkaline SC pH 11; TSp pH 11 and TSa pH 11. These powder were previously re-solubilised in water. Added water represented 10% (w:w) of the initial herring mince. Controls samples containing no additive, salt, Bovine Serum Albumin (BSA), or salt + BSA were also used. The content of salt and BSA were adjusted to 125 and 600 mg per kg of herring mince respectively, since it was the highest amount of salt and protein in the initial brines. All batches were kept in trays at 5 ºC and samples analysed on days 1, 4 and 7 of storage. At each sampling point, the samples were packaged in vacuum plastic bags and stored at -80 ºC until further analysis.

2.4. Storage Stability

2.4.1. Frozen herring coating

Total lipid content and peroxide value (PV)

Total lipids extract were obtained from 10 g herring mince or fillet with methanol/chloroform (1:1 v/v) according to the method of Bligh and Dyer (1959). PV was measured directly on the lipid extract (LEX) according to the method described by the International IDF Standards (1991) and expressed as milli equivalent (mEq) of O_2 per kg oil.

Volatile secondary oxidation products

The volatile secondary oxidation products were analysed only at day 4 for the herring mince and after 4 and 10 weeks for the glazed herring fillet using dynamic headspace according to Eymard, Baron, and Jacobsen (2009). An aqueous suspension of 10 g of fish powder was purged at 37 ºC in a water bath for 30 minutes with a nitrogen flow of 340 mL/min. The volatile compounds in the samples were collected on Tenax GR traps (Chromapack, Bergen op Zoom, The Netherlands). A Perkin–Elmer (Norwalk, CT) ATD-400 automatic thermal desorber system was used for thermally desorbing the collected volatiles from the Tenax traps using helium as a carrier gas (with a flow of 1.3 mL of helium/min). Thereafter volatiles were separated and quantified by gas chromatography-mass spectrometry (GC-MS), a DB 1701 column (30 x 0.25 mm, 1.0 µm; J&W Scientific, Folsom CA, USA) and the following programmes of temperatures were used: 35 ºC for 5 minutes, 35-90 ºC at 3 ºC min^{-1}, 90-240 ºC at 10 ºC min^{-1}, and finally hold at 240 ºC for 4 minutes. The GC–MS transfer line temperature was kept at 280 ºC. The mass-selective detector used ionisation at 70 eV in EI mode and 50 µA emission. The scans were performed in the mass range of 30–350 atomic mass units with a repetition rate of 2.2 scans/s. The compounds were identified by MS library searches and by comparing retention time and spectra with MS runs of external standards. For quantification

purpose, calibration curves were prepared by adding a mixture of ten selected standards in ethanol (concentrations 0.01-0.05 mg/g) directly on Tenax tubes (1 μL) and performing thermal desorption and GC-MS analysis under the conditions described above for the samples.

2.4.2. Fresh herring mince

PV and volatile secondary oxidation products were also used for monitoring lipid oxidation in fresh herring mince.

Thiobarbituric reactive substances (TBARS)

Fish and mince samples were analysed for TBARS using the methodology described by Vyncke (1975). Results were expressed as μmol malondialdehyde equivalents (MDA) per kg of muscle.

Protein oxidation

Protein carbonyls were measured on the herring mince as described by Levine, Williams, Statdman and Shacter (1994). Results are expressed in nmol carbonyls per mg of protein.

2.5. Statistical analysis

The data for pH, mass balance, soluble proteins and salt content were subjected to One-way ANOVA. Data of coating storage and mince herring experiment were analysed by multifactor ANOVA of each variable, taking into account treatment and time. All analyses were performed in triplicate. Fisher LSD (Least Significant Difference) test was applied for determining group differences at 95% significance level. Statgraphics Centurion XVI was used for carrying out the statistical analysis.

3. RESULTS

3.1. Characterisation of brines

Different brines (SC, TSa and TSp) and their acidic and basic counterparts (pH 2 and pH 11, respectively) were characterised for the biochemical composition (protein, salt concentration and protein profile using SDS-PAGE). The initial pH of the brines was found to be in the range of 5.96 to 6.05.

Yield (**Table 1**) ranged from 42 to 56% for unmodified brines, 35 to 49% for acidic- and 60 to 64% for basic–solubilised brine, respectively. Yield decrease can be due to the loss of solubilised proteins into the floating layer and sediments during centrifugation. Protein solubilisation into the floating layer has been reported to be lower for herring compared to other food such as mackerel and chicken (Undeland et al., 2002). The differences were explained by the structure of the acidified/alkalised herring homogenates, which creates a large floating layer and sediment with high water content. In fresh fish, the solubility of muscle proteins can be greater than 95% of the total proteins but herring brine contains salt which solubilises some proteins (salting in) whilst some others might precipitate (salting out). The theory behind acid or alkaline protein isolates is based on the formation of net, respectively positive or negative charge on the proteins, and consequently electrostatic forces are created, which drive the molecules apart from each other, enhancing solubilisation.

The analysis of soluble protein in the initial brines shows the highest amount in TSp (50.8±3.5 mg/mL), followed by TSa (41.0±2.68 mg/mL) and SC (37.9±3.15 mg/mL) (**Table 1)**. Marinating time is a crucial factor to determine the final amount of total nitrogen, both protein and non-protein. Nitrogen diffuses from herring into the brine during ripening (Szymczak & Kolakowski, 2012). SC had the shortest time of ripening, approximately 9 months, compared to TSa and TSp, which were

marinated for more than 16 months and this could explain the difference in protein content in the brines, which has also been reported previously (Gringer et al., 2014). The largest protein yield was obtained for the basic pH treatment, followed by neutral pH and acid pH. It can be concluded that solubilisation of protein from herring brine at pH 2 is not very efficient. A possible cause for this behaviour could be the low pH used which is lower than those found in literature, and in agreement with reports from Marmon and Undeland (2013), who reported that a pH below 2.5 decreased herring muscle protein solubility. Contrary to our results, protein recovery yield by acid solubilisation is usually higher than by alkaline solubilisation (Nolsøe & Undeland, 2009). Despite the low pH selected for our solubilisation, another explanation could be based on the high amount of salt contained in the samples, and a possible interaction between salt and pH, as reported by Kim and Park (2008) in Alaska pollock protein isolates, where salt induced salting out of unfolded protein.

Table 1: Yield expressed in % of initial volume before centrifugation and soluble protein of the recovered brine SC, TSa, TSp and their acidic (pH 2) and alkaline (pH 11) solutions.

	Yield (%, v:v)	**Soluble protein (mg/mL)**
SC	$56.67\pm4.16_{cdf}$	$37.95\pm3.15_{d}$
TSa	$42.00\pm3.46_{ab}$	$41.08\pm2.68_{d}$
TSp	$52.00\pm7.2_{bcd}$	$50.83\pm3.56_{f}$
SC pH2	$49.08\pm7.83_{ab}$	$2.73\pm0.57_{a}$
TSa pH2	$47.72\pm1.49_{bc}$	$3.95\pm0.77_{a}$
TSp pH2	$35.44\pm2.13_{a}$	$22.91\pm4.56_{b}$
SC pH 11	$64.04\pm9.85_{f}$	$30.17\pm1.98_{c}$
TSa pH 11	$60.20\pm0.71_{df}$	$34.13\pm3.32_{cd}$
TSp pH 11	$63.51\pm5.29_{f}$	$37.77\pm3.28_{cd}$

Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different ($p>0.05$).

The salt content for all samples ranged between 12 to 15% (data not shown). Alkaline solutions had significantly ($p<0.05$) lower salt concentration than acid solutions and both significantly lower than the unmodified brines. NaCl is known to increase muscle protein solubility, even if the solubility of thin and thick filament protein is largely different, salt affects the pH shift solubilisation of protein. Interaction between salt and protein and the impact of salt on protein unfolding and solubility in muscle food is still a major debate (Puolanne & Halonen, 2010). Analysis of the protein profiles through SDS-PAGE (**Figure 1**) was performed for initial and alkaline treated brines, as the acid treated samples were discarded from further studies due to their low protein yield.

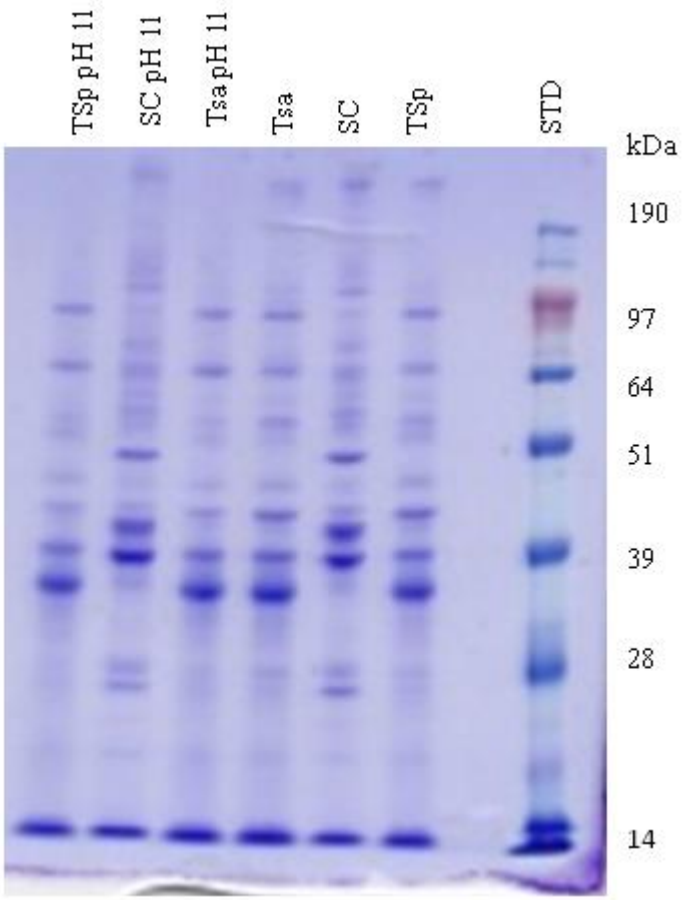

Figure 1: SDS-PAGE of initial brines TSa, TSp and SC and their alkaline (pH 11) solutions. STD: prestain standard seablue with indicated molecular weight kDa.

For all unmodified and alkaline solutions studied, myosin did not appear in any sample as no band was visible at around 200 kDa, being probably extensively degraded during the processes. Less myosin heavy chain (about 190 kDa) was solubilized from samples subjected to alkaline pH-shift. Despite the amount of scientific literature describing the fish marinating process, there is a lack of information on the qualitative and quantitative protein that is transferred to the brine during the marinating process (Szymczak & Kolakowski, 2012). Clear differences in the pattern of protein molecular weights profiles are observed between the SC permeates and both TSp and TSa. In general, SC showed higher molecular weight protein than the other two types of brines (TSp and TSa) which can be attributed to the high amount of proteolytic enzyme in these samples compared

to SC (Gringer at al., 2014). SC showed an intense fragment around 45 kDa and another around 49 kDa, but did not show the intense band revealed by TSp and TSa profiles at approximately 37 kDa. This different protein pattern was also in agreement with previous results (Gringer et al., 2014). For TSa and TSp lighter bands were observed in the high molecular weight region above 100 kDa.

3.2. Frozen herring coating

Rancidity during storage for frozen herring fillets coated with brine (SC, TSp, TSa) was measured by primary and secondary oxidation products: peroxide values (PV) and volatiles, respectively. Average oil content of herring used in this experiment was 20% (wet weight). PV showed early lipid oxidation (**Figure 2**). Control sample, fillets vacuum packed at -80 °C upon arrival to the laboratory, had 0.82 mEq per kg oil and after 4 weeks of storage a protective effect of brine glazing was observed when comparing PV to non-coated samples.

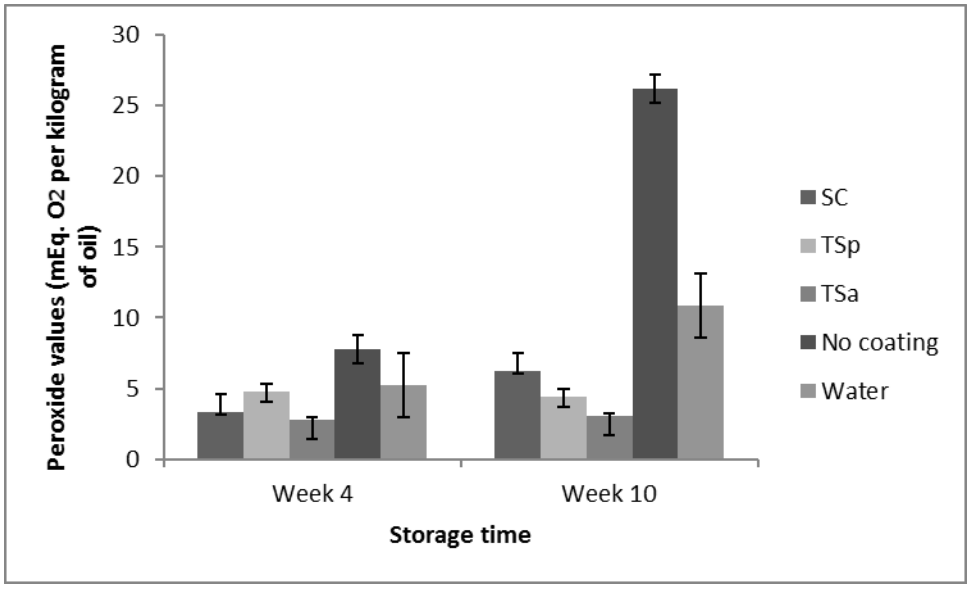

Figure 2: Peroxide value (PV) (mEq of O_2 per kilogram of oil) during storage at -10°C of brine coated frozen fillets for 4 and 10 weeks.

Brine seemed more potent to act as an antioxidant compared to water coating. These differences between PV of coated and un-coated samples were more evident after 10 weeks of storage. PV of TSp and TSa brine-glazed samples after 10 weeks of storage were significantly (p<0.05) lower than those of SC samples, and protein and peptide content (higher in TSp and TSa brines than in SC brine) may have contributed to this effect. A recent work (Gringer et al., 2014) also reported that TSp and TSa had higher iron chelating and radical scavenging activities than SC, which could contribute to their ability to be better coating agent than SC and water. Other authors (Kakatkar et al., 2004; Rodríguez-Turienzo et al., 2011) also reported that the protein glazing limited the lipid oxidation in fishery products during frozen storage and were a better protection than water glazing. However, more investigations are needed to reveal the exact mechanisms. Coatings of salmon with whey protein isolate and acetylated monoglyceride also reduced lipid oxidation during frozen storage (Stuchell & Krochta, 1995). It was further suggested that protein coating reduced the diffusion of oxygen and the availability of oxygen to the fish surface. It is possible that in herring brine, a combination of salt and protein change the structure of the frozen water on the surface of

the fish allowing less oxygen to penetrate the muscle tissues thereby preventing oxidation. Protein solutions have been the subject of patents (Kelleher 2006, 2011) in the process of protein isolate and have been applied to acidic solutions of muscle protein or dry powders into foods to retain moisture during cooking, prevent fat absorption during frying and prevent dehydration among other effects. Herring brines are acidic with pH value around 6 and contain large amount of protein and peptide and therefore can also be considered for such applications and more investigations are needed to demonstrate their potential. The concentration of volatile secondary products such as hexanal, 1-penten-3-ol, 2.4-heptadienal and heptanal after 4 and 10 weeks of storage are shown in **Table 2** and these results are in agreement with those for primary oxidation products (PV).

Table 2: Development of volatiles (ng per g mince) during storage at -10 °C of brine coated frozen fillets for 4 and 10 weeks.

	Hexanal		**1-penten-3-ol**		**2.4 heptadienal**		**Heptanal**	
	4 Weeks	**10 Weeks**	**4 Weeks**	**10 Weeks**	**4 Weeks**	**10 Weeks**	**4 Weeks**	**10 Weeks**
SC	$_{A}119.0^{ab}$	$_{A}181.6^{ab}$	$_{A}72.9^{ab}$	$_{A}92.7^{a}$	$_{A}99.6^{a}$	$_{B}240.8^{b}$	$_{A}2.2^{ab}$	$_{A}7.6^{ab}$
	±8.6	±59.9	±20.5	±29.5	±21.4	±58.1	±0.6	±5.8
TSp	$_{A}104.2^{ab}$	$_{A}132.1^{a}$	$_{A}75.60^{ab}$	$_{A}69.6^{a}$	$_{A}109.8^{a}$	$_{A}161.8^{ab}$	$_{A}1.4^{a}$	$_{A}3.5^{a}$
	±29.7	±49.8	±6.1	±12.7	±40.5	±18.0	±0.5	±1.3
TSa	$_{A}76.5^{a}$	$_{A}165.9^{ab}$	$_{A}50.03^{a}$	$_{A}70.3^{a}$	$_{A}81.5^{a}$	$_{A}127.2^{a}$	$_{A}1.2^{a}$	$_{A}3.2^{a}$
	±23.7	±69.6	±17.0	±15.7	±9.5	±36.1	± 0.5	±1.6
No Coating	$_{A}156.\ 8^{b}$	$_{A}278.2^{b}$	$_{A}83.\ 3^{b}$	$_{A}177.2^{b}$	$_{A}232.\ 7^{b}$	$_{A}320.0^{c}$	$_{A}3.6^{\ b}$	$_{A}15.6^{b}$
	±49.6	±102.6	±8.2	±82.3	±23.9	±63.7	±1.0	±10.0
Water	$_{A}73.1^{a}$	$_{A}149.8^{ab}$	$_{A}56.\ 3^{a}$	$_{B}112.7^{ab}$	$_{A}112.\ 3^{a}$	$_{A}124.3^{a}$	$_{A}1.8^{a}$	$_{A}4.8^{a}$
	±41.7	±64.1	±18.3	±8.8	±59.3	±28.7	±2.3	±3.7
Vacuum Packed*	22.8		26.6		32. 5		0.3	
	±1.6		±5.5		±6.0		±0.1	

*Control sample, fillets vacuum packed at -80 °C upon arrival to the laboratory.
Values (mean ± standard deviation. n=3) followed by the same uppercase letter in same row are not significantly different in the same volatiles (p>0.05).
Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different (p>0.05).

The alcohol 1-penten-3-ol is one the major oxidation products in herring mince among volatiles analysed and our results are in accordance with Sampels, Åsli, Vogt, and Mørkøre (2010). Hexanal and 1-penten-3-ol have been identified as good markers for early lipid oxidation and correlated with rancid off-flavour. Hexanal is representing the omega-6 whilst 1-penten-3-ol is representing the omega-3 fatty acid degradation products.

These results are in agreement with those for primary oxidation products (PV). After 4 weeks, although not significantly ($P<0.05$), the level of 2,4-heptadienal was lower in brine glazed fillets compared to water glazed, whilst no difference was observed for the other analysed volatiles. After 10 weeks, TSp and TSa seemed to protect better against oxidation than SC. The mixture of compounds present in the brines such as organic acids, natural antioxidants from the fish or inherent to the spices (SC and TSp) added during processing of marinated herrings, protein and peptide with antioxidant activity, makes it difficult to access the exact nature of the active compounds. Elias Kellerby, and Decker (2008) reviewed the antioxidant activity of protein and peptides and indicated that hydrolysis with the formation of peptides lead to higher antioxidant properties, as TSa and TSp occurred. Nevertheless, more research is needed, including purification, structure identification and investigation of the mechanisms responsible for the antioxidative activity in herring brines.

3.3. Fresh herring mince

The impact of addition of brine or their basic counterpart as additive was tested during storage of herring mince and the results for protein (protein carbonyls) and lipid (PV and TBARS) oxidation are presented in **Table 3**.

Table 3: Carbonyls content (nmol per mg of protein), Peroxide value (PV) (mEq per kg of oil) and Thiobarbituric Acid Reactive Substances (TBARS) (µmol malondialdehyde (MDA) per kg of muscle) for herring minced stored at 5ºC and added 1000 mg/kg of SC, TSa, TSp brine or their alkaline pH 11 counterparts or 125 mg per kg salt (NaCl) and/or 600 mg per kg bovine serum albumin (BSA).

	Day 1			Day 4			Day 7		
	Carbonyls	**PV**	**TBARS**	**Carbonyls**	**PV**	**TBARS**	**Carbonyls**	**PV**	**TBARS**
SC	$_{A}1.27\pm0.05^{ab}$	$_{A}4.05\pm1.09^{abc}$	$_{A}66.44\pm1.7^{cdf}$	NM	$_{B}7.33\pm0.44^{a}$	$_{A}71.88\pm1.62^{ab}$	$_{B}3.43\pm0.53^{a}$	NM	$_{A}73.25\pm9.51^{ab}$
TSp	$_{A}3.53\pm0.09^{d}$	$_{B}5.99\pm0.25^{df}$	$_{A}44.35\pm2.77^{a}$	NM	$_{C}8.53\pm0.52^{ab}$	$_{B}79.41\pm1.87^{bc}$	$_{B}6.49\pm0.30^{abc}$	NM	$_{C}92.13\pm6.58^{bcd}$
TSa	$_{A}1.27\pm0.13^{ab}$	$_{A}2.93\pm1.55^{a}$	$_{A}41.68\pm3.69^{a}$	NM	$_{B}9.83\pm2.43^{abc}$	$_{B}81.48\pm1.87^{bc}$	$_{C}5.52\pm0.14^{ab}$	NM	$_{B}84.05\pm7,71^{abc}$
TSa pH 11	$_{A}5.29\pm0.54^{f}$	$_{A}4.65\pm0.40^{bcd}$	$_{A}49.43\pm4.26^{ab}$	NM	$_{C}10.17\pm0.23^{abc}$	$_{C}94.17\pm1.65^{a}$	$_{B}7.97\pm1.41^{bc}$	NM	$_{D}121.61\pm1,69^{f}$
TSp pH 11	$_{A}2.65\pm0.53^{f}$	$_{A}5.12\pm0.16^{bcdf}$	$_{A}65.38\pm3.80^{cdf}$	NM	$_{B}18.8\pm2.22^{d}$	$_{B}102.44\pm4.67^{f}$	$_{B}8.27\pm1.09^{c}$	NM	$_{B}105.06\pm1,62^{df}$
SC pH 11	$_{A}2.24\pm0.19^{bc}$	$_{A}4.52\pm0.61^{abcd}$	$_{A}75.10\pm1.86^{f}$	NM	$_{B}13.51\pm0.23^{c}$	$_{A}63.15\pm1.66^{a}$	$_{C}7.22\pm0.17^{abc}$	NM	$_{A}68.39\pm6,10^{a}$
Salt	$_{A}1.96\pm0.22^{ab}$	$_{A}2.99\pm0.11^{a}$	$_{A}70.99\pm2.40^{df}$	NM	$_{B}8.43\pm1.00^{ab}$	$_{B}87.51\pm1.96^{cd}$	$_{C}4.32\pm0.03^{abc}$	NM	$_{B}91.89\pm1.82^{bcd}$
BSA	$_{A}3.28\pm0.93^{cd}$	$_{A}5.43\pm0.59^{cdf}$	$_{A}60.99\pm0.57^{cd}$	NM	$_{B}8.60\pm0.78^{ab}$	$_{B}83.65\pm2.38^{c}$	$_{C}8.27\pm0.49^{bc}$	NM	$_{B}82.99\pm1.55^{abc}$
Salt+BSA	$_{A}1.25\pm0.05^{a}$	$_{A}3.70\pm0.30^{ab}$	$_{A}65.14\pm5.44^{cdf}$	NM	$_{B}12.14\pm3.36^{bc}$	$_{B}98.62\pm1.39^{f}$	$_{B}5.78\pm0.15^{abc}$	NM	$_{B}94.80\pm9.15^{cd}$
No Additive	$_{A}1.66\pm0.06^{ab}$	$_{A}6.34\pm0.58^{f}$	$_{A}57.28\pm5.30^{bc}$	NM	$_{B}11.84\pm1.62^{bc}$	$_{C}91.57\pm5.33^{cd}$	$_{C}13.77\pm0.09^{d}$	NM	$_{D}117.42\pm1.34^{f}$

NM: not measured. Values (mean ± standard deviation. n=3) followed by the same uppercase letter in same row are not significantly different (p>0.05).
Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different (p>0.05)

The formation of carbonyl compounds from amino acid side chains is probably the most outstanding result of metal-ion catalysed oxidation of myofibrillar protein (Lund, Heinomen, Baron & Estévez, 2011). Initial carbonyl value (unprocessed herring minced) was 0.56 mmol/g of protein. As expected, the formation of protein carbonyls increased over storage time. Carbonyl content was significantly higher in herring with alkalinised brines especially in TSa. These results differ from Marmon and Undeland (2013); these authors did not find protein oxidation as measured by carbonyl content as a consequence of the alkaline pH-shift processing. The numerical value of carbonyls was significantly lower in minced herring containing SC brines compared to TSp and TSa in unmodified brines. Protein oxidation is in agreement with lipid oxidation results. This may be due to the different process to obtain the SC brine where fillets are marinated in brine compared to the TSa and TSp where the headed fish is processed. According to our results, samples without any additives showed the highest carbonyl value at the end of storage, while including salt prevented protein oxidation. This last result is in contrast with the findings by Kanner, Harel and Jaffe (1991), which showed a pro-oxidative effect of salt. In this regard, Andersen et al. (2007) stated that salt can be either antioxidative or pro-oxidative in muscle food.

In general, PV indicated higher levels of lipid oxidation in all alkaline-solubilised brine compared to initial brine, a trend which was also confirmed with other lipid oxidation parameter such as TBARS. Among samples, SC showed lower PV compared to TSp and TSa. This might be explained by the results from Vareltzis et al. (2011) demonstrating that strong pro-oxidants, such as heme protein which are known to be present in the brine, are less bound to membrane and thus more pro-oxidative. SC, TSa and TSp had lower PV and TBARS compared to the control without any additive from day 1, indicating that as an additive, brine is able to prevent lipid oxidation. However, there were no significant differences between samples and no significant difference when compared

with sample with salt and/or protein. In contrast, all brines seemed to present some antioxidant potential when examining TBARS. From 4 days of storage, the oxidative stability order was SC > TSp > TSa. However, the brine samples were comparable to salt and/or protein added samples. Both protein and salt played a protective role toward oxidation, but a non-synergistic effect was found when salt and protein were added together. Several research papers have showed the protective effect of protein toward lipid oxidation and it is well-known that proteins and peptides can have antioxidant activities (Elias, Kellerby, & Decker, 2008). At day 7, SC, TSa and TSp appeared to be preventing oxidation compared to alkalinized brines and/or controls, and among their basic counterparts only SC seemed to show this effect. Peptides obtained from alkaline isolate hydrolysates from tilapia muscle proteins have been shown to possess some antioxidant activity with increasing degree of hydrolysis, but extensive hydrolysis is also known to reduce the antioxidative potential (Raghavan & Kristinsson, 2008). This could explain the loss of antioxidative activity in TSa and TSp compared to SC. Indeed, the traditional barrel-salted process method (TSp and TSa) includes a step where the headed fish is stored for 6 months in brine, which can result in the transport of haemoglobin and other pro-oxidative compounds from the fish into the brine. It is also known the process induces hydrolysis of the muscle protein due to the presence of proteases. The basic treatment of the brine might have further induced protein and peptide hydrolysis in TSa and TSp up to an extent that resulted in a loss of activity.

The brines in ranking order of antioxidant capacity when considering both protein and lipid oxidation are SC > TSa > TSp. These results differ from the glazing experiment where TSa and TSp were better antioxidants than SC, demonstrating that the application is an important part of the antioxidant potential and that antioxidant activity should always be tested in a matrix based set-up/experiment, as also suggested by others Farvin, Grejsen and Jacobsen (2012). Evaluation of

volatile secondary oxidation products with identification and quantification in the herring mince was performed on day 4 and the results are presented in **Table 4**.

Table 4: Development of volatiles (ng per g minced) for herring mince stored 4 days at 5 ºC after addition of 1 g per kg of SC, TSp and TSa or their alkaline (pH 11) lyophilized brines or Salt (NaCl) 125 mg per kg and/or bovine serum albumin (BSA) 600 mg per kg.

	Hexanal	**1-penten-3-ol**	**2.4 heptadienal**	**Heptanal**
SC	15.97±1.32^{bc}	154.75±14.91^{ab}	3.71±0.07^{a}	5.02±0.32^{abc}
TSp	23.39±1.64^{d}	218.92±13.20^{cde}	6.37±0.04^{cd}	7.96±1.18^{d}
TSa	14.58±1.49^{abc}	205.61±9.84^{bcd}	4.78±0.41^{ab}	6.04±0.10^{bcd}
TSa pH 11	16.76±2.70^{bc}	174.55±31.41^{abc}	5.32±0.82^{bc}	4.40±0.27^{ab}
TSp pH 11	24.98±6.50^{d}	265.81±77.39^{f}	7.51±1.07^{d}	7.15±0.26^{cd}
SC pH 11	14.33±0.80^{bc}	179.84±20.39^{bc}	4.79±0.44^{ab}	7.06±0.25^{cd}
Salt	14.02±0.40^{abc}	149.50±8.16^{ab}	4.12±0.26^{a}	3.38±0.14^{a}
BSA	12.10±1.51^{ab}	159.50±4.74^{ab}	3.88±0.25^{a}	3.51±0.40^{ab}
Salt+BSA	9.70±1.14^{a}	120.35±11.28^{a}	4.36±0.74^{ab}	4.71±0.46^{ab}
No additive	16.72±0.60^{c}	237.40±19.83^{de}	4.09±0.75^{a}	3.02±0.11^{a}

Values (mean ± standard deviation. n=3) followed by the same lowercase letter in same column are not significantly different (p>0.05).

The volatiles analysed at day 4 all showed a similar tendency. In general, SC had the lowest concentration of volatiles while the highest levels were found in TSp, both unmodified and basic pH-solubilised. Among controls, there were only significant differences between samples without additive and the rest of the samples (i.e. salt, BSA and salt+BSA) in the case of hexanal and 1-penten-3-ol where protein (BSA) and salt could have played a protective role preventing lipid oxidation. The results observed were in agreement with the data in **Table 2** indicating a ranking order from lowest to highest presence of volatiles related to lipid oxidation as following: SC > TSa > TSp.

4. CONCLUSIONS

In summary, this investigation revealed the possibility of using herring brines by-products as natural antioxidant coating agents or additives. The use of brines as a glaze for frozen herring could represent an alternative to water glazing especially for TSa and TSp. Hence, this by-product can be used as natural protection for frozen fish fillets in fish prone to oxidation such as herring. Tested as antioxidant ingredient in herring minced, the brines in all cases showed a very good antioxidant activity when compared to the control, containing no additive. However, the salt and protein controls also showed a good antioxidant effect. Nevertheless, herring mince SC brine was the most effective in retarding lipid and protein oxidation as it resulted in low level of PV, TBARS, volatiles and carbonyls. Alkaline pH-solutions did not show any further benefit compared to unmodified brines. The direct use of protein solutions isolated from herring marinating brines seems feasible for preventing the oxidation of fresh herring mince, although it seems that salt and protein present in the brine play a key role in the observed antioxidant effect. Nevertheless the exact nature of the antioxidants in the brine still needs to be revealed. Furthermore the brines showed different behaviour depending on the application, with SC being better suited for mince and TSa and TSp better suited for coating of frozen fish. Thus, the results of the present study suggest the use of brines can be successfully employed as coating/glazing and natural ingredient to prevent oxidation in frozen herring fillets and chilled herring mince respectively demonstrating that this waste can be turned into a resource.

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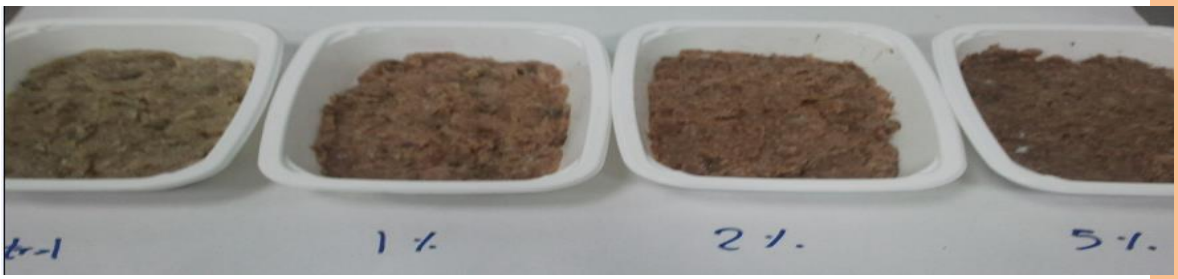
Chapter 2

Carob seed peel as natural antioxidant in minced Atlantic horse mackerel



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Carob seed peel as natural antioxidant in minced Atlantic horse mackerel

ABSTRACT

Carob seeds are used for carob bean gum extraction, where normally the carob seed peel (CSP) is lost. Recently, novel processing methods allow obtaining CSP as a by-product. CSP has not been previously characterised or studied for food applications. The aims of the present study were to characterise CSP and to evaluate its effectiveness as natural antioxidant in ground Atlantic horse mackerel (*Trachurus trachurus)*.

Proximate composition, phenolic content, phenolic profile and antioxidant capacity of CSP were determined. Atlantic horse mackerel mince was formulated with three different CSP concentrations (0% - control, 1%, 2% and 5% w/w) and stored at 4 ºC over 4 days.

CSP, which showed high phenolic content, was effective in preventing lipid and protein oxidation of minced mackerel. CSP reduced the peroxide value, conjugated dienes and trienes, TBARS, protein carbonyls and increased α-tocopherol levels of fish mince, showing a concentration-dependent antioxidant effect in fish mince. Fish samples with CSP showed better physicochemical parameters (pH, a_w, TVBN) and lower perceived off-odours (fishy and rancid) over storage than control.

In conclusion, carob seed peel could be used as natural antioxidant additive as it showed antioxidant properties and prevented protein and lipid oxidation when applied in a food product (minced fish).

Keywords: By-product valorisation; natural antioxidant; fish; carob; oxidation.

1. INTRODUCTION

Food processing industries generate large quantities of waste. Nowadays, efforts are focussed on reducing waste through the use of more efficient processes and upgrading the waste into added-value by-products, for instance, food ingredients. Carob tree (*Ceratonia siliqua*) grows in Mediterranean countries, being Spain the first carob fruit world producer with up to 40,000 tons in 2012 (FAOSTAT, 2014). The carob fruit consists of two main parts, the pod and the seeds. Traditionally, the primary uses of carob pods were animal feed and cocoa substitute; also, the seeds are industrially used for carob bean gum extraction (E-410) (Roseiro et al., 2013). This gum is added to a variety of products as thickener, stabiliser or flavouring. In the process of gum extraction different by-products are generated. A novel process allows the peel or cuticle, accounting for the 30-33% of the seeds, to be separated intact as a by-product (Gharnit, El Mtili, Ennabili, & Sayah, 2006).

The use of other carob by-products as value-added products, such as carob germ flour (Durazzo, Turfani, Narducci, Azzini, Maiani, & Carcea, 2014), the whole unprocessed fruit (Rababah et al., 2013), fruit extracts (Bastida et al., 2009) or the kibbles without seeds (Roseiro et al., 2013), has already received attention; however, the carob seed peel has not been previously characterised or used in food formulation. Other peel by-products, such as potato peel, have already shown antioxidant activity with potential food applications (Farvin, Grejsen, & Jacobsen, 2012).

Atlantic horse mackerel (*Trachurus trachurus*) is one of the most important pelagic fisheries in the southern Europe. This species contains high levels of long chain omega-3 polyunsaturated fatty acids (PUFAs), essential nutrients with well-known beneficial effects on human health. This fatty acid composition is also responsible for some inconvenience when processing Atlantic horse mackerel due to an increased susceptibility to oxidation (Farvin, et al., 2012). Many natural

antioxidants, such as rosemary extract (Vareltzis, Koufidis, Gavriilidou, Papavergou, & Vasiliadou, 1997), phenolics from grape and olive oil by-products (Medina, Gallardo, González, Lois, & Hedges, 2007) and potato peel extract (Farvin et al., 2012) have already been tested for preventing oxidation in Atlantic horse mackerel.

OBJETIVES

The aims of the present study were to characterise the proximate composition, phenolic composition, antioxidant and antimicrobial properties of carob seed peel, and to evaluate the effectiveness of carob seed peel as natural antioxidant in minced Atlantic horse mackerel.

2.MATERIALS AND METHODS

2.1. Chemicals

For the determination of total fibre content a kit from Megazyme Int. (Wicklow, Ireland) was used. All the chemicals were obtained from Panreac Química S.A (Barcelona, Spain). Folin-Ciocalteau phenol reagent, Gallic Acid, Trolox, Catechin, 1-diphenyl-2-picrylhydrazyl (DPPH), Fluorescein, 2,2`-azobis (2-methyl-propionamidine dihydrochloride) and the rest of standards for phenolic profile were purchased from Sigma-Aldrich (Madrid, Spain). For the minced Atlantic horse mackerel storage experiment, all the reagents were also from Sigma-Aldrich. All the HPLC grade solvents were supplied from Lab-Scan (Dublin, Ireland). Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB) and peptone were purchased from Difco laboratories (Difco laboratories, Sparks, MD).

2.2. Carob seed peel (CSP) characterisation

2.2.1. CSP preparation

Carob seed peel was kindly donated by Alimentaria Adín S.L. (Paterna, Valencia, Spain). The CSP was ground using a grinder (Ika-Werke M20, Staufen, Germany), and sieved to obtain a particle size smaller than 300 μm. The CSP was stored at 4 ºC until further analyses.

2.2.2. Proximate composition

Moisture content was gravimetrically determined (AOAC, 1995). Nitrogen content was analysed by the Kjeldahl technique (AOAC, 1995) and protein content was determined by multiplying N by 6.25. Ash content was determined by heating in a furnace to 550 °C for 24 h (AOAC, 1990). Total fat of dried samples was extracted with petroleum ether (BP 40-60 ºC) in an extracting unit Soxtec System 2055 Tecator (FOSS, Hillerød, Denmark) and gravimetrically determined. Carbohydrates were estimated as difference. Total fibre content (soluble and insoluble fibre) was determined according to the AOAC enzymatic method 985.29 (AOAC, 1995) using the reagent kit K-TDFR. All proximate composition determinations were performed in triplicate.

2.2.3. Extract preparation

Two grams of CSP were weighed out and mixed with 50 mL methanol:water (50:50, v/v). Extraction was carried out by stirring for 60 min at 60 ºC. Subsequently, it was filtered using Whatman nº1 filter paper. The filtrate was made up to 50 mL with methanol:water (50:50, v/v) in a volumetric flask and stored in dark until further analysis. This procedure was modified from the one described by Rababah et al. (2013). All determinations on CSP extract were performed in triplicate.

2.2.4. Determination of total phenolic content

Total phenols were measured using the Folin-Ciocalteu method (Slinkard and Singleton 1977). Results were expressed as mg gallic acid equivalent (GAE) per 100 g d.w. using a calibration curve with gallic acid as standard (9.8 µM to 70 µM).

2.2.5. Determination of condensed tannins

The method described by Porter, Hrstich and Chan (1985) was followed for the determination of condensed tannins in the extracts. The results were expressed as mg leucocyanidin equivalent (LE) per 100 g d.w.

2.2.6. Determination of radical DPPH-scavenging activity

The effect of antioxidant activity on DPPH was estimated according to the procedure described by Brand-Williams, Cuvelier and Berset (1995) with modifications. Results were expressed as µmoles Trolox Equivalents (TE) per g of sample.

2.2.7. ORAC (oxygen radical absorbance capacity)

The procedure was based on Ou, Hampsch-Woodill and Prior (2001). Results were expressed as µmoles Trolox Equivalents (TE) per g of sample.

2.2.8. Total Soluble Solids

The total soluble solids were measured at room temperature (20 ºC) using a digital refractometer (ATAGO, Fukuoka, Japan). Results were expressed as Brix degrees (ºBrix).

2.2.9. Phenolic profile by HPLC-DAD analysis

A 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, degasser, autosampler and a diode array detector (DAD) was used for the identification and quantification of various phenolic compounds in the samples. A C18 reverse phase analytical column (150 mm x 4.6 mm internal diameter; particle size, 5 μm) (Teknokroma Analitica S.A., Barcelona, Spain) was used for the separation. The binary mobile phase consisted of a 6% acetic acid in 2 mM sodium acetate buffer (Solvent A, pH 2.55, v/v) and acetonitrile (solvent B), and the gradient program was as follows: 0% B to 15% in 35 min, 15% B to 75% in 20 min, and 75% B to 100% B in 5 min, and then 10 min more at 100% B. There was a 10 min post run going back to the starting conditions for reconditioning. The flow rate was 1.0 mL/min for a total run of 85 min the injection volume was 10 μL for all samples. All standards were dissolved in methanol.

The detection was done using at 270 nm for gallic acid, 280 nm for flavanols (catechin and epicatechin), naringenin and naringenin 7-O-glucoside, 320 nm for hydroxycinamic acids (caffeic and coumaric acids) and for chlorogenic and ferulic acids and 360 nm for the flavonols miricetin, quercetin, luteolin and kaempferol. Compounds were tentatively identified by similar retention times and UV-vis spectra to those of standards.

2.2.10. Antimicrobial properties

The sources of bacteria used in the present study had previously described by Friedman, Henika and Mandrell (2002). *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* were streaked on TSA and incubated overnight at 37 ºC. One isolated colony was picked from each TSA plate and inoculated into a tube with 5-mL TSB at 37 °C for 24 h with agitation. Each inoculum was prepared by serially diluting (10×) in 0.1% peptone water.

Overlay diffusion tests were used for antimicrobial assays. To test antimicrobial effect of CSP and FG (Gelatin films)/CSP against three pathogenic microorganisms, 100 μL of 10^5 colony-forming units (CFU/mL) of each inoculum were uniformly spread onto TSA plates and left to dry for 5 min at room temperature. A 10 mm diameter sterile filter paper disk was placed at the center of each plate, 20 μL of FG/CSP solutions (diluted with water, 50/50, v/v) was placed on top of each disk. The plates were incubated at 37 °C for 48 h. The inhibition radius around the filter paper (colony-free perimeter) was measured in triplicate with a digital caliper (Neiko Tools, Ontario, CA, USA), and the inhibition area was then calculated in square millimeters. Antimicrobial activity of FG with different level of CSP against *L. monocytogenes* was measured using the same methodology, only replacing the filter paper disc with different FG/CSP discs (12 mm diameter). FG/CSP disks were placed over the agar with the film´s shiny side down.

2.3. Evaluation of CSP as natural antioxidant in Atlantic horse mackerel

2.3.1. Minced Atlantic horse mackerel storage experiment

Six kilograms of Atlantic horse mackerel (*Trachurus trachurus)* were bought in a fish market (Carrefour) in Valladolid, Spain. Fish was manually skinned and minced using a blender with a 7 mm exit pore (Lacor 69067, Guipúzcoa, Spain). Samples were randomly allocated into 4 batches: Control, 1 % CSP, 2 % CSP and 5 % CSP. CSP was dispersed in cold water and added to the fish samples. The moisture of CSP-containing samples was adjusted to match that of the original muscle. All batches were kept in trays at 4 ºC and samples analysed on days 0, 1, 2 and 3. The whole experiment was performed twice; all analyses were performed in triplicate.

2.3.2. Composition of Atlantic horse mackerel

Moisture content was gravimetrically determined according to AOAC (1995). Total lipids were extracted from 10 g of sample with methanol/chloroform (1:1, v/v) according to the method of Bligh and Dyer (1959).

2.3.3. pH and water activity

To determine pH, 10 g of fish flesh was homogenised with 100 mL of distilled water, filtered and then the pH filtrate was measured at room temperature with a pH-meter model 507 (CRISON, Barcelona, Spain). Water activity was measured with a water activity meter Aqualab 4TE (Decagon Devices Inc, Pullman, WA, USA).

2.3.4. Fatty acid composition

Fatty acids were analysed on the Bligh and Dyer extract obtained from minced Atlantic horse mackerel at day 0. The chloroform phase of the extract was evaporated to dryness under nitrogen. The remaining lipid phase was dissolved in 1 mL of hexane and methylated with 100 μL of 0.5 M methanolic KOH for 10 min at room temperature. The upper layer was transferred to a 2 mL vial. Analysis of fatty acid methyl esters (FAME) was carried out on a gas chromatograph Agilent 7890A equipped with a DB-23 column 60 m x 0.32 mm, (0.25 μm film thickness) and a flame ionisation detector. Helium was used as the carrier gas. The oven temperature was programmed to 50 ºC for the first 7 min and increased up to 200 ºC at a rate of 25 ºC per min, then the temperature was further increased to 230 ºC at a rate of 3 ºC per min and held for 26 min. Injection and detector temperatures were 250 ºC and 280 ºC, respectively. One microliter of the hexane extract was injected in split mode (ratio 25:1), and FAME's were identified by comparison of retention times with those of the standard (37 FAME's mix, Supelco, Sigma-Aldrich).

2.3.5. Peroxide Value (PV)

PV was measured directly on the Bligh & Dyer extract according to the method described by the International IDF Standards (1991). Results were expressed in milliequivalents of O_2 per kg of lipid.

2.3.6. Conjugated hydroperoxides (dienes and trienes)

Conjugated hydroperoxides were measured on the Bligh & Dyer extract dissolved in hexane, as described by Undeland, Stading and Lingnert (1998). The absorbance was measured at 234 nm and 268 nm and results were calculated as mmol of hydroperoxides per kg of lipid.

2.3.7. Thiobarbituric acid reactive substances (TBARS)

Samples were analysed using the methodology described by Vyncke (1975). Results were expressed as μmol malondialdehyde (MDA) per kg of muscle.

2.3.8. Tocopherol

Tocopherol content was determined on the Bligh and Dyer lipid extracts by HPLC using an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA), equipped with a fluorescence detector according to AOCS Official method (1992). Results are expressed in μg tocopherol per g muscle.

2.3.9. Free Fatty Acids (FFAs)

Ten grams of Bligh and Dyer extract were used to determine the FFAs content. FFAs were determined by titration with 0.1 M NaOH after adding ethanol (15 mL) to the Bligh and Dyer extract, using phenolphthalein as an indicator. FFAs content was calculated as oleic acid percent (AOCS, 1998).

2.3.10. Protein carbonyls

Proteins carbonyls were measured as described by Levine, Williams, Statdman & Shacter (1994). Results were expressed in nmol of carbonyls per mg of protein.

2.3.11. Total Volatile Basic Nitrogen (TVBN)

TVBN was determined according to the method described by Regulation (EC) 2005/2074 (EC, 2005). Results were expressed as mg TVBN per 100 g muscle.

2.3.12. Sensory analysis

Sensory evaluation was performed by a trained panel composed of ten people. The following odour descriptors: fishy, rancid and ammonia were scored on a -5 to 5 scale, compared against a control at day 0 which was regarded as of value 0. Approximately 20 g of each sample placed in a container were presented in randomised order to panellists after keeping for 15 min at room temperature to reach equilibrium between the minced Atlantic horse mackerel and the headspace of the sample container.

2.3.13. Colour

The colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured using a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japan). The illuminant was D65 (colour temperature of 6504 K) and the standard observer was 10º. Measurements were taken on the samples packaged in transparent plastic bags at six different points.

2.4. Statistical analysis

Data were analysed by one-way ANOVA. Fisher LSD (Least Significant Difference) test was applied for determining group differences at 95% significance level. Statgraphics Centurion XVI was used for carrying out the statistical analysis.

3. RESULTS

3.1. Analysis of carob seed peel

3.1.1. Proximate composition and total soluble solid

CSP composition in moisture, protein, fat and ash were 15.30% (±0.20), 4.86% (±0.40), 0.31% (±0.01) and 3.70% (±0.30), respectively. Carbohydrates were calculated by difference as 75.83% of the product. CSP presented 6.80 ºBrix of total soluble solids, which consisted above all of soluble mono- and disaccharides. As it was expected, CSP composition was completely different to other by-products from carob bean gum extraction, such as germ or pod. Dakia, Wathelet and Paquot (2007) characterised germ obtained by different treatments. These results showed that germ consisted basically of protein (54.7-67.1%). In addition, fat was significant higher in germ (7.1-8.2%) compared to CSP.

The main characteristic of CSP is its high content in dietary fibre. The dietary fibre content of this by-product was 61.64% (±0.32). This value is relatively high when compared with other similar by-products susceptible to be used as antioxidant ingredients for their valorisation, such as for example apple peels (Massini, Rico, Martín-Diana, & Barry-Ryan, 2013). Fibre content is not commonly present in animal origin food such as seafood products, so the fibre contribution would be an extra benefit to the final product, besides its antioxidant capacity.

3.1.2. Total Phenolic Content (TPC), Condensed Tannins and Antioxidant Capacity (DPPH & ORAC)

The carob seed peel showed a total phenolic content of 1722±84.4 mg GAE per 100 g d.w. Durazzo et al. (2014) found TPC values for the complete seed in the range of 2428.28 mg GAE per 100 g d.w. These authors also obtained values of TPC for the germ and the endosperm of 1924.70 and 71.03 mg GAE per 100 g d.w., respectively. Bearing in mind that the endosperm, the fraction with the lowest value of TPC within the seed's fractions, as reported by Durazzo et al. (2014), accounts for the 42-46% of the weight of the seed, it would be expected for the other two fractions to have higher TPC content than the complete seed. As the germ TPC is below the average of that of the whole seed, the cuticle or peel would finally be supposed to bear the highest concentration of total phenols. This is not the case for our result (1722 mg GAE per 100 g d.w.), which is lower than the value obtained for the complete seed by Durazzo et al. (2014), and this imbalance could be explained either through variety differences, or by the two different extraction methods used, and in this sense, a significantly higher TPC for CSP might be expected when including a second extraction step with 70% acetone as in the methodology by the referred authors (Durazzo et al., 2014).

Furthermore, CSP showed higher TPC when compared to other by-products that have been tested for similar valorisation strategies, as for example potato peel (68.7 ± 5.7 mg GAE per 100 g d.w.), in Farvin et al. (2012). The condensed tannin content of CSP was 1350 (±50) mg LE per 100 g d.w. This result was significantly higher that the results found by Rababah et al. (2013) in the carob fruit.

The DPPH and ORAC results for CSP were 687.59 (±64.25) and 428.01 (±24.43) μmoles TE per g d.w., respectively. The existing data on the carob germ are very scarce, and there is no report, as far as the authors could know, on characterisation of CSP antioxidant capacity. Roseiro et al. (2013)

worked with carob pods, and found a similar range of TPC (580 to 2710 mg GAE per 100 g of dry mass) as the one found in the present work, but lower range for antioxidant activity (27 to 130 μmol TE per g dry mass).

3.1.3. Phenolic profile by HPLC-DAD analysis

The phenolic profile was determined by HPLC-DAD and identified phenolic compounds quantified with the use of standards. Besides tannins, flavonoids and flavonols accounted for the majority of compounds, mainly catechin (360 mg per 100 g), quercetin (90 mg per 100 g) and epicatechin (6 mg per 100 g). Catechins, also the major polyphenolic compounds in green tea, were reported to exert lipoxygenase inhibitory action in mackerel muscle (Banerjee, 2006). Medina et al. (2007) evaluated the antioxidant effectiveness of catechins as pure compounds in minced fish muscle, and found catechin as the most efficient among the four catechins studied.

Among benzoic acids, 136 mg per 100 g corresponded to gallic acid. This compound was previously found in carob pulp kibbles (Roseiro et al., 2013). Other phenolic compounds present in CSP included flavones such as a luteolin derivative (229 mg per 100 g) detected at 360 nm.

Comparison of the CSP phenolic composition with other studies was difficult, as CSP has not been previously characterised. Apart from that, different extraction conditions led to different phenolic profiles. Another difficulty to compare results is the differences in the phenolic pattern depending on the season, variety or region.

3.1.4 Antimicrobial activity of CSP

CSP was tested against three important foodborne pathogens such as *Escherichia coli* 0157:H7, *Salmonella enterica* and *Listeria monocytogenes*. Both CSP power and dilution did not inhibit the growth of these three pathogenic bacteria (**Figure 1**). The absence of surrounding clear zones in the

overlay test served as evidence that the CSP did not have inhibitory activity against aforementioned bacteria.

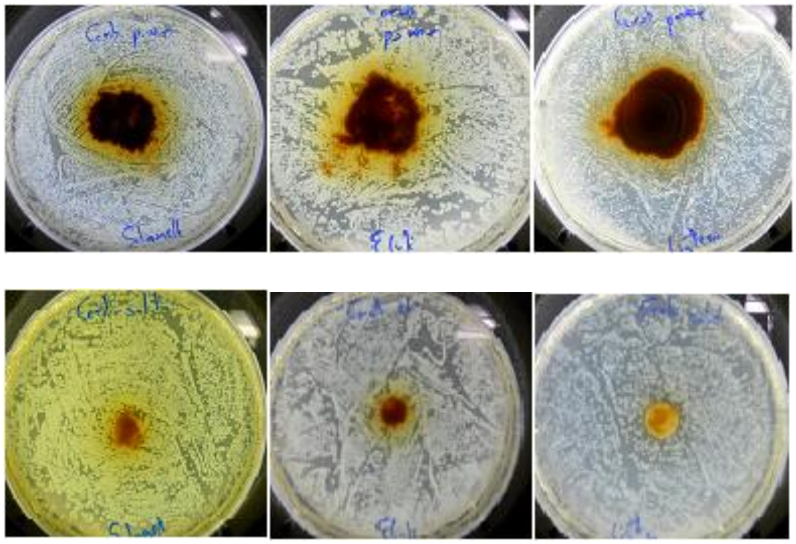

Figure 1: Photographs of overlay diffusion test using CSP powder (above) and dilution (below) of *S. enterica*, *E.coli* and *L. monocytogenes*.

This finding is different from Tassou, Drosinos and Nychas (1997) and Aissani, Coroneo, Fattouch and Caboni (2012) who demonstrated antimicrobial activity of carob against *Listeria monocytogenes*. Nevertheless, they tested different carob constituents, mainly fruit sections and leaves, respectively. Apart from that, extract was used instead of the powder. Aissani et al. (2012) reported that the antimicrobial mechanism of carob leaves against *L. monocytogenes* was the inhibition of proline dehydrogenase (PDH) by means of polyphenols, which prevented bacterial growth. Taking into account the antimicrobial activity of the different types of polyphenols, gallic acid played an important role (Aissani et al., 2012). However, this compound is a minority in CSP, according to our previous analyses. In conclusion, the use of different parts of carob linked to extraction methods produced distinct mixtures of phenolic compounds and consequently different antimicrobial activities.

3.2. Minced Atlantic horse mackerel storage experiment

3.2.1. General characteristics of minced fish (fat, moisture, pH and aw)

Moisture and fat content of the minced fish were found to be in the range of 70.56-72.66 and 4.80-7.03 g per 100 g respectively. Both results agreed with previous studies on Atlantic horse mackerel (Aubourg, Lehmann & Gallardo, 2002). Fat and moisture content did not change over storage and no differences were found among samples.

The initial pH values (6.67-6.80) were higher than other found in literature (Farvin et al., 2012; Tzikas, Papavergou, Soultos, Ambrosiadis, & Georgakis, 2009), differences which could be due to normal species variability or the catching method.

Samples with 5% CSP significantly showed the lowest pH values (mainly pH 6.67) at the beginning of storage, when compared to the rest of the samples. During chilled storage, there was a significant pH decrease with all treatments. This reduction can be attributed probably to the growth of lactic acid bacteria and lactic acid formation (Picouet, Cofan-Carbo, Vilaseca, Ballbé, & Castells, 2011). Control samples maintained pH levels similar to 1% and 2% up to day 2, and higher than the rest of the samples by the end of the storage (day 3).

Water activity (a_w) values ranged from 0.986 to 0.992. There was not a clear trend over storage. Nevertheless, control started with lower values, but at the end of storage (day 3) no differences were observed among samples (data not shown).

3.2.2. Fatty acid composition

The proportion of saturated fatty acids in the total lipids fraction of Atlantic horse mackerel was 27.91% of the total fatty acids, whereas the mono and polyunsaturated fatty acids constituted 29.81% and 32.42% respectively. This is in agreement with the previously reported high sensitivity to oxidation of this fish's oil (Eymard, Baron, & Jacobsen, 2009). Docosahexaenoic acid (DHA, C22:6 n-3) was the most abundant (18.02%) of the polyunsaturated fatty acids, whereas the major saturated fatty acid was palmitic acid (C16:0) and oleic acid (C18:1, n-9) the main monounsaturated fatty acid, with a percentage over the total fatty acid content of 18.62% and 21.74%, respectively.

3.2.3. Peroxide value (PV)

The peroxide value (PV) gradually increased in all samples (**Figure 2**), as storage progressed until day 2. Between day 2 and 3, PV levels remained steady, with the exception of 5% CSP. The steady levels between day 2 and 3 may be explained by the change in formation and decomposition rates of peroxides, as these are primary oxidation products and tend to decompose in secondary products (Pereira de Abreu, Losada, Maroto & Cruz, 2010).

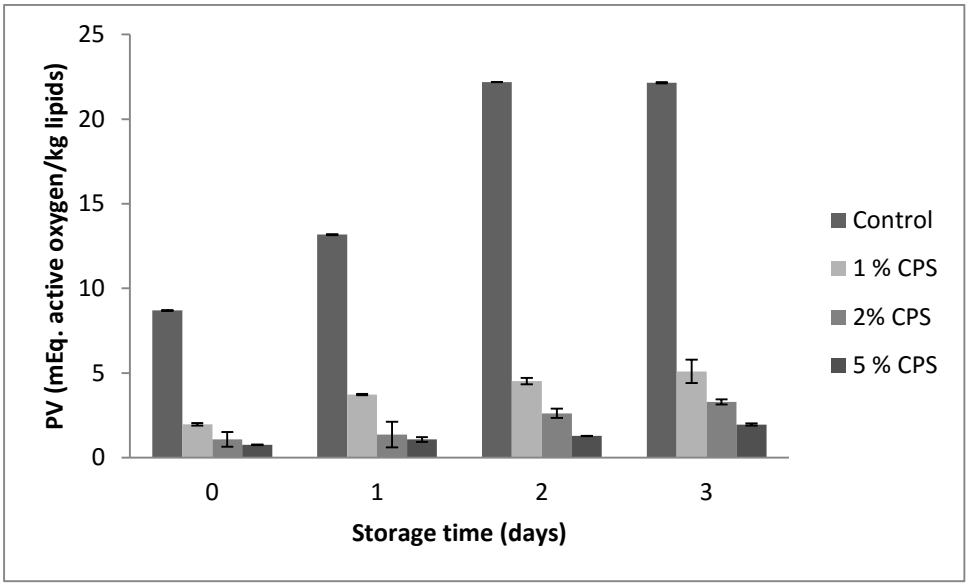

Figure 2: Peroxide value (PV) of minced horse mackerel with different concentrations of carob seed peel (CSP) during storage at 4 ºC.

From day 0, immediately after preparation of samples, PVs were lower in all CSP samples when compare to control samples. In studies with other by-products, initial PVs were also lower immediately after the preparation in the samples where white grape antioxidant dietary fibre (WGDF) was added to Atlantic horse mackerel minced muscle (Sánchez-Alonso, Jiménez-Escrig, Saura-Calixto, & Borderías, 2008). This could be explained by the importance of adding antioxidant as early as possible to foodstuffs to achieve maximum protection against oxidation. Mincing affects lipid oxidation due to cellular disruption and subsequent activation of lipoxygenase. Lipoxygenase can initiate the oxidation of polyunsaturated fatty acids to produce acyl hydroperoxides (Joaquin, Tolasa, Oliveira, Lee & Lee, 2008).

In summary, all samples containing CSP showed greater stability toward oxidation during all storage period. Significant differences (p<0.05) were also observed due to CSP concentration, decreasing PVs with increasing CSP concentrations.

3.2.4. Conjugated dienes and trienes

The levels of conjugated dienes were significantly lower ($p<0.05$) in samples with CSP compared to those of control samples at second day of storage. In the case of trienes, these significant differences were found after 1 day of storage (**Table 1**). The use of CSP significantly reduced the dienes and trienes formation over the 3-days storage.

Table 1: Dienes and trienes (mmol of hydroperoxides per kg of lipid) of minced horse mackerel with different concentrations of carob seed peel (CSP) during storage of at 4 ºC.

	Day 0	**Day 1**	**Day 2**	**Day 3**
Dienes				
Control	$^{A}22.75\pm0.48_{a}$	$^{A}24.51\pm1.93_{a}$	$^{A}29.48\pm1.42_{b}$	$^{B}57.05\pm1.02_{b}$
1 % CSP	$^{A}24.02\pm0.48_{a}$	$^{A}24.17\pm0.43_{a}$	$^{A}24.50\pm0.08_{a}$	$^{A}25.09\pm0.07_{a}$
2% CSP	$^{A}19.02\pm3.86_{a}$	$^{A}22.28\pm10.48_{a}$	$^{A}22.03\pm1.28_{a}$	$^{B}26.95\pm1.28_{a}$
5 % CSP	$^{A}18.77\pm0.15_{a}$	$^{B}22.08\pm0.55_{a}$	$^{B}22.03\pm1.05_{a}$	$^{C}24.50\pm1.06_{a}$
Trienes				
Control	$^{A}2.34\pm0.11_{a}$	$^{B}4.62\pm0.08_{b}$	$^{B}4.61\pm0.13_{b}$	$^{B}4.36\pm0.46_{b}$
1 % CPS	$^{A}2.65\pm0.01_{a}$	$^{A}2.33\pm0.23_{a}$	$^{A}2.35\pm0.01_{a}$	$^{B}3.50\pm0.28_{a}$
2% CPS	$^{A}2.38\pm1.17_{a}$	$^{A}2.86\pm1.12_{a}$	$^{A}2.17\pm0.42_{a}$	$^{A}2.05\pm0.03_{a}$
5 % CPS	$^{A}1.28\pm0.16_{a}$	$^{A}2.22\pm0.10_{a}$	$^{A}2.19\pm0.11_{a}$	$^{A}2.07\pm0.68_{a}$

Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same row are not significantly different ($p>0.05$).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same column, for each parameter, are not significantly different ($p>0.05$).

Sánchez-Alonso et al. (2008) found that differences in trienes formation due to antioxidant concentration increased over storage time in frozen minced fish muscle treated with red grape antioxidant fibre. On the other hand, the peroxide values (**Figure 2**) showed significant differences when using different CSP concentrations; in this regard, Marmesat, Morales, Velasco, Ruiz-Méndez & Dobarganes (2009) concluded that conjugated dienes and trienes values perform as oxidation indicators with much lower sensitivities than the peroxide value, and in this sense, although

differences between CSP samples and control were observed, they were not significant between samples with increasing CSP concentrations.

3.2.5. Thiobarbituric acid reactive substances (TBARS)

Initial TBARS values of all groups ranged from 22.98 (control) to 15.67 (2% CSP) µmol MDA per kg of muscle (**Figure 3**). TBARS showed a gradual increase during chilled storage for all samples. As TBARS are related to secondary oxidation products, they accumulate and, as expected, increased along all the storage time evaluated.

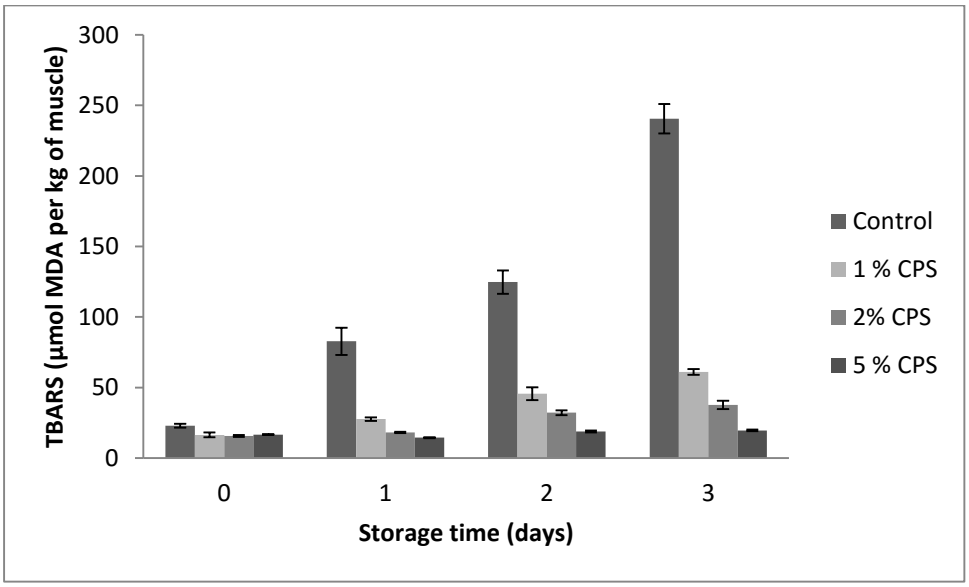

Figure 3: Thiobarbituric Acid Reactive Substances (TBARS), quantified as malondialdehyde (MDA), of minced horse mackerel with different concentrations of carob seed peel (CSP) during storage at 4 ºC.

There were significant differences between control and samples containing CSP from day 0, that could be due to the protective effect of added antioxidant (Bastida, Sánchez-Muniz, Olivero, Pérez-Olleros, Ruiz-Roso, & Jiménez-Colmenero, 2009). Over storage, the inclusion of CSP in Atlantic horse mackerel minced muscle exerted an antioxidant effect dependent on the concentration. This parameter has shown to be a good index of sensory rancidity development in seafood (Sánchez-Alonso et al., 2008).

3.2.6. Tocopherol content

The levels of tocopherol decreased during storage. Tocopherols are reduced due to its antioxidant activity, scavenging radicals that could otherwise damage lipids and proteins. Initial tocopherol content ranged from 8.67 to 13.80 μg per g sample (data not shown); these results were similar to those obtained by Farvin et al. (2012). At day 0, there were no significant differences between the concentrations of tocopherol within different CSP concentration groups. From day 2 of storage, there was a significant preservation of tocopherol in samples with 5% CSP. At the end of storage period, samples with 5% CSP showed the highest tocopherol content (10.17 μg tocopherol per g of muscle) compared to control, 1% CSP and 2% CSP with a concentration of 7.45, 7.11 and 7.37 μg tocopherol per g of muscle, respectively. In brief, only the supplement of the Atlantic horse mackerel minced muscle with 5% CSP was effective for limiting the loss of α-tocopherol.

3.2.7. Free fatty acids (FFAs)

FFAs were measured as an estimation of lipolysis. The initial FFA content ranged from 0.70 to 0.98% (data not shown), similar to values found by Tzikas et al. (2009) in *Trachurus mediterraneus*. In the present study, FFAs content remained constant in all experiment and no differences were found between samples. This trend differed from other oxidative markers (PV, conjugated dienes and trienes, TBARS, tocopherol). Farvin et al. (2012) indicated that free fatty acids might not be reliable oxidative status indicators.

3.2.8. Protein oxidation: Determination of protein carbonyls

The protein carbonyls of minced Atlantic horse mackerel with different concentrations of CSP are shown in **Figure 4**. The formation of carbonyl compounds from amino acid side chains is probably the most outstanding result of metal-ion catalysed oxidation of myofibrillar protein (Lund, Heinomen, Baron & Estévez, 2011). As expected, the formation of protein carbonyls increased over

storage time. This rapid development of protein oxidation during storage is favoured by the previous mincing, being protein oxidation induced by heme protein (Eymard et al., 2009).

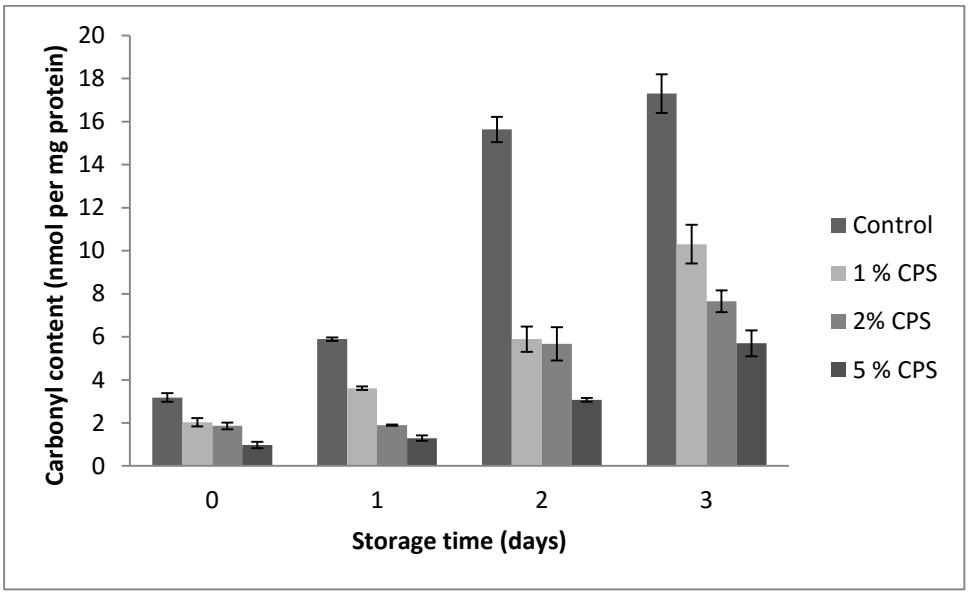

Figure 4: Carbonyl content of minced horse mackerel with different concentrations of carob seed peel (CSP) during storage at 4 ºC.

CSP was effective in preventing protein carbonyls development, when compared to control. The effectiveness of CSP was depending on their concentration. CSP has a high content of phenolic compounds, which has been proposed by Siebert, Troukhanova and Lynn (1996) as inhibitors of the oxidation of proteins, either by preventing the lipid oxidative reactions, binding to the proteins, or forming complexes with them. These results are consistent with lipid oxidation products. Many phenolic-rich plants and extracts have also demonstrated antioxidant effect on proteins (Lund et al., 2011).

3.2.9. Total Volatile Basic Nitrogen (TVBN)

TVBN measurements (**Figure 5**) were low at the beginning of the period of storage, with values in accordance with data reported by literature for freshly caught fish, which are typically between 5 and 20 mg TVBN per 100 g (Vareltzis et al., 1997).

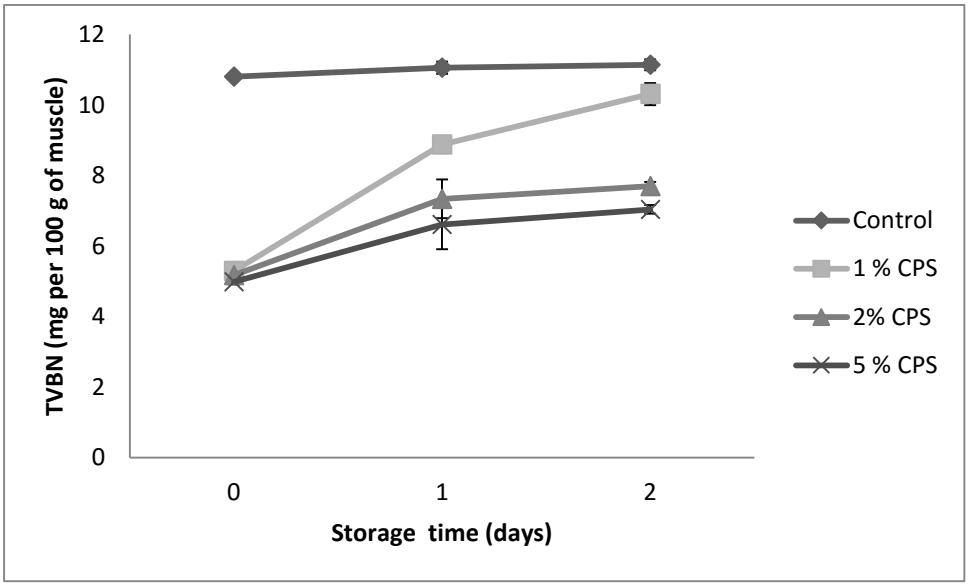

Figure 5: Total volatile basic nitrogen (TVBN) of minced horse mackerel with different concentrations of carob seed peel (CSP) during storage at 4 ºC.

Results showed an increase in TVBN for all samples over storage. Nevertheless, TVBN in any treatment exceeded the legal limit established by European Union of 25-35 mg of TVBN in 100 grams of muscle. Significant differences (p<0.05) between control and samples treated with antioxidant (1% CSP, 2% CSP and 5% CSP) occurred. CSP reduced the TVBN formation depending on CSP concentration. TVBN increase is related to spoilage by bacteria and activity of endogenous enzymes (Vareltzis et al., 1997).

3.2.10. Sensory properties

Sensory analysis is the best available method to understand changes caused by lipid oxidation which are perceived by humans. Control samples had the highest scores in fishy and rancid odour respect to samples with CSP (data not shown). Rancid odour development is well documented during chilled storage of fatty fish species (Ólafsdóttir & Jónsdóttir, 2010). The sensory results were also consistent with a reduction of oxidation (PV, hydroperoxides, TBARS, carbonyls) in samples with CSP compared to control. Nevertheless, panellists were not able to see differences among

samples with different CSP concentrations. Concerning ammonia odour, this was only detected in control samples. These data agreed with TVBN values. A possible explanation is that TVBN in seawater fish comes mainly from ammonia (Clucas, 1982).

3.2.11. Colour

Sample colour values expressed as lightness (L*), redness (a*) and yellowness (b*) are shown in **Table 2**. Lightness (L*) significantly increased over storage. This behaviour of the lightness parameter has previously been observed in salmonids, representing discolouration of the flesh (Robb, Kestin, & Warris, 2000).

Table 2: Colour parameters: Lightness (L*), Redness (a*), Yellowness (b*) minced horse mackerel with different concentrations of carob seed peel (CSP) during storage at 4 ºC.

L*	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Control	$^{A}22.10\pm0.76_{b}$	$^{B}33.63\pm0.52_{b}$	$^{C}39.03\pm0.05_{a}$	$^{B}32.41\pm1.23_{a}$
1 % CPS	$^{A}32.71\pm1.91_{c}$	$^{A}31.77\pm2.14_{b}$	$^{B}36.79\pm3.41_{a}$	$^{B}37.20\pm1.94_{c}$
2% CPS	$^{A}16.50\pm1.38_{a}$	$^{B}28.58\pm0.38_{a}$	$^{C}37.90\pm0.70_{a}$	$^{C}34.71\pm3.35_{b}$
5 % CPS	$^{A}21.16\pm0.44_{b}$	$^{B}38.59\pm0.66_{c}$	$^{B}33.82\pm0.66_{a}$	$^{B}32.91\pm0.84_{ab}$
a*				
Control	$^{C}4.65\pm0.52_{a}$	$^{B}2.20\pm0.44_{a}$	$^{B}1.89\pm0.01_{a}$	$^{A}1.14\pm0.02_{a}$
1 % CPS	$^{C}6.66\pm0.48_{c}$	$^{B}3.68\pm0.28_{b}$	$^{A}2.86\pm0.68_{b}$	$^{A}2.73\pm0.15_{b}$
2% CPS	$^{C}8.61\pm0.59_{d}$	$^{B}3.78\pm0.09_{b}$	$^{B}3.35\pm0.62_{c}$	$^{A}2.43\pm0.25_{b}$
5 % CPS	$^{C}5.97\pm0.70_{b}$	$^{B}4.06\pm0.71_{c}$	$^{B}3.84\pm0.05_{c}$	$^{A}2.73\pm0.11_{b}$
b*				
Control	$^{A}2.10\pm0.55_{a}$	$^{B}3.97\pm0.66_{b}$	$^{A}1.50\pm0.01_{a}$	$^{A}1.48\pm0.08_{a}$
1 % CPS	$^{B}4.33\pm0.27_{b}$	$^{A}3.49\pm0.45_{b}$	$^{AB}3.74\pm0.49_{b}$	$^{A}3.56\pm0.09_{b}$
2% CPS	$^{D}5.14\pm0.96_{b}$	$^{A}2.44\pm0.43_{a}$	$^{C}4.02\pm0.07_{b}$	$^{B}3.14\pm0.14_{b}$
5 % CPS	$^{A}4.92\pm0.73_{b}$	$^{A}3.33\pm0.61_{ab}$	$^{A}4.24\pm0.16_{b}$	$^{A}4.34\pm0.29_{c}$

Values (mean ± standard deviation, n=6) followed by the same uppercase letter in same row are not significantly different (p>0.05).
Values (mean ± standard deviation, n=6) followed by the same lowercase letter in same column, for each parameter, are not significantly different (*p*>0.05).

There was a significant reduction ($p<0.05$) in redness in all the groups over storage. In this sense, the loss of redness can be related to oxidation of heme proteins, haemoglobin and myoglobin, which are red in their reduced form and brown in their oxidised ferric form. Colour results agreed with oxidative measurement (PV, hydroperoxides, TBARS, tocopherol), where the addition of CSP reduced the minced Atlantic horse mackerel oxidation. At days 1 and 2 of storage, higher values of a* were observed in samples with increasing CSP concentrations. This effect was not observed at day 3, where all CSP-added samples resulted in similar a* values, although significantly higher than the control (this last difference was observed over the entire storage).

Yellowness values (b*) changed significantly when CSP was added, but did not differ within samples with different percentage of CPS. Different from a*, in the case of b* did not give any information about oxidation changes. Usually, surface colour (a* and b*) changes are related to oxidation development, Sánchez-Alonso et al. (2008) found that yellowness was related to the oxidised heme proteins (met-heme proteins), which achieved a brown colour.

4. CONCLUSIONS

Carob seed peel is characterised by high fibre and polyphenol content, the later contributing to its antioxidant potential. Nevertheless non antimicrobial activity was detected against three foodborne pathogens tested.

Tested as natural antioxidant in minced fish, CSP showed antioxidant activity and was able to significantly inhibit the lipid oxidation (PV, hydroperoxides, TBARS, rancid odour), reduce loss of α-tocopherol, colour modification and protein oxidation (carbonlys), and improve other chemical parameters such as TVBN.

These results indicate that CSP, a by-product from carob bean gum extraction, could be used as natural antioxidant in minced fish (chilled storage). However, further studies are necessary to evaluate its potential health benefits.

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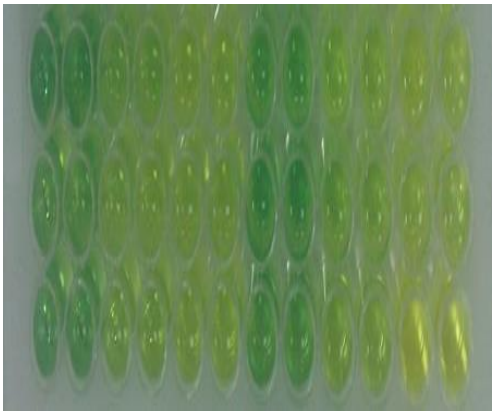
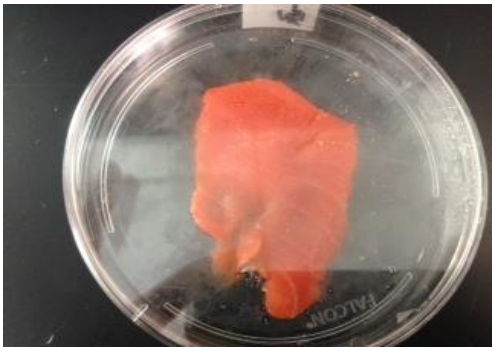
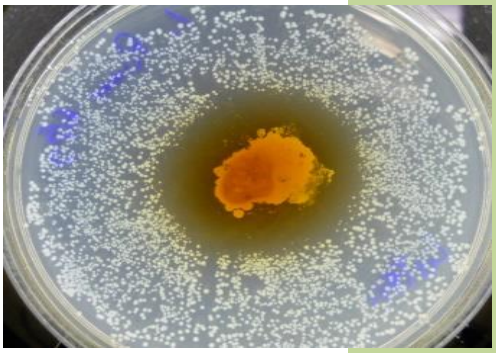
Chapter 3

Olive leaf as antimicrobial and antioxidant ingredient in fish products







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Olive leaf as antimicrobial and antioxidant ingredient in fish products

ABSTRACT

There is a growing interest in the replacement of synthetic food antioxidants by natural extracts. Olive leaf is an abundant by-product of olive oil industry. Olive leaf products were evaluated as antimicrobial/antioxidant ingredients directly applied on chilled fish or through edible films for smoked fish preservation.

Olive leaf powder (OLP) showed a high antioxidant capacity and fiber content. OLP and its water/ethanol extract (OLPE) were tested against *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enterica* using agar diffusion test. OLP and OLPE only showed antibacterial activity against *L. monocytogenes* in agar diffusion tests.

The antioxidant capacity of OLP in chilled minced muscle was investigated. Concentrations of 0 % (control), 1.5% (1.5% OLP), 3.5% (3.5% OLP) and 4.5% (4.5% OLP) were added to minced horse mackerel and stored at 4 ºC over 3 days. The addition of OLP increased significantly the antioxidant capacity of minced fish and correlated with the delayed lipid oxidation over storage. The effectiveness of OLP was concentration-dependent in all parameters studied.

Furthermore, fish gelatin films (FGF) with different levels of OLPE (0, 1.88, 3.75 and 5.63 % w/w) were formulated and their total soluble phenolics (TSP) and antioxidant capacity, colour and water vapor permeability (WVP) were studied. Antimicrobial and antioxidant activity increased with increasing OLPE concentration in FGF. Lightness was not significantly modified with increasing OLPE addition in the films. In contrast, a^* was reduced and b^* increased directly with OLPE concentration. Addition of 3.75 and 5.63 % of OLPE to FGF increased WVP of the films.

FGF with 5.63 % OLPE were selected to test against *L. monocytogenes* inoculated smoked salmon. These films significantly reduced the growth of *L. monocytogenes* on smoked salmon

during storage. The results showed OLP as a potential natural antioxidant and antimicrobial in fish products. Besides, this study highlights for the first time the effectiveness of FGF incorporating OLPE for diminishing the growth of *L. monocytogenes* on smoked salmon.

Keywords: Olive leaf; olive leaf extract; by-products valorisation; antioxidant capacity; antimicrobial; minced horse mackerel; gelatin films; smoked salmon; *Listeria monocytogenes*.

1. INTRODUCTION

Olive leaves are an important by-product of the olive oil industry. Olive leaves are generated during the pruning of olive trees, and also are an industrial residue from olive oil extraction. Specifically, 10 % of the total weight of olives arriving to the mills is represented by this by-product (Herrero et al., 2010). Olive orchards are typical in the Mediterranean countries and are spreading around the world. In fact, more than 8 million hectares are cultivated worldwide (Peralbo-Molina & Luque Castro, 2013), with each olive tree producing around 25 kg of by-products (twigs and leaves) annually (Niaunakis & Halvadakis, 2006). The antioxidant (Pereira et al., 2007; Apostolakis, Grigorakis, & Makris, 2014; Mylonaki, Kiassos, Makris, & Kefalas, 2008; Lee & Lee 2010) and antimicrobial (Pereira et al., 2007; Markin, Boskou, & Andrikopoulus, 2003) activities of olive leaf powder (OLP) and their extracts (OLPE) have been widely studied.

Due to its antioxidant properties, many works have investigated the use of olive leaf as natural antioxidant directly incorporated in oil (Chiou et al., 2007; Malheiro et al.,2013; Delgado-Adámez, Franco Baltasar, Ayuso Yuste, & Martín-Vertedor, 2014), in muscle model system (Hayes et al., 2009) and in meat products (Nieto, O'Grady, Gutierrez & Kerry, 2010; Hayes, Allen, Brunton, O'Grady, & Kerry, 2011; Gök & Bor, 2012). To the best of our knowledge there is little information concerning the impact of olive leaf as natural antioxidant in fish. Pazos, Alonso, Fernández-Bolaños, Torres and Medina (2006) applied purified hydroxytyrosol, the major phenolic compound in olive oil mill waste, in frozen horse mackerel fillets.

Horse mackerel (*Trachurus trachurus*) is one of the most important pelagic fisheries in the southern Europe. This species contains high levels of long chain omega-3 polyunsaturated fatty acids (PUFAs), essential nutrients with well-known beneficial effects on human health. This fatty acid composition is also responsible for some inconvenience when processing

horse mackerel due to an increased susceptibility to oxidation (Farvin, Grejsen, & Jacobsen, 2012). Many natural antioxidants, such as rosemary extract (Vareltzis, Koufidis, Gavriilidou, Papavergou & Vasiliadou, 1997), grape procyanidins, hydroxytyrosol and propyl gallate (Pazos et al., 2006), hydroxycinnamic acids and catechins (Medina, Gallardo, González, Lois & Hedges, 2007) and potato peel extract (Farvin et al., 2012) have already been tested for preventing oxidation in horse mackerel.

As far as we know, there are no literature data concerning the impact of olive leaf as additive in fish. Fish mince is an excellent model system to study processing for short periods of time. Oxidation is accelerated due to the decrease in particle size, oxygen incorporation and loss of compartmentation (Medina et al, 2012).

In developed countries, foodborne illnesses are caused mainly by *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*. Nevertheless, the effectiveness of OLP and OLPE against these important foodborne pathogens, particularly *Listeria monocytogenes*, has not been tested. Nowadays, consumers are demanding natural compounds to replace synthetic additives. OLP, as a food additive, may adversely affect palatability. The incorporation of these natural compounds into edible films appears to be a good strategy to reduce the amounts and consequently minimize astringency and bitterness. In addition, edible films with added OLP produce a gradual liberation of the additive to the food throughout its shelf life (Campos, Gerchenson, & Flores, 2011). Very few reports of OLPE inclusion in films exist (Erdohan, Çam, & Turhan, 2013; Khalil, Ismail, El-Baghdady, & Mohamed, 2013; Marcos et al., 2014). OLPE, incorporated into polylactic acid (PLA), had antimicrobial activity against *Staphylococcus aureus* (Erdohan et al, 2013). Khalil et al. (2013) studied the antibacterial activity of silver nanoparticles synthesized using OLPE. Antioxidant properties of biodegradable films (Ecoflex® and Ecoflex®-polylactic acid PLA) with α-tocopherol and OLPE were studied by Marcos et al. (2014).

L. monocytogenes has a relatively high incidence in ready-to-eat (RTE) products. The processing of cold-smoked salmon does not include any recognizable critical point to prevent the growth of *L. monocytogenes* and thus ensure safety of this product (Ye, Neetoo, & Chen, 2008). The use of edible films with antimicrobial bioactive compounds or extracts to inhibit *L. monocytogenes* has been reported in some works (Ye et al., 2008; Jiang, Neetoo, & Chen, 2010; Tammineni, Ünlü, & Min, 2013; Neetoo & Mahomoodally 2014).

OBJECTIVES

The objectives of this study were:

1. Characterize the olive leaves in regards to its proximate composition, phenolic composition, total polyphenols and antioxidant properties and also testing the antimicrobial activity of OLP and OLPE against three important foodborne pathogens: *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*.
2. Study the effect of addition of the olive leaves on chilled minced horse mackerel analysing its antioxidant properties, the prevention of oxidation and sensory characteristics.
3. Evaluate antioxidant, antimicrobial and physical properties of fish gelatin films (FGF) with different OLPE concentrations, and finally; to test the effectiveness of a selected film with an optimized OLPE concentration against *L. monocytogenes* in smoked salmon.

2. MATERIALS AND METHODS

2.1. Chemicals

Fish gelatin was kindly donated by Mr. Richard Norland (Norland Products Inc., Cranbury, NJ). Folin-Ciocalteau phenol reagent, Trolox, 1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, ABTS [2,2´-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)], and AAPH solution (2,2`-azobis (2-methyl-propionamidine) dihydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile and methanol, ethanol, chloroform, hexane, isopropanol, glycerol, formic acid (88%, ACS reagent grade), and hydrochloric acid were purchased from Fisher Scientific Ltd. (Fair Lawn, NJ). Luteolin 7-glucoside, apigenin 7-glucoside, verbascoside and oleuropein standards used for HPLC analysis were obtained from Sigma–Aldrich (St. Louis, MO). Trypticase Soy Agar (TSA), Trypticase Soy Broth (TSB), Oxford Medium Base (OMB) and peptone were purchased from Becton, Dickinson and Company (Sparks, MD).

2.2. Olive leaf powder (OLP) characterization

2.2.1. Preparation of olive leaf powder (OLP)

Mission olive leaves were collected manually from an orchard in Pleasanton, CA. Leaves were washed in a Kenmore series 400 triple action agitator washer (Sears, Roebuck and Co., Chicago, IL) with cold tap water in a 40 min washing cycle. Drained leaves were blanched for 10 s with boiling water in a Groen TDB7-40 steam kettle (Groen, Jackson, MI) at a ratio of 0.012 kg of leaves per L of water. Blanched leaves were dried in a steam-heated cabinet hot–air drier (Procter& Schwartz, Inc., Horsham, PA) with air down circulation at 80 °C for 35 min over perforated S.S. trays. Olive leaf powder (OLP) was produced by pre-grinding dry olive leaves in a Cuisinart blender and milling through a 0.25 mm S.S. screen in a model 3010-014 cyclone sample mill (UDY Corp., Fort Collins, CO). OLP was packaged in metallized flat pouches with nitrogen flushing before hot-sealing and stored at 2 °C until

being used for extraction. Fifty grams of OLP were mixed with 250 mL of 60 % ethanol at 50 % speed using a PC-351 Corning hot plate stirrer (Corning Glass Works, Corning, NY) for 72 h at ambient temperature in a 500 mL Erlenmeyer flask covered with a cork stopper and wrapped with Parafilm. The final mixtures were centrifuged in a Sorvall RC, 5C Plus with a SA-600 rotor at 29.000 *g* for 10 min at 4 ºC (Kendro Laboratory Products, Newtown, CT). Supernatant was evaporated in a Büchi RE rotary evaporator (Brinkmann Instruments, Inc., Cranberry Township, PA) at 40 °C. Final samples (OLPE) were then lyophilized in a VirTis Ultra 25EL freeze-drier, packaged in metallized flat pouches with nitrogen flushing before hot-sealing and stored at 4 °C until further analysis.

2.2.2. Proximate composition of OLP

Moisture content was gravimetrically determined (AOAC, 1995). Ash content was determined by heating in a furnace at 550 °C for 24 h (AOAC, 1990). Total fat was extracted with petroleum ether in a Soxtec extraction System 2055 Tecator (FOSS, Hillerød, Denmark) and gravimetrically determined. Carbohydrates were estimated by difference. Total fibre content (soluble and insoluble) was determined with an enzymatic method (985.29, AOAC, 1995). All proximate composition determinations were performed in triplicate.

2.2.3. Phenolic profile of OLP

OLP (500 mg) was placed in a screw cap test tube with 7 mL of methanol. The mixture was sonicated for 10 min in a Branson 8200 ultrasonic cleaner (Branson Ultrasonics Corp., Danbury, CT). After 20 min the mixture was sonicated for an additional 10 min. The sample was stored overnight in a refrigerator. The mixture was centrifuged and extracted three times with 5 mL aliquots of methanol (total of three extractions). The combined extract was evaporated to dryness with a nitrogen stream. The residue was dissolved in 10 mL of methanol with 1 % acetic acid.

HPLC characterization of phenolic constituents in OLP was performed on a Hewlett-Packard 1100 Series HPLC system consisting of an 1100 Series quaternary pump, an 1100 Series vacuum degasser, a manual injector (model 7725i, Rheodyne, now IDEX Health & Science LLC, Rohnert Park, CA), equipped with a 20 μL sample loop, an 1100 Series thermostated column compartment, and an 1100 Series diode array detector. The instrument was controlled and data were processed by HP ChemStation for LC 3D. The analytical column was a 250×4.6 mm i.d. Betasil C18 (5 μm; Keystone Scientific Inc., now Thermo Hypersil-Keystone LLC, Bellefonte, PA). The column was maintained at 30 °C. Separations were carried out using a modification of a previously reported method (Cardoso, Guyot, Marnet, Lopez-da-Silva, Renard, & Coimbra, 2005). Elution was performed using mobile phase A (2.5% acetic acid in water) and mobile phase B (acetonitrile). The gradient system was 0 min, 97% A; 4 min, 91% A; 15 min, 85% A; 75 min, 79% A; 80 min, 70% A and 85-90 min, 10% A. The flow rate was 1 mL per min. The detection was done using 320 nm for luteolin-7-glucoside, apigenin-7-glucoside and verbascoside and 280 nm for oleuropein. The extracts were filtered through a 0.45 μm filter before injection.

2.2.4. Total Phenol (TP) and antioxidant capacity of OLP (olive leaf powder) and OLPE (olive leaf extracts)

Extract preparation

Total phenols, oxygen radical absorbance capacity and ferric reducing antioxidant power were evaluated on olive leaf extracts (OLPE). The method described by Sánchez-Alonso Jiménez-Escrig, Saura-Calixto and Borderías, (2008) was followed for extract preparation. DPPH radical scavenging capacity and Trolox equivalent antioxidant capacity were evaluated directly on olive leaf powder (OLP). All determinations on OLP and OLPE were performed in triplicate.

Total Phenols (TP)

Total phenols were measured using the Folin-Ciocalteu method (Slinkard & Singleton, 1977). Results were expressed as mg Gallic Acid per g sample (d.w.) using a calibration curve with gallic acid as standard (9.8 μM to 70 μM).

Determination of the Oxygen radical absorbance capacity (ORAC)

The procedure was based on a previously reported method (Ou, Hampsch-Woodill, & Prior, 2001). Results were expressed as mmol Trolox Equivalent (TE) per g sample (d.w.).

Ferric ion reducing antioxidant power (FRAP)

The reducing power was determined according to the procedure described by Pereira et al. (2008). Results were expressed as μmol Fe Equivalent per g sample (d.w.).

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity and Trolox Equivalent Antioxidant Capacity (TEAC).

The measurement of total antioxidant capacity, including the contribution of insoluble matter to total antioxidant capacity, was determined following the procedure proposed by Serpen Capuano, Fogliano, and Gökmen (2007). One mg of OLP was diluted into 100 mg of cellulose powder prior to measurement. DPPH results were expressed as percentage of inhibition of the DPPH radical compared to a control with no OLP and TEAC as mmol TE per g sample (d.w.).

2.2.5. Antimicrobial activity against pathogenic bacteria

The sources of bacteria used in the present study had been previously described by Friedman, Henika, and Mandrell (2002). *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* were streaked on TSA and incubated overnight at 37 ºC. One isolated colony was picked from each TSA plate and inoculated into a tube with 5 mL TSB at 37 °C for 24 h with agitation. Each inoculum was prepared by serially diluting (10×) in 0.1 % peptone water.

Overlay diffusion tests were used for antimicrobial assays. To test antimicrobial effect of OLP and OLPE against three pathogenic microorganisms, 100 μL of each inoculum with concentration of 10^5 colony-forming units (CFU per mL) was uniformly spread onto TSA plates and left to dry for 5 min at room temperature. A 10 mm diameter sterile filter paper disk was placed at the center of each plate, 20 μL of OLP/OLPE solutions (diluted with water, 50/50, v/v) was placed on top of each disk. The plates were incubated at 37 °C for 48 h. The inhibition radius around the filter paper (colony-free perimeter) was measured in triplicate with a digital caliper (Neiko Tools, Ontario, CA, USA), and the inhibition area was then calculated in square millimeters.

2.3. Evaluation of OLP as natural antioxidant in horse mackerel

2.3.1. Minced horse mackerel storage experiment

Six kilograms of horse mackerel (*Trachurus trachurus)* were bought in a fish market in Valladolid, Spain. Fish was manually skinned and minced using a blender with a 7 mm exit pore (Lacor 69067, Guipúzcoa, Spain). Samples were randomly allocated into 4 batches: Control, 1.5% OLP, 3% OLP and 4.5% OLP. OLP was dispersed in cold water and added to the fish samples. The moisture of OLP-containing samples was adjusted to match that of the original muscle. All batches were kept in trays at 4 ºC and samples analysed on days 0, 1, 2 and 3. The whole experiment was performed twice; all analyses were performed in triplicate.

2.3.2. Physicochemical markers

Proximate composition of horse mackerel

Moisture content was gravimetrically determined according to AOAC (1995). Total lipids were extracted from 10 g of sample with methanol/chloroform (1/1, v/v) according to the method of Bligh and Dyer (1959).

Colour

Colour parameters, lightness (L*), redness (a*) and yellowness (b*), were measured using a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japan). Measurements were taken on samples packaged with transparent plastic bags at ten different points.

2.3.3. Oxidation markers

Peroxide Value (PV)

PV was measured directly on the Bligh & Dyer (1959) extract according to the International IDF Standards method (1991). Results were expressed in mEq. active oxygen per kg lipids.

Thiobarbituric acid reactive substances (TBARS)

Samples were analyzed as described by Vyncke (1975). Results were expressed as μmol malondialdehyde (MDA) per kg of muscle.

Protein carbonyls

Proteins carbonyls were measured as described by Levine, Williams, Stadtman, & Shacter (1994). Results were expressed in nmol carbonyl per mg of protein.

Tocopherol

The tocopherol content was determined according to the AOCS official method (1992). Results were expressed in μg α-tocopherol per g of muscle.

2.3.4. Antioxidant markers

One gram of horse mackerel mince was extracted following the method of Sánchez-Alonso et al. (2008). All determinations (TP, ORAC, FRAP, DPPH and TEAC) on horse mackerel mince were performed in triplicate. Different from OLP, DPPH and TEAC was determined from the extract instead of quencher method. The effect of antioxidant activity on DPPH was estimated according to the procedure described by Brand-Williams Cuvelier, and Berset (1995) and as modified by Al-Duais, Müller, Böhm and Jetschke (2009). Results were expressed as percentage of inhibition of the DPPH radical compared to a control with no

extract. TEAC was carried according Re et al. (1999). Results were expressed as μmol Trolox Equivalents (TE) per g muscle (d.w.).

2.3.5. Sensory analysis

Sensory evaluation was performed by a trained panel composed of ten people. The following odour descriptors: fishy, rancid and ammonia were scored on a 0 to 9 scale, being 0 non-existent and 9 the maximum. Approximately 20 g of each sample placed in a container were presented in randomized order to panelists after keeping for 15 min at room temperature to reach equilibrium between the minced horse mackerel and the headspace of the sample container.

2.4. Antimicrobial activity of Fish Gelatin Films with Olive Leaf Extract on *Listeria monocytogenes* in Ready-to-Eat Smoked Salmon.

2.4.1. Preparation of fish gelatin films (FGF) with OLPE

FGF with OLPE were prepared by mixing 75 g per L of fish gelatin aqueous solution with glycerol (5 g per L biopolymer, as plasticizer). Afterwards, 0, 1.88, 3.75 and 5.63 w of OLPE /w of gelatin (%) were incorporated into filmogenic solutions. The solutions were mixed for 5 min at 500 rpm in a PMC series 730 DataPlate digital hot plate/magnetic stirrer (Barnstead Thermolyne Corp., Hampton, NH) and heated in a Cole-Parmer mod. 12504-50 water bath (COLPE-Parmer Instruments Co., Chicago, IL) at 60 °C for 15 min. The solutions were mixed again for 5 min. These steps were repeated 4 times before the gelatin solution was degassed under vacuum to avoid microbubble formation. Films were cast on 29×29 cm glass plates using a 35 mil (1 mil = 0.0254 mm) gap draw down aluminum bar to spread the filmogenic solutions onto a flat Mylar film to facilitate the removal of films after overnight drying at room temperature (25 °C). Films were peeled from the Mylar after drying and stored on layers of aluminum foil in zip-locked plastic bags at 4 °C and 65 % RH until

physical, chemical, and antimicrobial properties were evaluated. Unless otherwise mentioned all analysis on the films was performed in triplicate.

2.4.2. Antimicrobial activity of FGF with OLPE

Antimicrobial activity of FGF with different level of OLPE (0, 1.88, 3.75 and 5.63 % w/w) against *L. monocytogenes* was measured using the methodology previously described in olive leaf powder (OLP) characterization, only replacing the filter paper disc with different FGF discs (12 mm diameter). FGF disks were placed over the agar with the film´s shiny side down.

2.4.3. Total soluble phenols (TSP) and antioxidant capacity of OLP, OLPE and OLPE films

The procedure for TSP analysis was adapted from Swain and Hillis (1959) with slight modifications (Du, Olsen, Avena-Bustillos, Friedman, & McHugh, 2011). The amount of TSP in each sample was determined by a standard curve (0-0.375 mg per mL of gallic acid), and expressed as mg gallic acid equivalents (GAE) per g dry weight tissue.

Method used for antioxidant capacity of phenolic compounds was adapted from Brand-Williams et al. (1995) with slightly modification (Du et al., 2011). The antioxidant capacity was calculated by measuring the decrease in absorbance of samples as compared to the methanol samples, and quantifying as µg Trolox equivalents from a standard curve developed for Trolox (0-0.75 mg per mL), and expressed as mg Trolox per g dry weight tissue.

2.4.4. Colour of OLP, OLPE and OLPE films

Colour of OLP, Japan). The instrument was calibrated with a white tile standard (L^* = 93.97, a^* = −0.88 and b^* = 1.21). To measure the colour of films, a white surface was used as background. Six determinations were carried out per sample per treatment. OLPE and OLPE films was measured using a Minolta CR-400 colorimeter (Minolta Inc, Tokyo, Japan).

2.4.5. Water vapor permeability of films

Water vapor permeability (WVP) of FGF with different concentrations of OLPE was determined according to the method described by McHugh, Avena-Bustillos, and Krochta (1993). Eight determinations were made per sample per treatment.

2.4.6. Inhibition of Listeria monocytogenes in cold smoked salmon

The same method mentioned above for preparation of bacteria inoculums used in overlay diffusion tests was used to prepare *L. monocytogenes* inoculums. Overnight bacterial culture was serially diluted (10×) in 0.1 % peptone water to obtain 10^7 CFU per mL inoculums for inoculation of smoked salmon. Enumerations of *L. monocytogenes* were done by plating on TSA and OMB for 48 h at 37 ºC.

Smoked salmon without preservatives was purchased in a local supermarket (Andronico's in Berkeley, CA). Salmon slices were cut into 5.7 x 2.5 cm pieces (weighing approximately 10 g) and were randomly allocated into 4 batches: Negative control (without inoculation, day 0 only), positive control, FGF and FGF with 5.63 % OLPE (w/w). On the surface of each piece of salmon slice, 100 µL of inoculum was spot (small inoculum drop) inoculated and placed in a sterile 100 mm diameter, 15 mm depth Petri dish. The inoculated samples (Positive control, FGF without OLPE, and FGF with OLPE) were dried under the biosafety hood for 30-60 min. Pieces of 6x3 cm FGF (film control) and OLPE film samples were wrapped to cover the whole top inoculated salmon surface. Samples were stored at 23 °C, 58 % RH for 6 days.

Salmon samples were taken at day 0, 1, 2, 4 and 6 for enumerating the survival of *L. monocytogenes*. Three replicates were used at each sampling interval for each treatment. Samples were homogenized in 50 mL 0.1 % peptone water using a stomacher mod. 400 Seward laboratory blender (Seward Laboratory Systems Inc., Davie, FL) on high speed for 2 min, prior to serial (10x) dilution with 0.1 % peptone water. Duplicate 100 µL (spread plating) or 20 µL (spot plating) samples were then plated onto OMB. In addition to plating

0.1 mL of the lowest dilution, 1 mL was distributed over four plates (0.25 mL each) to improve the detection limit. Plates were counted at 24-48 h after incubation at 37 ºC. Results were reported as the log of the number of survivors per gram of salmon. Higher microbial counts were obtained on negative control (day 0) samples, therefore OMB plates were chosen as the selective media for the enumeration of *L. monocytogenes* on smoked salmon in the storage study.

2.5. Statistical analysis

Experiment data were analyzed by one-way ANOVA. Fisher LSD (Least Significant Difference) test was applied for determining group differences at 95 % significance level. Statgraphics Centurion XVI was used for carrying out the statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Olive leaf powder (OLP) characterization

OLP composition in moisture, fat and ashes were 2.26% (±0.11), 5.09% (±0.20) and 6.14% (±0.37), respectively. Carbohydrates were calculated by difference as 75.83% of the product. These values were in accordance with the findings of Niaounakis and Halvadakis (2006). The dietary fiber content of this by-product was 36.03% (±2.17). Fiber content is not commonly present in animal origin food such as seafood products, so the fiber contribution would be an extra benefit to the final product, besides its antioxidant capacity.

Several polyphenols were quantified in OLP, being Oleuropein the main compound, followed by flavonoids such as luteolin-7-glucoside and apigenin-7-glucoside. An hydroxycinnamic acid derivative, verbascoside, was also found in olive leaves, in agreement to other studies reported (Pereira et al., 2007; Benavente-García, Castillo, Lorente, Ortuño, & Del Río, 2008; Herrero et al., 2011; Rahmanian, Jafari, & Wani, 2015). Apart from these antioxidants, OLP also contained α-tocopherol (238.97±4.03 μg per g dried samples).

Different antioxidant assays were carried out (TP, ORAC, FRAP, DPPH and TEAC). TP content was 20.24±0.62 mg of gallic acid (GAE) per g dried OLP. Similar results were found by Makris et al. (2007) and Nieto et al. (2010) with TP values of 20.58 and 17.00 mg gallic acid (GAE) per g, respectively. The antioxidant potential of OLP measured by ORAC and FRAP were 1.06±0.05 mmol TE per g OLP (d.w.) and 7.64±0.25 μmol Fe Equivalent per g OLP (d.w.) respectively.

DPPH and TEAC were determined directly on the olive leaf powder (OLP), without previous extraction; on the other hand TP, ORAC and FRAP were determined on the extracts of olive leaf (OLPE), as antioxidant tests are usually carried out. OLP had 63.13±1.39% of DPPH inhibition and 33.62±1.34 mmol TE per g OLP d.w., measured against ABTS radical (TEAC). These results are higher than data previously reported (Herrero *et al*., 2011; Botsoglou *et al*., 2014). This increase in antioxidant capacity compared to that obtained by other authors may be due to the contribution of non-extractable matter to total antioxidant activity, which is taken into account in direct determination. This difference due to methodology is more relevant for vegetables rich in insoluble fiber such as OLP.

Among the antioxidant effectiveness of phenolic groups in OLP, Benavente-García et al. (2000) indicated the following relative antioxidant capacity measured by TEAC: verbascoside > oleuropein > luteolin-7-glucoside > apigenin-7-glucoside. In this work, the effect of the structure element on the antioxidant activity of OLP compound was related. Verbascoside has a great antioxidant capacity due to two catechol structures. Antioxidant activity of oleuropein is mainly due to hydroxytyrosol moiety. The minor antioxidant capacity of luteolin-7-glucoside can be explained due to the glycosylation of the 7-hydroxyl group of flavones reducing the antioxidant activity. Apart from that, apigenin-7-glucoside only had a single hydroxyl group. Consequently the electron donor capacity diminished at greater extend. Benavente-García et al. (2000) demonstrated that the effectiveness of OLP

was attributed to the synergistic effect of combined phenolic compounds in OLP. Similarly, Lee and Lee (2010) studied the antioxidant activity contribution of individual phenolic compounds in the olive leaf extract and demonstrated that the antioxidant capacity of the combined compounds was higher than those of the individual phenolic compounds such as oleuropein.

As shown in **Figure 1,** OLPE has approximately 6 times higher oleuropein concentration than OLP. This fact indicated the high efficiency of the extraction method used for OLPE in this study.

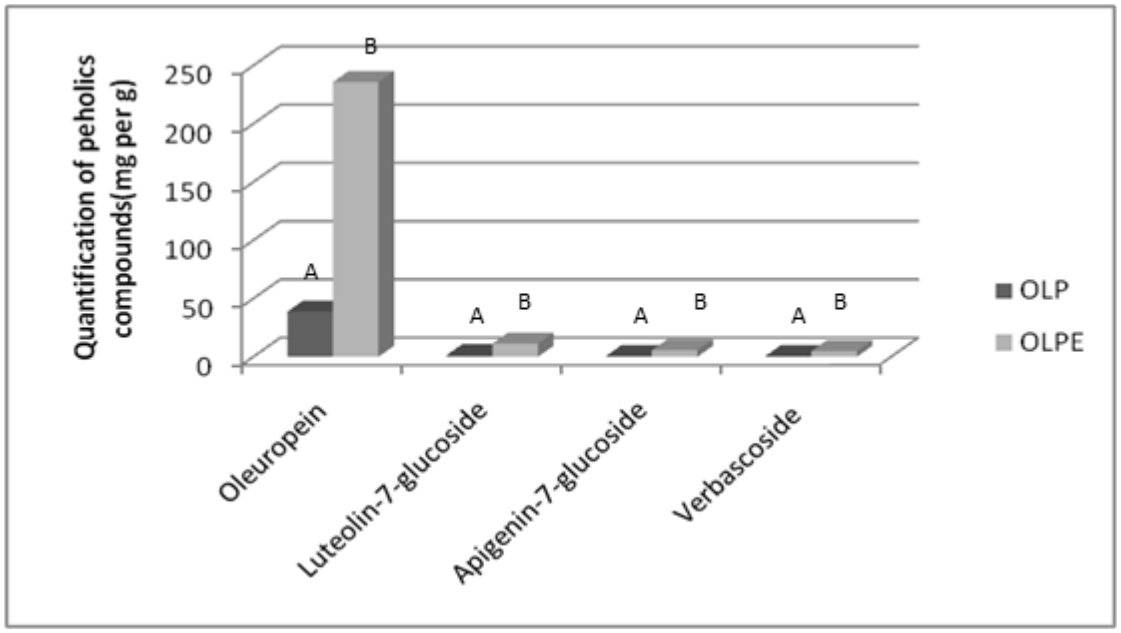

Figure 1: Four major phenolics compounds mg per g in the olive leaf powder (OLP) and olive leaf powder extract (OLPE). Bars for each phenolic compound with different uppercase letter are significantly different between OLP and OLPE ($p< 0.05$).

The optimization of the extraction process also played an important role. In this sense, we did not obtain the maximum theoretical total phenolics calculated by Mylonaki et al. (2008). In our study, ethanol was selected as the most appropriate solvent. Although methanol achieved a high total phenolics yield it is not a food-grade solvent.

3.2.Antimicrobial activity of OLP and OLPE

OLP and OLPE were tested against three important foodborne pathogens, *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*. OLP and OLPE powder only inhibited the growth of *L. monocytogenes* (**Figure 2**).

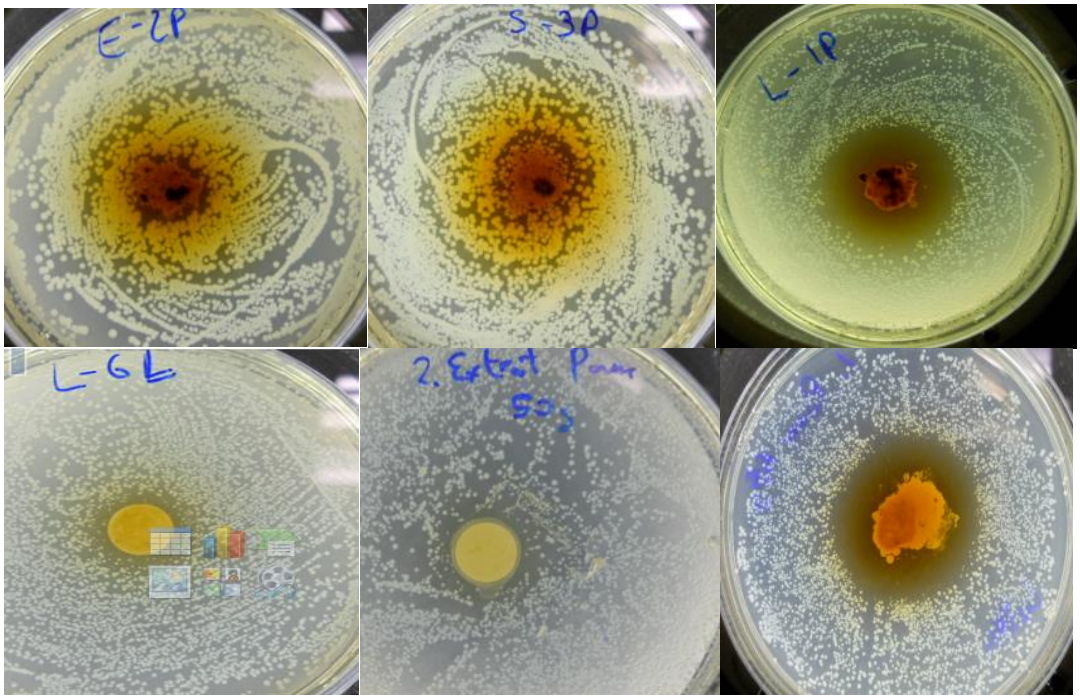

Figure 2: In the top row, OLP powder activity against *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*. In the bottom row FGF with 0.5 g OLP/g gelatin, and 0.5 g OLPE/g gelatin, and OLPE alone against *Listeria monocytogenes*.

Several studies have shown similar results (Dorman & Deans 2000; Sivarooban, Hettiarachchy, & Johnson, 2008; Du et al., 2011). Differences in microbial cellular wall and membrane structure are probably the main cause for this behavior, having the Gram-positive single membrane microorganisms lower capacity to resist the acidification and loss of the membrane gradient. Friedman et al. (2013) also found that olive juice powder was more effective against *L. monocytogenes* than *E. coli* and *S. enterica*. In agreement with our results, Lee and Lee (2010) did not find antimicrobial activity of OLPE against *E. coli*. These

findings differed from those obtained by Khalil et al. (2002), where silver ions with OLPE had antimicrobial activity against *E. coli* in well diffusion method. Also, Sudjana et al. (2009) found similar inhibitory activities for *S. enterica* and *E. coli*, as they did for *L. monocytogenes* using a commercial olive leaf extract (4 mg/mL oleuropein).

OLPE showed higher inhibition, 70 times in 100 % powder and 42 times in 50 % powder (diluted with water), on *L. monocytogenes* than OLP. The inhibitory zones produced by the overlay test with *L. monocytogenes* were higher at 24 h than at 48 h. Similar results with lower inhibitory zones after 48 h when compared to 24 h were found by Du, Olsen, Avena-Bustillos, McHugh, Levin, and Friedman (2009), but they did not have any apparent explanation for this effect.

Previous works have shown the antimicrobial activity *in vitro* of water extracts from olive leaves (Markin et al., 2003; Pereira et al., 2007) and the existence of synergistic effect of its components (Lee & Lee, 2010). These authors reported antimicrobial activity against a range of bacterial species, including those that in this work were not sensitive to OLPE. This result may be due to differences in the extraction procedure and the solvent used (a mixture of ethanol/water in this case).

3.2. Evaluation of OLP as natural antioxidant in horse mackerel

3.2.1. Physicochemical markers

Moisture content was found to be in the range of 70.81(±1.34) to 74.25(±0.12) g per 100 g. These results agreed with previous studies on Atlantic horse mackerel (Aubourg, Lehmann, & Gallardo, 2002). In all samples, a water loss took place over storage.

The fat content of minced horse mackerel ranged from 2.27(±0.05) to 2.95(±0.11) g per100 g. Similar levels have been previously described (Aubourg et al., 2002; Sanjuás-Rey, Barros-Velázquez, & Aubourg, 2011). No significant differences due to storage time or treatment were observed.

Sample colour values expressed as lightness (L*), redness (a*) and yellowness (b*) are shown in **Table 1**. The addition of OLP diminished the lightness (L*) of the samples. OLP applied in increasing concentrations respectively decreased and increased a* and b* colour parameters.

Table 1: Colour parameters: Lightness (L*), Redness (a*), Yellowness (b*) of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage of at 4 ºC.

L*	**Day 0**	**Day 1**	**Day 2**	**Day 3**
Control	$^{A}34.46(\pm1.82)_{b}$	$^{B}42.54(\pm0.51)_{c}$	$^{B}40.75(\pm2.61)_{b}$	$^{B}42.98(\pm1.91)_{c}$
1.5 % OLP	$^{A}32.21(\pm2.36)_{a}$	$^{A}34.26(\pm6.09)_{a}$	$^{A}34.56(\pm4.83)_{a}$	$^{A}33.86(\pm7.15)_{a}$
3.5 % OLP	$^{A}34.32(\pm1.56)_{b}$	$^{A}35.22(\pm3.71)_{b}$	$^{A}35.26(\pm3.17)_{a}$	$^{A}35.78(\pm3.35)_{ab}$
4.5 % OLP	$^{AB}38.11(\pm0.02)_{c}$	$^{A}37.11(\pm0.79)_{b}$	$^{AB}35.85(\pm6.47)_{a}$	$^{B}38.37(\pm1.89)_{b}$
a*				
Control	$^{A}4.55(\pm0.23)_{d}$	$^{A}4.31(\pm0.39)_{d}$	$^{A}4.59(\pm1.30)_{c}$	$^{B}5.86(\pm0.70)_{d}$
1.5 % OLP	$^{B}1.51(\pm0.13)_{c}$	$^{AB}0.88(\pm0.07)_{c}$	$^{A}0.32(\pm0.07)_{b}$	$^{A}0.61(\pm0.08)_{c}$
3.5 % OLP	$^{A}-2.35(\pm0.69)_{b}$	$^{A}-1.71(\pm0.88)_{b}$	$^{A}-2.27(\pm0.54)_{a}$	$^{B}-0.66(\pm0.08)_{b}$
4.5 % OLP	$^{A}-4.41(\pm0.02)_{a}$	$^{B}-2.79(\pm0.68)_{a}$	$^{B}-2.61(\pm0.50)_{a}$	$^{C}-1.85(\pm0.15)_{a}$
b*				
Control	$^{A}9.95(\pm0.43)_{a}$	$^{A}11.26(\pm1.67)_{a}$	$^{A}10.77(\pm2.50)_{a}$	$^{A}11.29(\pm0.50)_{a}$
1.5 % OLP	$^{A}16.52(\pm0.20)_{b}$	$^{A}17.24(\pm2.96)_{b}$	$^{A}17.32(\pm2.25)_{b}$	$^{A}16.35(\pm1.42)_{b}$
3.5 % OLP	$^{A}19.74(\pm0.50)_{c}$	$^{A}20.19(\pm1.78)_{c}$	$^{A}19.57(\pm1.32)_{bc}$	$^{A}20.73(\pm1.40)_{c}$
4.5 % OLP	$^{A}22.74(\pm0.03)_{d}$	$^{A}20.41(\pm1.72)_{d}$	$^{B}19.43(\pm3.31)_{c}$	$^{B}23.67(\pm1.78)_{d}$

Values (mean ± standard deviation, n=10) followed by different uppercase letter in same row are significantly different (p<0.05).
Values (mean ± standard deviation, n=10) followed by different lowercase letter in same column are significantly different (p<0.05).

3.2.2.Oxidation markers

Lipid damage markers were measured to evaluate the potential protective effect of OLP on minced horse mackerel. PV showed a gradual increase in all samples (**Figure 3**) as storage progressed, especially within days 2 and 3. Samples with higher content of OLP (3% and 4%) had lower PVs compared to the control immediately after preparation (day 0).

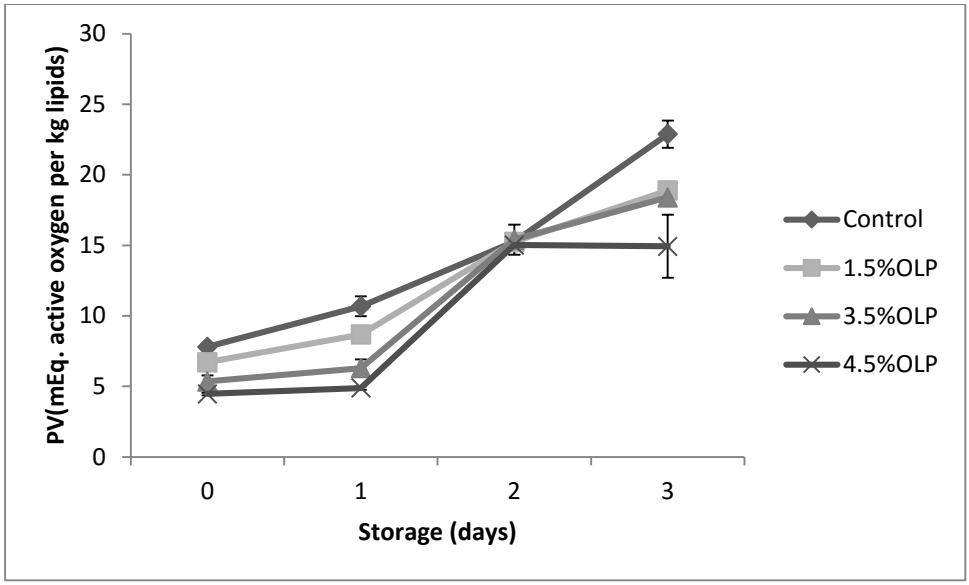

Figure 3: Peroxide value (PV) of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

A similar protective effect immediately after addition of grape pomace was observed by Sánchez-Alonso et al. (2008) on minced horse mackerel muscle. Adding the antioxidant as early as possible to foodstuffs is of prime importance to achieve maximum protection against oxidation, as it could be observed from the differences between control and the rest of the samples. Horse mackerel has a high susceptibility to oxidation because its muscle contains large amounts of haemoglobin (pro-oxidant) and polyunsaturated fatty acids (Richard & Hultin, 2002). Furthermore, the mincing process rapidly affects lipid oxidation due to cellular disruption and subsequent activation of lipoxygenases, which can initiate polyunsaturated fatty acid oxidation (Joaquin, Tolasa, Oliveira, Lee, & Lee, 2008). These results are in accordance with previous work by Malheiro et al. (2013), who reported that the use of olive leaf extract in soybean oil microwave-heated reduced PV formation.

Secondary lipid oxidation products were monitored as TBARS. TBARS of differently treated minced horse mackerel are shown in **Figure 4**.

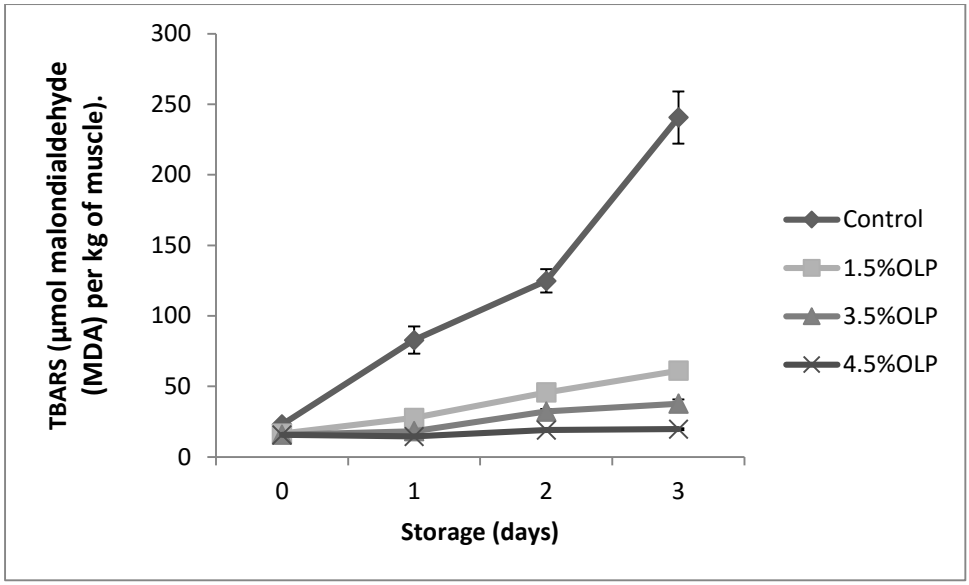

Figure 4: Thiobarbituric Acid Reactive Substances (TBARS), quantified as malondialdehyde (MDA), of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4ºC.

As expected, TBARS showed a significant increase over storage. Also, a dose-dependent effect of OLP on TBARS was observed. Similar results, showing concentration-dependent protective effect of olive leaf extract on lipid oxidation in food models, have been previously reported (Hayes et al., 2009). The addition of 250 µg of olive leaf extract per g reduced the TBARS formation in pork sausages (Hayes et al., 2011). Olive leaf extract (500 and 1000 ppm) also showed an effective role in meatballs in TBARS content over storage (Gök & Bor, 2012). In contrast, Nieto et al. (2010) did not find any effect of olive leaf extract on lipid stability.

The formation of carbonyls compounds from amino acid side chains is probably the most outstanding result of metal-ion catalyzed oxidation of myofibrillar protein (Lund, Heinonen, Baron, & Estévez, 2011). The changes of protein carbonyls in minced horse mackerel over 3 days of refrigerated storage are illustrated in **Figure 5**.

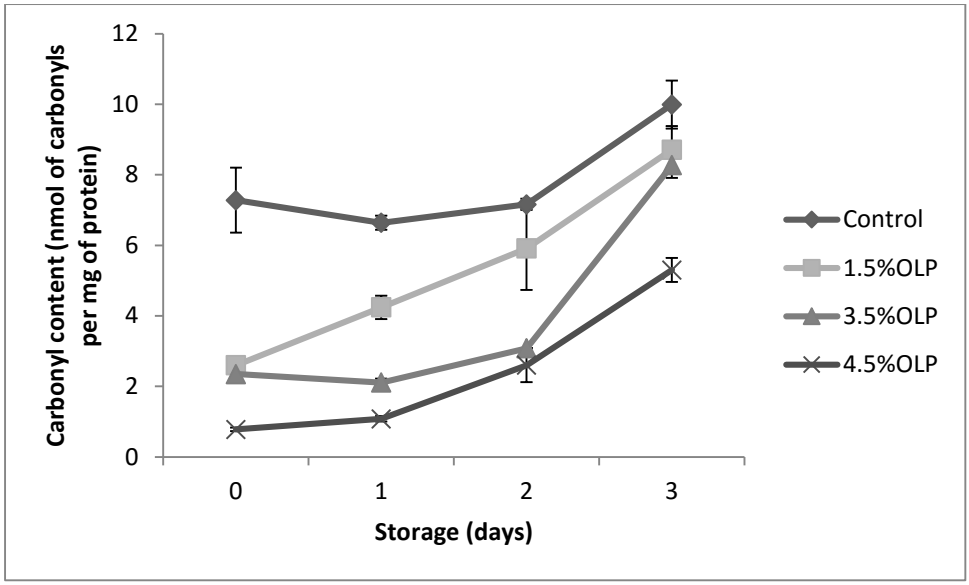

Figure 5: Carbonyl content of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

Initially, carbonyl values (7.28±0.92-0.78±0.05) were in accordance with previous investigations in minced horse mackerel (Eymard, Baron, & Jacobsen, 2009). The content of protein carbonyls increased in all groups over storage. This rise could be attributed to the mincing process. Mincing releases the naturally present pro-oxidants in the muscle, such as heme proteins (Eymard et al., 2009). Carbonyl content was significantly higher in control samples and the addition of OLP was effective in reducing carbonyl formation depending on its concentration. In fact, at days 2 and 3, there were no significant differences between control and 1.5% of OLP minced horse mackerel. Siebert, Troukhanova, and Lynn (1996) proposed that polyphenols act as inhibitors of protein oxidation, either by preventing lipid oxidative reactions, by binding to proteins, or by forming complexes with them.

In our mackerel samples, the levels of α-tocopherol were significantly increased with OLP increasing concentration, and OLP better maintained α-tocopherol levels over storage (**Table 2**). No α-tocopherol was detected in control and 1.5% of OLP from day 2. Malheiro et al. (2013) also found that the addition of olive leaves protected the α-tocopherol loss in vegetal oil.

Table 2: α-tocopherol content (μg per g) of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

	Day 0	**Day 1**	**Day 2**	**Day 3**
Control	$^{B}3.10\pm0.01_{a}$	$^{A}0.53\pm0.02_{a}$	ND	ND
1.5 % OLP	$^{B}3.78\pm0.04_{ab}$	$^{A}0.68\pm0.07a$	ND	ND
3.5 % OLP	$^{B}4.06\pm0.48_{b}$	$^{A}1.29\pm0.15_{b}$	$^{A}1.12\pm0.14_{a}$	$^{A}0.93\pm0.11_{a}$
4.5 % OLP	$^{C}5.15\pm0.44_{c}$	$^{B}2.00\pm0.12_{c}$	$^{AB}1.60\pm0.10_{b}$	$^{A}1.40\pm0.03_{b}$

Values (mean ± standard deviation, n=3) followed by different uppercase letter in same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by different lowercase letter in same column are significantly different ($p<0.05$).
ND: Not detected.

3.2.3. Antioxidant markers

The effectiveness of OLP as antioxidant was evaluated using several assays (TP, ORAC, FRAP, DPPH and TEAC)**.** The addition of OLP significantly increased total phenol content and antioxidant capacity of fish samples. Samples with OLP had higher antioxidant values along the storage period, being this effect concentration dependent. Results at day 0 were shown at **Table 3**.

Table 3: Antioxidant capacity: Total Phenols (TP) expressed as gallic acid equivalent (GAE), Oxygen Radical Absorbance Capacity (ORAC) expressed as Trolox Equivalent (TE), Ferric ion Reducing antioxidant Power (FRAP), DPPH and Trolox Equivalent Antioxidant Capacity (TEAC) of minced horse mackerel with different concentrations of olive leaf power (OLP) at day 0.

	TP (mg GAE per g dw)	**ORAC** (µmol TE per g dw)	**FRAP** (µmol Fe equivalent per g dw)	**DPPH** (% radical inhibition)	**TEAC** (µmol TE per g dw)
Control	$2.33\pm0.08_a$	$17.90\pm1.70_a$	$0.09\pm0.00_a$	$11.24\%\pm0.57_a$	$25.31\pm2.15_a$
1.5 % OLP	$6.46\pm0.41_b$	$48.04\pm1.74_b$	$0.33\pm0.00_b$	$33.62\%\pm1.85_b$	$48.07\pm1.50_b$
3.5 % OLP	$16.60\pm2.18_c$	$76.62\pm0.44_c$	$0.63\pm0.01_c$	$40.65\%\pm2.72_c$	$59.46\pm0.54_c$
4.5 % OLP	$22.40\pm0.44_d$	$91.62\pm6.28_d$	$0.84\pm0.01_d$	$49.58\%\pm1.41_d$	$61.77\pm0.46_d$

Values (mean ± standard deviation, n=3) followed by different lowercase letter in same column are significantly different ($p<0.05$).

A reduction of the antioxidant capacity of the samples over storage was expected. Significant reductions in TP and ORAC were observed from day 1 (**Figure 6.a** and **6.b**). These decreases were in accordance with increasing lipid (PV) and protein (carbonyls) oxidation markers.

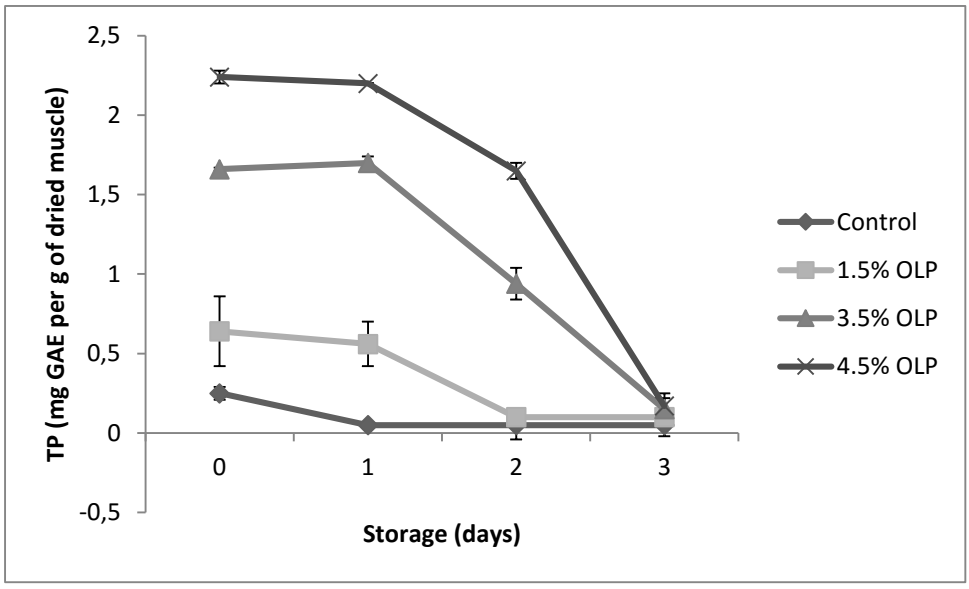

Figure 6.a: Total Phenols (TP) of mince horse mackerel with 0, 1.5, 3.5 and 4.5 % of olive leaf power (OLP) during storage at 4 ºC.

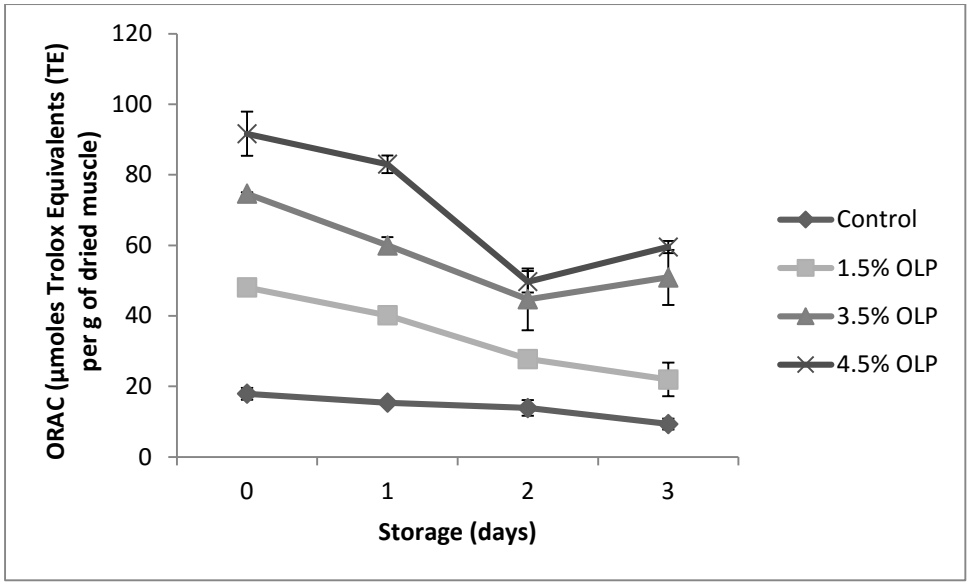

Figure 6.b: Oxygen radical absorbance capacity (ORAC) of mince horse mackerel with 0, 1.5, 3.5 and 4.5 % of olive leaf power (OLP) during storage at 4 ºC.

During oxidation, antioxidants act in various ways, binding metal ions, scavenging radicals and decomposing peroxides (Sánchez-Alonso et al., 2008). The distribution of the antioxidants in the food system affected significantly their effectiveness (Pazos et al., 2006). The mincing causes the release of haemoglobin, one important endogenous catalysts of oxidation. The addition of OLP as additive in horse mackerel mince demonstrated antioxidant protection due to the localization of the OLP where oxidation takes place.

3.2.4. Sensory markers

Sensory results (fishy, rancid and ammonia odor) are shown in **Figure 7.** In all cases, a progressive increase in odour was detected by the panel over storage in all samples. A preserving effect with OLP addition can be concluded from the odour evaluation results.

In fishy odour (**Figure 7.a**), panellists were only able to detect differences at the end of storage among samples with different OLP concentration (day 3). Nevertheless, control samples had the highest scores in fishy odour respect to samples with OLP.

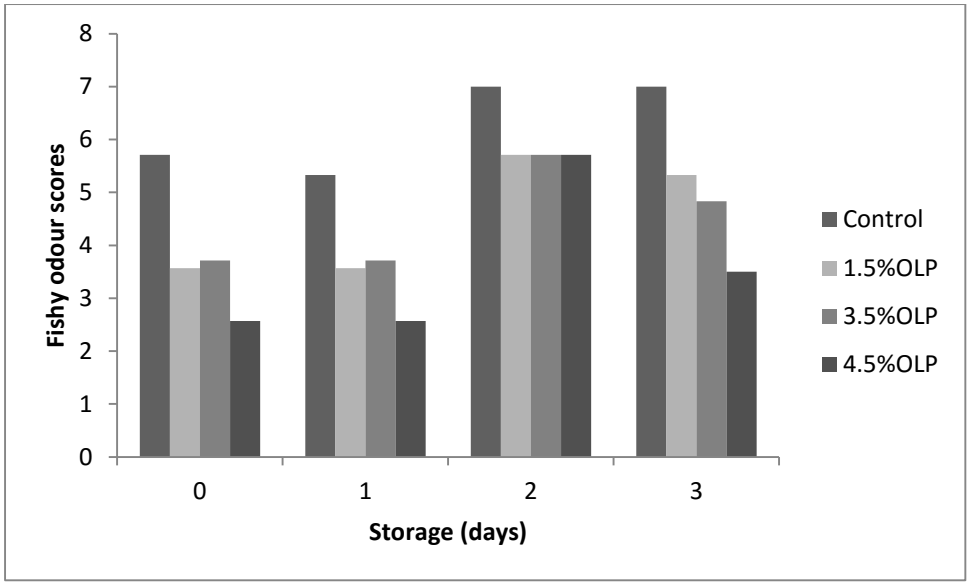

Figure 7.a: Fishy odour scores of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

Rancid odour (**Figure 7.b**) development is well documented during chilled storage of fatty fish species (Ólafsdóttir & Jónsdóttir, 2010). The sensory results were also consistent with a reduction of oxidation (PV, TBARS, carbonyls) in samples with OLP, compared to control samples. From day 2, significant differences in rancid scores occurred, with increasing values related to decreasing OLP concentration, being control the samples with highest rancid.

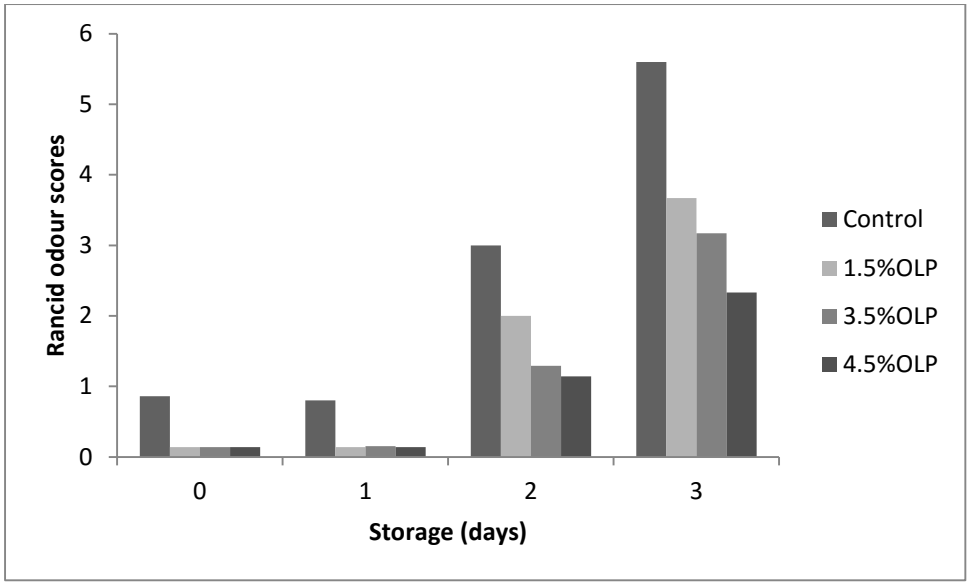

Figure 7.b: Rancid odour scores of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

The ammonia odour (**Figure 7.c**) increased sharply over storage, while it increased only slightly in OLP samples. Ammonia odour reflected the decomposition of muscle, mainly proteins, amines and nucleic bases.

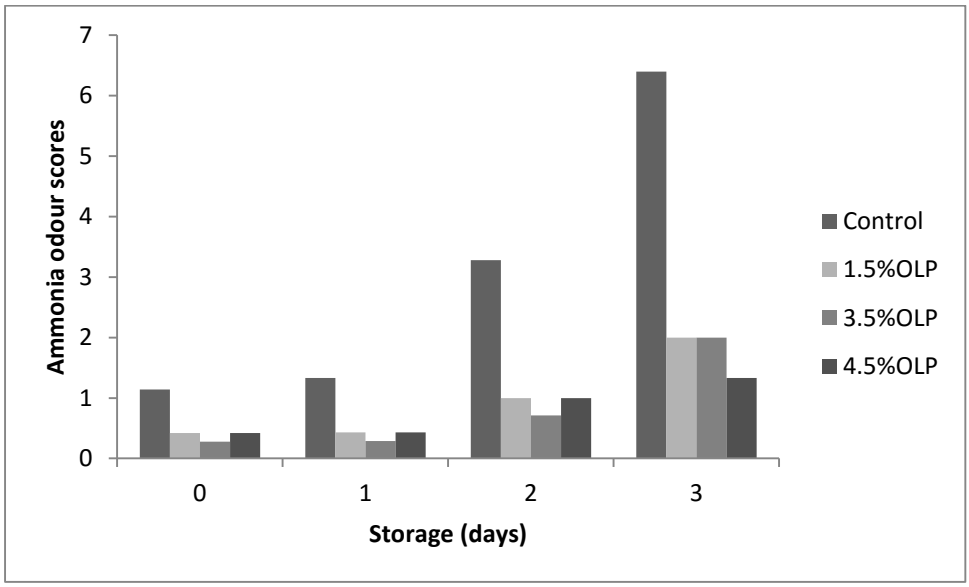

Figure 7.c: Ammonia odour scores of minced horse mackerel with different concentrations of olive leaf power (OLP) during storage at 4 ºC.

The inclusion of OLP avoided the development of off-odours (fishy, rancid and ammonia). Additionally, some panelists noted a pleasant "herb" and "green" aroma due to OLP addition.

3.3. Antimicrobial Fish Gelatin Films with Olive Leaf Extract for Inactivation of *Listeria monocytogenes* on Ready-to-Eat Smoked Salmon

3.3.1. Antimicrobial activity of FGF with different level of OLPE

FGF without OLPE used as control did not inhibit the growth of *L. monocytogenes* (**Figure 8**), and neither did FGF with 1.88 w/w (%). As illustrated in **Figure 8**, FGF discs with increased amount of OLPE, from 3.75 to 5.63 w/w (%), caused a significant increase in the inhibitory zones from 367.7 (±27.9) to 432.3 (±22.2) mm^2 after 24 h. After 48 h, the inhibitory zones of FGF with 3.75 and 5.63 w/w (%) were reduced to 154.5 (±20.0) and 211.0 (±50.7) mm^2, respectively. Based on these results, FGF with 5.63 w/w (%) was selected for testing inhibition of *L. monocytogenes* in smoked salmon. The overlay test simulated food wrapping and might suggest what could happen when wrapping films contact contaminated food surfaces.

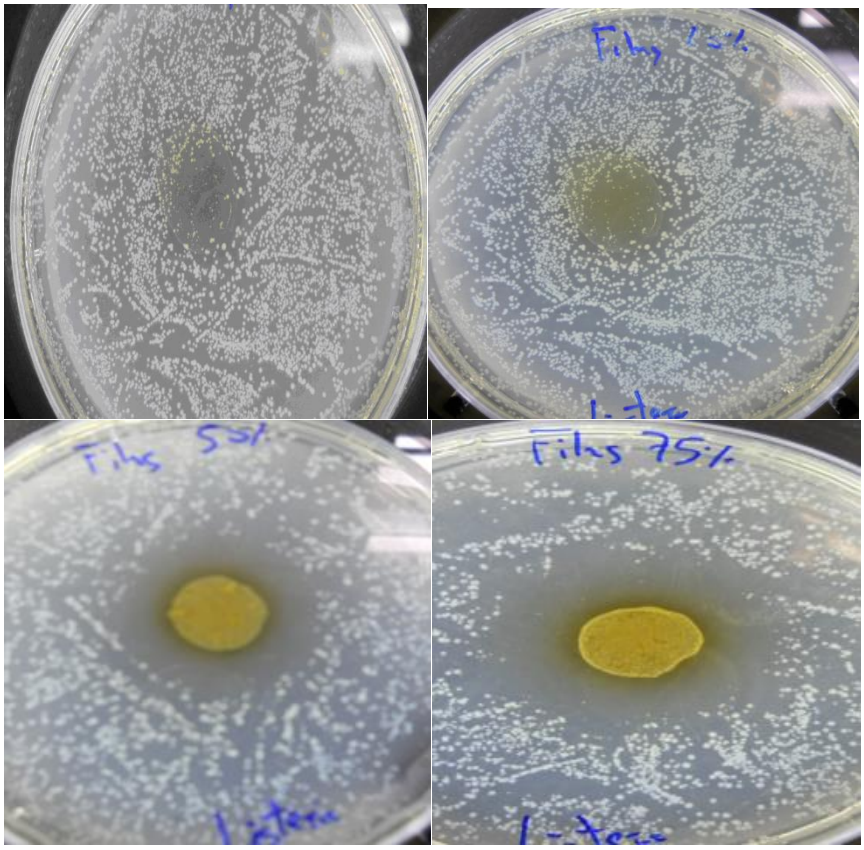

Figure 8: Antimicrobial activity of Fish Gelatin Films (FGF) with 0 (top left), 1.88 (top right), 3.75 (bottom left) and 5.63 % (w/w) (bottom right) of olive leaf power extract (OLPE) per gelatin against *Listeria monocytogenes*.

3.3.2.Total soluble phenols (TSP) and antioxidant capacity of OLPE films

TSP and antioxidant capacity of OLPE films are shown in **Table 4**. TSP of OLPE films significantly increased with increasing OLPE concentration. The same trend was observed in the antioxidant capacity. FGF (control film) without OLPE had some antioxidant capacity, possibly due to the presence of amino acids such as glycine and proline in the fish gelatin (Mendis, Rajapakse & Kim, 2005). A good correlation (R^2= 0.921) was found between TSP and antioxidant capacity of OLPE films. It is clear that phenols can break the free radical chain, contributing to the final antioxidant activity (Lee & Lee 2010).

Table 4: Total soluble phenols and antioxidant capacity of Fish Gelatin Films (FGF) with different levels of olive leaf power extract (OLPE).

OLPE concentration (% w/w)	Total soluble phenols (mg Gallic Acid per g d.w.)	Antioxidant Capacity (mg Trolox per g d.w.)
0	0.31 (±0.04)^{A}	0.95 (±0.08)^{A}
1.88	8.52 (±0.49)^{B}	80.87 (±11.15)^{B}
3.75	22.37 (±1.49)^{C}	164.56 (±1.54)^{C}
5.63	26.69 (±0.43)^{D}	173.77 (±5.11)^{C}

Values (mean ± standard deviation. n=3) followed by different uppercase letters in the same column are significantly different ($p < 0.05$).

3.3.3. Colour of OLP, OLPE and OLPE films

The low impact of OLPE on film colour (**Table 5**) is an important factor, because colour changes cause limitations for the practical use of many natural compounds in food. The effects of different levels of OLPE inclusion on colour of FGF are shown in Table 3. There were not significant differences in *L** values among different levels of OLPE (0, 1.88, 3.75 and 5.63 w/w (%) in FGF). Whereas, *a** and *b** values were significantly modified with OLPE addition. *a** was reduced and *b** increased directly with the concentration of OLPE added in FGF. From this data, it is clear that OLPE addition caused more greenness (reduction of a*) and yellowness (increase of b*) of the films.

Table 5: Colour parameters of OLP, OLPE and Fish Gelatin Films (FGF) with different levels of OLPE.

Sample I. D.	Colour		
	L*	***a****	***b****
OLP	68.60 (±0.19)^{B}	11.94 (±0.70)^{F}	29.01 (±0.81)^{E}
OLPE	58.14 (±0.18)^{A}	6.40 (±0.09)^{E}	22.68 (±0.18)^{D}
FGF	90.33 (±6.93)^{C}	-0.20 (±0.03)^{D}	-0.34 (±0.07)^{A}
1.88 % w/w OLPE	92.82 (±2.06)^{C}	-3.46 (±0.16)^{C}	13.15 (±0.95)^{B}
3.75 % w/w OLPE	90.64 (±0.26)^{C}	-4.17 (±0.25)^{B}	18.15 (±2.90)^{C}
5.63 % w/w OLPE	90.09 (±1.03)^{C}	-4.89 (±0.08)^{A}	28.68 (±1.40)^{E}

Values (mean ± standard deviation. n=6) followed by different uppercase letter in the same column are significantly different ($p < 0.05$).

3.3.4.Water vapour permeability of OLPE films

There were not significant differences in relative humidity (% RH) among OLPE films (**Table 6**). Ideally, to compare water vapor permeability (WVP) of the films, films need to have the same % RH. Difference in % RH will cause difference in the driving force for water diffusion (Du et al., 2009).

Table 6: Water vapour permeability (WVP) of Fish Gelatin Films (FGF) with different levels of OLPE

OLPE Concentration (% w/w)	Thickness (mm)	RH (%)	Permeance (g/kPa.h.m^2)	WVP (g-mm/kPA.h.m^2)
0	0.041(±0.003)^{A}	83.79(±0.67)^{NS}	19.35(±0.96)^{NS}	0.73(±0.12)^{A}
1.88	0.034(±0.005)^{A}	82.13(±2.66)	21.84(±3.99)	0.66(±0.09)^{A}
3.75	0.094(±0.016)^{C}	84.50(±2.63)	18.42(±3.77)	1.68(±0.10)^{C}
5.63	0.072(±0.006)^{B}	83.40(±1.63)	19.93(±2.35)	1.44(±0.27)^{B}

Values (mean ± standard deviation. n=8) followed by different uppercase letter in the same column are significantly different ($p < 0.05$). NS indicates no significant differences in the column

The addition of 1.88 w/w (%) OLPE to the FGF did not change the WVP compared to control FGF. Nevertheless, FGF with 3.75 and 5.63 w/w (%) had significant higher WVP (Table 4). WVP of films depends on both molecular diffusion coefficient and solubility of water in the matrix (McHugh et al., 1994). As there was not any difference in permeance values among films with different OLPE concentration, the increase in WVP may be due to the difference in thickness between OLPE films. Films with 3.75 w/w (%) had significant higher thickness than those with 5.63 w/w (%) (**Table 6**). Hydrophilic films, such as gelatin films, often exhibit positive slope relationships between thickness and WVP (McHugh et al., 1993). Films with 5.63 w/w (%) were selected to evaluate the inhibition of *Listeria* in smoked salmon. One of the main characteristics of an ideal edible film for storage of smoked salmon would be low WVP. FGF with 5.63 w/w (%) had significant lower WVP than those films with 3.75 w/w (%).

3.3.5.Inhibition of Listeria in smoked salmon

The effects of FGF with or without OLPE on the growth of *L. monocytogenes* on inoculated smoked salmon are presented in **Figure 9**. Smoked salmon was checked for initial background microorganisms and was found to contain no background *L. monocytogenes* in day 0 negative control samples. Samples were stored at room temperature instead of refrigerated temperature to shorten the time needs for testing the anti-listerial effect of OLPE films. The initial concentration of *L. monocytogenes* on inoculated smoked salmon ranged from 6.13 to 6.2 log CFU per g. *L. monocytogenes* grew rapidly to 7-8 log CFU per g during the first two days of storage in all samples (positive control, FGF and 5.63 w/w (%) film). This trend was in accordance with the finding of Ye et al. (2008) which demonstrated the ability of this pathogen to grow rapidly at room temperature. During the storage period, no reduction in *L. monocytogenes* was observed in positive control and FGF samples, while 5.63

w/w (%) films started to show a decrease in *L. monocytogenes* on day 6. FGF were able to substantially slow down *L. monocytogenes* compared to positive control samples at day 1.

Storage time (days)	0	1	2	4	6
Positive control	Ba	Cb	Bc	Bd	Bc
FGF	Ba	Bb	Bc	ABc	Bc
FGF with OLPE	Aa	Ab	Ac	Ac	Ab

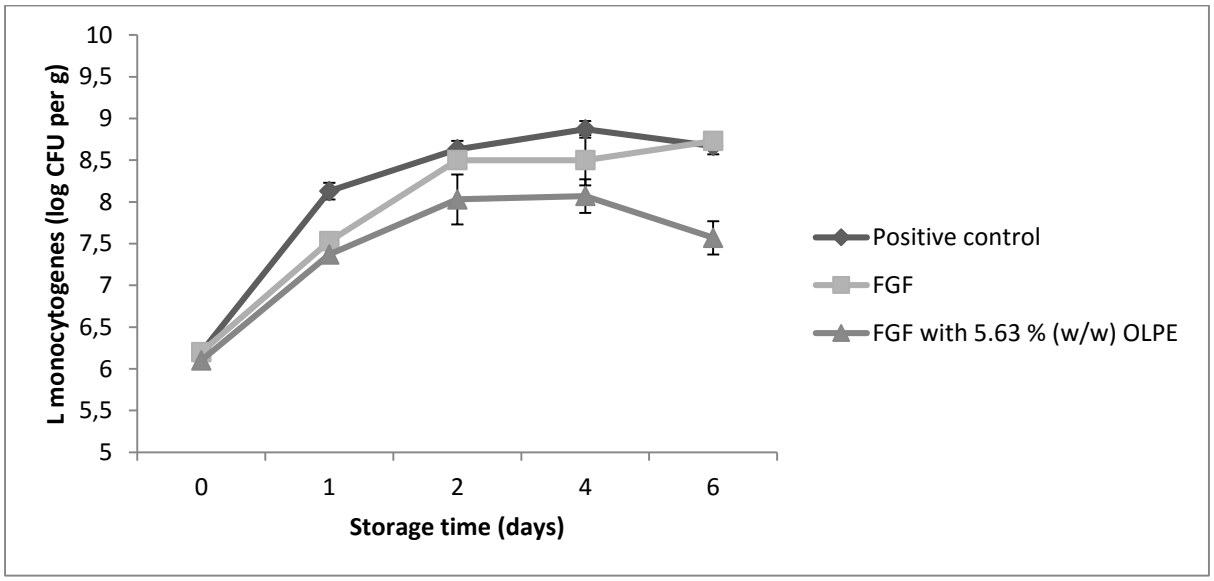

Figure 9: Effect of Fish Gelatin Films (FGF) with 5.63 w/w (%) of olive leaf power extract (OLPE) on the growth of *Listeria monocytogenes* on inoculated cold-smoked salmon during 6 days of storage at 23 °C.

Our results were in agreement with those reported by Song et al. (2014). The initial efficiency of FGF can be attributed to its location on the surface of salmon, where major microbial concentrations occurred. As storage progressed, FGF were solubilized. Hence, FGF did not have any anti-listerial activity themselves (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010) and their initial activity was linked on the food surface localization. FGF with 5.63 w/w (%) were able to significantly reduce the growth of *L. monocytogenes* in smoked salmon in respect of positive control and FGF. Several researchers had already shown that OLPE can inhibit the growth of *L. monocytogenes* in the *in vitro*

assays. Nevertheless, in this study, it was demonstrated for the first time that FGF with OLPE can reduce *L. monocytogenes* growth on food, specifically, in a ready-to-eat (RTE) product, such as cold-smoked salmon, where the prevalence of this microorganism is relatively high. The application of FGF with OLPE would provide an additional hurdle to the growth of *L. monocytogenes*. These results indicated that OLPE, a by-product of olive oil industry, could be used in edible films for RTE seafood due to its anti-listerial activity.

4.CONCLUSIONS

Olive leaf power (OLP) is rich in total soluble polyphenols, with significant antioxidant capacity regardless of the method of analysis used: TP, ORAC, FRAP, DPPH and TEAC. Oleuropein, luteolin 7-glucoside, apigenin 7-glucoside and verbascoside were the main phenolic compounds detected in OLP. This by-product was highly effective against *L. monocytogenes*. These properties suggest that OLP could be used as a food ingredient directly applied on food or through edible films.

Tested as antioxidant ingredient in minced fish, OLP possessed notable antioxidant activity which increased the TP and antioxidant capacity of the fish. The addition of different concentrations of OLP reduced lipid oxidation, loss of α-tocopherol and protein oxidation. In the majority of the oxidation parameters tested, the effectiveness of OLP was concentration-dependent.

In comparison with OLP, olive leaf power extract (OLPE) has significant higher phenols content, antimicrobial and antioxidant activities. The highest antimicrobial activity against *L. monocytogenes* and the lower water vapor permeability of fish gelatin films (FGF) were achieved with the highest tested concentration of olive leaf power extract (5.63 % w/w). FGF with this concentration of extract slowed *L. monocytogene*s growth on inoculated RTE cold-smoked salmon. Therefore, the results suggested that OLP and OLPE have great potential for use as functional ingredients. Besides, OLP and OLPE have other advantages such as low-

cost due to their waste-stream nature. In addition, edible FGF containing OLPE could be an effective tool to inhibit the growth of *L. monocytogenes* in RTE seafood products.

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Chapter 4

Edible films and high pressure processing on the shelf life of trout fillets: Microbiological and physicochemical aspects

This chapter has been partially published in the Journal of the Science of Food and Agriculture (Albertos et al., 2015) and presented as poster communications in WEFTA meeting (9-11 of October of 2013, Tromsø, Norway), EFFoST Anual Meeting (12-15 of November of 2013, Bolonia, Italy) and EFFoST Anual Meeting (25-28 of November of 2014, Uppsala, Sweden).

Edible films and high pressure processing on the shelf life of trout fillets: Microbiological and physicochemical aspects

ABSTRACT

The inhibitory effect of chitosan films with clove oil (0 to 50 g per kg) was evaluated on a range of ten representative food spoilage and pathogenic bacteria. The antioxidant properties of films were also studied.

The bacteria most sensitive to the films was *Shewanella putrefaciens*, and the most resistant resulted *Aeromonas hydrophila*, since inhibition of the last was apparent only at 50 g kg^{-1} clove essential oil (CEO). Films with 20 g per kg CEO inhibited nine out often of the bacteria tested. Total phenolic content and antioxidant capacity of edible films increased as the CEO concentration did it, up to 20 g per kg.

Chitosan films with 20 g per kg CEO were combined with high pressure (HPP) processing as treatment for trout fillets and changes in physicochemical parameters and microbial load were evaluated at 4 ºC over 22 days of storage. The films reduced the weight loss and water activity compared to fresh and treated samples (HPP and cooking). All treatments reduced microbial spoilage, as reflected from total volatile basic nitrogen (TVBN). Results showed that all microbial load markers (total aerobic mesophilic, lactic acid bacteria and total coliform), except for coliform counts of the trout fillets covered with chitosan films were lower than those of HPP treated samples, and similar to cooked samples. Both, cooking and HPP affected lipid oxidation (TBARS, rancid odour), colour (L* and a* parameters) and overall appearance (colour intensity, off-colour and surface defects). However, changes occurred at lesser extent on pressurised samples. In this sense, the application of chitosan films reduced lipid

oxidation, especially using the films with CEO addition. However, sensory panel detected the persistent odour of CEO containing films.

The use of 20 g per kg CEO-chitosan films showed a further improvement in the shelf life of trout fillets when compared to that obtained with the HPP and cooking treatment. Besides, the use of chitosan films in trout fillets also enhanced the quality of trout over storage, without unfavourable sensory changes.

Keywords: Edible films; clove essential oil; high pressure processing; trout; chitosan.

1. INTRODUCTION

The use of edible coating and films allows extending the shelf life of many food products. Chitosan has been widely used as hydrocolloid to constitute edible films due to its biodegradability, biocompatibility, antimicrobial and antioxidant activities and non-toxicity. Chitosan films have selective permeability to gases (CO_2 and O_2) and good mechanical properties (Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez & Fernández-López, 2013). However, due to their hydrophilic nature, chitosan films do not provide an effective control of moisture transfer, a property that is often desirable for edible film applications.

Essential oils have been widely used as flavouring agents in food since the earliest recorded history. Nowadays, they attract increasing attention due to their antimicrobial and antioxidant properties (Holley & Patel, 2005). Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén and Montero (2010) tested the antimicrobial activity of different essential oils, finding that clove oil had the highest inhibitory effect over a range of microorganisms. Its principal constituent, eugenol, is the main responsible for CEO's antimicrobial properties (Burt, 2004). Similar results were found by

Alboofetileh, Rezaei, Hosseini and Abdollahi (2014), where the qualitative antimicrobial activities of six selected essential oils against three common pathogenic foodborne bacteria were tested. After that, the most effective essential oils were incorporated in alginate/clay nanocomposite films with the aim of evaluating their antibacterial activity against *Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus*. In both assays clove essential oil was the second most effective, only behind marjoram. The highest concentration tested (15 g per kg) inhibited the growth of the three pathogens.

Minimally processed food products are in increasing demand due to convenience and fresh-like organoleptic characteristics. The best strategy for maintaining the balance between safety and quality of this type of products is the application of multiple barriers against microbial growth (hurdle technology) (Leitsner, 2000). Although products subject to High Pressure Processing (HPP) generally present a superior quality compared to products that have undergone conventional thermal pasteurisation, many HPP potential applications would require long treatment time and high pressure intensity to ensure an adequate inactivation level of pathogens and spoilage microorganisms. Furthermore, it is well documented that HPP processing may induce lipid oxidation (Cheftel and Culioli, 1997; Medina-Meza, Barnaba & Barbosa-Cánovas, 2014). This effect depends on factors like pressure level, fish species and subsequent storage conditions. However, very little information exists on HPP effect on seafood oxidation, especially lipid oxidation (Yagiz, Kristinsson, Balaban & Marshall, 2007).

A hurdle strategy approach can lead to a reduction of the intensity of the treatments, avoiding the negative effects and enhancing synergistic antimicrobial interactions. Available evidence suggests the existence of such synergistic effect. Ross, Griffiths, Mittal and Deeth (2003) pointed that timing of antimicrobial addition is important,

which should be present during pressurisation for additive or synergistic effects with HPP to occur. Raouche, Mauricio-Iglesia, Peyron, Guillard and Gontard (2011) proposed a mechanism of a pressure triggered uptake of antimicrobial compounds by HPP sublethally injured microorganisms. The effectiveness of the synergistic effect between essential oils and HPP decreases on foods when compared to results in buffered solutions. The incorporation of these natural compounds to edible films seems to be a good strategy to reduce the amount of essential oil through a gradual release of the additive to the food along its shelf life (Campos, Gerschenson & Flores, 2011). In addition, the efficiency is improved due to the localisation of the antimicrobial activity on the surface of foods (Aasen, Markussen, Møretrø, Katla, Axelsson & Naterstad, 2003).

These combined techniques can be useful in the preservation of the fish, since it is a very perishable commodity. Preservation technologies are specially required to maintain highly perishable food such as fatty fish. Rainbow trout (*Oncorhynchus mykiss*) contains a high proportion of polyunsaturated fatty acids (PUFAs), making it highly susceptible to oxidation and development of unpleasant off-flavours (Baron, Kasgard, Jessen & Jacobsen, 2007). Andevari and Rezaei (2011) used gelatin coatings with cinnamon essential oil, efficiently preserving the quality of refrigerated rainbow trout fillets over a period of 15 days. Nowzari, Shábanpour and Ojagh (2013) showed no differences between the application method (coating or film wrap) in the antimicrobial efficiency of gelatin-chitosan films on refrigerated trout fillets. A synergistic increase in the shelf life of chilled trout was found by Günlu, Sipahioglu and Alpas (2014) by the use of HPP and chitosan films, being more effective the films alone than HPP.

The combination of HPP and edible films with antioxidant and/or antimicrobial properties as hurdle strategy for food products has been recently proposed, and very few

reports exist about its application on fish (Gómez-Estaca, Montero, Jiménez & Gómez-Guillén, 2007; Ojagh, Núñez-Flores, López-Caballero, Montero & Gómez-Guillén, 2011; Núñez-Flores, Castro, López-Caballero, Montero, & Gómez-Guillén, 2013; Günlu et al., 2014). There are no previous reports on the combination of HPP and edible film formulated with chitosan including clove essential oil.

OBJETIVES

The purpose of the study was to combine high pressure processing (HPP) and chitosan edible films with clove essential oil and to determine the effects of these three selected hurdles on the shelf life of rainbow trout (*Oncorchynchus mykiss*) fillets. An optimal concentration of 20 g per kg clove essential oil was previously selected on the basis of previously evaluated *in vitro* antimicrobial and antioxidant effects.

2. MATERIALS AND METHODS

2.1. Chemicals

All the chemicals used in the formulation of films were food grade quality aditio-Panreac products (Panreac Química, Barcelona, Spain). Other reagents (gallic acid, 37 FAME mix, Supelco) were purchased to Sigma-Aldrich (Sigma Aldrich Chemical Co Steinheim, Germany). All the solvents were HPLC grade (Lab-Scan, Dublin, Ireland).

All culture media used for the antimicrobial activity determination of chitosan films with different concentration of CEO were supplied by Oxoid (Basingstone, Hampshire, England). In the microbiological analysis on the shelf life of fish, buffered peptone water was obtained from AES (Cambourg, France) and culture media (PCA, MRS and VRBL) from Biolife (Milan, Italy).

2.2. Formulation of the films

The films were prepared by aqueous solution of 15 g per kg of chitosan and 10 g per kg acetic acid. The mixture was stirred at 40 ºC in an enzymatic digester (GDE, Velp Scientifica, Italy) for 2 hours to obtain a homogenous solution. Afterwards, 5 g of glycerol per kg of biopolymer were added as plasticiser. Food grade clove essential oil (CEO) was incorporated in proportion of 5, 10, 15, 20 and 50 g per kg of filmogenic solution, and 2 g of Tween 80 per kg of biopolymer as emulsifying agent. The oil-added film forming solutions were homogenised at 13.400xg (Ultraturrax IKA T25 digital, IKA-Werke, Germany) keeping in ice bath to avoid overheating. Later, solutions were degassed using an ultrasound bath (Selecta SA, Spain) for 30 min and kept overnight at room temperature in darkness. The films were obtained by casting 20 mL in 90 mm-diameter Petri dishes and dried at 42 ºC in an air-forced incubator (Biosan ES-20, Biogen Científica SL, Madrid, Spain) for 15 h. Prior to analyses, the films were conditioned in desiccators over a saturated solution of KBr (58% relative humidity).

2.3. Antimicrobial activities of chitosan films with different concentrations of CEO

2.3.1. Microbial species

The antimicrobial activity of chitosan films with or without clove oil was tested over ten microorganisms obtained from Spanish Type Culture Collection (CECT) and Public University of Navarra (UPNA), listed in **Table 1**.

Table 1: Culture conditions of the selected microorganisms.

Microorganism	Bacterial strain	Culture media	Incubation Temperature	Incubation Time (hours)
Enterococcus faecalis	CECT 481	Brain Heart Infusion broth (BHI)	37 ºC	24
Lactobacillus sakei	CECT 4808	Man, Rogosa and Sharpe (MRS) in 8% CO_2	30 ºC	24
Leuconostoc mesenteroides	CECT 219	Man, Rogosa and Sharpe (MRS)	30 ºC	24
Listeria innocua	CECT 910	Brain Heart Infusion broth (BHI)	37 ºC	48
Staphylococcus warneri	CECT 236	Nutrient Agar (NA) I	37 ºC	24
Aeromonas hydrophila	CECT 5173	Tryptic Soy Agar (TSA)	30 ºC	48
Escherichia coli	CECT 434	Tryptic Soy Agar (TSA)	37 ºC	48
Pseudomonas fragi	CECT 378	Nutrient Agar (NA) II	30 ºC	24
Shewanellla putrefaciens	UPNA 5346	Nutrient Agar (NA) II	25 ºC	48
Vibrio alginolyticus	UPNA 521	Nutrient Agar (NA) I+3%NaCl	26 ºC	24

CECT: Spanish Type Culture Collection. UPNA: Public University of Navarra.

Five of these bacteria were chosen as specific fish spoilage and pathogenic microorganisms (*Lactobacillus sakei, Pseudomonas fragi*, *Shewanella putrefaciens*, *Aeromonas hydrophila* and *Vibrio alginolyticus*) and the rest of them as general spoilage and pathogenic bacteria (*Listeria innocua*, *Escherichia coli*, *Staphylococcus warneri*, *Enterococcus faecalis* and *Leuconostoc mesenteroides*).

2.3.2. Agar disc diffusion method

A suspension of each microorganism was spread on the solid medium plates (0.1 mL of 10^7 CFU per mL suspension). Culture conditions of the selected microorganisms are presented in **Table 1**.

Circular pieces of the different films (13 mm diameter) were laid onto the plate´s surface and, after incubation, the clear zone surrounding the films (inhibition area) was considered as measurement of the antimicrobial activity. The inhibition area was measured as the diameter of the colony-free perimeter (including the disk), using a digital caliper (Neiko Tools, Ontario, Calif., U.S.A.). All the tests were performed in triplicate.

2.4. Antioxidant capacity of chitosan films with different concentrations of CEO

2.4.1. Extract preparation

A 200 mg piece of conditioned film was placed in a polypropylene tube containing 30 mL of methanol, the lid tightly closed and the content stirred at 3000 xg for 90 minutes at room temperature. After centrifugation at 3214 xg for 10 minutes the supernatant was collected for antioxidant capacity and total phenol determinations. Measurements were carried out in triplicate.

2.4.2. Total Phenols (TP)

Total phenols were measured using the Folin-Ciocalteu method (Slinkard & Singleton, 1977). Results were expressed as mg Gallic Acid Equivalents (GAE) per g sample using a calibration curve with gallic acid as standard (9.8 μM to 70 μM).

2.4.3. ORAC (Oxygen Radical Absorbance Capacity)

The procedure was based on Ou, Hampsch-Woodill and Prior (2001). Results were expressed as μmoles Trolox Equivalents (TE) per g sample.

2.4.4. TEAC (Trolox Equivalent Antioxidant Capacity).

The analysis was carried according Re, Pellegrini, Proteggente, Pannala, Yang and Rice-Evans (1999). Results were expressed as μmoles TE per g sample.

2.5. Effect of chitosan films with CEO and HPP on the shelf life of fish

2.5.1. Preparation and treatments of fish samples

Gutted aquaculture rainbow trouts (*Oncorhynchus mykiss*) were provided by aquaculture farm IPEASA (Villa de Fuentidueña, Segovia, Spain). After skinning and filleting, they were cut into pieces of 5.7 x 2.5 mm (weighing approximately 10 g) and randomly allocated into 7 batches: fresh fish (Fresh), chitosan film (Film), chitosan film

with 20 g kg^{-1} of clove oil (Clove), cooked (Cook), high pressure processing (HPP), chitosan film and high pressure treatment combination (HF) and chitosan film with 20 g kg^{-1} of clove oil and high pressure treatment combination (HC). Pieces of the samples for film treatments (film, clove, HF and HC) were individually wrapped with 90 mm-diameter films with the help of tweezers in hygienic conditions. Afterwards, all samples (fresh, film, clove, HPP, HF and HC) were placed in co-extruded polyamide/polyethylene (30/130 μm thickness) flexible bags with oxygen permeability of 30 mL mm^{-2} day^{-1} bar^{-1} and water vapour transmission of 1.4 g m^{-2} day^{-1} (Industrias Pargón, Salamanca, Spain), and vacuum sealed. A slightly different procedure for cooked samples was followed: within the bags the samples were placed in a convection oven at 90 ºC for 15 min, blast chilled to 4 ºC and vacuum sealed.

After vacuum packaging, high pressure processed samples (HPP, HC and HF) were treated in a high pressure unit Wave 6000/135 (NC Hyperbaric, Burgos, Spain) for 10 minutes at 300 MPa at 12ºC. All treatments were stored at 4 ºC until analysis at days 0, 6, 9, 15 and 22. The assay was made twice. All analyses were performed in triplicate.

2.5.2. pH

A 10 g sample of fish flesh was homogenised in 100 mL of distilled water and the mixture filtered. The pH (pH-meter model 507, CRISON, Barcelona, Spain) of the filtrate was measured at room temperature.

2.5.3. Water activity (a_w)

Water activity was measured with an Aqualab 4TE water activity meter (Decagon Devices Inc, Pullman, WA, USA).

2.5.4. Drip loss

Drip loss (%) was gravimetrically calculated (Mexis, Chouliara, & Kontominas, 2009) by difference between the fillet of rainbow trout at the beginning and at the end of the storage time (days 0 and 22).

2.5.5. Total Volatile Basic Nitrogen (TVBN)

TVBN was determined according to the method described by EC Regulation 2005/2074 (EC, 2005). Results were expressed as mg N per 100 g muscle.

2.5.6. Proximate composition

Initial composition of trout fillets was determined at day 0. Moisture content was gravimetrically determined (AOAC, 1995). Protein content was analysed by the Kjeldahl technique (AOAC, 1995) (N=6.25). Ash content was determined by heating to 550 °C for 24 h (AOAC, 1990). Total fat was extracted with petroleum ether (Soxtec extraction, System 2055 Tecator, FOSS, Hillerød, Denmark).

2.5.7. Fatty acids composition

Total lipids were extracted from 10 g samples with methanol:chloroform (1:1, v:v) according to the Bligh and Dyer method (1959). The chloroform phase of the Bligh and Dyer (B&D) extract at day 0 of trout fillets was evaporated under nitrogen, the lipid phase dissolved in hexane (1 mL), and methylated with 0.5M methanolic KOH (100 μL) for 10 min. The upper layer was analysed for fatty acid methyl esters (FAME) in a gas chromatograph (Agilent 7890A) equipped with a DB-23 column (60 m x 0.32 mm, 0.25 μm film thickness) and a flame ionisation detector. Helium was used as the carrier gas. The oven temperature was programmed at 50 ºC for the first 7 min and increased up to 200 ºC at a rate of 25 ºC/min; the temperature was further increased to 230 ºC at a rate of 3 ºC min^{-1} and held for 26 min. Injection and detector temperatures were 250 ºC and 280 ºC, respectively. One microlitre of hexane extract was injected in split mode (25:1), and FAMEs identified from standard's retention times.

2.5.8. Microbiological analysis

Fish samples (10 g) were aseptically transferred into bags (Microgen, Surrey, United Kingdom) with 90 mL of sterile Buffered Peptone Water and homogenised with a pulsifier for 90 s (Pul 100E, Microgen, Surrey, United Kingdom). For each sample, appropriate serial decimal dilutions were prepared in Buffered Peptone Water solution (1 g per L) for the following microorganism counts:

(i) Total aerobic mesophilic were determined using Tryptic Glucose Yeast Agar (PCA) after incubation at 30 ºC for 72 h ± 3 h.

(ii) Lactic acid bacteria on double-layer Man Rogosa Sharpe medium (MRS) incubated at 30 ºC for 72 h ± 3 h.

(iii)Total coliform on double-layer Violet Red Bile Agar (VRBL) incubated at 37 ºC for 24 h± 2 h.

2.5.9. Thiobarbituric acid reactive substances (TBARS)

Samples were analysed using the methodology described by Tarladgis, Watts, Younathan and Dugan (1960). Analyses were performed in triplicate and results were expressed as μg malondialdehyde (MDA) per g muscle.

2.5.10. Hexanal

Hexanal content in fish samples was determined by Headspace Gas Chromatography/Mass spectrometry (GC/MS) using Solid Phase Dynamic Extraction (SPDE). Three grams of fish samples were placed in 20 mL vials with 100 μL of cyclopentanone as internal standard. Headspace volatile were sampled by SPDE with 90% dimethylsiloxane and 10% active carbon, under the following conditions: preincubation time: 1 min; incubation temperature: 70 ºC; syringe temperature: 75 ºC; number of extraction strokes: 50; extraction fill/eject speed: 40 μL per s; pre-desorption time: 30 s; desorption gas volume: 500 μL; desorption flow speed: 15 μL per s.

GC analysis were accomplished by HP 6890N Series instrument (Agilent Technologies) equipped with HP-5MS capillary column (60 m x 0.32 mm, 0.25 um film thickness) and a 5973i mass spectrometer detector (Agilent Technologies) working with the following temperature programme: 40 ºC to 240 ºC at 3 ºC per min; injector and detector temperatures: 250 ºC; carrier gas helium at 1 mL per min in splitless mode.

Identification of the compounds was based by their retention time and the comparison of their MS with the library spectra present in Wiley and National Institute of Standards and Technology (NIST) databases. All determinations were performed in triplicate.

2.5.11. Colour

Colour parameters, lightness (L*), redness (a*) and yellowness (b*), were measured using spectrophotometer (Minolta CM-2002, Osaka, Japan). The illuminant was D65 and standard observer 10º. Measurements were taken on samples packaged with transparent plastic bags at six different points.

2.5.12. Sensory analysis

A trained panel consisting of 12 panellists were recruited from the department of Biotechnology and Food Science of the Burgos University, Spain, for their previous experience in sensory analysis. All the tests were carried out in a sensory laboratory equipped with individual booths (ISO 8589:1988). The samples were coded and randomly presented to panellists. The following descriptors were studied: colour intensity, off-colours, surface defects, drip loss, fishy odour intensity and off-odours. The panellists were asked to evaluate these descriptors in comparison with control fish, ranking the samples between -4 (lower values than control) to 4 (higher values than control), scoring 0 if samples did not differ from control (Fresh).

2.6. Statistical analysis

The statistical analyses were performed using Statgraphics Centurion XVI. One-way analyses of variance were carried out. Differences between pairs of means were assessed on the basis of confidence intervals using Fisher LSD (Least Significant Difference) with a level of significance of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Properties of chitosan films with different concentration of CEO

3.1.1. Antimicrobial activities

Chitosan films without clove oil did not produce inhibition halos (**Table 2**), although antimicrobial activity was detected as growth inhibition in the area under the films.

Table 2:Antimicrobial activity of the chitosan films with increasing clove oil concentrations, expressed as the diameter of the clear zone surrounding the films (mm) in agar disc diffusion tests.

	Clove oil (g per kg)					
Microorganısı	**0**	**5**	**10**	**15**	**20**	**50**
E. faecalis	N.E	N.E	N.E	N.E	20.33±0.50^{a}	20.00±0.00^{a}
L. sakei	N.E	N.E	N.E	20.00±0.20^{a}	17.67±4.00^{a}	24.00±2.65^{b}
L. mesenteroides	N.E	16.74±3.46^{a}	20.65±1.15^{b}	21.67±0.58^{b}	22.50±0.71^{b}	26.67±2.08^{c}
L. innocua	N.E	15.01±4.04^{a}	20.31±1.15^{b}	21.67±1.15^{b}	23.00±1.73^{c}	26.67±2.08^{c}
S. warneri	N.E	N.E	N.E	N.E	20.00±0.00^{a}	20.03±0.58^{a}
A. hydrophila	N.E	N.E	N.E	N.E	N.E	20.33±1.15^{a}
E. coli	N.E	16.74±3.46^{a}	22.33±0.58^{b}	22.67±0.58^{b}	24.33±1.10^{b}	27.67±0.58^{c}
P. fragi	N.E	N.E	15.49±5.20^{a}	26.50±0.71^{b}	28.50±0.70^{b}	31.00±1.00^{b}
S. putrefaciens	N.E	32.40±3.54^{a}	35.47±2.12^{a}	40.67±0.58^{b}	42.67±0.50^{b}	48.00±1.00^{c}
V. alginolyticus	N.E	N.E	N.E	N.E	23.67±1.50^{a}	27.33±1.53^{b}

N.E: Inhibition zone non exhibited.
Values (mean ± standard deviation, n=3) followed by the same letter in same row are not significantly different ($p>0.05$).

Similar results were reported by Wang et al. (2011) who argued that only dissolved chitosan particles, and not those that form films, could diffuse to create an inhibition area. Coma, Martial-Gros, Garreau, Copinet, Salin and Deschamps (2002) explained that the chitosan positive charges may compete with Ca^{2+} for the negatively charges of the bacterial membrane. The inhibition areas, therefore, are formed only by the diffusion of the essential oil compounds. Films prepared with increasing oil concentrations exhibited higher inhibitory effect on the tested bacteria; four of the species tested already showed inhibition halos with 5 g per kg clove oil films. Hosseini, Razavi and Mousavi (2009) showed that clove oil was effective against both gram-positive and gram-negative bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Pseudomonas aeruginosa*) at concentration of 15 g per kg. In this work, we did not find antimicrobial inhibition zone (Table 2) against *V. alginolyticus*, *A. hydrophila*, *S. warneri* and *E. faecalis*at 15 g per kg CEO concentration. However, this concentration was effective in showing inhibition of the rest of the bacterial species tested. On the other hand, Wanget al. (2011) did not find significant inhibition of growth for *E. coli* and *S. aureus*, two of the genera tested in the present work, with clove oil concentrations under 50 g per kg.

Growth inhibition of all the microorganisms tested was observed with 20 g per kg clove oil concentration, with the exception of *A. hydrophila*. This bacterium was only inhibited when the highest concentration (50 g per kg) was used.

Previous studies (Ouattara, Simard, Holley, Piette & Bégin, 1997; Mangena & Muyima, 1999; Hosseini, Razavi, Mousavi, Yasaghi & Hasansraei, 2008) have shown higher antimicrobial efficiency of essential oils on gram-positive bacteria than on gram-negative bacteria. Other authors have not found such relation between morphology (Gram-negative and Gram-positive) and sensitivity to essential oil in bacterial growth

(Gómez-Estaca et al, 2010; Dorman & Deans, 2000; Chaieb et al., 2007) as occurred in our work. CEO showed relatively low antimicrobial activity against some gram-positive bacteria such as *E. faecalis*, *S. warneri* and *L. sakei*. In this way, Holley and Patel (2005) explained the greater resistance of the lactic acid bacteria (LAB) to be related to their better ability to deal with conditions of osmotic stress and more effective response to K^+ efflux caused by many of these antimicrobials.

Gómez-Estaca et al. (2010) among a range of microorganisms tested, found *S. putrefaciens* as the most sensitive bacteria to clove oil either alone or formulated in film; this microorganism was also the most sensitive as observed in our results. Gómez-Estaca et al. (2010) also showed no differences in sensitivity to clove oil between *E. coli* and *L. innocua*; a similar result was also observed in our work. Previous works showed low sensitivity of *Pseudomonas* spp. to clove oil (Gómez-Estaca et al., 2010; Ouattara et al., 1997; Mangena & Muyima, 1999). In contrast, our results (**Table 2**) showed a strong inhibition of the clove essential oil-containing films on *P. fragi* (highest inhibition after *S. putrefaciens*). Holley and Patel (2005) reviewed the antimicrobial activity of plant essential oils stating that the genus *Pseudomonas* show consistently high resistance to these types of antimicrobials. On the other hand, Gómez-Estaca, López De Lacey, Gómez-Guillén, López-Caballero and Montero (2009) reported the effectiveness of films incorporated with CEO against four microorganisms: *L. acidophilus*, *P. fluorescens*, *L. innocua* and *E. coli*. In general, percentages of inhibition were similar to those reported here, with the exception of *Lactobacillus*, although Gómez-Estaca et al. (2009) tested *L. acidophilus* instead of *L. sakei*. Differences may be due to variability between species within the same genus, which could be occurring also with differences observed in this work and literature regarding the *Pseudomonas* genus.

According to the classification by Ponce, Fritz, Del Valle and Roura (2003) of the sensitivity of microorganisms, our results showed that against 20 g per kg clove oil chitosan films, *A. hydrophila* was not sensitive, *V. alginolyticus, S. warneri* and *E. faecalis* were very sensitive, and the rest of microorganisms tested (*L. sakei, P. fragi, S. putrefaciens, L. innocua, E. coli* and *L. mesenteroides*) were extremely sensitive. The only microorganism not inhibited by the 20 g per kg CEO film, *A. hydrophila*, is highly sensitive and does not develop resistance to HPP treatments (Vanlint, Rutten, Michiels & Aertsen, 2012). Furthermore, a ranking test previously carried out comparing fish samples with films with CEO at different concentrations showed significantly lower acceptability of the samples incorporating 50 g per kg CEO films when compared to the rest (20 g per kg or lower) (data not shown). After these results, the films with a clove oil concentration of 20 g per kg were chosen as optimal for the following study of fish preservation.

3.1.2. Antioxidant capacities

Total phenols and antioxidant capacity (ORAC and TEAC) of chitosan film were shown in **Table 3**.

Table 3: Total phenols and antioxidant capacity of chitosan films with different concentration of CEO.

CEO (g per kg)	Total Phenols (mg GAE per g)	ORAC (μmoles TE per g)	TEAC (μmoles TE per g)
0	0.44±0.02^{a}	1.84±0.10^{a}	0.02±0.00^{a}
5	0.83±0.09^{b}	3.29±0.43^{b}	0.08±0.01^{b}
10	1.21±0.11^{bc}	4.33±0.76^{b}	0.12±0.00^{c}
15	1.73±0.15^{c}	6.65±0.26^{c}	0.19±0.01^{d}
20	1.98±0.28^{d}	8.28±0.34^{d}	0.23±0.01^{e}
50	2.06±0.29^{d}	9.76±0.53^{e}	0.24±0.01^{e}

Values (mean ± standard deviation, n=3) followed by the same lowercase letter are not significantly different (p>0.05).

Total phenolic content and antioxidant capacity of edible films increased with increasing CEO concentrations, up to 20 g per kg. Although no previous studies on the antioxidant activity of chitosan films with CEO were found, similar behaviour has been observed for chitosan films formulated with other essential oils such as *Zataria multiflora* and *Thymus* spp. essential oils (Moradi, Tajik, Rohani & Oromiehie, 2011; Ruiz-Navajas et al., 2013). A further significant increase in antioxidant capacity ($p<0.05$) from 20 and 50 g per kg CEO was observed with ORAC, but not with the rest of antioxidant assays. A possible explanation was proposed by Moradi et al. (2011). Films with high amount of CEO (50 g per kg) have a hydrophobic character, which may have reduced the migration of the phenolic compounds from the film into the sample in the antioxidant assays with the exception of ORAC.

Chitosan films without CEO had some phenolic content and antioxidant capacity (**Table 3**). Our results were in agreement with Moradi et al. (2011) and Ruiz-Navajas et al. (2013). The scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH_2^+) groups to form stable macromolecule radicals and the NH_2 groups can form ammonium (NH_3^+) groups by absorbing a hydrogen ion from the solution (Ruiz-Navajas et al., 2013).

Phenols are very important constituents of CEO, being eugenol the major constituent. Phenols have scavenging ability due to their hydroxyl groups (Gülçin et al., 2004). The antioxidant activity of CEO according to Jirovetz, Buchbauer, Stoilova, Stoyanova, Krastanov and Schmidt (2006) may be based on various mechanisms, such as scavenging radicals and chelation.

It was found good correlation between total phenols and antioxidant activity in chitosan films with CEO with the different methods carried out to evaluate antioxidant capacity, all above $r^2=0.96$.

3.2. Effect of films and HPP on shelf life of trout fillets

3.2.1 Properties of fresh trout fillets

3.2.1.1. Proximate composition

Average of moisture, protein, fat and ash in rainbow trout fillets were 75.62% (±1.12), 18.63% (±1.33), 4.65% (±0.70) and 1.10% (±0.06), respectively. Similar results were reported in aquaculture rainbow trout by Andevari and Rezaei (2011).

3.2.1.2. Fatty acid composition

The proportion of saturated fatty acids in the lipid fraction was 16.69%, whereas the mono and polyunsaturated fatty acids constituted 37.75% and 32.42% of the total fatty acids, respectively. Docosahexaenoic acid (DHA, C22:6 n-3) was the most abundant (11.11%) of the polyunsaturated fatty acids, whereas the major saturated fatty acid was palmitic acid (C16:0), and oleic acid (C18:1, n-9) the main monounsaturated fatty acid, with a percentage over the total fatty acid content of 10.16% and 31.97%, respectively. This is in agreement with data previously reported by Rebolé et al. (2015).

3.2.2.Effect of chitosan films with CEO and HPP on trout fillet preservation

3.2.2.1. pH

The pH evolution along time (**Table 4**) agrees with results reported by Mexis et al. (2009) in rainbow trout fillets stored at 4 ºC.

Table 4: pH of rainbow trout fillets subjected to different treatments along storage at 4ºC.

Treatment/Day	0	6	9	15	22
Fresh	$^{c}6.95\pm0.02_{A}$	$^{b}6.86\pm0.02_{C}$	$^{b}6.83\pm0.03_{B}$	$^{d}7.11\pm0.03_{DE}$	$^{a}6.61\pm0.03_{C}$
Film	$^{d}7.09\pm0.01_{B}$	$^{b}6.88\pm0.01_{C}$	$^{b}6.84\pm0.04_{B}$	$^{c}7.00\pm0.02_{C}$	$^{a}6.43\pm0.02_{B}$
Clove	$^{b}6.87\pm0.09_{A}$	$^{a}6.67\pm0.10_{B}$	$^{c}7.10\pm0.05_{D}$	$^{b}6.83\pm0.09_{B}$	$^{a}6.63\pm0.04_{C}$
Cook	$^{d}7.18\pm0.06_{B}$	$^{b}6.89\pm0.01_{CD}$	$^{c}6.98\pm0.01_{C}$	$^{d}7.17\pm0.01_{E}$	$^{a}6.69\pm0.03_{CD}$
HPP	$^{d}7.11\pm0.02_{B}$	$^{b}6.97\pm0.02_{D}$	$^{b}6.95\pm0.04_{C}$	$^{c}7.04\pm0.02_{CD}$	$^{a}6.75\pm0.03_{D}$
HF	$^{b}7.16\pm0.04_{B}$	$^{b}7.13\pm0.01_{E}$	$^{b}7.06\pm0.01_{D}$	$^{c}7.32\pm0.04_{F}$	$^{a}6.49\pm0.06_{B}$
HC	$^{b}6.92\pm0.02_{A}$	$^{ab}6.31\pm0.01_{A}$	$^{ab}6.61\pm0.01_{A}$	$^{ab}6.50\pm0.02_{A}$	$^{a}6.22\pm0.16_{A}$

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same column are significantly different ($p<0.05$).
Fresh: fresh fish, Film: Chitosan Film, Clove: Chitosan Film with Clove Essential Oil, Cook: Cooking treatment, HPP: High Pressure Processing, HF: HPP + Chitosan Film, HC: HPP + Clove Film.

The pH values decreased from day 0 to day 6 of storage, increasing from day 6 to day 15. This later increment in pH could be due to alkaline compounds which are formed from protein and nucleotide decomposition in the muscle during post-mortem period (Mexis et al., 2009). From day 15 of storage, pH values diminished, probably due to the growth of lactic acid bacteria and lactic acid formation (Picouet, Cofan-Carbo, Vilaseca, Ballbè & Castells, 2011)

Picouet et al. (2011) found an increase in pH values for salmon samples treated with sous-vide cooking; in this regard, we observed significantly higher pH of the cooked samples when compared to fresh at days 0 and 9. Also, high pressure processing resulted in higher pH values when compared to fresh samples (except for day 15). This increase in pH can be explained as a decrease in available acid groups of pressure or heat-denatured proteins (Picouet et al., 2011; Cruz-Romero, Kelly & Kerry, 2008). However, this effect of higher pH values was not observed in the samples where the high pressure processing was combined with films containing CEO; these samples maintained the lowest pH values over storage, when compared to the rest of the samples. This effect was not observed either with the chitosan film and HPP combined (HF) or the sample with CEO film.

3.2.2.2. a_w

Water activity plays an important role in the fish spoilage and the growth of microorganisms. In this sense, the effect in a_w of the differences treatments were studied and the relation between a_w and other spoilage indicator were established. The a_w values did not show a clear trend over storage time (**Table 5**).

Table 5:A_w of rainbow trout fillets subjected to different treatments along storage at 4ºC.

Treatment /Day	**0**	**6**	**9**	**15**	**22**
Fresh	$^{ab}0.9901\pm0.0008_{C}$	$^{b}0.9905\pm0.0007_{D}$	$^{a}0.9893\pm0.0006_{CD}$	$^{b}0.9908\pm0.0007_{BC}$	$^{b}0.9909\pm0.0001_{B}$
Film	$^{ab}0.9880\pm0.0012_{AB}$	$^{ab}0.9879\pm0.0010_{C}$	$^{a}0.9858\pm0.0024_{A}$	$^{b}0.9889\pm0.0018_{B}$	$^{ab}0.9885\pm0.0006_{A}$
Clove	$^{a}0.9868\pm0.0007_{A}$	$^{a}0.9867\pm0.0010_{B}$	$^{a}0.9878\pm0.018_{ABC}$	$^{b}0.9902\pm0.0004_{BC}$	$^{a}0.9876\pm0.0011_{A}$
Cook	$^{a}0.9877\pm0.0014_{AB}$	$^{b}0.9897\pm0.0011_{CD}$	$^{b}0.9904\pm0.0006_{D}$	$^{b}0.9908\pm0.0007_{BC}$	$^{b}0.9912\pm0.0000_{B}$
HPP	$^{a}0.9893\pm0.0006_{BC}$	$^{a}0.9886\pm0.0007_{BCD}$	$^{a}0.9888\pm0.0011_{BCD}$	$^{b}0.9923\pm0.0005_{C}$	$^{b}0.9909\pm0.0007_{B}$
HF	$^{a}0.9876\pm0.0012_{AB}$	$^{a}0.9866\pm0.0002_{B}$	$^{a}0.9868\pm0.0007_{AB}$	$^{a}0.9890\pm0.0035_{B}$	$^{a}0.9891\pm0.0003_{A}$
HC	$^{b}0.9865\pm0.0003_{A}$	$^{a}0.9832\pm0.0020_{A}$	$^{b}0.9861\pm0.0006_{A}$	$^{b}0.9862\pm0.0007_{A}$	$^{b}0.9879\pm0.0012_{A}$

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same column are significantly different ($p<0.05$).
Fresh: fresh fish, Film: Chitosan Film, Clove: Chitosan Film with Clove Essential Oil, Cook: Cooked treatment, HPP: High Pressure Processing, HF: HPP + Chitosan Film, HC: HPP + Clove Film.

The treatment combining CEO film and high pressure processing produced the lowest a_w values over storage, with significant differences to the rest of samples on days 6 and 15. HPP, Cook and Fresh samples showed the highest values over storage. Intermediate values were observed for the rest of samples with film (except for HC). As a general pattern, the use of films reduced the a_w of the samples. Gómez-Estaca et al. (2010) found an increase in water solubility of films when CEO was incorporated to their

formulation. Chitosan films absorbed some moisture from the fillets due to their hydrophilic nature. This decrease in the a_w can be related to minor microbial counts in samples with films (Film, Clove, HF and HC).

3.2.2.3. Weight Loss

The changes in weight loss were evaluated at days 0 and 22 (**Table 6**).

Table 6: Drip loss (%) of rainbow trout fillets subjected to different treatments at the beginning and at the end of storage at 4ºC.

Treatment/Day	0	22
Fresh	$^{a}4.86\pm1.51_{B}$	$^{a}6.67\pm1.55_{B}$
Film	$^{a}1.01\pm0.49_{A}$	$^{a}1.42\pm0.82_{A}$
Cook	$^{a}15.24\pm0.60_{C}$	$^{b}16.12\pm3.14_{C}$
Clove	$^{a}2.53\pm1.83_{A}$	$^{a}1.68\pm0.55_{A}$
HPP	$^{a}6.10\pm1.09_{B}$	$^{a}6.70\pm0.90_{B}$
HF	$^{a}2.69\pm0.96_{A}$	$^{a}3.39\pm0.13_{A}$
HC	$^{a}2.01\pm1.09_{A}$	$^{a}1.71\pm0.52_{A}$

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same row are significantly different (P<0.05).
Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same column are significantly different (P<0.05).
Fresh: fresh fish, Film: Chitosan Film, Clove: Chitosan Film with Clove Essential Oil, Cook: Cooked treatment, HPP: High Pressure Processing, HF: HPP + Chitosan Film, HC: HPP + Clove Film.

Drip loss values of cooked samples significantly increased over storage (p<0.05). Although not significant differences were found in the rest of the samples over storage, it was observed an increase in all treatments, except for those where clove essential oil was used (Clove and HC).

Regarding the effect of treatments, Cook had the highest drip loss values, followed by HPP and Fresh samples. There were not differences in drip loss values between Fresh and HPP. No significant differences were observed within the rest of samples, where films were applied either alone or in combination with high pressure treatments (Film, Clove, HF and HC). In this sense, both films (with or without CEO) reduce drip loss in

samples submitted to HPP. This effect is produced by the water absorption carried out by chitosan films as it was previously mentioned.

3.2.2.4. Microbiological evolution

Aerobics initial counts (day 0) were low as compared with other authors' results (Chytiri et al., 2004; González-Rodríguez, Sanz, Santos, Otero & García-López, 2001), befitting fresh and correctly manipulated samples (**Figure 1**).

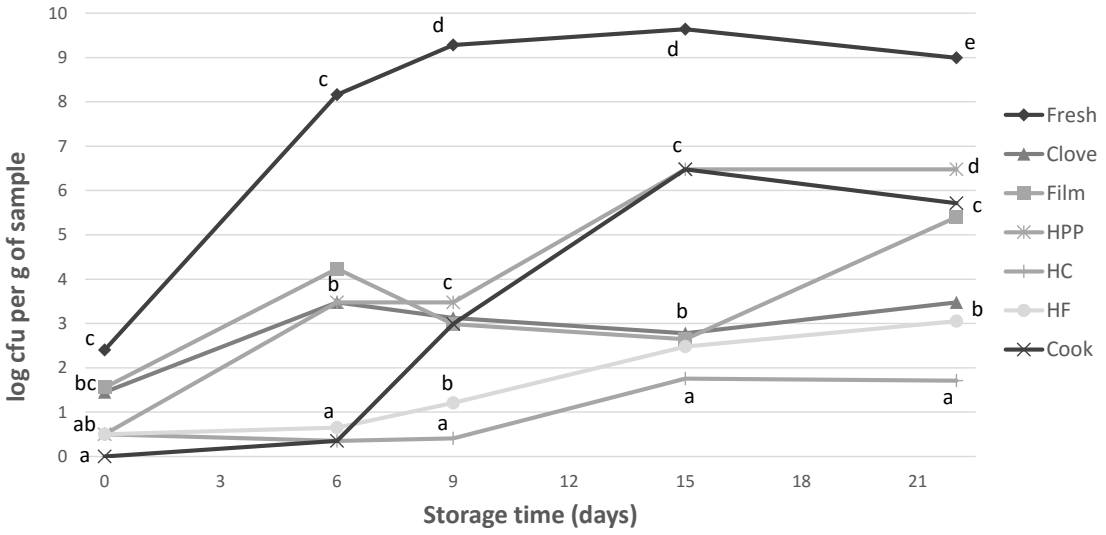

Figure 1: Total aerobic mesophilic counts in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days. Different letters have mean values that are significantly different ($p< 0.05$) due to the treatment.

One-way ANOVA results showed significant increases ($p<0.05$) in aerobic counts (**Figure 1**) of Fresh samples at day 6, with values exceeding those recommended for saleability (10^6 CFU per g) (Commission Regulation (EC) 2073/2005). Fresh fish (Fresh) samples showed (in **Figure 1**, **Figure 2** & **Figure 3**) the highest counts ($p<0.05$) for all the microorganisms evaluated (aerobic, lactic acid bacteria and coliforms), as expected.

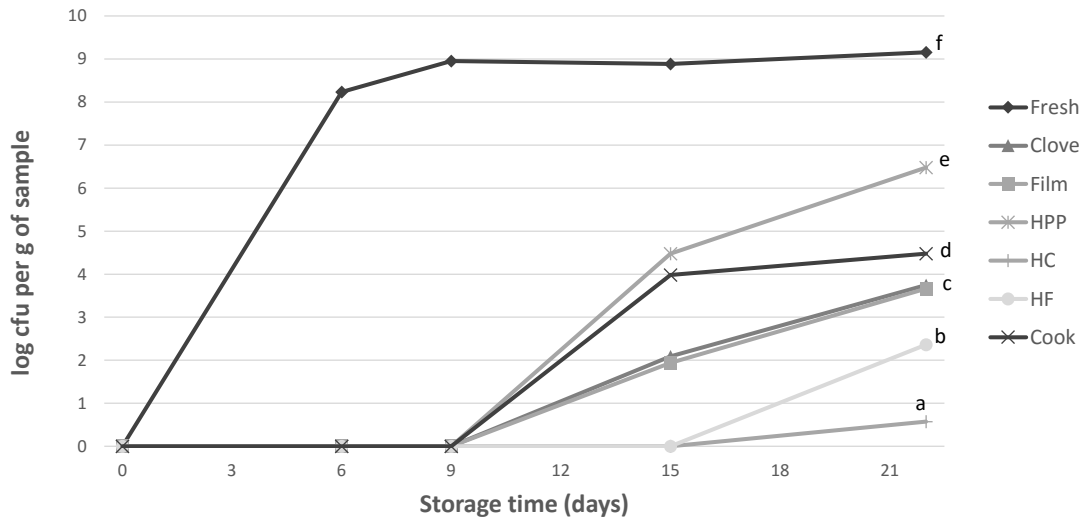

Figure 2: Total coliform counts in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days. Different letters have mean values that are significantly different ($p< 0.05$) due to the treatment.

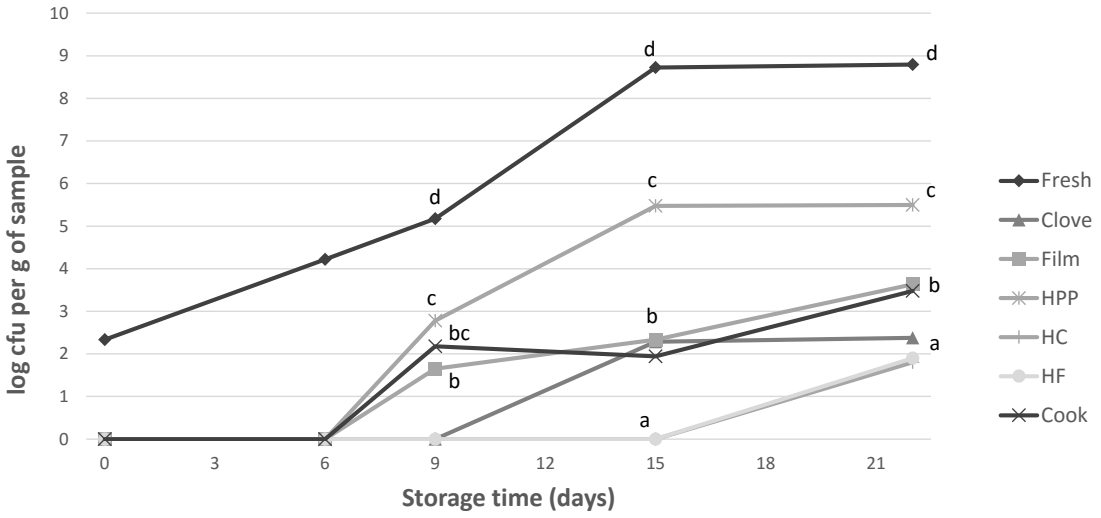

Figure 3: Lactic acid bacteria counts in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days. Different letters have mean values that are significantly different ($p< 0.05$) due to the treatment.

The results showed significant reductions of the microbial load due to application of any of the treatments, although HPP resulted in the smallest microbial reduction, obtaining

significantly lower microbial loads when HPP was combined with any of the films. The HPP conditions (300 MPa, 10 min) were intentionally lower than those recommended by Yagiz et al. (2007) in order to achieve an effective reduction of aerobic microorganisms (450 - 600 MPa, 15 min). The milder HPP conditions selected in this work were intended to obtain effective but not excessive treatments when combining HPP and films, in order to minimise the impact on the organoleptic properties of the final product.

The application of films exerted a protective effect when compared to fresh samples, and this antimicrobial effect is even higher when films are combined with HPP. CEO film (Clove) maintained lower microbial counts than film with no CEO (Film), although this effect was not statistically significant (**Figure 1**). The inhibitory effect of films formulated with CEO on spoilage bacteria growth during fish storage was reported by Gómez-Estaca et al. (2010). Fernández-Pan, Mendoza and Maté (2013) pointed out the potential of CEO for food preservation, as applied in films, although they used different polymeric base (whey protein) and obtained better results with rosemary essential oil. The antimicrobial effect of the essential oils can be based on their bioactive compounds' hydrophobic nature, which allows altering the cell membrane and rendering it more permeable (Sikkema, Bont & Poolman, 1994). Not only the purposely-added essential oil but the polymeric base of the film can also exert intrinsic bioactivity, as it occurred in our results. Other authors working with chitosan did not find the same result. Moradi et al. (2011) obtained significant antimicrobial effect on mortadela type sausages when applying chitosan films incorporating essential oils, but no effect was observed with the chitosan film without the oils. On the other hand, the application of films has not always resulted in microbial growth reduction (Bolumar, Andersen & Orlien, 2011; Marcos, Aymerich, Monfort & Garriga, 2008; Ojagh et al., 2011). Gomez-Estaca et al. (2007)

came to a similar conclusion when working with gelatin and chitosan films with oregano and rosemary essential oils on sardine, and suggested the more promising combination of films and HPP. Similarly, Ojagh et al. (2011) compared the effect of HPP (similar conditions to our work: 300 MPa, 10 min) at two temperatures in combination with gelatin-lignin films on salmon. These authors found no antimicrobial effect of the films, and the only effect was attributed to the HPP treatment. Nuñez-Flores et al. (2013) reported an additive antimicrobial effect of the combination of HPP and gelatin-lignosulphonate films, although the sole use of films did not exert an antimicrobial effect. In our results also some significant additive effect was observed with the use of HPP and films (with CEO) in mesophilic aerobic (**Figure 1**) and coliform (**Figure 2**) counts. Cooking did not improve the microbial load when compared to any of the film treatments, and except for the samples with film alone (Film), the microbial levels of the samples with CEO film (Clove) and films combined with HPP (HF and HC) resulted lower than those for the cooking samples. These higher microbial load reduction in the samples where film was used could be also explained by the lower levels of drip loss and a_w when compared to those with no film (Fresh, Cook, HPP). Also, the more likely localisation of the microbial contamination on the surface of the trout fillets, as their structure was preserved, could also explain the high efficiency of all the film treatments found in this work. Similar results were found by Aasen et al. (2003). Nevertheless, the unequal results that can be found in the effectivity of films formulated with essential oils can also be attributed to differences in the formulation of the films, even differences between batches of essential oils, the heterogeneity of populations of the strains used in the *in vitro* tests, or synergistic or antagonistic effects of food components, which make necessary the tailoring of film formulation for any specific application (Burt, 2004).

3.2.2.5.TBARS

Lipid oxidation was studied using the TBARS method to monitor levels of secondary oxidation products formed (**Table 7**).

Table 7: Thiobarbituric acid reactive substances (µg malondialdehyde per gram of muscle) of trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days.

Treatment/Day	0	6	15	22
Fish	$_{A}0.059\pm0.007^{a}$	$_{B}0.252\pm0.002^{b}$	$_{AB}0.136\pm0.003^{b}$	$_{C}0.403\pm0.004^{c}$
Film	$_{A}0.042\pm0.007^{a}$	$_{A}0.031\pm0.002^{a}$	$_{A}0.031\pm0.020^{a}$	$_{B}0.272\pm0.023^{b}$
Clove	$_{A}0.015\pm0.009^{a}$	$_{A}0.020\pm0.007^{a}$	$_{A}0.025\pm0.011^{a}$	$_{B}0.062\pm0.007^{a}$
Cook	$_{A}0.463\pm0.098^{b}$	$_{A}0.674\pm0.100^{c}$	$_{A}0.703\pm0.065^{c}$	$_{A}0.476\pm0.011^{cd}$
HPP	$_{A}0.037\pm0.008^{a}$	$_{B}0.050\pm0.003^{a}$	$_{D}0.730\pm0.007^{c}$	$_{C}0.572\pm0.006^{d}$
HF	$_{A}0.056\pm0.004^{a}$	$_{A}0.053\pm0.010^{a}$	$_{A}0.084\pm0.006^{ab}$	$_{B}0.256\pm0.04^{b}$
HC	$_{A}0.014\pm0.002^{a}$	$_{AB}0.022\pm0.009^{a}$	$_{A}0.012\pm0.001^{a}$	$_{B}0.038\pm0.006^{a}$

Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same row are not significantly different (p<0.05).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same column are not significantly different (p<0.05)

In many cases, the level of TBARS remained practically unchanged until the end of storage time (day 22). During the storage, TBARS value was under the limit beyond which fish would normally develop an undesirable odour (1-2 µg MDA per g) (Souza et al., 2010). This fact can be attributed to the presence of carotenoids, which can act as a strong endogenous antioxidant system in rainbow trout (Ojagh et al., 2011). The initial TBARS values of trout fillets varied a lot in literature ranging from 0.07 µg MDA g^{-1} (Hamzeh & Rezaei, 2012) to 10.43 µg MDA per g (Chytiri et al., 2004; Mexis et al., 2009). These differences could also depend on the filleting process, which may affect rancidity levels of fish samples, probably as a result of exposure of the fish lipids to atmospheric oxygen, accelerating oxidation (Chytiri et al., 2004).

Cooking treatment induced the highest TBARS increase at the beginning of storage. Nevertheless, TBARS levels remained constant along storage time. Similar results were found by Ojagh et al. (2011). These increment in the TBARS values in cooked samples may have occurred due to the high temperature that promotes lipid peroxidation,

increasing MDA levels (Weber et al., 2008). The specific mechanism was firstly proposed by Frankel (1991). High temperatures have sufficient energy to break covalent bonds in the acyl backbone to form a variety of lipid alkyl radicals and these radicals react to form lipid oxidation products.

In pressurised samples (HPP), TBARS levels increased from day 15 of storage. There were not significant differences between HPP and Cook samples after that day. This trend also was found by Gómez-Estaca et al. (2007), Ojagh et al. (2011) and Günlu et al. (2014). A possible explanation can be that HPP induces changes in fish muscle, increasing asthaxantine availability (Yagiz et al., 2007), a powerful antioxidant, which could have counterbalanced the pro-oxidative effect of high pressure reported previously by several authors (Cheftel & Culioli, 1997; Gómez-Estaca et al., 2009; Yagiz et al., 2007). Medina-Meza et al. (2014) reviewed the effect of HPP in the degradation of the lipid fraction of food. According to these authors, pressure treatment below 300 MPa has little effect on lipid oxidation, being this linearly increased above the mentioned pressure level. In the case of fish products, lipid autoxidation seems to occur at lower pressure (Medina-Meza et al., 2014).

Coating with chitosan edible film enriched with CEO (Clove) lowered oxidation rate in the same way as using HPP together with clove. Use of natural plant extracts in lipid oxidation prevention has been widely described in literature. In fact, Jirovetz et al.(2006) showed that clove oil had a protective role in oxidative processes through TBARS determination. Nevertheless, there are few reports based on edible films formulated with plant extracts. In addition, most of them are focused on physical, chemical and/or antimicrobial properties, not on their antioxidant effect (Gómez-Estaca et al., 2007). Andevari and Rezaei (2011) also demonstrated the higher efficacy of

coating enriched with other essential oil, such as cinnamon, compared to gelatin coating alone, on the oxidation of rainbow trout over chilled storage.

Chitosan films (Film) prevented lipid oxidation to a lesser extent than films with CEO did. According to our results, films exerted a protective role in lipid oxidation, when compared to cooked, HPP and control (Fresh) samples at the end of storage (day 22). Souza et al. (2010) also found a protective effect on chitosan coated compared to control samples. Nevertheless, not synergistic effect was observed in HF respect to Film samples. Our findings differed from Günlu et al. (2014), where HF had significant lower TBARS values than chitosan covered samples (Film).

3.2.2.6. Hexanal concentration

The concentration of hexanal is shown in **Table 8**.

Table 8: Hexanal concentration (ng per g) in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days.

Treatment/Day	6	9	15
Fish	$^{a}0.0360\pm0.0020^{A}$	$^{c}2.2148\pm0.1723^{C}$	$^{a}0.9682\pm0.0935^{B}$
Film	$^{a}0.0032\pm0.0014^{A}$	$^{a}0.3987\pm0.0103^{A}$	$^{a}1.6691\pm0.3320^{B}$
Clove	$^{a}0.0295\pm0.0028^{A}$	$^{a}0.1162\pm0.0045^{B}$	$^{a}0.2500\pm0.0427^{C}$
Cook	$^{c}0.8965\pm0.1237^{A}$	$^{a}0.4714\pm0.0007^{A}$	$^{b}15.143\pm3.8699^{B}$
HPP	$^{b}0.2591\pm0.1310^{A}$	$^{b}1.2427\pm0.2835^{AB}$	$^{a}3.9276\pm0.9381^{B}$
HF	$^{a}0.0611\pm0.0485^{A}$	$^{a}0.1672\pm0.0070^{A}$	$^{a}2.3055\pm0.2792^{B}$

Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same row are not significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same column are not significantly different ($p<0.05$).

Initiation of lipid oxidation in fish is generally associated with polyunsaturated fatty acids in phospholipids of muscle cell membranes (Decker & Xu, 1998). Hexanal has been identified as a good marker for early lipid oxidation and correlated with rancid off-flavour (Timm-Heinrich, Eymard, Baron, Nielsen & Jacobsen**,** 2013).

Hexanal concentration significantly increased over storage time. It was remarkably the different pattern in hexanal concentration of fish, which reached the highest value at day 9, decreasing from this date. From these results it is clear that cooking procedures significantly increased hexanal concentration in relation to the rest of treatments. Moreira, Valente, Castro-Cunha, Cunha and Guedes de Pinho (2011) also obtained the highest hexanal concentration in Senegalese sole muscle submitted to cooking.

High pressure also induced oxidation, but to a lesser extent than cooking. Similar results were found by Beltran, Pla, Yuste and Mor-Mur (2003), where hexanal values of cooked (90 ºC for 15 minutes) and pressurized (300 MPa for 30 minutes at 20 ºC) minced chicken were compared. The mechanism of pressure-induced oxidation is related to the effect on the cell membrane. The pattern of hexanal generation was very similar that to the observed in TBARS values. Nevertheless, hexanal was less sensitive to monitor lipid oxidation compared to TBARS, differing from Bragnolo Danielsen and Skibsted (2005). No significant differences between coated (Film and Clove) and uncoated (Fresh) fish samples were found. This is consistent with the results from Kim, Yang, Noh, Chung and Min (2012), where the volatile profiles of coated with defatted mustard meal film and uncoated smoked salmon were not distinguishable.

3.2.2.7. Total Volatile Basic Nitrogen (TVBN)

TVBN in trout fillets subjected to different treatments and stored at 4 ºC are shown in **Table 9**.

Table 9: Total volatile basic nitrogen (mg N 100 per g of muscle) of rainbow trout fillets subjected to different processing treatments and stored at 4ºC for 22 days.

Treatment/Day	0	6	9	15	22
Fish	$_{A}11.90\pm0.99^{c}$	$_{B}18.2\pm1.62^{c}$	$_{B}18.57\pm0.65^{f}$	$_{C}21.00\pm2.13^{d}$	$_{D}22.4\pm1.30^{c}$
Film	$_{A}11.02\pm0.8^{bc}$	$_{BC}14.70\pm0.99^{b}$	$_{C}15.31\pm0.98^{d}$	$_{C}16.01\pm0.99^{c}$	$_{AB}12.6\pm0.63^{a}$
Cook	$_{A}7.00\pm0.98^{a}$	$_{B}15.4\pm0.01^{b}$	$_{B}15.59\pm0.70^{a}$	$_{BC}16.10\pm0.98^{c}$	$_{C}18.2\pm1.98^{bc}$
HPP	$_{A}8.4\pm0.02^{ab}$	$_{B}12.6\pm0.99^{a}$	$_{B}12.6\pm1.19^{b}$	$_{C}15.54\pm0.59^{b}$	$_{D}18.90\pm0.98^{c}$
HF	$^{A}7.00\pm1.98^{a}$	$^{B}14.40\pm1.40^{b}$	$^{B}14.45\pm0.32^{c}$	$^{B}14.47\pm0.81^{a}$	$^{B}15.4\pm1.61^{Ab}$

Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same row are not significantly different (p<0.05). Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same column are not significantly different (p<0.05)

Trout fillets without any treatment (fresh) presented the highest initial TVBN values. This was in accordance with data reported in the scientific literature, similar levels between 5 and 20 mg TVBN per 100 g have previously been found by Rezaei and Hosseni (2008). An increase in TVBN values was observed during storage in all the treatments. Throughout storage, TVBN did not exceed the legal limit established by European Union of 25-35 mg of TVBN in 100 grams of muscle (EC, 2005) for any of the treatments. Nevertheless, Chytiri, Chouliara, Savvaidis & Kontominas (2004) reported TVBN values of 18.31 mg N per 100 g for filleted trout samples at the time of sensory rejection. Based on the above, it could be concluded that chitosan film prevented the deterioration of trout fillets, as compared to raw fish (Fresh), cooked and HPP samples. In fact, at the end of storage high pressure treatment samples showed 18.90 mg N per 100 g, while TVBN value in HPP treatment samples covered by chitosan film was 15.4 mg N per 100g. Souza et al. (2010) also found the protective effect of chitosan coated samples compared to control in the increase of TVBN. These differences were attributed to the antimicrobial activity of chitosan, reducing the

bacterial population and consequently their capacity for oxidative deamination. Günlu et al. (2014) described a significant synergistic effect in the use of HPP and chitosan films, not observed in our results. Nevertheless, they used a higher storage time (44 days).

3.2.2.8. Colour

Table 10 shows the evolution of colour parameters along 22 days of storage.

Table 10: Colour parameters of rainbow trout fillets subjected to different processing treatment and stored at 4ºC for 22 days.

Treatment/Day	0	6	9	15	22
Fresh	L*35.61±0.47	L*38.32±4.27	L*37.60±4.71	L*37.19±5.90	L*36.46±3.28
	a*10.38±0.66	a*12.72±4.17	a*10.44±3.33	a*15.95±2.47	a*12.02±2.45
	b* 9.58±1.70	b*14.30±3.45	b*11.73±3.04	b*19.18±3.18	b*11.44±2.45
Film	L*32.15±2.29	L*35.3±3.01	L*36.92±6.63	L*33.36±2.75	L*36.77±2.12
	a*11.47±0.76	a*13.54±3.52	a*12.97±5.4	a*17.50±1.57	a*10.97±2.29
	b* 9.43±0.62	b*13.18±3.84	b*13.39±2.45	b*18.67±4.11	b*13.41±3.05
Clove	L* 42.02±4.17	L* 43.96±2.31	L* 42.60±4.86	L* 39.80±5.33	L*48.57±2.74
	a* 8.81±1.06	a* 13.97±3.71	a*11.39±1.67	a*11.27±2.37	a*9.52±0.69
	b*12.70±6.11	b*17.27±3.96	b*10.54±1.04	b*11.36±1.13	b*11.27±3.07
Cook	L* 62.95±3.58	L* 60.60±3.53	L*61.27±5.67	L*60.56±5.49	L*61.47±5.86
	a*14.61±2.92	a*6.53±1.55	a*6.89±4.12	a*6..71±0.68	a*8.54±0.77
	b*16.75±3.37	b*12.03±3.02	b*13.36±6.8	b*8.08±0.30	b*14.19±2.63
HPP	L*52.99±4.64	L*58.4±2.06	L*56.38±1.94	L*54.76±3.63	L*56.441.67
	a*15.69±1.51	a*15.59±0.51	a*14.02±4..36	a*10.46±3.34	a*11.77±1.80
	b*14.09±3.09	b*12.89±1.22	b*15.05±3.57	b*12.51±4.45	b*11.64±1.57
HF	L*53.46±1.90	L*54.07±2.73	L*54.43±2.87	L*52.25±2.07	L*50.48±5.05
	a*10.96±1..64	a*9.03±2.60	a*10.21±3.66	a*12.71±2.99	a*10.19±2.90
	b*16.28±2.59	b*14.42±2.88	b*15.66±3.22	b*14.19±2.83	b*12.96±1.78
HC	L*53.43±1.03	L*54.07±2.73	L*55.10±2.77	L*52.25±2.07	L*50.48±5.05
	a*10.96±1..64	a*9.03±2.60	a*10.21±3.66	a*12.71±2.99	a*10.19±2.90
	b*16.28±2.59	b*14.42±2.88	b*15.66±3.22	b*14.19±2.83	b*12.96±1.78

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same column are significantly different ($p<0.05$).
Fresh: fresh fish, Film: Chitosan Film, Clove: Chitosan Film with Clove Essential Oil, Cook: Cooked treatment, HPP: High Pressure Processing, HF: HPP + Chitosan Film, HC: HPP + Clove Film.

Lightness (L*) significantly increased in cooked and HPP treated samples. This increment as a result of cooking is well documented (Ojagh et al., 2011; Picouet et al., 2011; Rodriguez-Turienzo et al., 2011). HPP also induced an increase in the opacity of fish muscle with a loss of its raw appearance (Gómez-Estaca et al., 2009; Yagiz et al., 2007; Ojagh et al., 2011). Matser, Stegeman, Kals, and Bartels (2000) reported lightness (L*) increment at pressures beyond 150 MPa. Films did not exert a protective role in changes produced by HPP. Chitosan film (Film) did not induce changes compared to uncoated (Fresh) samples in L* parameter over storage.

Cooked samples presented the most pronounced reduction in redness (a*). These results were in agreement with Ojagh et al. (2011). The reduction in a* can be explained by the thermal degradation of carotenoids (mainly astaxanthin and canthaxantin). On the other hand, carotenoids are bound to some myofibrillar proteins and thus, the degree of protein denaturation may also influence colour changes (Rodriguez-Turienzo et al., 2011). No significant changes were found between HPP and film-treated samples (Film, Clove, HF and HC). Similarly, yellowness (b*) did not suffer modifications with the different treatments.

3.2.2.9 Sensory analysis

Sensory analysis is the best available method to understand changes caused by lipid oxidation which are perceived by humans. **Figures 4** , **5** and **6** show the mean values for the sensory characteristics of trout fillets (fishy odour, strange odour, drip loss) at 0, 6, 9, 15 and 22 days of chilled storage at 4 ºC. Sensory analysis was intended to determinate differences between treated samples and untreated (control) fish over storage.

Fishy odour gradually increased in all samples (**Figure 4**), as the storage progressed until day 15. From this point, a decrease took place. Among treatments, fishy odour at the end of storage was qualified as rancid in samples submitted to cooking (cook). In fact, these samples also had the highest hexanal values. Volatile organic compounds are responsible for the overall aroma of fish products. Some of them contribute to positive sensory attributes but other produce "off-flavours". In relation to HPP, lipid rancid odour has been hardly detected. Although TBARS values in HPP samples exceeded the organoleptic detectable thresholds of off-flavour in muscle foods from day 15 (Gray, Gomaa & Buckley, 1996). The combination of films and HPP did not contribute to improve the fishy odour.

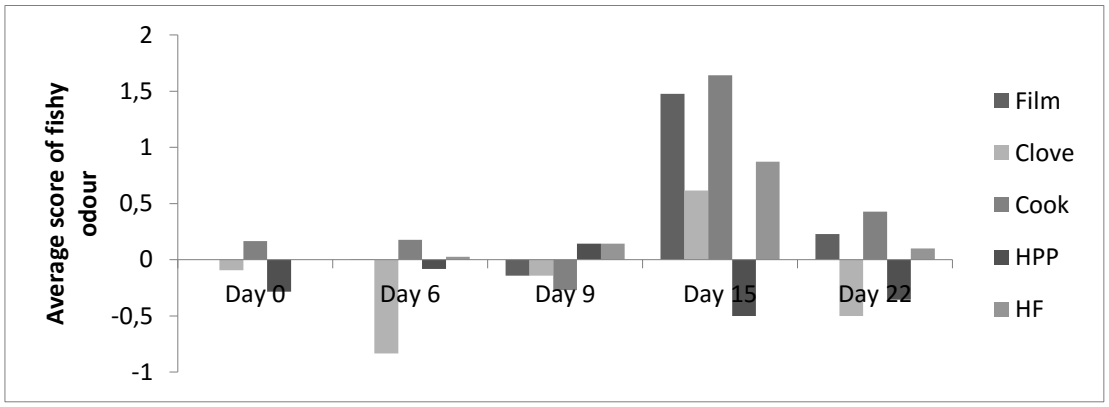

Figure 4: Average score of fishy odour in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days compared to untreated trout (Fresh) which correspond score of 0.

Results of off-odour (**Figure 5**) showed that a strange odour was detected by the panellists in samples covered with CEO (Clove). Other essential oils such as thymol, applied as coating, have also a strong odour and they did not produce unfavourable changes according to sensory analysis (Chamanara, Shabsnpour, Gorgin & Khomeiri, 2012). These authors used a different application method (coating instead of film) and half the essential oil concentration in the films as in our work. Nevertheless, chitosan

films made no unfavourable change in odour. Odour was described as herbal by the sensory panel in the present work

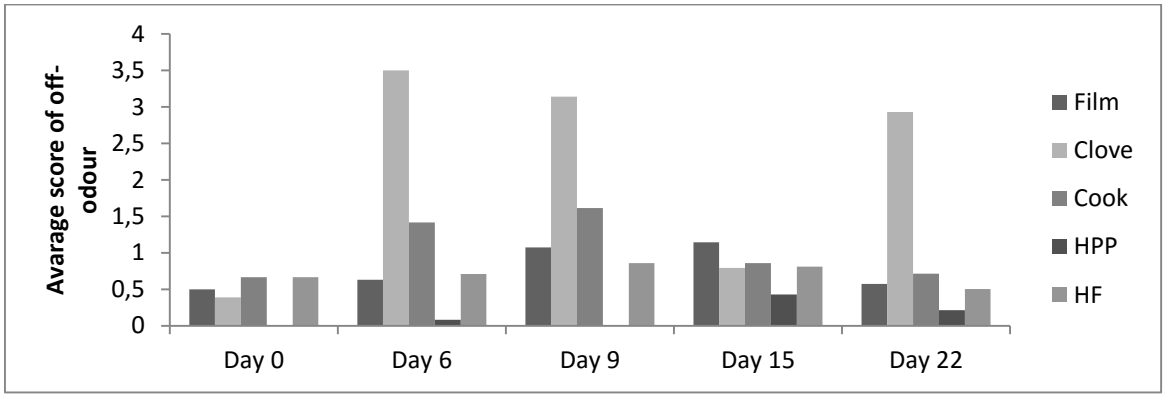

Figure 5: Average score of off-odour in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days compared to untreated trout (Fresh) which correspond score of 0.

Panellists perceived significantly more drip loss in cook samples when compared with the rest of treatments (**Figure 6**) and increased over storage. HPP also had effect on drip loss at lesser extent to cooking. The use of films (with or without CEO) protected from drip loss probably through the water absorption ability of the films.

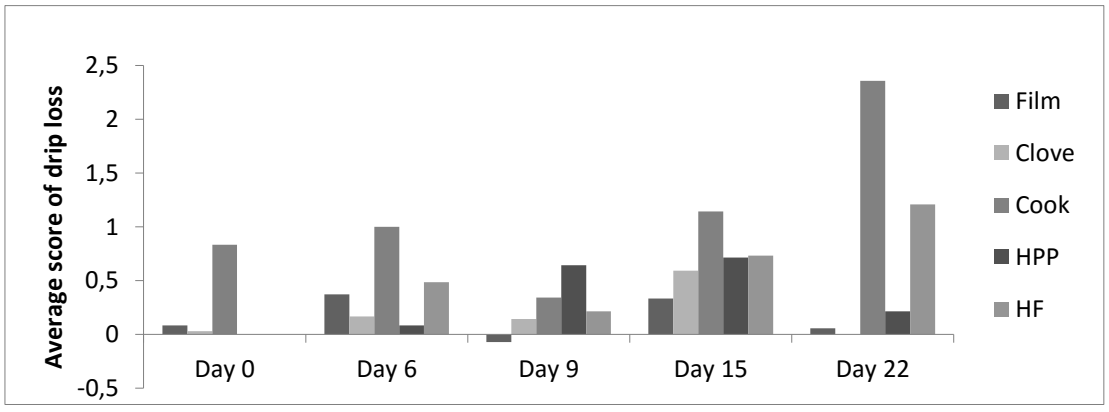

Figure 6: Average score of drip loss in rainbow trout fillets subjected to different processing treatments and stored at 4 ºC for 22 days compared to untreated trout (Fresh) which corresponds to 0.

Concerning to colour, sensory analyses (data not shown) were in accordance with instrumental measurement. Cooking treatment caused the most negative changes among

samples. Samples submitted to cooking presented white areas typical of cooked appearance. In fact, some panellist unequivocally identified cooked samples. Over 15 days of storage, whiteness turned into yellowness according to panellist. Cooked samples presented a porous surface, with some cracks on their surface; these surface defects increased over storage. HPP also caused colour changes, to a lesser extent than cooking treatment. A pink appearance was induced by the HPP treatment. As storage time increased, brown spots appeared at the edge of the HPP samples. The use of chitosan films (Film) did not adversely affect the colour. Regarding sensory analysis results, chitosan films appear more suitable to increase the shelf life of refrigerated trout.

4.CONCLUSIONS

The first part of this work determined the most appropriate concentration of clove oil as 20 g per kg for its formulation into chitosan films. This film showed *in vitro* antimicrobial activity on nine of the ten studied microorganisms, microorganisms selected as representative for fish spoilage bacteria. On the other hand, phenolic compounds and antioxidant capacity of films increased with increasing clove oil concentration, up to 20 g per kg.

The use of chitosan films (with and without clove oil) reduced the drip loss and water activity of trout fillets, when compared to fresh, cooked and high pressure treated (300 MPa, 10 min) samples. Film application was comparable from a microbiological point of view to cooking and better than high pressure treatment. A clear trend in the data suggested a synergistic effect of films and high pressure treatment in inhibiting mesophilic aerobic, lactic acid and coliform bacteria.

Both, HPP and cooking treatments induced lipid oxidation and changes in organoleptic markers (colour parameters and sensory properties). Nevertheless, they were effective

reducing TVBN compared to untreated trout. The combination of HPP and films would be a promising technology to reduce the negative effects of HPP on lipid oxidation and sensory parameters. The inclusion of clove essential oil significantly reduced the lipid oxidation, according TBARS measurements compared to chitosan films, without modification of colour. However, clove oil caused strange odour in the fish.

In conclusion, the use of films on trout fillets, alone or in combination with high pressure processing, shows to be a suitable non-thermal treatment for trout fillets, while maintaining a similar shelf life than that obtained with thermally treated methods.

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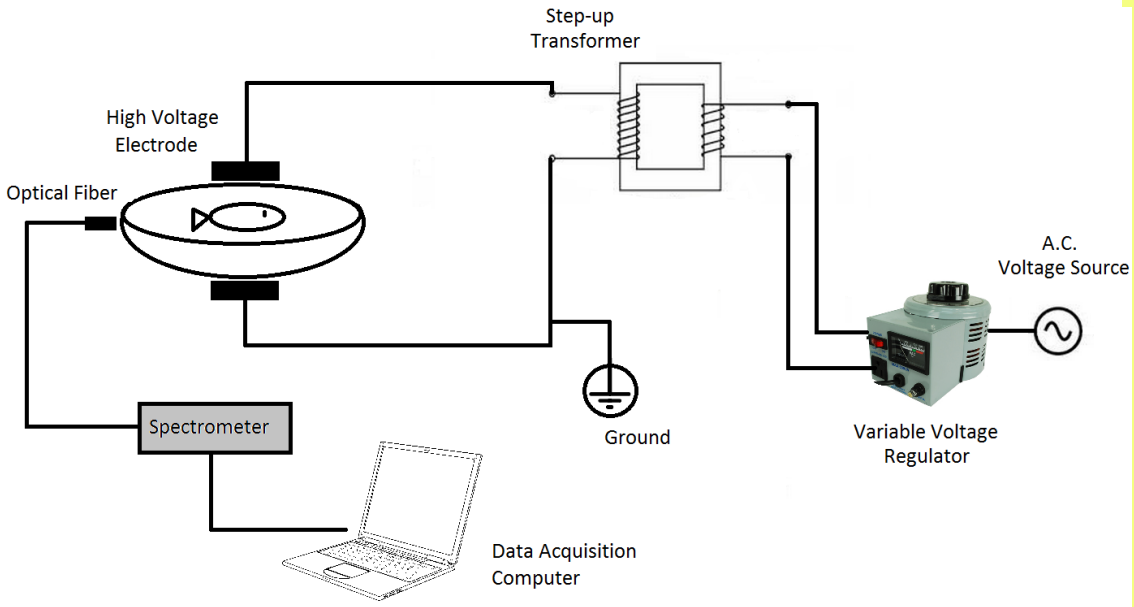
Chapter 5

Effects of atmospheric cold plasma treatment on microbiological and physicochemical characteristics of oily fish fillets.



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Agriculture and Food Development Authority

Effects of atmospheric cold plasma treatment on microbiological and physicochemical characteristics of oily fish fillets.

ABSTRACT

This study investigates the immediate (24 h) and over storage (11 days) effects of atmospheric cold plasma (ACP) on microbiological and physicochemical characteristics of pelagic oily fish. Firstly, the effect of ACP with variable voltage (70 kV and 80 kV) and treatment time (1, 3 and 5 min) on mackerel fillets after 24 h was reported. Secondly, Atlantic herring was treated with ACP at 70 kV and 80 kV for 5 min and studied over chilled storage (11 days).

Within 24 h after ACP treatment, spoilage bacteria (Total aerobic psychrotrophic, *Pseudomonas* and LAB) of mackerel fillets were significantly reduced. Significant increases in lipid oxidation parameters (PV, dienes) were observed in ACP-treated samples. Both treatment intensity (voltage) and time had great effects on microbiological characteristics and lipid oxidation. Nevertheless, no changes in pH and colour (with the exception of L*) were recorded as a function of ACP. These results suggest that ACP could be employed as a means of enhancing shelf life of fish. However, ACP increased lipid oxidation and further investigation is needed in order to explore posssible technology limitations or other noticeable negative effects over product shelf life.

Regarding the observable effects of ACP treatment over fish shelf life, results showed that microbial load (Total aerobic mesophilic, total aerobic psychrotrophics, *Pseudomonas*, LAB and *Enterobacteriaceae*) was lower in treated than in control samples over storage. Of the two voltage used (treatment time was set at 5 min), 80 kV was more effective than 70 kV, resulting in significantly lower microbial counts,

although other quality markers (TBARS, colour) indicated 70 kV should be employed instead undesired changes over storage.

Keywords: Atmospheric pressure cold plasma; Atlantic mackerel; Atlantic herring; shelf life; lipid oxidation.

1.INTRODUCTION

Atlantic mackerel (*Scomber scombrus*) and Atlantic herring (*Clupea harengus*) are the most important pelagic fisheries in Europe, containing high levels of long chain polyunsaturated fatty acids (PUFA). Despite the health benefits of PUFA, these species are underutilised due to their highly susceptibility to oxidation, which is directly related to the production of off-flavours and off-odours (Harris & Tall, 1994). The activity of microorganisms is the main factor limiting the shelf life in fresh fish (Olafsdottir et al., 1997). Great efforts have been made to enhance shelf life in fresh Atlantic mackerel and herring. For this purpose technologies such as freezing (Aubourg, Rodríguez, & Gallardo, 2005; Aubourg, Stodolnik, Stawicka, & Szczepanik, 2006; Hamre, Lie, & Sandness, 2003), packaging (Randell, Hattula, & Ahvenainen, 1997; Özogul, Taylor, Quantick, & Özogul, 2000; Lyhs, Lahtinen, & Schelvis-Smit, 2007), high pressure (Senturk & Alpas, 2013; Karim et al., 2011) or the combination of these technologies (Torres, Vázquez, Saraiva, Gallardo, & Aubourg, 2013) have been used.

Atmospheric cold plasma (ACP) is an emerging technology which has recently received attention for decontamination of foods. Cold plasma at atmospheric pressure often is characterized by a net gas temperature close to ambient and a thermodynamic disequilibrium between the temperature of neutral and electron species (Misra, Keener, Bourke, Mosnier, & Cullen, 2014). One of the most efficient method for producing the ozone is through dielectric barrier discharges (DBD) (Misra, Kaur, Tiwari, Kaur, Singh,

& Cullen, 2015). Previous studies have demonstrated the application of DBD to generate atmospheric cold plasma inside sealed packages filled with air with the application of sufficiently high voltages (Misra, Patil et al., 2014; Misra, Ziuzina, Cullen, & Keener, 2013; Pankaj, Misra, & Cullen, 2013). ACP is generated when high voltage is applied across the electrodes; this discharge generates energetic electrons that dissociate oxygen molecules by direct impact. This single O atom combines with oxygen molecules (O_2) to form ozone gas (Misra et al., 2014)

The effectiveness of ACP in meat inoculated with pathogenic bacteria has been demonstrated (Noriega, Shama, Laca, Díaz, & Kong, 2011; Kim, Yun, Jung, Jung, Choe, & Jo, 2011; Rød, Hansen, Leipold, & Knøchel, 2012; Kim, Yong, Park, Choe, & Jo, 2013), whereas the application of ACP for controlling spoilage bacteria growth has received little attention. Nevertheless, the major cause of fish spoilage is microbial growth and metabolism resulting in the formation of amines, sulphides, alcohols, ketones and organic acids with unpleasant and unacceptable off-flavours (Gram & Dalgaard, 2002). Gram and Dalgaard (2002) defined "specific spoilage microorganisms (SSO)" as the fraction of the total microflora that causes fish spoilage. On chilled fish, psychrotolerant Gram-negative bacteria (*Pseudomonas* spp and *Shewanella* spp) and lactic acid bacteria (LAB) were predominant. Thus, the quantification of these microorganisms is important in order to determinate the shelf life of these products.

The suitability of ACP for high lipid containing food is doubted. These products are susceptibility to oxidation due to the formation of hydroxyl acids, keto acids, short-chain acids and aldehydes (Misra, Tiwari, Raghavarao, & Cullen, 2011). The effect of ACP on food quality in meat products has not been studied extensively. Further studies should be conducted to assess the potential use of ACP in these types of products. Kim

et al. (2011) did not find significant changes (pH, TBARS, microscopic observation) except for colour, mainly L* value increase, when working on bacon. Rød et al. (2012) observed that TBARS values of all plasma-treated samples increased after treatment as well as over storage time, but plasma did not exert influence on colour. Kim et al. (2013) found lower TBARS values at day 0 in ACP-treated bacon, as compared to untreated samples, but then TBARS increased as storage progressed. Also, significant reductions in sensory quality parameters were observed in ACP-treated samples.

In summary, the potential use of ACP on fish products is here reported for the first time, as far as the authors are aware. Although, ACP has been widely evaluated to pathogenic microorganism reduction; further studies would be desirable to know the effect of ACP technology on product microbiological and physicochemical characteristics.

OBJECTIVES

Therefore, the objectives of this study were to:

(I) Investigate the effects of different conditions (voltages and times) of ACP treatment on the fish spoilage bacteria reduction, lipid oxidation and physicochemical characteristics of Atlantic mackerel fillets.

(II) Characterize the microbiological and physicochemical changes and lipid oxidation of herring during chilled storage for 11 days at 4 ºC after ACP treatment.

2. MATERIAL AND METHODS

2.1. Chemicals

All the chemicals were analytical grade obtained from Sigma-Aldrich (Wicklow, Ireland). Culture media were supplied by Oxoid (Basingstoke, UK).

2.2. Product characteristics

Six kilos of unskinned fillets of Atlantic mackerel (*Scomber scombrus*) and ten kilos of Atlantic herring (*Clupea harengus*) were purchased in Stevie Connolly Seafood (Dublin, Ireland). Both species were caught in early and late February 2015, respectively.

2.3. In-package plasma treatment

Each fillet was packaged in commercial 270 µm-thick polyethylene terephthalate trays (150 mm x 70 mm x 35 mm) sealed with a high barrier 50 µm film. In brief, the package was placed between two circular aluminium plate electrodes (outer diameter=158 mm) over polypropylene (PP) dielectric layers (2 mm thickness), as shown in Figure 1. The system was set-up to a voltage transformer (Phoenix Technologies, Inc, USA). More detailed description of experimental plasma system was made by Misra et al. (2014). Treatments were done in duplicate.

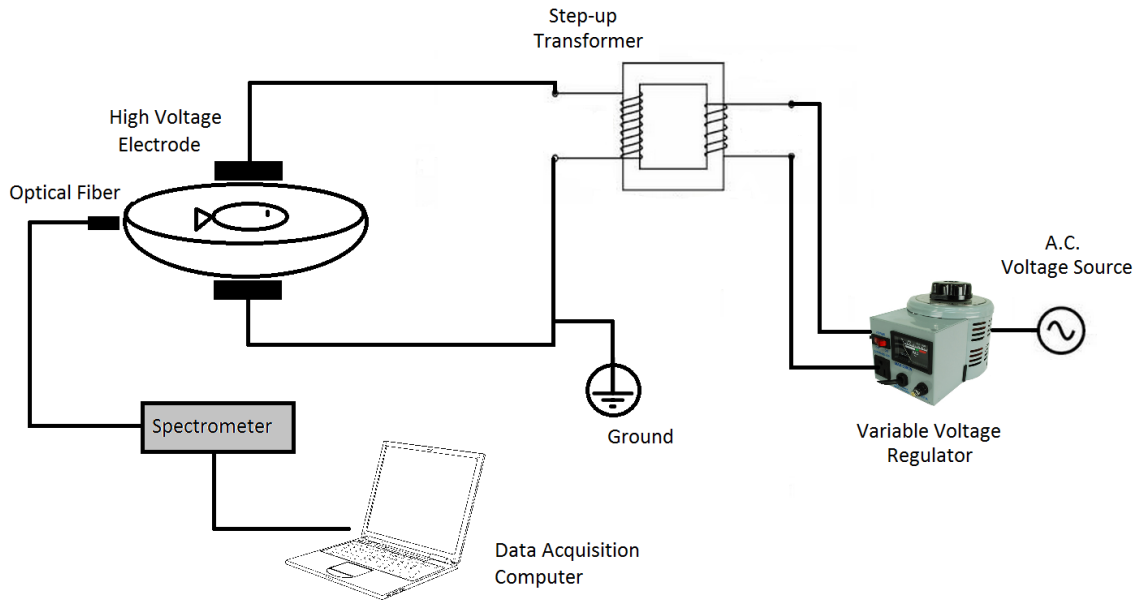

Figure 1: Schematic of the experimental set-up for indirect ACP plasma treatments of Atlantic mackerel fillets. The cold plasma is generated inside the tray. The fillets are placed outside the inter-electrode space.

For the first assay, two discrete voltages of 70 and 80 kV were applied across the electrodes for 1, 3 and 5 min. In the second assay, the previously selected conditions were used: voltages of 70 and 80 kV for 5 min.

In both experiments, all treatments were stored at 4 ºC. In the first assay, Atlantic mackerel fillets were analysed after 24 days of storage, whereas Atlantic herring fillets were analysed on days 1, 3, 6, 9 and 11 of storage. The complete assay was duplicated and all analyses were performed in triplicate.

2.4 Effects of atmospheric pressure cold plasma treatment on microbiological and physicochemical characteristics of Atlantic mackerel (*Scomber scombrus*) fillets

2.4.1 Microbiological analysis

Fish samples (10 g) were aseptically transferred into bags (Seward 80 bags, United Kingdom) with 90 mL of sterile maximum recovery diluent (MRD) and homogenised with a Stomacher blender for 5 min (Seward, London, UK). For each sample, appropriate serial decimal dilutions were prepared in MRD for the following microorganism counts:

(i) Total aerobic mesophilic were determined using Tryptic Glucose Yeast Agar (PCA) with 1% NaCl after incubation at 30 ºC for 72 h.

(ii) Total psychrotrophic bacteria on 1% NaCl PCA spread plates, incubated at 15 ºC for 72 h.

(iii) Lactic acid bacteria on double-layer Man Rogosa Sharpe medium incubated at 30 ºC for 72 h.

(iv) *Pseudomonas* on spread plates of Pseudomonas Agar Base with added CFC (Cetrimide, Fucidine, Cephalosporine) supplement for *Pseudomonas* spp. incubated at 25 °C for 48 h.

2.4.2 pH

The pH of fillets was measured at room temperature using a portable pH meter (Orion Research Inc., Boston, MA 02129, USA).

2.4.3 Moisture and total lipid content of Atlantic mackerel

Moisture content was gravimetrically determined according to AOAC (1995). Total lipids were extracted from 10 g samples with methanol/chloroform (1:1, v:v) according to the Bligh and Dyer method (1959).

2.4.4 Lipid oxidation

Peroxide Value (PV)

PV was measured directly on the Bligh & Dyer extract according to the method described by the International IDF Standards (1991). Results were expressed in milliequivalents of O_2 per kilogram of oil.

Conjugated hydroperoxides (Dienes)

Conjugated hydroperoxides were measured on the Bligh & Dyer extract dissolved in hexane, as described by Undeland, Stading and Lingnert (1998). The absorbance was measured at 268 nm and results were calculated as mmoles of hydroperoxides per kilogram of oil.

Thiobarbituric acid reactives substances (TBARS)

Samples were analysed using the methodology described by Vyncke (1975) on a 5% trichloracetic acid extract of the fish muscle. Results were expressed as mg of malondialdehyde (MDA) per kilogram of fish.

2.4.5 Colour

The colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured using a colorimeter (Colour Quest XE Hunter Lab, Northants, UK). The illuminant was D65 (colour temperature of 6504 K) and the standard observer was 10º. The colorimeter was standardised using a light trap and a white calibration plate. Measurements were taken on the samples packaged in transparent plastic bags at three different points.

2.5 Microbiological and physicochemical changes of herring (*Clupea harengus*) during chilled storage for 11 days at 4ºC after cold plasma treatment

2.5.1 Microbiological analysis

Analyses were performed as described in 2.4.1. In addition, *Enterobacteriaceae* were determined on double-layer Violet Red Bile Glucose (VRBG) incubated at 37 ºC for 24 h.

2.5.2 Physicochemical parameters

The pH of fillets, TBARS and colour were measured as described in 2.4.2, 2.4.4 and 2.4.5, respectively.

2.6 *Statistical analysis*

The data for the first assay (Atlantic mackerel) were subjected to One-way ANOVA. Data of Atlantic herring fillets over storage were analysed by multifactor ANOVA of each variable, taking into account treatment and time. Fisher LSD (Least Significant Difference) test was applied for determining group differences at 95% confidence level. Statgraphics Centurion XVI was used for carrying out the statistical analysis.

3. RESULTS

3.1 Effects of atmospheric pressure cold plasma treatment on microbiological and physicochemical characteristics of Atlantic mackerel (*Scomber scombrus*) fillets

3.1.1 Microbiological analyses

Figure 2 shows the changes in the microbial flora of Atlantic mackerel submitted to ACP. The initial total aerobic mesophilic bacteria (control) were 4.1±0.07 log CFU per g. The initial bacterial concentration ranged between 3.0 and 5.0 log CFU per g on filleted fish obtained from retail establishment (Dalgaard, Gram & Huss, 1993; Chytiri, Paleologos, Savvaidis & Kontominas, 2004). There was not significant (P>0.05) reduction by ACP on total aerobic mesophilic bacteria. On the other hand, psychrotrophic bacteria, LAB and *Pseudomonas* counts were significantly (P<0.05) reduced due to ACP.

Total aerobic psychrotrophic bacteria incubated at 15ºC were higher in Atlantic mackerel compared to total aerobic mesophilic bacteria. Similarly, Nuñez-Flores, Castro, López-Caballero, Montero & Gómez-Guillén (2013) showed differences in total microorganism counts depending on incubation temperature related to the nature of psychrotrophic microbiota predominant in fish. The reduction of total aerobic

psychrotrophic bacteria was more dependent on plasma exposure time than on voltage applied. In any case, the most effective reduction was achieved using the highest voltage and time (80 kV, 5 min).

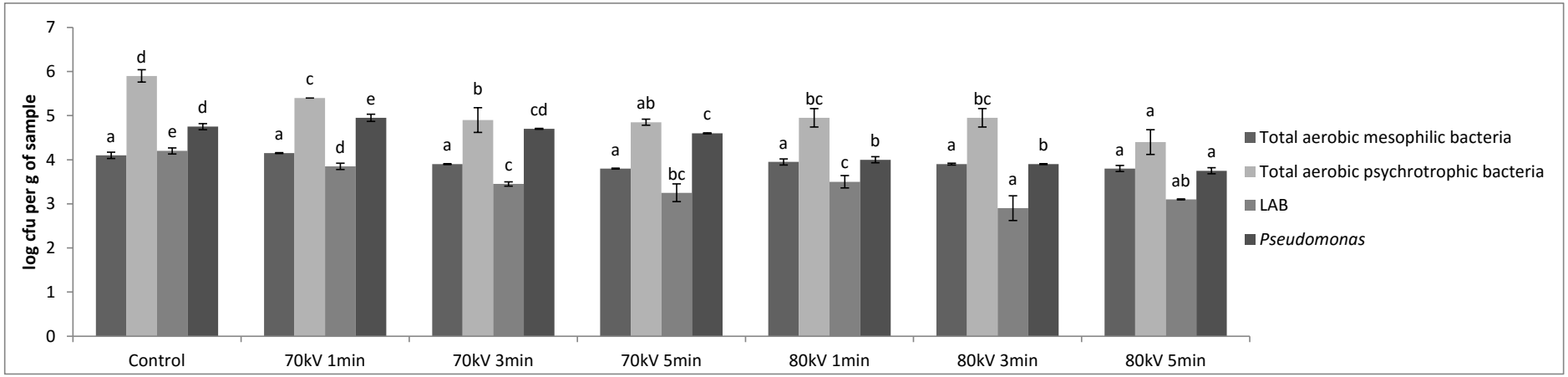

Figure 2: Total aerobic mesophilic bacteria, psychrotrophic bacteria, LAB and *Pseudomonas* (log cfu per g of samples) counts in Atlantic mackerel subjected to different ACP treatments. Different letters have values that are significantly different ($p<0.05$) due to treatment effect, for the same group of microorganisms.

LAB counts were lower than *Pseudomonas* counts. LAB were more dominant after some days of storage. This fact can be explained due to the inhibition of other bacteria by the formation of lactic acid and bacteriocins (Gram & Dalgaard, 2002). Besides, LAB can grow under both anaerobic and aerobic conditions, as in our study occurred. Other authors found *Pseudomonas* the dominant microflora in filleted chilled fish (Chytiri et al., 2004). Both microorganisms (LAB and *Pseudomonas*) were more significantly reduced depending on the voltage applied than the exposure time. Kim et al. (2011) studied the effect of atmospheric pressure plasma on inactivation of pathogens (*L. monocytogenes*, *E. coli* and *S. typhimurium*). Concerning LAB and *Pseudomonas*, the previous work along with the present study, demonstrated that plasma treatment at high voltage was effective for reducing fish spoilage microorganism over short time periods.

In this work, input power and exposure time were the main factors in microorganism reductions. ACP generate reactive species (ROS) such as atomic oxygen (O) and hydroxyl radicals (OH) and they can react with almost all bacteria cell resulting in damage to DNA proteins, lipids and membranes (Kim et al., 2011; Kim et al., 2013).

3.1.2 pH

The pH values were similar than other found in the literature in Atlantic mackerel (Senturk & Alpas, 2013). Atlantic mackerel did not show any clear trend in pH behaviour after plasma exposure (**Figure 3**).

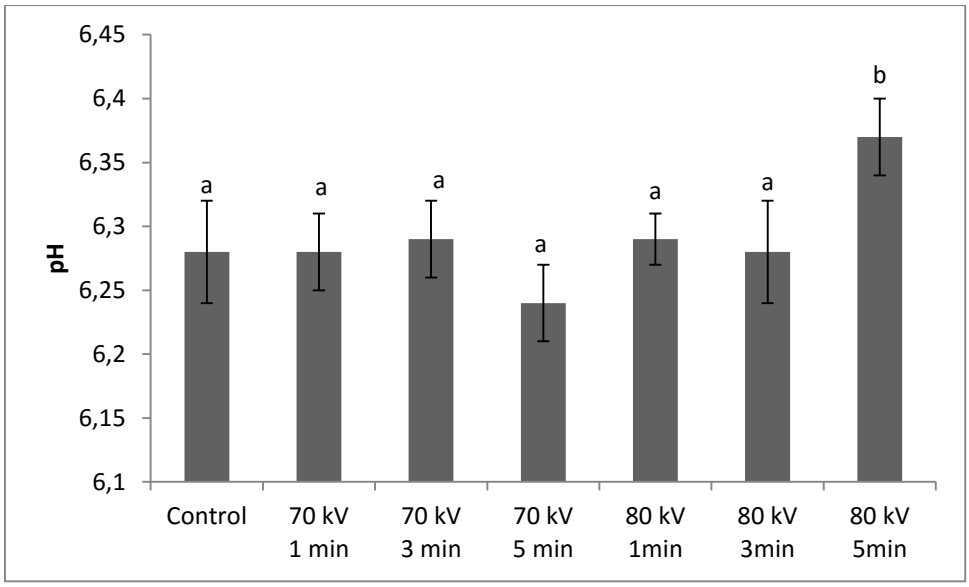

Figure 3: pH of Atlantic mackerel fillets submitted different ACP treatments.

Values (mean ± standard deviation. n=3) with different lowercase letter are significantly different (p<0.05).

There were no differences on the pH levels of Atlantic mackerel after ACP treatments with the exception of 80 kV at 5 min. These results were consistent with Kim et al. (2011) and Ulbin-Figlewicz, Brychcy and Jarmoluk (2013), where cold plasma did not induce pH changes.

The pH values of the samples submitted to 80 kV at 5 min were clearly the highest. Furthermore, there was not apparent relationship between pH and bacterial growth. As occurred in this case, other authors have also previously reported pH not as a good

indicator of microbial growth and fish freshness (Alfaro, Hernández, Baliño-Zuazo, & Barranco, 2012). Nevertheless, Senturk and Alpas (2013) specified that fresh Atlantic mackerel with pH higher than 7.00 was considered to be spoiled. All treatments tested maintained samples under this critical limit.

3.1.3 Moisture and total lipid content of Atlantic mackerel

Moisture and fat content of Atlantic mackerel were found to be in the range of 69.91-68.11 and 14.14-14.9 g 100 g^{-1}, respectively. Both results agreed with previous studies on Atlantic mackerel (Torres, Vázquez, Saraiva, Gallardo, & Aubourg, 2013; Aubourg, Rodríguez, & Gallardo, 2005). Fat and moisture content did not change among treatments.

3.1.4 Lipid oxidation

Primary oxidation was followed by PV and dienes assessment (**Table 1**).

Table 1: Lipid oxidation markers (Peroxide Value, Dienes) of Atlantic mackerel submitted to different ACP treatments.

	PV (mEq. active oxygen per kg lipids)	**Dienes** (mmol of hydroperoxides per kg lipid)
Control	6.89±0.00a	1.42±0.19a
70 kV 1min	8.97±0.74a	2.17±0.28ab
70 kV 3min	21.87±0.59b	2.37±0.12b
70 kV 5min	35.44±6.16c	2.78±0.55b
80 kV 1min	17.59±3.23b	1.46±0.36a
80 kV 3min	35.75±0.09c	2.25±0.30b
80 kV 5min	37.57±2.49c	5.56±1.33c

Values (mean ± standard deviation. n=3) followed by different lowercase letters in same column are significantly different (p<0.05).

Control samples had 6.89 milliequivalents of O_2 per kilogram of oil. Ozogul and Balikei (2013) also reported similar initial PV value. A significant (P<0.05) primary oxidation (PV and Dienes) development was observed for ACP treatment. Comparison of different voltages (70 kV and 80 kV) and exposure time (1, 3 and 5 min) led to the conclusion that higher intensities and time significantly increased the oxidation. Similarly, Joshi et al. (2011) suggested that lipid oxidation is proportional to the amount of plasma energy applied. Van Durme, Nikiforov, Vandamme, Leys, & De Winne (2014) also revealed that cold plasma caused the formation of several volatiles related to lipid oxidation. ACP can generate reactive species that have strong oxidation capacity.

TBARS values ranged from 0.74±0.01 to 0.75±0.00 mg of malondialdehyde (MDA) per kilogram of fish**.** There were not significant differences (P>0.05) between control and samples submitted to ACP treatment. As secondary oxidation products, they

accumulated and, as expected, TBARS increased along all the storage time evaluated. Therefore, even if differences of TBARS were observed over storage (11 days), after 24 h they were not evident. The effect of ACP on lipid oxidation measured through TBARS values was not clarified. Whereas, Rød et al. (2012) and Kim et al. (2013) reported higher TBARS values of plasma treated samples than those of control on bresaloa and pork loin. Kim et al. (2011) found lower TBARS values of plasma-treated bacon compared to non-treated control at day 0. This trend reverted over storage; plasma treatment produced higher TBARS values than non-treated control.

3.1.5 Colour

Colour has a direct influence on acceptance of fish and influences the consumers´ decision to buy (Rodriguez-Turienzo, Cobos, Moreno, Caride, Vieites & Diaz, 2011). The impact of ACP on the colour of Atlantic mackerel is shown in **Table 2**.

Not a clear trend was found between any plasma treated samples depending on voltage or exposure time, similar to Rød et al. (2012). However, a significant decrease in L* (lightness) took place as a consequence of ACP compared to untreated (Control) samples. Kim et al. (2011, 2013) observed L* value decrease on bacon and pork loin treated with ACP. There were not differences between ACP treated samples and untreated (control) in a* and b* values. These results indicated that the ACP did not markedly influence the colour.

Table 2: Colour (L*, a* and b*) of Atlantic mackerel submitted to different ACP treatments.

	L*	a*	b*
Control	57.42±2.15d	3.67±0.63ab	14.12±2.75ab
70 kV 1min	53.72±2.98bc	4.48±0.33c	13.26±1.56ab
70 kV 3min	51.70±0.50ab	3.96±0.06bc	13.21±0.55ab
70 kV 5min	53.87±1.26bc	4.19±0.29bc	15.13±0.12b
80 kV 1min	55.37±0.86cd	3.19±0.35a	15.15±0.42b
80 kV 3min	50.65±0.93a	4.4±0.49c	13.44±0.15ab
80 kV 5min	55.91±0.47cd	3.97±0.28bc	11.52±2.32a

Values (mean ± standard deviation. n=3) followed by different lowercase letter in same column are significantly different (p<0.05).

3.2 Microbiological and physicochemical changes of herring (*Clupea harengus*) during chilled storage for 11 days at 4 ºC after cold plasma treatment

3.2.1 Microbiological analyses

Both ACP treatments (70 kV and 80 kV for 5 min) reduced the microbial population (Total aerobic mesophilic, total aerobic psychrotrophics, *Pseudomonas*, LAB and *Enterobacteriaceae*) of Atlantic herring fillets over storage. The antibacterial activity of ozone in fish has been previously reported by other author (Bono, & Badalucco, 2012; Campos, Rodríguez, Losada, Aubourg, & Barros-Velázquez, 2005; Gonçalves, 2009). Initially, total aerobic mesophilic (**Figure 4**) in all Atlantic herring fillets were 10^4 cfu per g. Similar values have also been reported by other authors in herring (Randell et al., 1997; Özogul et al., 2000; Lyhs et al., 2007). All treatments, with the exception of the highest voltage (80 kV for 5 min), had aerobic counts which exceeded the acceptable

limit (10^6cfu g^{-1}) after 6 of storage. After ACP treatment, there were only significant reduction between treated (70 kV and 80 kV) and untreated (Control) samples without differences depending on voltages. Nevertheless, higher voltage exerted a significant reduction in total aerobic mesophilic population on Atlantic herring fillets.

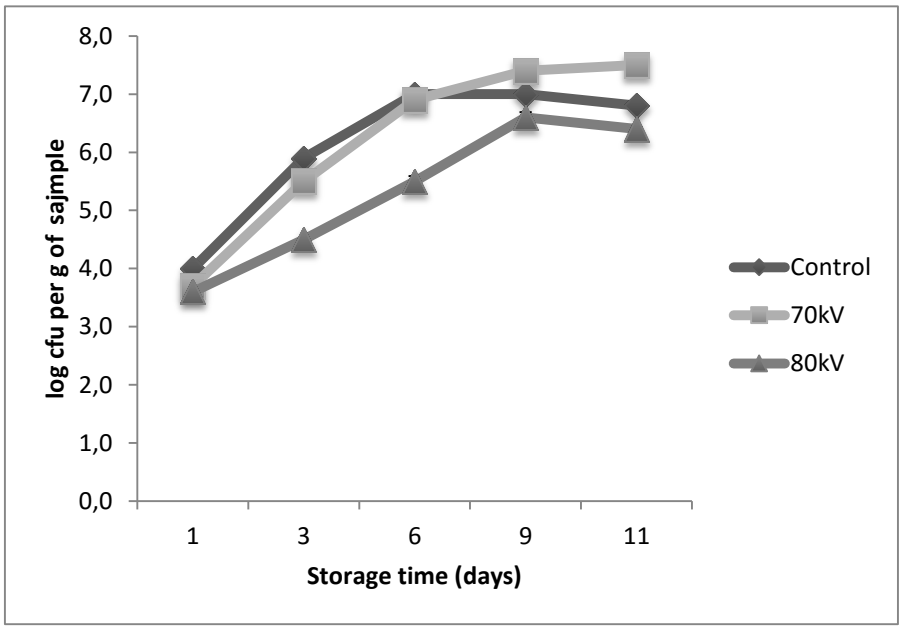

Figure 4: Total aerobic mesophilic (log cfu per g) counts in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

Total aerobic psychrotrophic (**Figures 4 & 5**) in Atlantic herring fillets showed higher counts than total aerobic mesophilic. Initial psychrotrophic counts (4.95±0.07 - 4.05±0.06 cfu per g) were consistent with Lyhs et al. (2007). These results suggested that psychrotrophic is the microflora predominant in the fish (Nuñez-Flores et al., 2013). In this sense, the psychrotrophic counts of all samples reached acceptable limit by day 6. From this day, only the highest voltage (80 kV for 5 min) was effective to maintaining the psychrotrophic counts within acceptable limits.

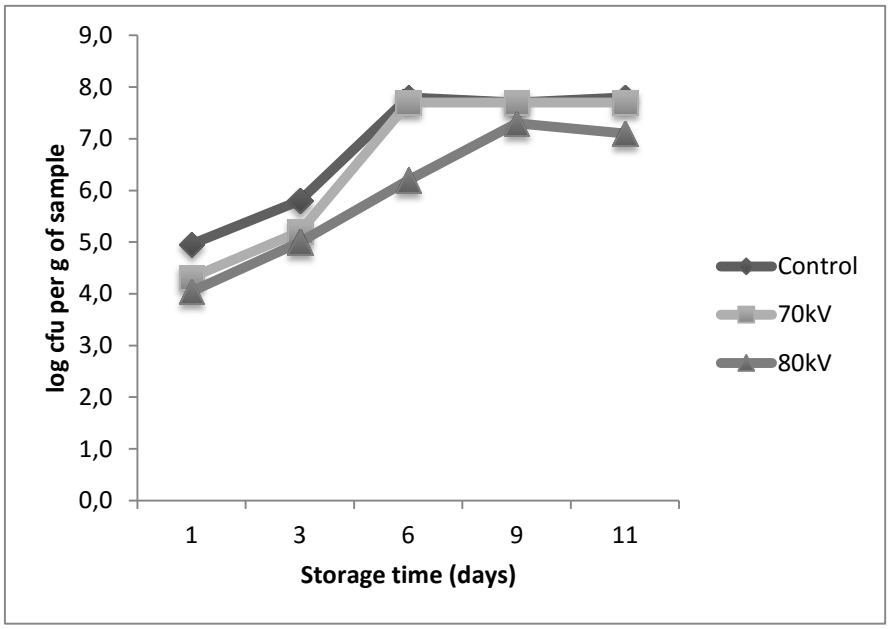

Figure 5: Total aerobic psychrotrophic (log cfu per g) counts in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

Pseudomonas were dominant in Atlantic herring fillets over storage (**Figure 6**). Initially, both ACP treatments (70 kV and 80 kV for 5 min) showed significant (P<0.05) effectiveness against *Pseudomonas*. This effect was dependent on the voltage used. Higher voltage (80 kV for 5 min) resulted in greater inactivation efficiency (at days 1, 3 and 6). As shown in Figure 6, *Pseudomonas* growth stopped from day 6. This fact can be explained because *Pseudomonas* are strictly aerobic microorganism and are unable to survive in the absence of oxygen (Mexis, Chouliara, & Kontominas, 2009).

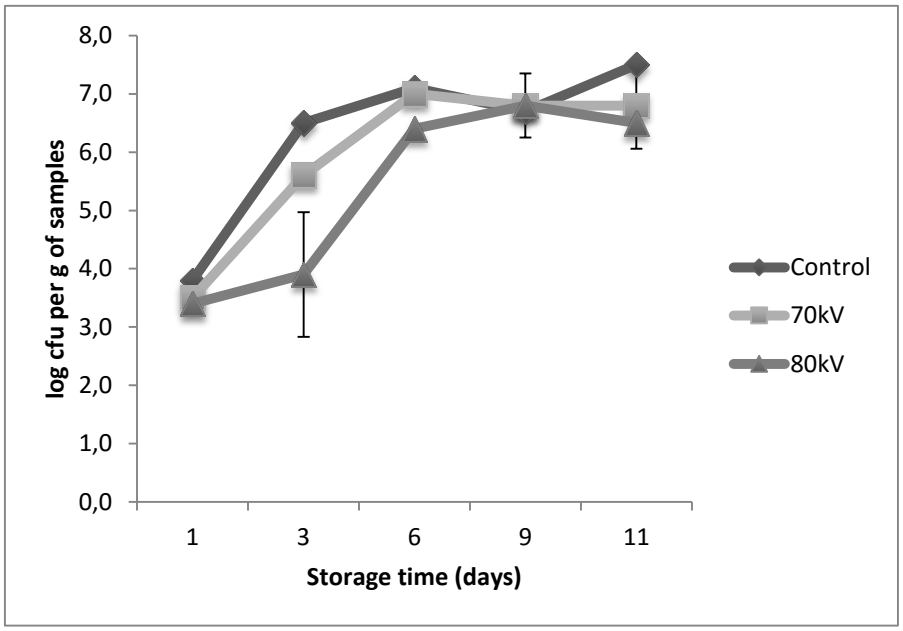

Figure 6: *Pseudomonas* (log cfu per g) counts in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

LAB were also part of the natural microflora of fresh Atlantic herring fillets **(Figure 7**). The initial counts were from 2.10±0.01 log cfu per g (80 kV for 5 min) to 3.80±0.71 log cfu per g (Control) on day 1, and reached counts from 5.10±0.02 log cfu per g (80 kV for 5 min) to 6.05±0.07 log cfu per g (Control) on day 11. These findings showed a reduction of LAB as a result of ACP. At the beginning of storage (days 1 and 3), only high voltage of ACP (80 kV for 5 min) was effective in the reduction of LAB. From day 6, treatment of 70 kV resulted in a reduction of LAB compared to the control. In fact, there were not differences in the voltage used at the end of storage.

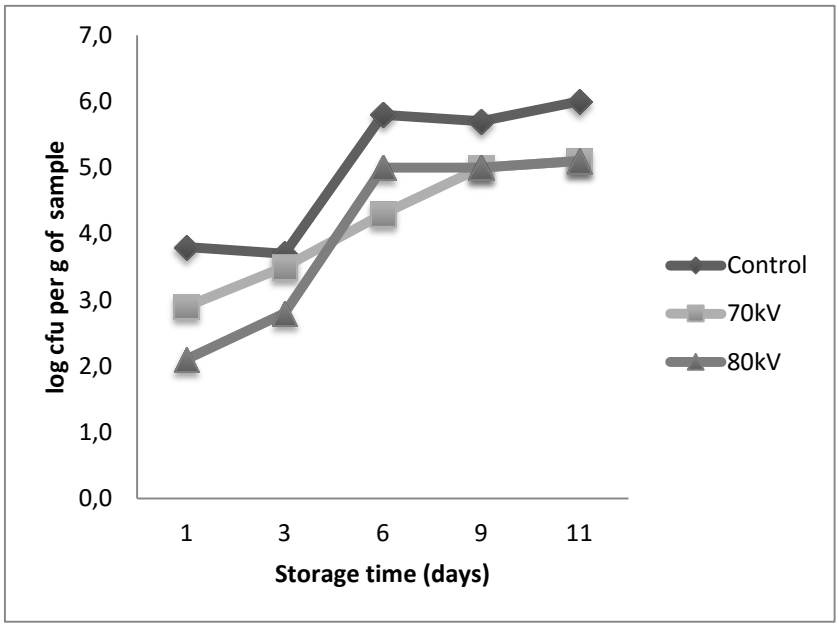

Figure 7: LAB (log cfu per g) counts in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

Enterobacteriaceae presence is a hygiene indicator. According to Lyhs, Lahtinen and Schelvis-Smit (2007), the maximun limits for aceptibility of unpacked herring would be 10 log cfu per g counts. Treated samples (70 kV, 80 kV) fullfilled with this requirement over storage (**Figure 8**). This bacterial group was the least dominant compared to the rest of the microorganisms studied. Similarly, Chytiri et al. (2004) reported that *Enterobacteriaceae* grew less than other Gram-negative psychrotrophic spoiling bacteria.

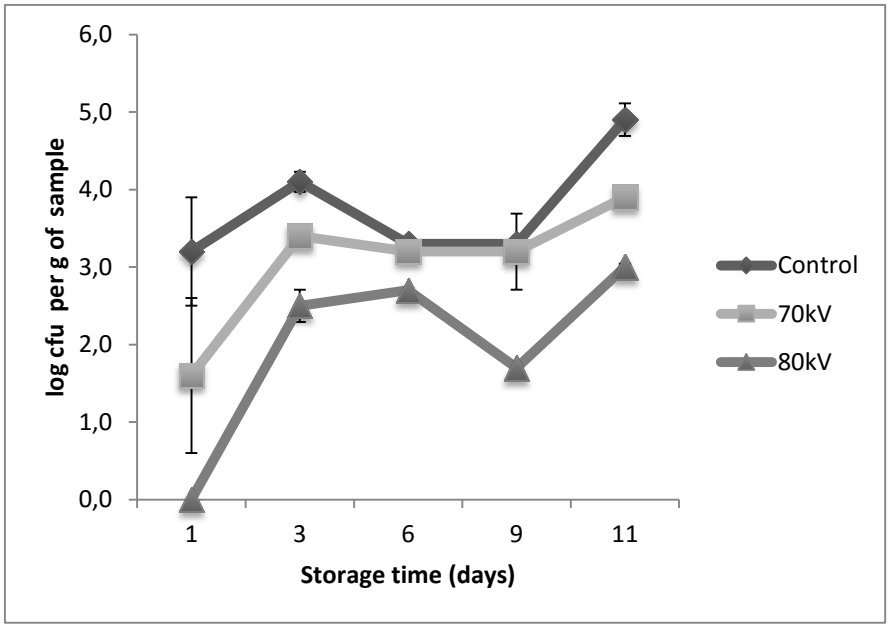

Figure 8: *Enterobacteriaceae* (log cfu per g) counts in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

3.2.2 pH

Changes on the pH levels of Atlantic herring fillets after ACP are shown in **Table 3**.

Table 3: pH in Atlantic herring fillets subjected to different processing treatments and stored at 4ºC for 11 days.

	Day 1	**Day 3**	**Day 6**	**Day 9**	**Day 11**
Control	$^{A}6.23\pm0.01_{c}$	$^{A}6.31\pm0.07_{b}$	$^{C}7.04\pm0.01_{c}$	$^{C}7.02\pm0.01_{b}$	$^{B}6.90\pm0.01_{a}$
70 kV 5min	$^{A}6.12\pm0.01_{a}$	$^{A}6.14\pm0.01_{a}$	$^{B}6.82\pm0.01_{b}$	$^{C}7.02\pm0.03_{b}$	$^{D}7.32\pm0.07_{c}$
80 kV 5min	$^{A}6.14\pm0.00_{b}$	$^{B}6.20\pm0.01_{ab}$	$^{C}6.73\pm0.03_{a}$	$^{D}6.90\pm0.01_{a}$	$^{E}7.16\pm0.01_{b}$

Values (mean ± standard deviation, n=3) followed by different uppercase letters in the same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by different lowercase letters in the same column are significantly different ($p<0.05$).

After ACP, a decrease in pH was observed. These results were consistent with Kim et al. (2013). Lower pH in samples submitted to ACP can be atributed to acidogenic molecules normally generated in air plasma.

Furthermore, pH values increased over storage up to day 9, whereas this rise occurred to a lesser extent in ACP treated samples, specially with the highest voltage (80 kV for 5 min). This increment in pH could be due to alkaline compounds that are formed from protein and nucleotide decomposition in the muscle during the post-mortem period (Mexis et al., 2009). Consequently, ACP may have exerted a protective effect, reducing protein decomposition. A storage-treatment interaction was observed, and at the end of storage, control samples showed the lowest pH values. pH values were also in agreement with LAB counts, which may have contributed to pH decrease due to lactic formation (Picouet, Cofan-Carbo, Vilaseca, Balbé, & Castells, 2011).

3.2.3 Thiobarbituric acid reactive substances (TBARS)

Atlantic herring is more susceptible to oxidation than most of other fish species (Hamre et al., 2003). In this sense, it is a suitable food matrix to study the effect of ACP on lipid oxidation. Several authors showing inconclusive results in lipid oxidation damage of plasma (Kim et al., 2011; Bae, et al., 2015; Jayasena et al., 2015) have highlighted the necessity of more studies to be conducted in order to clarify the effect of ACP on lipid oxidation of muscle foods. Possible pro-oxidant effect of ozone on fish constituents has not been sufficiently studied up to now (Gonçalves, 2009).

TBARS are related to secondary oxidation products; they accumulate and, as expected, increasing values were observed in all treatments over storage (**Table 4**).

Table 4: TBARS in Atlantic herring fillets subjected to different processing treatments and stored at 4 ºC for 11 days.

	Day 1	**Day 3**	**Day 6**	**Day 9**	**Day 11**
Control	$^{A}0.17\pm0.01_{a}$	$^{B}0.36\pm0.01_{a}$	$^{C}0.48\pm0.01_{a}$	$^{D}0.60\pm0.00_{a}$	$^{E}0.65\pm0.00_{a}$
70 kV 5min	$^{A}0.17\pm0.01_{a}$	$^{B}0.36\pm0.00_{a}$	$^{C}0.48\pm0.01_{a}$	$^{D}0.63\pm0.00_{b}$	$^{E}0.75\pm0.00_{b}$
80 kV 5min	$^{A}0.19\pm0.00_{b}$	$^{B}0.42\pm0.00_{b}$	$^{C}0.53\pm0.00_{b}$	$^{D}0.78\pm0.01_{c}$	$^{D}0.77\pm0.00_{c}$

Values (mean ± standard deviation, n=3) followed by different uppercase letter in same row not significantly different ($p<0.05$). Values (mean ± standard deviation, n=3) followed by different lowercase letter in same column, for each parameter, are significantly different ($p<0.05$).

Differences in TBARS values between control and 70 kV/5 min samples were not significant ($P>0.05$) until the end of storage, mainly day 9. Gray, Gomaa and Buckley (1996) established that TBARS values above 0.5 mg MDA kg^{-1} are detectable as off-flavour by sensory panels. Consequently, in 80 kV/5 min samples exceeded these values from day 6 of storage, and from day 9 in the rest of samples. Ozone applied as ozonated water to sanitising fish was described as a potent oxidizer (Crapo, Himelbloom, Vit, & Pedersen, 2004). Gonçalves (2009) reported that ozone is one of the more powerful antioxidants. This high chemical activity is attributted to a stable electronic configuration which impels to look for electrons from other molecules.

Therefore, ACP at 70 kV for 5 min could be employed as a preservation while technology retaining product quality.

3.2.4 Colour

Colour value changes in herring fillets treated with ACP plasma over storage are shown in **Table 5**. After 24 hours of ACP treatment, the luminosity of herring fillets was reduced. In this regard, several works (Kim et al., 2011; Kim et al., 2013) indicated L* values decrease with ACP plasma treatment. This drop of L* values of the herring fillets surface was proportional to the voltage applied. Samples submitted to ACP maintained steady levels of lightness over storage, different from control.

Table 5: Colour parameters: Lightness (L*), Redness (a*), Yellowness (b*) in Atlantic herring fillets subjected to different processing treatments and stored at 4ºC for 11 days.

L*	**Day 0**	**Day 3**	**Day 6**	**Day 9**	**Day 11**
Control	$^{BC}56.38\pm0.26_{c}$	$^{C}55.19\pm3.76_{a}$	$^{BC}54.92\pm0.94_{ab}$	$^{A}49.58\pm4.32_{a}$	$^{AB}52.41\pm1.66_{a}$
70 kV 5 min	$^{A}52.08\pm0.16_{b}$	$^{A}50.86\pm0.60_{a}$	$^{B}59.88\pm0.97_{b}$	$^{A}49.99\pm6.05_{a}$	$^{A}49.73\pm3.09_{a}$
80 kV 5 min	$^{A}50.21\pm0.02_{a}$	$^{A}51.21\pm2.03_{a}$	$^{A}50.10\pm6.17_{a}$	$^{B}58.41\pm0.94_{b}$	$^{A}51.74\pm0.59_{a}$
a*					
Control	$^{AB}3.05\pm0.05_{a}$	$^{A}0.75\pm0.14_{c}$	$^{AB}2.37\pm0.64$a	$^{AB}1.62\pm0.62_{a}$	$^{B}3.85\pm0.72_{a}$
70 kV 5 min	$^{BC}3.79\pm0.03_{b}$	$^{A}0.24\pm0.06_{b}$	$^{AB}1.49\pm0.45$a	$^{BC}2.41\pm0.41_{a}$	$^{C}4.42\pm0.43_{a}$
80 kV 5 min	$^{CD}4.40\pm0.03_{c}$	$^{A}0.15\pm0.03_{a}$	$^{BC}3.04\pm2.37$a	$^{AB}2.03\pm0.71_{a}$	$^{D}5.35\pm1.50_{a}$
b*					
Control	$^{BC}12.02\pm0.07_{c}$	$^{B}11.47\pm0.51_{a}$	$^{C}13.72\pm1.77_{b}$	$^{A}7.87\pm1.02_{a}$	$^{D}15.83\pm1.37_{b}$
70 kV 5 min	$^{A}11.15\pm0.06_{b}$	$^{AB}13.26\pm0.78_{b}$	$^{AB}14.21\pm0.66_{b}$	$^{AB}12.37\pm2.65_{b}$	$^{B}14.74\pm1.62_{b}$
80 kV 5 min	$^{A}10.61\pm0.04_{a}$	$^{A}12.15\pm0.69_{ab}$	$^{A}10.06\pm1.57_{a}$	$^{B}16.12\pm1.82_{c}$	$^{A}11.14\pm1.27_{a}$

Values (mean ± standard deviation, n=6) followed by the same uppercase letter in same row are not significantly different ($p>0.05$).
Values (mean ± standard deviation, n=6) followed by the same lowercase letter in same column, for each parameter, are not significantly different ($p>0.05$).

Colour results (a*, b*) after ACP (day 1) showed correlation to oxidative parameters (TBARS). Higher a* values (less redness) in ACP treated samples could be related to oxidation of heme proteins, haemoglobin and myoglobin, which are red in their reduced ferrous form and brown in their oxidised ferric form. Furthermore, an increase in yellowness (b*) in samples submitted to ACP due to the oxidised heme proteins (met-heme proteins), which achieved a brown colour. This differences were also observed at days 3 and 9. Furthermore, control and 70 kV samples tended to more yellowness (increased b*) over storage, while 80 kV caused a higher increase in b* just after treatment (day 1), but with less fluctuations over storage.

4. CONCLUSIONS

ACP is shown to be a suitable treatment for reducing the spoilage bacteria (Total aerobic psychrotrophic bacteria, *Pseudomonas* and LAB). Treatment voltage and time were both found to have significant effects on microbial inactivation. Processing conditions of ACP treatment (voltage and time) rendered Atlantic mackerel more

susceptible to lipid oxidation. In addition, ACP does not affect adversely physicochemical parameters such as pH and colour.

Tested ACP for Atlantic herring fillets preservation, the microbial growth decreased over chilled storage. The use of higher voltage (80 kV for 5 min) was more effective inhibiting microbial growth. Nevertheless, the less intense treatment (70 kV for 5 min) would be required in order to reduce negative effects on quality parameters such as lipid oxidation or colour for its use on Atlantic herring fillets.

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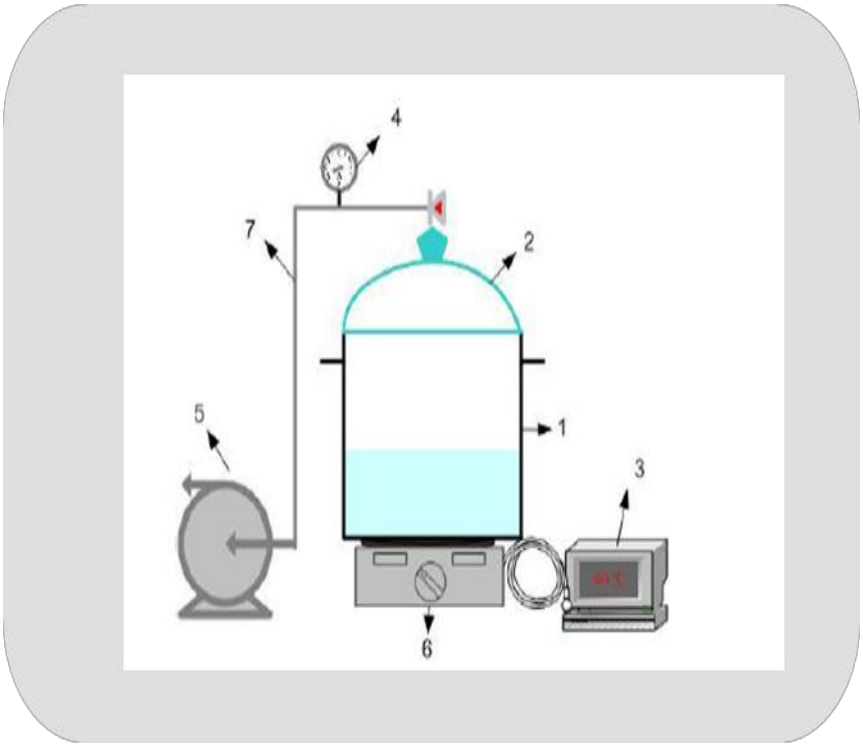
Chapter 6

Protective role of vacuum vs atmospheric frying on PUFAs' balance and lipid oxidation in mackerel patties



This chapter has been partially presented as poster communication (Albertos et al., 2015) in EuroFoodChem XVIII (13-16 of October of 2015, Madrid, Spain) as well as it has been submitted to Innovative Food Science and Emerging Technologies.

Protective role of vacuum vs atmospheric frying on PUFAs' balance and lipid oxidation in mackerel patties

ABSTRACT

Vacuum frying was compared to conventional frying as processing methods for mackerel patties, and their effects on proximate and fatty acid composition, and oxidative and organoleptic parameters were evaluated.

A temperature of 165 ºC was selected for conventional (atmospheric) frying; based on a similar thermal driving force (Δ65 ºC), a temperature of 107 ºC was used in the case of vacuum frying (80 mmHg, water boiling point 42 ºC). Frying times of 2, 4, 6, 8 and 10 minutes were investigated.

Results showed no significant differences in oil content between samples, although vacuum frying produced a significantly lower degradation of EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid), reducing the polyene index and maintaining a lower ω6/ω3 fatty acid ratio. Vacuum frying samples resulted in lower protein and ash content, whereas they showed significantly (p<0.05) higher moisture content.

The use of vacuum reduced the formation of peroxides and carbonyl derivatives. Tocopherol levels decreased in all samples regardless of the frying conditions used, although vacuum fried samples maintained higher tocopherol levels over storage. These samples also showed higher luminosity and lower a* and b* values, which are associated to lower non-enzymatic browning levels.

These results support the applicability of vacuum frying technology on fish patties since it prevents colour changes, improves juiciness and reduces oxidation due to the frying process.

Keywords: Vacuum; frying; mackerel; oxidation; fish patties.

1.INTRODUCTION

Deep frying or conventional frying is commonly used for fish cooking owing to unique sensory properties, highly appreciated by consumers (Dobarganes, Marqués-Ruiz, & Velasco, 2000). Nevertheless this culinary technique had some disadvantages, such as increment of fat content of the fish fillet (García-Arias, Álvarez Pontes, García-Linares, García-Fernández, & Sánchez-Muñiz, 2003), changes in its fatty acid profile (Sánchez-Muñiz, Viejo, & Medina, 1992; Sebedio, Ratnayake, Ackman, & Prevost, 1993; Moradi, Bakar, Motalebi, Syed Muhamad, & Che Man, 2011) and production of oxidised and polymerised lipid products as a result of frying oil degradation (Moreira, Castell-Perez, & Barrufet, 1999). In this sense, vacuum frying is an alternative process to conventional frying, which is carried out under pressures below atmospheric level, allowing the use of lower temperatures. This leads to several advantages in the final product, such as thermolabile nutrients' preservation, more natural colour and flavour and limited oil degradation. Nevertheless, it is unclear whether the reason for the observed reduction in oil content is due to the vacuum frying alone or due to the combined effect of pre-treatment and vacuum frying (Tarmizi & Niranjan, 2010).

The main applications of vacuum frying are those related to fruit and vegetable snacks development (Moreira, 2014) and very little research has been carried out on fish (Andrés-Bello, García-Segovia, & Martínez-Monzó, 2010; Chen, Zhang, & Fang, 2014). A few studies have focused on the effect of vacuum frying on proximate composition, colour, texture and/or sensory properties of fish (Andrés-Bello et al. 2010). Nowadays, it is widely accepted the health benefits of long-chain omega-3 polyunsaturated fatty acids (ω3 PUFA) highly present in fatty fish species, due to the role that these fatty acids play on cellular structure as main components of cell membranes and contribute to different membrane functions (Lee & Lip, 2003, EFSA,

2012). In general, dietary recommendations advise the consumer weekly consumption of one to two portions of fatty fish (ISSFAL, 2004). European Food Safety Authority (EFSA), based on cardiovascular risk considerations for European adults, recommends to consume between 250 and 500 mg/day of ω3 PUFA (EFSA, 2012) and the American Heart Association (AHA) recommends a daily intake of 400–500 mg of EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid) (Kris-Etherton, Harris, & Appel, 2002).

At the moment, fatty fish consumption is lower than the recommended intake by health authorities, especially in children populations. One strategy to increase the consumption among children consumers is through the development of new, more attractive, products, which may be attained through frying processing among other methodologies. On the other hand, the frying process exerts significant changes on the fat content, lipid fraction and fatty acids profile of fish, with special emphasis on DHA and EPA, which are easily oxidised, and in this sense vacuum frying may pose many advantages. However, to the best of our knowledge, no research to date has been reported on the effect of the use of this frying technology (under vacuum conditions) on the fatty acid composition and lipid oxidation (Zuta, Simpson, Zhao, & Leclerc, 2007).

Mackerel (*Scomber scombrus*) is a fish with high nutritional interest, being caught with other fish as bycatch, and in the past the muscle has been mechanically processed (Martelo-Vidal, Mesas, & Vázquez, 2012). However, at the moment there are equipments and processes to recover the fish proteins, increasing its use in commercially more attractive products (García-Sifuentes et al., 2009; Larrazábal, Escriche, & Camacho, 2010; Uresti, Téllez-Luis, Ramírez, & Vázquez, 2004). The use of mackerel for restructured products such as a frozen fillet ready for cooking and/or fish patties is an opportunity due to the volume produced, although the main

inconvenience would be its susceptibility to oxidation, especially after processing, due to the fatty acid composition, which is high in PUFAs.

The aim of this paper was to study the effect of vacuum versus conventional frying on mackerel patties through the analysis of oxidative parameters and their impact on the organoleptic properties.

2. MATERIAL AND METHODS

2.1. Chemicals

All the chemicals were analytical grade obtained from Panreac Química S.A (Barcelona, Spain) and Sigma-Aldrich Co (Madrid, Spain). All the solvents were HPLC grade (Lab-Scan, Dublin, Ireland).

2.2. Fish

Six kilos of Atlantic mackerel (*Scomber scombrus*) caught in early March 2014 were purchased in Carrefour (Valladolid, Spain). The average weight of mackerel was 200 g.

2.3. Frying process

Fillets were manually skinned and minced using a blender with a 7 mm exit pore (Lacor 69067, Guipúzcoa, Spain). Patties of 50 g were prepared manually with a round-shaped mould. All samples were processed immediately in order to avoid oxidative processes. The assay was made twice.

Vacuum frying (VF) and conventional frying (CF) procedures were performed in the same frying equipment. An electrically heated vacuum fryer (GASTROVAC®, International Cooking Concepts, Barcelona, Spain) which was slightly modified by connecting the frying stainless steel vessel to a rotary vacuum pump (Model RA 0025 F, BUSCH Ibérica SA, Granollers, Spain). For CF experiments the vacuum pump was switched off. In order to compare the effects of VF and CF, a same thermal driving force was used in both experiments. A CF temperature of 165 ºC was selected, and the

thermal driving force calculated as 65 ºC (temperature difference over the water boiling point). Taking into account the pressure and resulting boiling point used for vacuum frying conditions (80 mmHg and 42 ºC respectively), a temperature of 107 ºC was set for the experimental VF runs. Frying times of 2, 4, 6, 8 and 10 minutes were investigated for both types of frying process (VF and CF). The frying vessel had a capacity of 3 L and was filled with high-oleic sunflower oil (Casado Group, S.L.U, Valladolid, Spain), heated to the corresponding final temperature (107 ºC for VF and 165 ºC for CF), and maintained for 30 minutes previously to the frying procedure to ensure that the oil temperature was constant. The first batch of fried patties was discarded. Once the oil reached the temperature, samples were placed in the frying basket in a ratio of 50 grams per litre of oil and immersed in the frying oil. Once the patties were fried, the basket was raised and the vessel internal pressure restored to atmospheric conditions. The samples were allowed to cool down and the excess of oil was removed manually. Patties were packaged under vacuum in co-extruded polyamide/polyethylene (30/130 μm thickness) flexible bags with oxygen permeability of 30 mL mm^{-2} day^{-1} bar^{-1} and water vapour transmission of 1.4 g m^{-2} day^{-1} (Industrias Pargón, Salamanca, Spain), and stored at -80 ºC until further analyses. Three independent experimental procedures were carried out and the frying trials were randomised to avoid the effect of temperature modifications. All determinations were performed in triplicate.

2.4. Proximate analyses

Moisture content was determined gravimetrically (AOAC, 1997). Oil content was extracted with petroleum ether (BP 40-60 ºC) in an extracting unit Soxtec System 2055 Tecator (FOSS, Hillerød, Denmark) and gravimetrically determined. Nitrogen content was analysed by the Kjeldahl technique (AOAC, 1984) and protein content determined

by multiplying nitrogen by the factor 6.25. Ash content was determined by heating in a 550 °C furnace for 24 h (AOAC, 1990). Moisture, fat, protein and ashes were expressed in percentage (%).

2.5 Fatty acids composition and lipid oxidation

2.5.1.Lipid extraction

Lipids were extracted according to the method of Bligh and Dyer (1959).

2.5.2.Fatty acid composition (FA)

The fatty acid profile of the samples was determined from the Bligh & Dyer extracts. The lipid-containing chloroform phase was separated and evaporated to dryness under nitrogen. The remaining residue was dissolved in 1 mL of hexane and a methylation procedure carried out by adding 100 μL of 0.5 M methanolic KOH and leaving the reaction for 10 min at room temperature. The upper layer was transferred to a 2-mL vial. Analysis of fatty acid methyl esters (FAME) were carried out on a gas chromatograph Agilent 7890A (Agilent Technologies, PA, California, USA) equipped with a DB-23 column 60 m x 0.32 mm, (0.25 μm film thickness) (Agilent Technologies, Palo Alto, CA, USA) and a flame ionisation detector. Helium was used as the carrier gas. The oven temperature was programmed to 50 ºC for the first 7 min and increased to 200 ºC at a rate of 25 ºC/min; then, the temperature was increased to 230 ºC at a rate of 3 ºC/min and held for 26 min. Injector and detector temperatures were 250 ºC and 280 ºC, respectively. One μL of the hexane extract was injected in split mode (ratio 25:1), and FAMEs were identified by comparison of retention times with those of 37 FAME's standard mix (Supelco, Sigma Aldrich, CO). Polyene ratio was calculated on the basis of fatty acid composition, being ([20:5] + [22:6]) 100/ [16:0]. The ratio ω6/ω3 was also estimated.

2.5.3.Peroxide Value (PV)

PV was measured directly on the Bligh & Dyer extract according to the method described by the International IDF Standards (1991). Results were expressed in milliequivalents of O_2 per kilogram of oil.

2.5.4.Conjugated hydroperoxides (Trienes)

Conjugated hydroperoxides were measured on the Bligh & Dyer extract dissolved in hexane, as described by Undeland, Stading and Lingnert (1998). The absorbance was measured at 268 nm and results were calculated as mmoles of hydroperoxides per kilogram of oil.

2.5.5.Thiobarbituric acid reactive substances (TBARS)

Samples were analysed using the methodology described by Vyncke (1975) on a 5% trichloracetic acid extract of the restructured fish muscle.

Results were expressed as μmoles of malondialdehyde (MDA) per kilogram of minced fish.

2.5.6.Tocopherol content

The tocopherol content was determined on the Bligh & Dyer extract by HPLC, using an Agilent 1200 series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector. One gram of the Bligh & Dyer extract were evaporated under nitrogen and redissolved in 2 mL of hexane with 20 μL of tocopherol acetate as internal standard. An aliquot (10 μL) was injected onto a normal phase column (250 mm x 4.6 mm, 5 μm) (Teknokroma Analítica S.A, Barcelona, Spain). Elution was performed with an isocratic mixture of hexane/2-propanol (99.6:0.4; v:v) at a flow 1.3 mL/min. Detection was carried out using a UV detector at 295 nm and 284 nm for tocopherol acetate. Results were expressed in μg tocopherol per g of minced fish.

2.6. Protein oxidation

Protein carbonyls were measured as described by Levine, Williams, Statdman and Shacter (1994). Briefly, a sample of fish patties (0.5 g) was homogenised in 10 ml of 50 mM tris-buffer (pH: 7.4) containing 1 mM EDTA and 0.01 % BHT. 200 µL of the homogenate was precipitated with 50 µL of TCA (100 %). After centrifugation (12.000xg, 3 min) the pellet was incubated with 500 µL of 10 mM dinitrophenylhydrazine (DNPH) in 2 M HCl, in dark conditions for 1 hour. A blank was prepared for each sample (without DNPH) and incubated in 2 M HCl. The samples were precipitated with 50 µL TCA (100 %), centrifuged (12.000xg, 3 min) and the pellets washed three times with 1 mL ethanol/ethyl acetate 1:1 (v/v). The pellet was re-dissolved in 1 mL of 6 M guanidine chloride in 20 mM KH_2PO_4. The carbonyl content was determined by measuring the absorbance at 370 and 280 nm against guanidine as blank. Results were expressed in nmoles of carbonyl per mg of protein.

2.7. Colour

The colour parameters lightness (L*), redness (a*) and yellowness (b*) were measured using a reflectance spectrophotometer (Minolta CM-2002, Osaka, Japan). The illuminant was D65 (colour temperature of 6504 K) and the standard observer was 10º. The colorimeter was standardised using a light trap and a white calibration plate. Measurements were taken on the samples packaged in transparent plastic bags at six different points.

2.8. Sensory analysis

Preliminary analytical descriptive tests were used to discriminate between the sensory quality attributes of mackerel patties. Ten members of Agro Technological Institute (ITACyL) evaluated the samples. Before starting the sensory experiments, panellists were familiarised with the product and scoring methods. The training consisted of

demonstration exercises involving the examination of fish patties at different levels of deterioration and treatment. This procedure was repeated several times until a level of consistency in scoring was obtained. During the analyses, samples were presented in randomised order to minimise possible sequence influence.

Appearance (homogeneity surface, crispy appearance, oily surface), colour, odour (fishy odour, oily odour) and texture (crispy surface and degree of internal cooking) were analysed on a five point scale, where 1 indicated the lowest intensity of each descriptor and 5 the highest intensity.

2.9. Statistical analyses

Data of each variable were analysed by multifactor analysis of variance (multifactor ANOVA), taking into account temperature and time as independent factors. Fisher LSD (Least Significant Difference) test was applied for determining group differences at 95% of significant level.

3. RESULTS

3.1. Proximate composition

Table 1 shows the effect of frying type on moisture, fat, ash and protein content.

Table 1: Proximate composition (moisture, oil, protein and ash content) of fish patties submitted to

Vacuum Frying and Conventional Frying during different frying times.

	Vacuum Frying (VF)	Conventional Frying (CF)
Moisture (%)		
2 minutes	$^{A}55.08\pm2.46_{d}$	$^{A}56.44\pm2.45_{f}$
4 minutes	$^{B}45.95\pm1.84_{c}$	$^{A}37.80\pm2.84_{d}$
6 minutes	$^{B}42.95\pm4.77_{c}$	$^{A}32.26\pm3.60_{c}$
8 minutes	$^{B}30.85\pm3.58_{b}$	$^{A}22.66\pm3.71_{b}$
10 minutes	$^{A}16.72\pm3.08_{a}$	$^{A}11.73\pm2.10_{a}$
Oil content (%)		
2 minutes	$^{A}6.15\pm0.23_{a}$	$^{A}6.94\pm0.28_{a}$
4 minutes	$^{A}9.04\pm0.71_{ab}$	$^{A}8.58\pm0.11_{a}$
6 minutes	$^{A}13.04\pm3.29_{ab}$	$^{A}17.37\pm0.44_{b}$
8 minutes	$^{A}18.68\pm2.40_{b}$	$^{A}17.11\pm1.32_{b}$
10 minutes	$^{A}31.81\pm7.31_{c}$	$^{A}27.18\pm4.68_{c}$
Protein (%)		
2 minutes	$^{A}36.61\pm0.74_{a}$	$^{B}31.62\pm0.41_{a}$
4 minutes	$^{A}42.71\pm0.72_{b}$	$^{B}44.32\pm0.08_{b}$
6 minutes	$^{A}42.40\pm0.05_{b}$	$^{B}44.96\pm0.01_{b}$
8 minutes	$^{A}47.38\pm0.32_{c}$	$^{B}54.96\pm0.01_{c}$
10 minutes	$^{A}48.72\pm0.99_{c}$	$^{B}54.43\pm0.26_{c}$
Ash (%)		
2 minutes	$^{A}2.16\pm0.31_{b}$	$^{A}2.57\pm0.28_{a}$
4 minutes	$^{A}2.30\pm0.03_{b}$	$^{B}4.21\pm0.32_{b}$
6 minutes	$^{A}1.60\pm0.01_{a}$	$^{B}5.61\pm0.06_{c}$
8 minutes	$^{A}3.09\pm0.07_{c}$	$^{B}5.56\pm0.10_{c}$
10 minutes	$^{A}2.75\pm0.08_{c}$	$^{B}5.19\pm0.07_{c}$

Values (mean ± standard deviation, n=3) followed by different uppercase letter in same row are significantly different ($p<0.05$).
Values (mean ± standard deviation, n=3) followed by different lowercase letter in same column, for each parameter, are significantly different ($p<0.05$).

The increase in frying time resulted in a decrease of moisture content regardless of type of frying. No differences ($p>0.05$) in moisture content of the samples were observed at the shortest frying time (2 min). After two minutes, the use of VF led to samples with significantly ($p<0.05$) higher levels of moisture compared to CF, although this difference was not significant at the longest frying time tested of 10 min (final moisture content of 11.73 % and 16.72 % for CF and VF respectively).

The free water is rapidly removed within the first 2 min. Initially moisture content of mackerel was around 68%. After this period of time, differences between VF and CF can be attributed to product microstructure changes that occur during the depressurisation process. Consequently the transport properties would be affected in VF, allowing the water vapour accumulation in the headspace of the fryer (Mariscal &

Bouchon, 2008).

Oil content of the samples increased with frying time, with values ranging between 6.15 - 6.94 % after 2 min of frying time and 27.18 - 31.81 % after 10 min. No differences related to the use of atmospheric or vacuum conditions were observed (**Table 1**). These results are in accordance with the data published by other authors (Mariscal & Bouchon, 2008; Tarmizi & Niranjan, 2010; Yagua & Moreira, 2011). Tarmizi and Niranjan (2010) explained that oil content cannot be associated only to different pressure conditions, and there were other reasons, such as pretreatments or draining methods to eliminate surface oil. On the other hand, Andrés-Bello, García-Segovia and Martínez-Monzó (2010) reported significant oil uptake reduction in VF compared to CF in gilthead sea bream fillets. Also, Garayo and Moreira (2002) reported VF or CF processed products showing different oil uptake patterns. In the VF, the major oil uptake occurs during the pressurisation due to pressure differences between the surroundings and the pore. There is oil absorption too during cooling, to a lesser extent (Garayo & Moreira, 2002). In CF, oil uptake happens during the frying and cooling.

Other parameters such as proteins and ashes are shown in **Table 1**. Both nutrients increased in VF and CF as frying time increased, with values significantly higher ($P<0.05$) in CF than in VF. This increase over frying time was due to water loss, and similar results have been reported by Weber, Bochi, Ribeiro, Victório and Emanuelli (2008) and Zhang et al. (2013).

3.2. Fatty acid composition

Fatty acid composition of mackerel was modified during the frying process, as shown in

Table 2, most probably due to the absorption of frying oil (Sánchez-Muñiz et al., 1992).

In this study, the levels of oleic and linoleic acids increased in mackerel patties, as expected from the type of oil used, high oleic sunflower oil (**Table 2**).

Table 2: Fatty acid composition of fish patties submitted to Vacuum Frying and Conventional Frying during different frying times.

	Vacuum Frying					Conventional Frying				
	2 minutes	**4 minutes**	**6 minutes**	**8 minutes**	**10 minutes**	**2 minutes**	**4 minutes**	**6 minutes**	**8 minutes**	**10 minutes**
C14:0	$^{B}3.17\pm0.07_{a}$	$^{AB}2.48\pm0.07_{b}$	$^{A}1.54\pm0.05_{a}$	$^{AB}2.41\pm0.00_{b}$	$^{AB}1.83\pm0.09_{a}$	$^{D}2.22\pm0.20_{a}$	$^{C}1.53\pm0.28_{a}$	$^{BC}1.20\pm0.02_{a}$	$^{A}0.62\pm0.00_{a}$	$^{AB}0.92\pm0.01_{a}$
C16:0	$^{B}9.99\pm0.34_{a}$	$^{AB}8.78\pm0.00_{b}$	$^{A}7.33\pm0.78_{a}$	$^{AB}8.62\pm0.09_{b}$	$^{AB}7.85\pm0.55_{a}$	$^{C}8.63\pm0.00_{a}$	$^{B}7.54\pm0.54_{a}$	$^{A}6.75\pm0.04_{a}$	$^{A}6.15\pm0.28_{a}$	$^{A}6.45\pm0.02_{a}$
C16:1	$^{A}1.77\pm0.47_{a}$	$^{A}1.74\pm0.40_{a}$	$^{A}1.17\pm0.00_{a}$	$^{A}1.46\pm0.02_{a}$	$^{A}1.12\pm0.47_{a}$	$^{C}1.37\pm0.12_{a}$	$^{B}1.10\pm0.02_{a}$	$^{A}0.82\pm0.01_{a}$	$^{A}0.71\pm0.00_{a}$	$^{A}0.67\pm0.01_{a}$
C18:1 n9t	$^{A}0.00\pm0.00_{a}$	$^{A}0.00\pm0.00_{a}$	$^{AB}0.61\pm0.07_{a}$	$^{A}0.07\pm0.00_{a}$	$^{B}1.43\pm0.12_{a}$	$^{A}2.42\pm0.14_{b}$	$^{A}2.26\pm0.36_{b}$	$^{B}4.94\pm0.13_{b}$	$^{D}9.72\pm0.21_{b}$	$^{C}6.42\pm0.12_{b}$
C18:1n9c	$^{AB}48.80\pm4.21_{a}$	$^{A}47.89\pm5.43_{a}$	$^{BC}61.36\pm8.06_{a}$	$^{ABC}54.34\pm0.15_{a}$	$^{C}62.23\pm3.27_{a}$	$^{A}56.39\pm0.19_{b}$	$^{BC}66.3\pm0.9_{b}$	$^{B}65.7\pm0.04_{a}$	$^{C}68.12\pm1.31_{b}$	$^{BC}66.10\pm0.54_{a}$
C18:2n6c	$^{A}5.92\pm0.47_{a}$	$^{A}6.27\pm0.05_{a}$	$^{A}6.65\pm0.00_{a}$	$^{A}6.57\pm0.08_{a}$	$^{B}7.49\pm0.48_{a}$	$^{A}6.77\pm0.05_{a}$	$^{B}7.93\pm0.15_{b}$	$^{BC}8.20\pm0.01_{b}$	$^{CD}8.6\pm0.40_{b}$	$^{D}8.40\pm0.19_{a}$
C18:3n6	$^{D}6.12\pm0.21_{a}$	$^{CD}5.700.40_{b}$	$^{BC}5.2\pm0.00_{b}$	$^{B}5.00\pm0.15_{b}$	$^{A}0.36\pm0.01_{a}$	$^{D}5.10\pm0.06_{a}$	$^{C}3.22\pm0.41_{b}$	$^{BC}2.96\pm0.02_{a}$	$^{AB}1.90\pm0.32_{a}$	$^{A}2.25\pm0.06_{a}$
C20:4n6	$^{D}10.78\pm0.11_{b}$	$^{C}9.57\pm0.41_{b}$	$^{B}8.42\pm0.29_{b}$	$^{B}8.40\pm0.11_{b}$	$^{A}4.76\pm0.04_{b}$	$^{D}7.81\pm0.48_{a}$	$^{C}4.70\pm0.41_{a}$	$^{BC}4.25\pm0.03_{a}$	$^{AB}3.68\pm0.72_{a}$	$^{A}3.09\pm0.08_{a}$
C20:5n3	$^{B}2.19\pm0.00_{a}$	$^{B}1.99\pm0.01_{b}$	$^{A}1.33\pm0.02_{a}$	$^{B}2.00\pm0.05_{b}$	$^{A}1.01\pm0.06_{a}$	$^{D}1.99\pm0.14_{a}$	$^{C}1.24\pm0.12_{a}$	$^{BC}1.09\pm0.00_{a}$	$^{A}0.69\pm0.03_{a}$	$^{AB}0.80\pm0.02_{a}$
C22:6n3	$^{C}3.61\pm0.86_{a}$	$^{C}3.75\pm0.16_{b}$	$^{AB}2.11\pm0.08_{a}$	$^{BC}3.40\pm0.30_{b}$	$^{A}1.70\pm0.33_{a}$	$^{C}3.23\pm0.04_{a}$	$^{B}1.89\pm0.09_{a}$	$^{B}1.77\pm0.01_{a}$	$^{A}1.25\pm0.04^{a}$	$^{A}1.32\pm0.04_{a}$
Polyene Ratio	71.76	65.40	56.21	62.60	37.72	61.87	41.70	42.96	31.01	33.01
ω6/ω3 ratio	1.39	1.40	1.61	1.47	4.99	1.43	1.90	1.93	3.04	2.38

Values (mean ± standard deviation, n=3) followed by the same uppercase letter are not significantly different during frying times (p>0.05).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter are not significantly different depending on the type of frying (p>0.05).

Consequently, the levels of EPA and DHA decreased in all samples due to the frying process. This decrease was significantly ($P<0.05$) lower in VF compared to CF. Sebedio et al. (1993) explained that the reduction of PUFAs was presumably due to the exposure to air. The exposure time during the frying also affected significantly the PUFA levels, and longer time produced significant higher reduction compared with lower exposure times.

Polyene ratio also reflected the loss of ω3 PUFAs (C: 20:5 and C: 22:5) (**Table 2**). The use of VF resulted in lower polyene ratio values than in CF. Frying time affected more the polyene ratio in samples cooked using CF, with a reduction after 4 minutes higher than 33%, while for VF the decreased after 8 minutes was lower than 13 %.

The ratio ω6/ω3 in different frying and times is showed in **Table 2**. It is well-documented that this ratio increases during frying (Candela, Astiasarán, & Bello, 1998; García-Arias et al., 2003). There is not an agreement about the desirable ratio but high ratios have been linked to coronary diseases (Moradi et al., 2011). When processed with VF, the degradation of ω3 was lower than with CF, showing VF samples lower ω6/ω3 ratios than CF, although after 10 min these differences were not significant.

3.3. Lipid oxidation markers

Primary oxidation was measured as PV and conjugated hydroperoxides. PV values followed different trends over frying time depending on frying conditions, showing no differences depending on frying conditions for up to 4 min of frying time, and increasing in the case of CF after 6 minutes (**Table 3**).

Table 3: Lipid oxidation markers (Peroxide Value, Trienes, TBARS) of fish patties submitted to Vacuum Frying and Conventional Frying during different frying times.

	Vacuum Frying (VF)	**Conventional Frying (CF)**
Peroxide value (mEq of O_2 per kilogram of oil)		
2 minutes	$^{A}7.70\pm0.12_{a}$	$^{A}7.81\pm0.15_{a}$
4 minutes	$^{A}6.75\pm0.56_{a}$	$^{A}6.25\pm0.01_{a}$
6 minutes	$^{A}7.06\pm0.28_{a}$	$^{B}11.41\pm0.51_{b}$
8 minutes	$^{A}6.16\pm0.24_{a}$	$^{B}13.20\pm0.84_{b}$
10 minutes	$^{A}6.08\pm0.59_{a}$	$^{C}23.84\pm0.98_{b}$
Trienes (mmoles of hydroperoxides per kilogram of oil)		
2 minutes	$^{A}3.36\pm0.27_{a}$	$^{A}3.23\pm0.04_{a}$
4 minutes	$^{A}3.43\pm0.46_{a}$	$^{AB}4.44\pm0.48_{a}$
6 minutes	$^{A}3.82\pm0.13_{a}$	$^{A}4.19\pm0.39_{a}$
8 minutes	$^{A}4.15\pm0.19_{a}$	$^{A}4.01\pm0.06_{a}$
10 minutes	$^{A}4.71\pm0.09_{a}$	$^{B}5.76\pm0.76_{a}$
TBARS (µmol of malondialdehyde MDA per kilogram of minced fish)		
2 minutes	$^{A}7.57\pm0.48_{a}$	$^{C}14.71\pm0.68_{b}$
4 minutes	$^{A}8.46\pm0.07_{a}$	$^{A}6.97\pm0.44_{a}$
6 minutes	$^{D}19.12\pm1.12_{b}$	$^{AB}4.95\pm0.45_{a}$
8 minutes	$^{C}15.84\pm0.50_{b}$	$^{B}6.71\pm0.72_{a}$
10 minutes	$^{B}13.06\pm1.35_{b}$	$^{A}4.25\pm0.35_{a}$

Values (mean ± standard deviation, n=3) followed by the same lowercase letter in same row are not significantly different ($p<0.05$). Values (mean ± standard deviation, n=3) followed by the same uppercase letter in same column are not significantly different ($p<0.05$).

In summary, VF favoured lipid stability against oxidation, when compared to CF. Nevertheless, no studies evaluating lipid oxidation over frying times were found for fish. Sun-Waterhouse, Xue and Wadhwa (2013) reported that the use of lower temperatures produced lower TOTOX index, which included PV value, in deep-fried potatoes.

Other primary products of lipid oxidation, such as conjugated trienes, were analysed (**Table 3**). Trienes remained constant during frying, with no significant differences between VF and CF. A possible explanation for the lack of increase is that hydroperoxides would have decomposed due to the higher temperatures frying (Weber et al., 2008; Zhang et al., 2013). Marmesat, Morales, Velasco, Ruiz-Méndez and

Dobarganes (2009) concluded that conjugated dienes and trienes values perform as oxidation indicator with a much lower sensitivity than the peroxide value, conclusion that would be in agreement with the results showed in this work.

Secondary lipid oxidation was studied by the TBARS value (**Table 3**). At 2 minutes, TBARS values were significantly higher in CF compared to VF. TBARS showed a gradual decrease during frying in CF samples. Different behaviour was observed for VF samples, where TBARS values increased for up to 6 minutes, where the highest values of TBARS were detected. The decrease of TBARS in CF could be explained by a decrease of MDA content because the reaction of lipid oxidation compounds such as MDA with other molecules, mainly amino acids and peptides due to the high temperatures used (Meinert, Andersen, Bredie, Bjegegaard, & Aaslyng, 2007).

The level of tocopherol (**Figure 1**) in samples increased during frying time, both in VF and in CF. Significant differences ($P < 0.05$) were observed during frying time; after 6 minutes the levels of tocopherol increased significantly, and they remained stable until the end of the experiment (10 min). Regardless of frying time, samples cooked using VF had higher levels of tocopherol. VF protected better tocopherol degradation due to low temperature and low oxygen content during the frying.

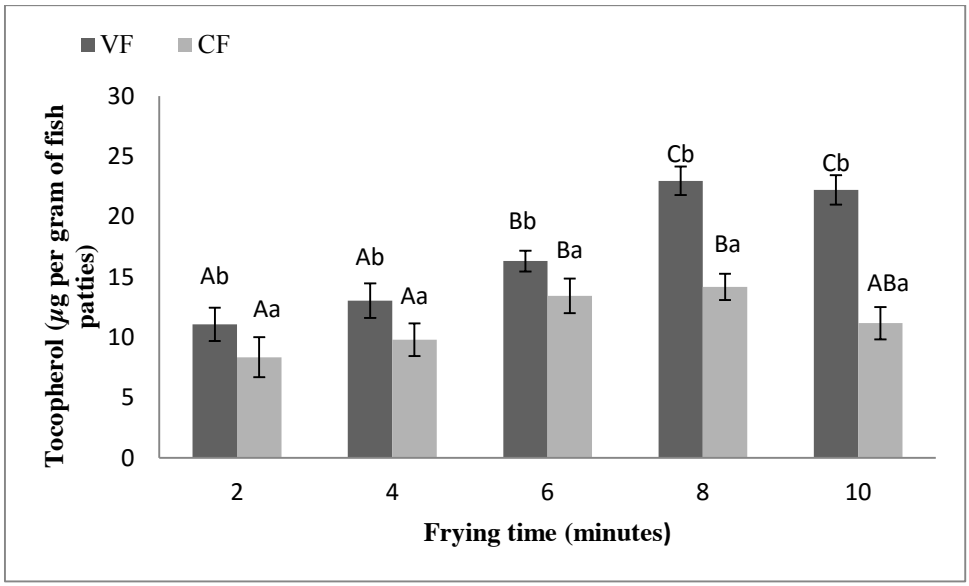


Figure 1: Tocopherol of fish patties submitted to Vacuum Frying (VF) and Conventional Frying (CF) during different frying times.

Values (mean ± standard deviation, n=3) followed by the same uppercase letter are not significantly different during frying time (p>0.05).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter are not significantly different depending on the type of frying (p>0.05).

3.4. Protein carbonyls

The formation of carbonyl compounds from amino acid side chains is probably the most outstanding result of metal-ion catalysed oxidation of myofibrillar protein (Lund, Heinomen, Baron, & Estévez, 2011). As expected, protein carbonyls were higher in CF than VF (**Figure 2**).

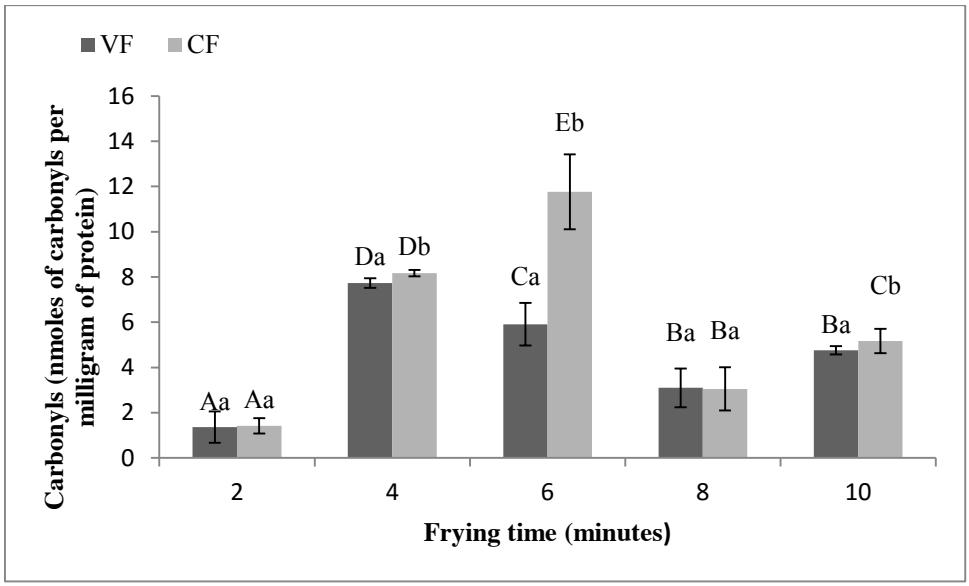


Figure 2: Carbonyls of fish patties submitted to Vacuum Frying (VF) and Conventional Frying (CF) during different frying times.

Values (mean ± standard deviation, n=3) followed by the same uppercase letter are not significantly different during frying time (p>0.05).
Values (mean ± standard deviation, n=3) followed by the same lowercase letter are not significantly different depending on the type of frying (p>0.05).

Nevertheless, the formation of protein carbonyls did not increase over the frying time. In fact, the highest value was obtained after 4 and 6 minutes in VF and CF respectively. This trend was similar to that obtain for TBARS values, where a TBARS degradation was produced. These results were difficult to compare as no studies showing results on carbonyl analyses in fried fish were reported, as far as the authors could find.

3.5. Colour

The effects of different types and time of frying on fish patty colour are shown in **Table 4**.

Table 4: Colour of fish patties submitted to Vacuum Frying and Conventional Frying during different frying times.

	Vacuum Frying (VF)	**Conventional Frying (CF)**
L*		
2 minutes	$^{B}38.78\pm5.51_{b}$	$^{A}36.63\pm5.39_{c}$
4 minutes	$^{B}38.62\pm5.77_{b}$	$^{A}36.38\pm6.26_{c}$
6 minutes	$^{A}34.67\pm3.23_{a}$	$^{A}34.19\pm3.66_{b}$
8 minutes	$^{B}34.07\pm5.45_{a}$	$^{A}30.14\pm4.33_{a}$
10 minutes	$^{B}33.67\pm6.69_{a}$	$^{A}31.07\pm4.63_{a}$
a*		
2 minutes	$^{A}3.32\pm0.15_{a}$	$^{B}4.12\pm0.14_{a}$
4 minutes	$^{A}3.37\pm0.28_{a}$	$^{B}4.36\pm1.36_{a}$
6 minutes	$^{A}2.94\pm0.34_{a}$	$^{B}4.08\pm1.01_{a}$
8 minutes	$^{A}2.91\pm0.77_{a}$	$^{A}3.96\pm0.51_{a}$
10 minutes	$^{A}3.07\pm0.25_{a}$	$^{B}3.71\pm0.70_{a}$
b*		
2 minutes	$^{A}6.58\pm0.09_{b}$	$^{A}6.84\pm0.77_{ab}$
4 minutes	$^{A}5.34\pm0.71_{ab}$	$^{B}7.58\pm1.93_{b}$
6 minutes	$^{A}6.16\pm0.13_{b}$	$^{A}5.69\pm1.07_{ab}$
8 minutes	$^{A}4.42\pm0.09_{a}$	$^{B}5.39\pm0.98_{a}$
10 minutes	$^{A}4.50\pm1.29_{a}$	$^{B}5.33\pm0.61_{a}$

Values (mean ± standard deviation, n=6) followed by the same uppercase letter in same row are not significantly different (p>0.05). Values (mean ± standard deviation, n=6) followed by the same lowercase letter in same column are not significantly different (p>0.05).

Lightness (L*) is one of the first attribute for acceptance by consumers, especially in the frying industry (Dueik & Bouchon, 2011). Lightness was significantly higher in VF than CF samples. A decrease of lightness (L*) was observed as result of the frying process. The decrease of lightness may be related to non-enzymatic browning reactions such as Maillard reaction (Mariscal & Bouchon, 2008), being the effect of temperature important in the rate of this reaction. These results were in accordance with Andrés-Bello et al. (2010) and Chen et al. (2014).

The green-red value (a*) of fried patties did not increase with frying time. Nevertheless, significant differences were found between VF and CF. Redness (a*) and yellowness (b*) shown significantly lower values ($P< 0.05$) in VF compared to CF. Similar results have been reported by Ándres-Bello et al. (2010) in vacuum fried fish. This increment was caused by a non-enzymatic browning during heating a high temperature (Richardson & Hyslop, 1985). To summarise, these results indicated that VF maintained better the original colour.

3.6. Sensory evaluation

There were no significant differences (Data not shown) concerning oiliness and oily odour for the patties prepared by these two frying methods (VF & CF). This was in agreement with the fat composition (**Table 1**), where oil uptake was similar in VF and CF. Samples cooked with VF showed less surface homogeneity and less colour intensity compared to CF. Sensory evaluation confirmed the results of instrumental measurement of colour, as CF patties were darker. In fact, the original colour of food was better retaining in VF due to the lower temperatures used. This minor degradation of colour in VF was due to the absence of Maillard reactions and oxidation during the process (Andrés-Bello et al., 2010). Visual appearance of fish patties under VF and CF after different frying times was shown in **Figure 3**.

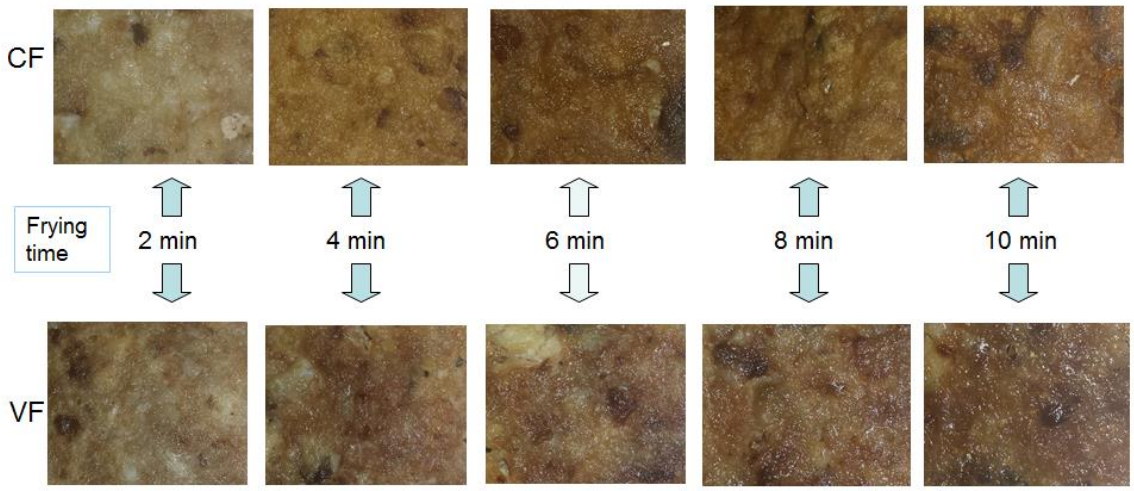


Figure 3: Visual appearance of fish patties submitted to Vacuum Frying (VF) and Conventional Frying (CF) at different frying times.

In both frying methods, as frying time increased, the patties were crunchier and more cooked. Probably associated to the major loss of water in CF patties compared to VF

(**Table 1**), the first showed a higher degree of crunchy appearance. In fact, CF samples fried for 6 minutes were overcooked. Overall, the use of VF maintained the patties juiciness.

4.CONCLUSIONS

Proximate composition, fatty acid profile and the intensity of oxidation of fish patties were affected by the frying method used. The vacuum frying protected ω3 PUFA (EPA and DHA) from degradation, improving the polyene ratio and reducing the content of peroxides and carbonyls. The moderate control of oxidation using vacuum was important on the improvement of the nutritional and organoleptic properties in the fish patties. The samples prepared with vacuum frying presented less modification of the colour and higher juiciness.

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CONCLUSIONS

This work has been focused on the use of natural ingredients, preferably by-products and novel technologies in order to obtain safe, natural and minimally processed fish products.

The following conclusions can be established from the experimental data:

- ✓ Brines, which were generated during ripening of marinated herrings, contained bioactive compounds with antioxidant capacity. These brines can be applied directly as natural antioxidant on frozen and fresh herring. Brines showed different efficacy depending on their application. TSa and TSc suited better as coating agent in frozen herring fillets whereas SC prevented oxidation better in fresh herring mince.
- ✓ Carob seed peel showed a great antioxidant capacity both *in vitro* assays and added as natural antioxidant in minced horse mackerel over chilled storage.
- ✓ Olive leaf is rich in polyphenols which provide antioxidant and antimicrobial properties. Olive leaf powder was succesfully used as antioxidant in minced chilled horse mackerel. Furthermore, olive leaf extract was applied through gelatin edible film on inoculated with *Listeria monocytogenes* on smoked salmon. These films slowed *L.monocytogenes* growth.
- ✓ Chitosan films with clove essential oil have high antioxidant and antimicrobial capacity. The use of chitosan films (with or without clove oil) with high pressure processing (300 MPa-10`) showed as a suitable treatment for the shelf-life, quality and sensory parameters of trout fillets. Films may be a valuable alternative to thermal treatments to maintain a similar shelf life.
- ✓ Atmospheric pressure cold plasma can be a promising technology to reduce the fish spoilage bacteria. Thus, fish shelf life was enlarged using this novel

technology according to experimental studies carried out. Nevertheless, fish fillets submitted to atmospheric pressure cold plasma became more susceptible to lipid oxidation. Further investigations are required to ensure the spoilage bacteria reduction without affecting quality properties such as oxidation.

- ✓ Vacuum frying demonstrated to be an useful technique to produce high-quality fish patties. Compared with atmospheric frying, vacuum frying protected better the loss of ω3 PUFA and the development of the oxidation. In addition, vacuum-treated patties present better colour and higher juiciness.

Consumers demand healthy, natural and minimally processed food, pushing the processing industry to constantly investigate novel treatments to provide those characteristics while ensure food safety. Fish holds high nutritional interest, with special emphasis on omega-3-polyunsaturated fatty acids, which however make it highly perishable.

Potential solutions are here attempted for improving the quality and extending the shelf life of fish, through emerging processing technologies, such as vacuum frying, edible coatings or cold atmospheric plasma, and/or agroindustrial by-product valorisation as natural antioxidants and antimicrobials, such as marinating brines, carob seed peel and olive leaves.

Among other findings, marinating brines, carob seed peel and olive leaves directly applied or/ and as coatings or films successfully showed antioxidant and antimicrobial activities in oily fish. High pressure combined with chitosan/clove edible films, extended the shelf life of trout fillets. Plasma was suitable treatment for reducing spoilage bacteria in fish. Finally, vacuum frying allowed obtaining convenience fish products with healthy attributes.

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