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Highlights

- The natural compounds used could replace synthetic antioxidants in food industry.
- The incorporation of antioxidant products into plastic matrices results in active films.
- The addition of these compounds into LDPE extensively inhibited lipid oxidation of salmon muscle.
- Active films containing tocopherols could be used to develop a promising active packaging to be applied in food preservation by food industry.

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DEVELOPMENT OF ANTIOXIDANT ACTIVE FILMS CONTAINING TOCOPHEROLS TO EXTEND THE SHELF LIFE OF FISH

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Abstract

With the purpose of develop active films for fish, five natural products with antioxidant properties containing tocopherols were selected and their antioxidant activity *in vitro* was tested using the DPPH method. In addition these antioxidants were also tested directly on the salmon muscle using the TBARS method. Besides, thermal degradation and differential scanning calorimetry tests allow us to select the products for incorporation into the polymer matrix. Then, two natural products have been selected and incorporated in low density polyethylene films. Film 2 and Film 3, which contain product C at 1 and 5 % respectively, presented a reduction in lipid oxidation up to 30 % for Film 2 and to 40 % for Film 3. Film 4, which contains 5 % of product D, reduced lipid oxidation during storage around 30-35 %. The results showed that Film 3 was the most effective to salmon conservation at long-term storage. These films could be used as future active packaging by the food industry for the salmon conservation to extend its shelf life.

Keywords: Lipid oxidation, natural antioxidants, tocopherols, active films, LDPE, salmon.

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1. Introduction

Seafood has recently received more attention from consumers as important source of nutritional components (unsaturated fatty acids, minerals and vitamins) that have positive benefits in nutrition and human health. The nutritional significance of fish and fish oils intake is due to their high content of essential long-chain omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which protect against heart disease, cancer, hypertension, rheumatoid arthritis, depression, diabetes, among other health benefits (Richards & Hultin, 2002; Sidhu, 2003). The high content of PUFAs in fish muscle, as well the presence of relevant prooxidant compounds, promote the development of enzymatic and non-enzymatic rancidity leading to organoleptic, physical and nutritional quality losses in food (Frankel, 1993; McClements & Decker, 2000; Erickson, 1997).

Lipid oxidation is the main cause of spoilage of fish during its processing and storage, being a key factor in the shelf life of food (Frankel, 1993; Frankel 1998b; Frankel, 1998a). As a consequence of this deterioration process, hydroperoxides are formed (primary oxidation) which are unstable and decompose relatively quickly into aldehydes, ketones, alcohols, acids, esthers or hydrocarbons (secondary oxidation). These secondary oxidative products are responsible for changes in color, texture, odor and flavor in food (McClements & Decker, 2000; Erickson, 1997).

The use of antioxidants is an effective way to prevent or minimize lipid oxidation in food products, delaying the formation of toxic oxidation products maintaining the nutritional quality and prolonging their shelf life (Huang, Ou, & Prior, 2005). Antioxidants act by inhibiting the oxidation of lipids and other molecules avoiding the damaging effects of oxidation in animal tissues. The major mechanisms of

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action in protecting the food from the oxidation process include the free radical scavenging and chain breaking by inhibiting the initiation or propagation phases of free radical chain reactions of lipid oxidation, metal chelation and singlet oxygen quenching (Huang, Ou, & Prior, 2005; Aidos, Masbernat-Martinez, Luten, Boom, & van der Padt, 2002).

Antioxidants may be present naturally as food components as in the case of tocopherols or added during food processing (Choe, & Min, 2009). In many situations, food processing is responsible for the loss of these endogenous compounds activity and required the addition of synthetic antioxidants such as 2 (3)-tert-butyl-4-methoxyphenol (BHA), butylated hydroxytoluene (BHT) and propyl gallate, which are widely used in the food industry. In recent years, several studies have shown evidence of some toxicity by synthetic antioxidants such BHT and BHA that may possess carcinogenic effects at high levels ~~(Wattenberg, 1986; Karl, 1984)~~ (EFSA, 2012; EFSA, 2011). For this reason, and for human health benefits, the use of natural antioxidants such as tocopherols, ascorbic acid or natural extracts with high levels of polyphenols are increasingly used as antioxidants in food. Besides, these natural compounds are being effective in the prevention and/or treatment of diseases caused by free radicals (Shahidi, & Zhong, 2010; Frankel, 1996).

Tocopherols are antioxidants extensively used as food additives by being effective, fat soluble and inhibitors of oxidation reactions because their efficient chain-breaking capacity, demonstrating effectiveness against lipid peroxidation and other oxidative situations caused by the presence of free radicals (Ohkatsu, Kajiyama, & Arai, 2001; Brigelius-Flohé, & Traber, 1999). To prevent and/or delay the food spoilage, antioxidants are directly added as food additives or could be added indirectly through the packaging material, using what is known as active packaging.

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Active packaging represents a new packaging concept with great interest in food industry. This system allows the release of the active components into the food and has shown great potential in the preservation of the food quality being an alternative to traditional packaging. Antioxidants such as BHT, BHA and α-tocopherol have been incorporated in plastic materials in order to protect the polymer matrix from degradation during processing. The α-tocopherol has been used in different situations to stabilize plastic materials such as low density polyethylene (LDPE) and polypropylene (PP) proving to be a good preservative of these materials (Wessling, Nielsen, & Giacin, 2000).

Nevertheless, the possibility of antioxidant compounds migrating from the plastic material being in contact with food has been observed. A study performed by Wessling, Nielsen, Leufvén, and Jägerstad, (1999) suggests that LDPE spiked with α-tocopherol can be used as active packaging, for foods with high fat content, releasing the antioxidant and extending the shelf life of the product.

The aim of this study is to evaluate and compare the antioxidant effectiveness of different natural antioxidant products obtained commercially which contains tocopherols, on the stability of salmon during refrigerated storage. Besides, the most suitable antioxidant for their incorporation into LDPE is selected. With the results obtained, the products to be used in the development of active packaging with antioxidant properties were selected and studies were conducted for assessment of their effectiveness in the delay of lipid oxidation in the salmon muscle.

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2. Materials and methods

2.1. Chemicals

2(3)-tert-butyl-4-hydroxyanisole (BHA) (98 %, CAS No. [25013-16-5]); butylated hydroxytoluene (BHT) (99.0 %, CAS No. [128-37-0]); sodium azide (99.0 %, CAS No. [26628-22-8]); 2-thiobarbituric acid (TBA) (≥98 %, CAS No. [504-17-6]) and trichloroacetic acid (TCA) (puriss. p.a. 99.5 %, CAS No. [76-03-9]) were purchased from Sigma-Aldrich (Steinheim, Germany). 1,1,3,3-tetraethoxypropane (TEP) (purum ≥95 % (GC), CAS No. [122-31-6]) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (TECHN ≥85 %, CAS No. [1898-66-4]) were supplied by Fluka Chemie AG (Buchs, Switzerland). Methanol (GC≥ 99.9%, CAS No. [67-56-1]); orthophosphoric acid (85 % GR for analysis, CAS No. [7664-38-2]) and ethanol (absolute for analysis, CAS No. [64-17-5]) were provided by Merck (Darmstadt, Germany). Irganox® 1076 (CAS No. [2082-79-3]) and Irgafos® 168 (CAS No. [31570-04-4]) were kindly supplied by Ciba-Geigy, Switzerland.

2.2. Commercial products containing natural antioxidants

The natural products with antioxidant activity used, TOCOBIOL®, TOCOBIOL® GL, NUTRABIOL®-T90, TOCOBIOL® PV, NUTRABIOL®-T50 PV, were supplied by Biotecnologías Aplicadas, S.L., BTSA, (Madrid, Spain). The presentation of the products TOCOBIOL ®, TOCOBIOL ® GL, NUTRABIOL ®-T90 was in oil form and products TOCOBIOL ® PV, NUTRABIOL ®-T50 PV were in

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133 powder form. Table 1 shows the composition of the different natural antioxidants tested.
134 This information was provided by the company.
135
136 *2.3. Materials*
137
138 *2.3.1. Fish sample*
139 Salmon (*Salmo salar*) was selected to be used in the antioxidant effectiveness
140 assays. The salmon was supplied to the laboratory, in frozen slices with a thickness
141 between 1 and 1.5 cm, by the company Elaborados FREIREMAR SA.
142 For the antioxidant effectiveness assessment, salmon was defrosted at room
143 temperature and its skin and bones removed from the flesh. To prevent microbial
144 spoilage during storage, a small quantity of sodium azide (approx. 1 mg) was added on
145 the surface of salmon.
146
147 *2.3.2. Processing of polymer formulations*
148 Several formulations of the antioxidant compounds with LDPE matrix were
149 prepared. The recipes of formulations are shown in Table 2. The matrix used was a low
150 density polyethylene (LDPE), provided by Repsol YPF (MFI; 20 g min^{-1}; density; 919
151 kg m^{-3}). Only the antioxidants compounds with a suitable thermal stability were mixed
152 with the polymer.
153 Blends with LDPE and antioxidants were prepared in an internal mixer
154 Brabender W50EHT at 150 ºC. Besides the natural antioxidants, the commercial
155 antioxidants IRGAFOS® 168 (secondary antioxidant) and IRGANOX® 1076 (primary
156 antioxidant) were also added in the usual proportions in industry in order to prevent the
157 oxidation of polymer matrix.

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In order to study the antioxidant effectiveness of the polymer formulations in the fish, active films with different antioxidant contents were processed. These films were also spiked with the commercial antioxidants IRGAFOS® 168 and IRGANOX® 1076 always at the same concentration of 0.2 and 0.4 % respectively. For the preparation of the extruded films, a Brabender DSE-20 twin-screw extruder with a flat die was used (the barrel and die temperature were 140 and 150 ºC respectively and the screw speed 45 rpm). The melted polymer formulations were cooled on a chilled roll and stretched in the machine direction. The chilled roll was kept at 80 ºC with a rotation speed of 1.2 rpm. The thickness of the extruded films was between 80 and 150 μm. Then, bags with dimensions of 6 cm x 6 cm were thermosealed from the extruded films.

2.4. Instruments

The concentration of 2-thiobarbituric acid reactive substances (TBARS) and the absorbance in the DPPH method were determined on a dual-beam spectrophotometer Uvikon XL, Bio-Tek Instruments, Milan, Italy.

On the extraction process of secondary oxidation compounds from fish muscle, a homogenizer T 25 digital ULTRA-TURRAX ® and a shaker MS2 Mini Vortex Shaker IKA ® were used.

Thermal stability of the antioxidant compounds were evaluated using a TGA-7 thermo balance (Perkin-Elmer).

The thermooxidative stability of the polymeric formulations (antioxidant product and polymer matrix) was assessed by Differential Scanning Calorimetry tests measuring its oxidation induction time (OIT). A Perkin–Elmer series 7 DSC isothermally was used to OIT measurements.

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183

184 *2.5 Methods*

185

186 *2.5.1. Determination of antioxidant effectiveness*

187 The methods employed in the evaluation of antioxidant activity were the 2,2-
188 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and the determination of
189 thiobarbituric acid reactive substances (TBARS) method.

190

191 *2.5.1.1. Free radical scavenging activity, DPPH assay*

192 The determination of antioxidant activity of different natural products was
193 carried out by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method,
194 which determines the hydrogen-donating antioxidant ability, according to the method
195 described by von Gadow, Joubert, and Hansmann (1997).

196 Standard solutions of the different antioxidant products were prepared in
197 methanol as well as two synthetic compounds with antioxidant properties usually used
198 in the food industry, BHA and BHT. The purpose to use these synthetic compounds was
199 to compare the activity of natural products with those that are highly active. All
200 solutions were prepared immediately before used and the concentration was within 0.1
201 to 3.5 g L^{-1} range. A methanolic solution of DPPH radical 3.6×10^{-5} M was prepared
202 daily and protected from the light. 50 µL of different methanolic solutions of
203 antioxidant product was added to 2 mL of DPPH solution and shaken. Absorbance was
204 determined at 515 nm in a spectrophotometer after 16 min. All determinations were
205 performed in triplicate. The absorbance decrease was converted to percentage of DPPH
206 inhibition (PI) according to the following equation:

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208

$$\text{PI} = \frac{(A_0 - A_{16})}{A_0} \times 100$$

209

210 Where,

211 A_0 is the absorbance of the control at initial time;

212 A_{16} is the absorbance of the sample at 16 minutes.

213

214 In order to obtain data of antioxidant activity comparable for all samples, the
215 concentration of product required to achieve a 50 % inhibition of the radical DPPH was
216 determined. This Equivalent Concentration 50 (EC_{50}) indicates the specific antioxidant
217 activity of the product expressed as g L^{-1}. EC_{50} values were obtained from the linear
218 regression curve data obtained from different concentrations of each compound used
219 against the percentage of free radical scavenging activity (PI %). The lower the EC_{50}
220 value, the higher is the antioxidant activity of the tested sample.

221

222 *2.5.1.2. Determination of thiobarbituric acid reactive substances – TBARS*

223 Malondialdehyde (MDA) is one of the largest relatively stable end products
224 generated from secondary lipid oxidation of polyunsaturated fatty acids (PUFA). This
225 compound reacts with 2-thiobarbituric acid forming a pink-colored compound which
226 was determined spectrophotometrically by measuring the absorbance at 530 nm (St.
227 Angelo, 1996).

228 TBARS were determined using the extraction procedure described by Witte,
229 Krause, and Bailey (1970) with slight modifications. Five grams of fish muscle, which
230 was added BHT (0.2 mg mL^{-1}), were extracted with 45.5 mL of extraction solution
231 containing 10 % trichloroacetic acid (TCA) in 0.02 M orthophosphoric acid to a final
232 volume of 50.0 mL. Samples were mixed in Ultra-Turrax 1 minute and then filtered

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233 through 0.45 µm nylon membrane filter. In a test tube with screw cap, 5 mL of extracted
234 solution and 5 mL of TBA solution (0.02 M) were homogenized by vortex and
235 afterward incubated in an oven at 100 °C for 40 minutes. Finally, samples were cooled
236 at room temperature, and the absorbance was measured by spectrophotometer at 530 nm
237 against a blank containing 5 mL of distilled water and 5 mL of TBA reagent.
238 MDA concentration was calculated using the calibration curve obtained using
239 1,1,3,3-tetraetoxipropano (TEP), a precursor of MDA, in a concentration range between
240 0 and 5 mg L^{-1}. Results were expressed as milligrams of malondialdehyde per kilogram
241 of sample (MDA mg kg^{-1} of salmon).
242
243 *2.5.2. Evaluation of antioxidant effectiveness of the different natural products in direct*
244 *contact with the salmon*
245 Different concentrations of natural products were added to different pieces of
246 salmon with a surface area of 3.1 × 4 cm^2 and weighing 15 ± 1 g.
247 Two different concentrations of each natural product (A, B, C, D and E) were
248 used. Antioxidant products solutions were prepared in ethanol. The concentrations
249 applied resulted in 30 mg dm^{-2} (A1, B1, C1, D1 and E1) and 125 mg dm^{-2} (A2, B2, C2,
250 D2 and E2) on the salmon surface.
251 Different pieces of salmon muscle additivated with the different compounds
252 were stored at 4 °C. Trial period was performed for 10 days and samples were collected
253 for TBARS determination at 3, 7 and 10 days.
254
255 *2.5.3. Evaluation of antioxidant suitability to incorporation in film*
256 In order to know the thermal stability of the antioxidant compounds, dynamic
257 experiments were conducted under oxidant atmosphere in a thermo balance. The

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antioxidant samples were tested from 50 ºC to 70 ºC at heating rate of 10 ~~ºC/min~~ ºC min^{-1}.

The thermooxidative stability of the polymeric formulations (antioxidant product and polymer matrix) was assessed measuring its oxidation induction time (OIT). The sample temperature was stabilized for 2 minutes at 200 ºC under inert atmosphere, which was subsequently switched to oxygen atmosphere to start the test. Analyses were carried out according to EN 728. The OIT was measured as the onset point at which the DSC thermogram suffers a suddenly drop respect to the instrument baseline. The maximum time in the experiments was 240 minutes.

2.5.4. Evaluation of antioxidant effectiveness of the films additivated with commercial products

To perform this study, different pieces of fish (± 20 g) were packed in individual LDPE bags containing different concentrations of selected antioxidant compounds. A LPDE film without natural antioxidants incorporated was used as control film.

Packed salmon was stored at 4 ºC for 21 days. Analyses of TBARS were performed at 4, 7, 11, 14, 18 and 21 days.

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3. Results and discussion

Several authors make distinction between antioxidant capacity and reactivity of the antioxidant compounds ~~(polyphenols)~~. The antioxidant capacity provides information about the duration of the antioxidant action, i.e. the ability to delay oxidative degradation, while the reactivity is characterized by the beginning of antioxidant process by a known concentration of an antioxidant or a mixture of antioxidants, determined by the reactivity of the/these antioxidant/s to free radicals in the corresponding reaction (Roginsky, & Lissi, 2005). For this reason, in this work, two analytical methods were used. An indirect method to determine the antioxidant products ability to free radical scavenging (DPPH) and a direct method which evaluates lipid oxidation in food where antioxidants were added.(TBARS).

In this study, three different assays were conducted. The first one was to evaluate the effectiveness of several commercial natural antioxidants, formulated with tocopherols, by adding different concentrations directly on the surface of the fish muscle. The second one was to study the different thermal properties of antioxidants in order to select the most suitable to process by extrusion. Finally, films additivated with the selected antioxidant products which showed better results in the previous mentioned test were evaluated.

3.1. Antioxidant properties of commercial products

The capacity to scavenge free radical is an antioxidant property of great significance as well as the quenching of the peroxide radicals terminating the peroxidation chain reaction (Brand-Williams, Cuvelier, & Berset, 1995). The data

obtained for the ability of DPPH radical scavenging by natural antioxidant products (Table 1) was compared with reference antioxidants BHA and BHT, usually used in food industry. ~~The EC_{50} values obtained for natural products A, B, C, D, E were 1.49 ± 0.075, 0.435 ± 0.050, 0.401 ± 0.062, 3.25 ± 0.095 and 0.704 ± 0.047 respectively, whereas for the synthetic antioxidants BHT and BHA were 2.53 ± 0.085 and 0.273 ± 0.025 g L^{-1} respectively.~~ Results of the antioxidant activity determined by DPPH method are shown in Table 3.The EC_{50} values obtained for the synthetic antioxidants used as reference, BHA and BHT, are in agreement with values found in the literature (Cruz, Moldes, Bustos, Torrado & Dominguez, 2007).

The results obtained indicate that all antioxidant extracts tested presented antioxidant activity and the natural product with highest radical scavenging activity was product C followed by B, E, A and D which had the lowest antioxidant activity among the products studied.

Except for D, all the products tested demonstrate higher antioxidant activity compared to BHT, synthetic antioxidant commonly used in food industry.

Antioxidants products B and C have displayed higher antioxidant activity presenting EC_{50} values of 0.435 and 0.401 g L^{-1} respectively, comparable to the reference synthetic antioxidant BHA which presented an EC_{50} of 0.273 g L^{-1}.

Comparing the three TOCOBIOL® products, the most active was B followed by A and finally D.

The highest antioxidant capacity presented by product B may be due to the fact that in its composition in addiction to 25 % of TOCOBIOL® product also includes 25 % of the synthetic antioxidant propyl gallate, used in the food industry due to its high antioxidant activity (Martinez-Tome, Jimenez, Ruggieri, Frega, Strabbioli, & Murcia,

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2001), and therefore showed greater antioxidant activity than the product A (EC_{50} of 1.49 g L^{-1}).

Product D was less effective in radical scavenging because it is composed by only 65 % of TOCOBIOL® product which is the responsible for its antioxidant activity.

Comparing both NUTRABIOL® products, C product presents the highest radical scavenging capacity to free radicals because is the one which contains highest percentage of tocopherols.

Of the two products tested in powder form, antioxidant E showed highest antioxidant capacity with an EC_{50} value of 0.704 g L^{-1} because of its high percentage of tocopherols (50.3 %). The same was observed for the oils, the product with highest proportion of tocopherols, product C (90.2 %), was the most effective, i.e. presented the highest antioxidant activity with an EC_{50} value of 0.401 g L^{-1}.

3.2. Antioxidant effectiveness of commercial products directly on salmon

To evaluate the effectiveness of these compounds directly on food, salmon was selected and lipid oxidation was evaluated during storage at 4 °C.

The study was divided into two parts and samples were analyzed at different days. In the first part of this study the antioxidant products in the oil form were tested and in the second one antioxidant products in powder form. In each assay there was a control test prepared as samples but without the addition of ~~commercial products~~ antioxidant products.

3.2.1. Evaluation of antioxidant effectiveness of the oil products

The antioxidant effectiveness of the different products tested in the salmon muscle was determined by the TBARS method. The data obtained with the 3 oils

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applied at 2 different concentrations used on the salmon muscle are shown in Table ~~3~~ 4a.

Lipid oxidation of salmon muscle samples increased during the storage in different degrees being the control test (without addition of antioxidant) which showed high TBARS values.

All antioxidants applied in this assay reduced lipid oxidation in salmon compared to the control test. At the lowest concentration of the study (30 $\mu g \cdot dm^{-2}$), product B showed better effectiveness, reducing notably lipid oxidation compared to control assay. For the C antioxidant, also at higher concentration of antioxidant employed in the salmon muscle (125 $\mu g \cdot dm^{-2}$) lower antioxidant effect was observed. After 10 days the antioxidant effect presented by the two concentrations of product C is approximately the same, with TBARS values around 2.4 mg MDA kg^{-1} sample, which corresponds to a reduction in lipid oxidation around 40 % respect to control assay. Analyzing the data obtained, it can be seen that the oil, ~~that~~ which presented better antioxidant effect in salmon is ~~B~~ product B, and for the two concentrations evaluated, the higher is the concentration used higher the antioxidant effectiveness. B1 and B2 concentrations reduced lipid oxidation about 70 % being B2 sample slightly more effective. Antioxidants A and B applied at the highest concentration, i.e. 125 mg dm^{-2} (A2 and B2), remain oxidation values below 1 mg kg^{-1} MDA of sample which is equivalent to a deterioration reduction in food around 75 % compared to control assay. ~~For compounds A and B it can be seen that the higher the concentration used the greater reduction of oxidation in fish was observed, while in the product C this effect was not observed.~~ For compounds A and B it can be seen that the higher the concentration used, the greater reduction of oxidation in fish was observed, while in the product C this effect was not observed. This could be due to that C was the compound with highest

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concentration of tocopherols (see Table 1) and presented the highest EC_{50} in the DPPH method (EC_{50} = 0.401 g L^{-1}), and subsequently, when applied to the food at the same concentrations that were used with other products, it could have a prooxidant effect caused by this high amount of tocopherols. Numerous authors have described this prooxidative effect of α-tocopherols after applying this product at high concentrations (Frankel, 1996; Brigelius-Flohé, & Traber, 1999; Huang, Frankel, & German, 1994). At very high concentrations, the prooxidant effect of α-tocopheroloccurs during the induction period of primary oxidation where hydroperoxides are formed in large amounts. This happens because at high concentrations, α-tocopherol loses its activity to inhibit the formation of these hydroperoxides demonstrating a low effectiveness in the primary oxidation inhibition. However this effect was not observed in the secondary oxidation where a significantly delaying of secondary products formation is verified (Huang et al., 1994; Tafazoli, Wright, & O'Brien, 2005; Tong, Sasaki, McClements, & Decker, 2000). Thus, the results obtained using the **C** antioxidant product could be due to a possible loss of activity of the compound as result of the large amount of tocopherols present in high concentrations because only a small amount of α-tocopherols consumed in the oxidation reaction and a large part remains available for further reactions that occur simultaneously and leads to an oxidation enhancement (Fuster, Lampi, Hopia, & Kamal-Eldin, 1998; Decker, Warner, Richards, & Shahidi, 2005).

3.2.2. Effectiveness of products in powder form

TBARS values obtained from salmon additivated with two commercial products in powder form with antioxidant properties, D and E, at two different concentrations are displayed in Table ~~3~~ 4b.

Analyzing the data obtained in this assay and, taking into account that the control was not much oxidized, which makes difficult the comparative, improvements were observed in the conservation of fish additivated with antioxidant products ~~in the concentrations~~ at the concentrations 1 and 2 (30 and 125 mg dm^{-2} respectively), except for the D2. Antioxidant effectiveness of product E in fish muscle increased with the increasing of the concentration employed. After 3 days of storage, the antioxidant effectiveness was higher than 50% compared to control test.

On the seventh day of trial, the degree of inhibition remained for the D1, E1 and E2 but in less percentage (52, 22 and 40 % respectively). Powder E showed an EC_{50} of 0.70 g L^{-1} in the DPPH method indicating the strong antioxidant/radical scavenger activity as well that the product had antioxidant activity *in vitro*. ~~This antioxidant effect was confirmed when applied directly into the salmon muscle and consequently might be considered as a possible extract to be used in fish conservation.~~ This antioxidant effect was confirmed when applied directly into the salmon muscle and consequently could be considered as a possible antioxidant product to be used in fish conservation.

Antioxidant D displayed the highest EC_{50} of the five compounds tested being the product which showed lower antioxidant activity in DPPH assay. However, added at concentration of 30 mg dm^{-2} (D1) on the sample of salmon demonstrated antioxidant effect in the 7 days of storage, while at higher concentration (D2) a possible prooxidant effect was observed because lipid oxidation did not decrease. Compound D seems to be an example of antioxidant that although it did not demonstrate great antioxidant activity by the indirect method (~~EC50~~ EC_{50}= 3.25 g L^{-1}), it was effective against lipid peroxidation of food (Table ~~3~~4b). Therefore, methods to determine free radical scavenging such as DPPH method are very useful to make a screening of the

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antioxidants, however to verify their effectiveness in food, methods to evaluate the food deterioration must be used.

The antioxidant effectiveness in food depends on a variety of factors such as polarity, solubility and metals quenching activity as well as the interaction with other food components (Roginsky, & Lissi, 2005; Decker et al., 2005). Antioxidant D presents a very similar behavior to that observed with the antioxidant oil **C** in terms of a possible pro-oxidant effect or inhibition of antioxidant activity when applied at high concentrations.

3.3. Thermal stability and antioxidant effectiveness of compounds

The thermal stability of the five antioxidants was evaluated and the most suitable ones, that is, which do not suffer thermal degradation at the LDPE processing temperature, were chosen for blending with polymer matrix.

Figure 1 displays the thermograms obtained from the TGA experiments. The **A** and B compounds have a lower thermal stability than others. The degradation process of A and B antioxidants start from 100 ºC, whereas in the other ones (C, D and E), it begins at 250 ºC (see onset in each curve) approximately. Keeping in mind that LDPE processing temperature is around 150 ºC (indicated by the arrow in the graph), the ~~A and B antioxidants~~ antioxidants A and B were ruled out to blend with the polymer.

Then, the antioxidant activity of the different LDPE / antioxidant blends was evaluated measuring the OIT by DSC. The oxidation induction time increases with the increase of antioxidant amount. This effect is observed with three antioxidants, C (Blends LDPE 1-3), D (Blends LDPE 4-6) and E (Blends LDPE 7-9) (Figure 2). Comparing same

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449 formulations, the antioxidant activity is quite similar in blends with antioxidant C and E
450 (the maximum time assayed) but it is much lower with D compound.
451 On the other hand, these results reveal a behavior of studied antioxidants as long-term
452 antioxidant. Therefore, OIT values increase with increasing natural antioxidant levels,
453 characteristic behavior of phenolic compounds. However phosphate stabilizers alone do
454 not increase the OIT of polyethylene in the temperature range of 180-210 ºC (Phease,
455 Billingham, & Bigger, 2000).

456 To prevent the matrix oxidation during the extrusion and the premature
457 consumption of the natural antioxidants, the two commercial antioxidants (IRGANOX®
458 1076 and IRGAFOS® 168) were added to the polymer blends. The OIT data of samples
459 LDPE 10 and LDPE 11 (the maximum time assayed) shown an available level of
460 antioxidant in the film to be released to fish.
461 Based on the results obtained, the natural antioxidants that should be selected to
462 incorporate in the films to be tested in the salmon packaging assay would be C and E.
463 Once these two products are constituted by the same active principle Nutrabiol® but in
464 different proportions, and for the purpose of testing two different products with different
465 compositions in antioxidant active compounds, the products selected to incorporate to
466 LPDE films were C and D. Thus, two different formulations in tocopherols can be
467 evaluated as well as their effectiveness against lipid peroxidation getting more
468 information about the possible application of these antioxidant products as ingredients
469 of active films.

470 In summary, the C and D antioxidants together with the commercial antioxidants
471 (Irgafos® 178 and Irganox® 1076 at 0.2 and 0.4 % respectively) were chosen to prepare
472 the LDPE active films. The active films formulations selected were: Film 2 and 3

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incorporated with natural product C at 1 % and 5 % respectively; Film 4 with natural product D at 5 %. Film 1 with only commercial antioxidants was used as control.

3.4 Evaluation of antioxidant effectiveness of the active films.

When incorporated into the plastic matrix, compounds with antioxidant activity will not be available as in previous assays, which were added directly into fish muscle. In addition, during films processing, that occurs at high temperatures and aggressive conditions, the active compounds could be reduced and its antioxidant capacity be affected. Therefore, based on the results of direct adding tests, commercial products which showed evidences of great effectiveness in inhibition of lipid oxidation in salmon were selected. Both of the products exhibited better results at lower concentrations and a possible pro-oxidant effect when applied at the highest concentration demonstrating their high activity.

Compound C showed a high ~~antioxidant activity applied~~ antioxidant activity when applied at low concentration, a high antioxidant activity by DPPH method and in its composition contains a high percentage of tocopherols. Therefore it was incorporated into the plastic matrix at two different concentrations (1 % and 5 %). Compound D was incorporated into the plastic matrix only at the highest concentration of 5 % because showed less antioxidant activity by DPPH method (EC_{50} = 3.25 g L^{-1}).

The antioxidant effect of films additivated with selected products was determined by the capacity to inhibit lipid oxidation in salmon muscle during storage at 4 °C by TBARS method.

Figure 3 shows the ~~malondialdehyde values (TBARS)~~ TBARS values, expressed as mg MDA Kg-1 of sample, obtained in the salmon muscle packed with the three films

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additivated with the natural products containing tocopherols compared to control film (Film 1). Results showed that lipid oxidation increased in all samples during storage but at different rates. Control test presented a maximum of oxidation of approximately 5.8 mg MDA kg^{-1}, while the samples packed with active films presented TBARS values ~~that varied over a range of~~ ranging within 3-4 mg MDA kg^{-1}.

Salmon packed with films spiked with antioxidant product **C** in the two concentrations of active ingredient applied at 1 and 5 % (Film 2 and Film 3 respectively), experimented an important lipid oxidation decrease, and the greatest antioxidant effect was verified at higher concentration ~~used~~ tested.

~~In the first days of assay, there were no important differences between the two concentrations used but after the 7th day differences were more remarkable.~~ At the first days of assay, no main differences between the two concentrations tested was observed, however, after the 7th day, differences were more remarkable. At 7th day, lipid oxidation was reduced around 25 % for Film 3, while for Film 2 this decrease was less than 10 % compared to control (Film 1). After day 11 of assay, Film 3 showed an oxidation decrease to around 40 %. In the same period less reduction, around 15 %, was observed for the Film 2**,** which at the end of the assay ~~oxidation~~ was decreased by 30 %. TBARS values of fish packed with Film 4 which contains 5 % of antioxidant product D also showed a significant reduction in lipid oxidation of salmon throughout entire assay compared to control, increasing food stability. During the first 7 days of study, a reduction around 40 % was observed to Film 4, and after that, oxidation decreasing remained constant around 30-35 % until the end of the assay.

Comparing the curves of the films spiked with the two products applied in this study at the same concentration (5 %), it can be pointed out that both films showed

522 important antioxidant effectiveness and there were ~~no important differences~~ no main
523 differences between the two compounds.

524 There was a small difference ~~in the first days~~ over the first days of analysis
525 where Film 4 (which contains TOCOBIOL®~~®~~ PV) was more effective than Film 3,
526 while at the end of the assay Film 3 is the most effective film. This may be due to the
527 high amounts of tocopherols presents in Film 3 (product C).

528 Active films used had shown an inhibitory effect on the lipid oxidation process
529 comparable to the effect observed with direct addition of antioxidants to the fish
530 muscle.

531 Results obtained with Films 2 and 3 confirm the effectiveness of compound C as
532 well as the higher concentration incorporated into the films greater the effectiveness of
533 them.

534 Results also confirmed the high antioxidant effect of compound D, which
535 although did not showed high antioxidant activity in the free radical scavenging method
536 (DPPH), it showed great effectiveness against salmon lipid oxidation.

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4. Conclusions

The results of antioxidant activity obtained by free radical scavenging DPPH method confirm that the natural products employed in this trial have important antioxidant effectiveness.

The order of free radical scavenging activity of the natural products tested was: NUTRABIOL®-T90 > TOCOBIOL® GL > NUTRABIOL®-T50 PV > TOCOBIOL® > TOCOBIOL® PV. Then, it can be said that considering free radical scavenging capacity, NUTRABIOL® products were the most active in both forms of powder or oil. These compounds constituted mainly by natural tocopherols could replace synthetic antioxidants in food industry.

The addition of these compounds extensively inhibited lipid oxidation of salmon muscle during the storage time, up to 70 %. These results indicate that these compounds could be used as food additives in order to prevent lipid oxidation during storage time.

After the incorporation of antioxidant products into plastic matrices, positive effects on reduction of lipid oxidation, up to 40 %, in fish were still observed, being able to be used for extending salmon shelf life.

These results indicate that these active films containing tocopherols could be used to develop a promising active packaging to be applied in food preservation by food industry.

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561

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Figure captions

Figure 1.

TGA thermograms of antioxidant compounds described in Table 1.

Figure 2.

OIT of LDPE blends described in Table 2.

Figure 3.

Lipid oxidation (TBARS) in salmon packed with antioxidant films stored at 4 °C during 21 days (Film 1: control; Film 2: 1% antioxidant C; Film 3: 5% antioxidant C; Film 4: 5% antioxidant D).

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e 1.

xidant products composition

tioxidant properties						**Powders with antioxidant properties**			
IOL®		**B – TOCOBIOL® GL**		**C – NUTRABIOL®-T90**		**D – TOCOBIOL®-PV**		**E – NUTRABIOL®-T50 PV**	
	18.6 %	Tocobiol:	25.0 %	Tocopherols	90.2 %	Tocobiol	65.0 %	Tocopherols	
	9.70 %	Tocopherols	18.6 %	Alpha-tocopherol	15.5 %	Tocopherols	18.6 %	Alpha-tocopherol	
	4.30 %	Sterols	9.70 %	Gamma and Beta-tocopherol	63.3 %	Sterols	9.70 %	Gamma and Beta-tocopherol	
des	19.8 %	Squalene	4.30 %	Delta-tocopherol	21.1 %	Squalene	4.30 %	Delta-tocopherol	
ble oil		Monoglycerides	19.8 %	Vegetable oil	9.80 %	Monoglycerides	19.8 %	Vegetable oil	
		Rest: vegetable oil				Rest: vegetable oil		Excipient:	
		Propyl gallate	25.0 %			Excipient:		Silica gel	
		Excipients:				Silica gel	35.0 %		
		Lecithin	35.0 %						
		Propylene glycol	15.0 %						

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Table 2.

LDPE formulations

	Natural antioxidants			Commercial antioxidants	
Formulations	C	D	E	IRGAFOS 168	IRGANOX 1076
LDPE 1	5%				
LDPE 2	1%				
LDPE 3	0.5%				
LDPE 4		5%			
LDPE 5		1%			
LDPE 6		0.5%			
LDPE 7			5%		
LDPE 8			1%		
LDPE 9			0.5%		
LDPE 10				0.2%	0.4%
LDPE 11	5%			0.2%	0.4%

Table 3.

Free radical scavenging activity

Antioxidant	EC_{50} (g L^{-1})
A	1.49 ± 0.075
B	0.435 ± 0.050
C	0.401 ± 0.062
D	3.25 ± 0.095
E	0.704 ± 0.047
BHT	2.53 ± 0.085
BHA	0.273 ± 0.025

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Table ~~3~~4a.

TBARS data obtained from salmon muscle in contact with different concentrations (30 and 125 mg dm^{-2}, for samples 1 and 2 respectively) of antioxidant oils (described in Table 1) during storage at 4 °C.

	TBARS (mg malondialdehyde kg^{-1} salmon muscle)						
Time (days)	Control	A1	B1	C1	A2	B2	C2
0	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12	0.31±0.12
3	2.2±0.21	0.83±0.41	1.2±0.12	2.2±0.41	0.61±0.05	0.49±0.09	2.4±0.38
7	3.9±0.17	2.6±0.04	0.57±0.08	2.0±0.29	0.68±0.09	0.63±0.10	2.9±0.16
10	3.9±0.17	3.5±0.14	1.0±0.21	2.4±0.05	1.1±0.25	0.79±0.12	2.3±0.01

Table ~~3~~4b.

TBARS values obtained from salmon muscle in contact with different concentrations (30 and 125 mg dm^{-2}, for samples 1 and 2 respectively) of antioxidant powders (described in Table 1) during storage time at 4 °C.

	TBARS (mg malondialdehyde kg^{-1} salmon muscle)				
Time (days)	Control	D1	E1	D2	E2
0	0.21±0.19	0.21±0.19	0.21±0.19	0.21±0.19	0.21±0.19
3	2.4±0.90	0.66±0.27	0.96±0.32	0.68±0.43	0.52±0.01
7	2.5±0.43	1.2±0.56	2.0±0.11	5.2±0.48	1.5±0.64

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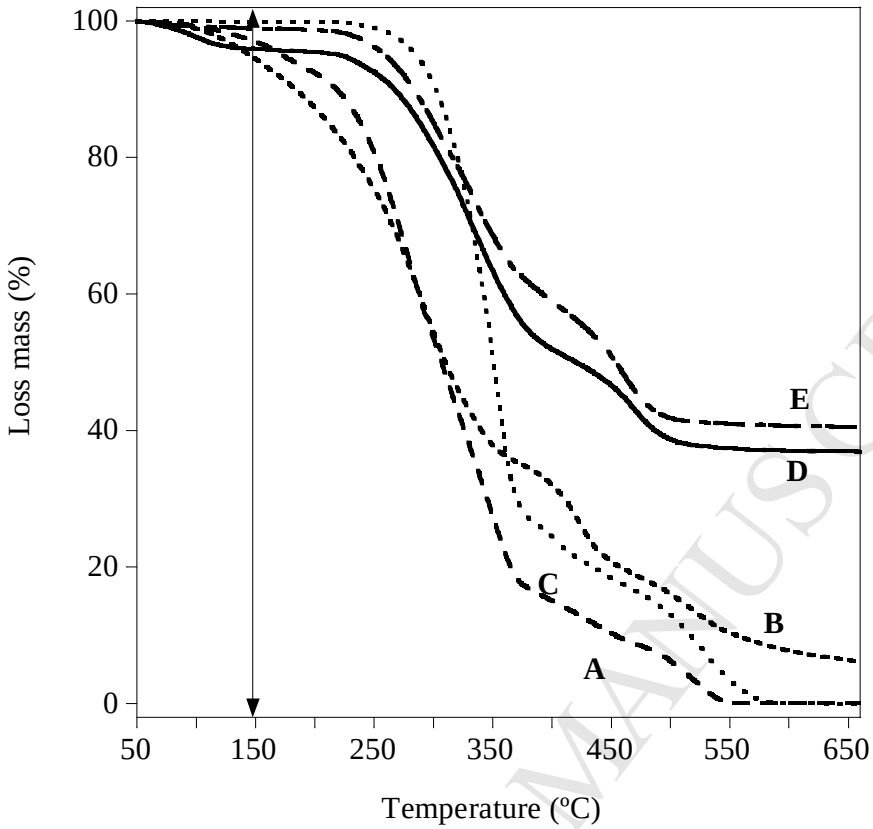

Figure 1.

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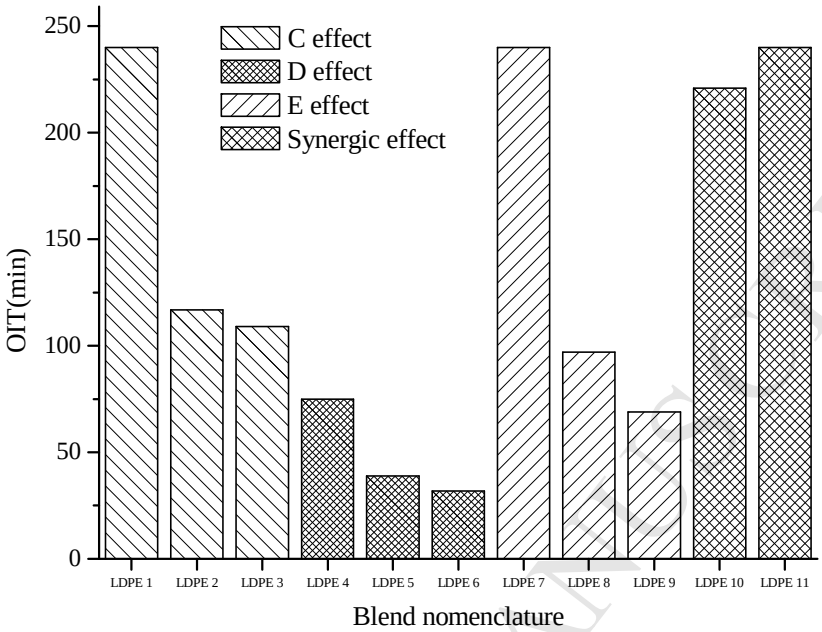

Figure 2

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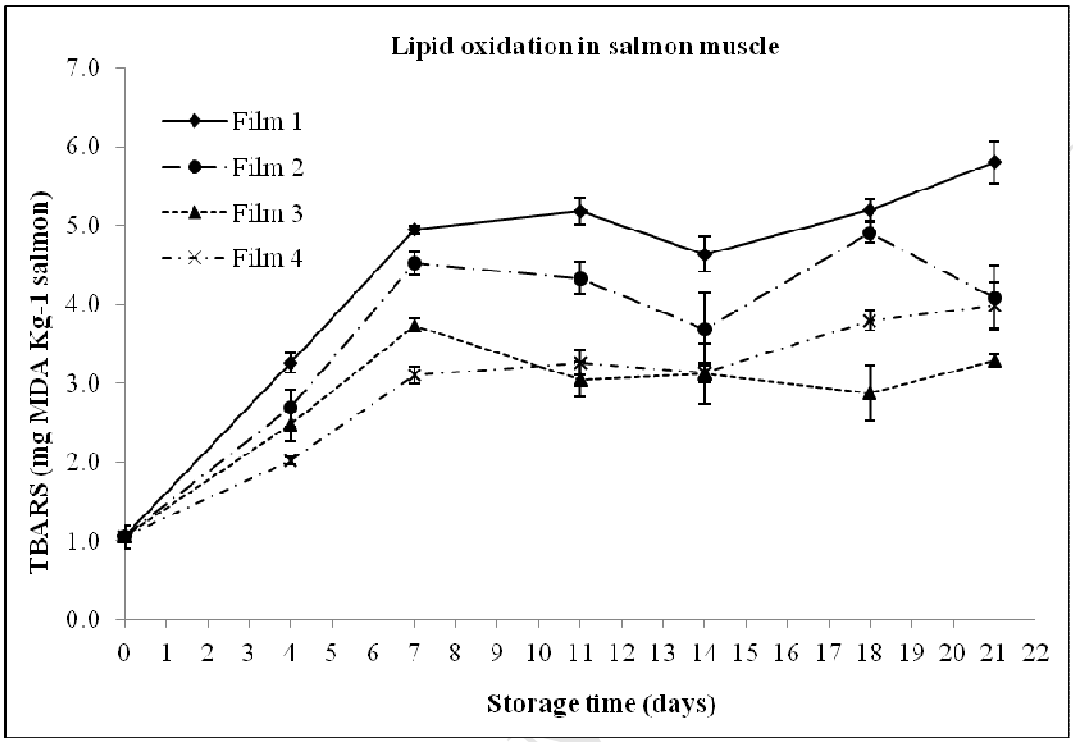

Figure 3.

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