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Effect of edible pectin-fish gelatin films containing the olive antioxidants hydroxytyrosol and 3,4-dihydroxyphenylglycol on beef meat during refrigerated storage.

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24

Abstract

The objective of this research was to evaluate the effect of the addition of two antioxidants naturally present in olives, hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG), to a pectin-fish gelatin edible film on the preservation of raw beef meat during refrigerated storage. A new composite film that included beeswax was also prepared, resulting in a reduction in the film's oxygen permeability. Results showed that the meat samples wrapped with film containing antioxidants reduced the formation of oxidation products in the form of thiobarbituric acid reaction substances (TBARS) compared with control film without antioxidants. HT added at 0.5% to the film with beeswax suppressed the lipid oxidation of beef meat during 7 days of storage at 4° C, possibly by the combined effect of acting as an oxygen barrier and the specific antioxidant activity. The interference of plasticizer agents (glycerol and sorbitol) incorporated to the film on the TBARS method was showed for the first time.

Keywords: Phenolic compound; pectin; skin fish protein; composite film; edible film; lipid oxidation; beeswax.

1. Introduction

Lipid oxidation is the major contributing factor to the deterioration of raw meat during storage, causing a reduction of quality and acceptability due to the development of an undesirable flavor and rancidity (Nielsen, Sørensen, Skibsted, & Bertelsen, 1997). Lipid oxidation can be controlled by the use of antioxidants. However, due to the potential health hazard of some synthetic antioxidants commonly used in the food industry, like butyl hydroxyanisole (BHA) or butyl hydroxytoluene (BHT), it may be desirable to replace these conventional antioxidants with natural anti-oxidative products.

Olives (*Olea europae*) and olive oil contain numerous polyphenols with excellent antioxidant properties. However, most phenols (about 98%) remain in the liquid-solid waste called *alperujo* that remains after the extraction of olive oil and only 2% are transferred to the oil. Therefore, *alperujo* is an abundant source of natural antioxidants. This olive oil by-product offers a practical and economic source of antioxidants that could replace synthetic substances for use in the food industry (Fernández-Bolaños, López, Fernández-Bolaños, & Rodríguez-Gutiérrez, 2008). In particular, hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) are two individual phenols abundant in olive fruit and olive oil with important antioxidant properties and their positive effects have been demonstrated on human health (Ciriminna, Meneguzzo, Fidalgo, Ilharco, & Pagliaro, 2016)(Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007).

Olive extract and hydroxytyrosol have been widely used in the food industry and many authors have reported their effectiveness in the reduction of the lipid oxidation of meat (Cofrades et al., 2011)(DeJong & Lanari, 2009) and fish (Medina, Sacchi, Biondi, Aubourg, & Paolillo, 1998)(Medina, Satué-Gracia, German, & Frankel, 1999)(Pazos, Alonso, Sánchez, & Medina, 2008). In contrast, few studies have been conducted until now on the functional properties of DHPG, an ortho-diphenol structurally similar to HT, with an additional hydroxyl in the β position. Moreover, most studies focused on the lipid oxidation

of meat or fish involve the direct addition of HT or phenolic extract to the muscle. To the best of our knowledge, the use of HT and DHPG as antioxidant components of edible films has not previously been reported.

Edible films and coatings help maintain the quality of meat products during their storage, mainly as a result of their ability to act as a water barrier, preventing dehydration, and an oxygen barrier, reducing lipid oxidation (Bonilla, Atarés, Vargas, & Chiralt, 2012)(Gennadios, Hanna, & Kurth, 1997). Furthermore, antioxidants can be added to the film formulation leading to better preservation since a high concentration of antioxidant is present on the surface of the meat (Gomez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009).

Films made from proteins and carbohydrates are excellent barriers to oxygen because of their tightly packed, ordered hydrogen-bonded network structure. However, in general, due to their hydrophilic nature, polymeric films generally exhibit limited water vapor permeability (WVP) barrier ability.

Pectin is a water-soluble hygroscopic polymer that is commonly used in the food and pharmaceutical industries as a thickening, coating and encapsulating material due to its gelling, stabilizing and thickening properties (Ahn, Halake, & Lee, 2017). However, relatively few studies have reported on the use of pectin as a source of edible coating for meat patties (Kang et al., 2007).

Gelatin is a protein based polymer widely used as a starting material for edible film formation. However, fish gelatin film showed poor water resistance properties (Nilsuwan, Benjakul, & Prodpran, 2016). Pectin-gelatin blended film offers advantages in terms of its mechanical properties and WVP with respect to films formed from pectin or protein alone (Di Pierro et al., 2013). In a previous work, we developed a pectin-gelatin film enriched with olive phenolic compounds HT or DHPG, with good mechanical and functional properties

that extended the shelf life of strawberries against molds with a significant delay in visible decay (Bermúdez-Oria, Rodríguez-Gutiérrez, Vioque, Rubio-Senent, & Fernández-Bolaños, 2017). This biopolymer-based film, combined with HT or DHPG, was an excellent oxygen barrier; however, its WVP was limited due to its hydrophilic nature. In this work, beeswax, a hydrophobic substance, was incorporated to assess the efficiency of the composite film to water-vapor transmission. Also, in the present study the development of pectin plus protein composite films with antioxidant capacity was studied in relation to meat preservation. The aim of this study was to evaluate and compare the antioxidant effect of two olive phenolic compounds (HT and DHPG), when added to pectin-fish gelatin films, on the oxidative stability of beef during refrigerated storage.

2. Materials and methods

2.1. Materials

HT and DHPG were extracted and purified from a by-product obtained from manufacturing of olive oil by a chromatographic system based on ion exchange, as described by Fernández-Bolaños, Guillén, Jiménez, Rodríguez, Rodríguez-Gutiérrez, & Lama-Muñoz. (2011) and Fernández-Bolaños et al. (2014). Citrus pectin with a high degree of esterification (53%), gelatin from fish skin (~95%), beeswax, and sorbitol were purchased from Sigma-Aldrich (St Louis, MO, USA). Glycerol was purchased from Panreac Química, S.A. (Barcelona, Spain). Commercial film of low-density polyethylene was acquired in a local supermarket.

2.2. Film preparation

Films were prepared by dissolving 0.5 g of citrus pectin and 0.5 g of fish gelatin in 20 ml of distilled water with continuous stirring at room temperature for 2 h. The pH of the mixture was first adjusted to 8 using 4 M NaOH then gradually reduced to 3–4 with 1 M HCl. To the polymer solution, 0.5g/g glycerol was added as a plasticizer and the mixture was stirred vigorously for 30 min. For the film with beeswax, 0.4 g/g beeswax was heated to 70 ° C until it melted and added to the polymer. The mixture was heated to 70 ° C and stirred vigorously for 15 min to allow correct homogenization of the beeswax mixture. After complete dispersion, the phenolic compounds HT and DHPG were added to the mixture at a concentration of 0.1–0.5% HT and DHPG with respect to dry polymers. Air bubbles were removed from the resultant gel by vacuum in kitasato during 1 h. The resultant gel was degassed and spread gently and very slowly on the surface of polypropylene film to avoid the formation of bubbles and then left to dry for 48 h at room temperature. The same methodology was used to prepare films containing no phenolic compounds (film control, without the addition of HT and DHPG).

2.3. Film thickness

Film thickness, expressed in μm , was measured using a micrometer (Baxlo 4000/Film) with an accuracy of 0.001 mm. Measurements were taken at five random sites on each film and for five films of each formulation. The mean and standard deviation were calculated for the evaluation of mechanical properties.

2.4. Oxygen permeability

The oxygen permeability (OP) of the films was analyzed using an oxygen transmission rate system (Mocom, USA) following the standard American Society for Testing and Materials method (ASTM, 2014). An effective area of 50 cm² was exposed to

permeation in test conditions of 25° C and 50% relative humidity. Assays were performed in triplicate and the mean values were expressed as $\text{cm}^3 \cdot \mu\text{m}/\text{m}^2 \cdot \text{d} \cdot \text{KPa}$.

2.5. Water vapor permeability

Water vapor permeability (WVP) was determined following the gravimetric method (ASTM, 2016). Film samples were sealed over a circular opening of an aluminum permeation container filled with anhydrous CaCl_2 . The cells were kept in a hermetically closed chamber containing an oversaturated solution of $\text{Mg}(\text{NO}_3)_2$ at 22 to 25° C in order to maintain a relative humidity difference of 95%. The WVP was determined from the slope obtained for the regression analysis of weight loss data versus time, once the steady state had been reached, divided by the film area. Results were expressed as $\text{g} \cdot \text{mm}/\text{h} \cdot \text{m}^2 \cdot \text{kPa}$. All tests were carried out at least in triplicate. An empty aluminum cup covered with the film was used as control.

2.6. Solubility of film components and bioactive compounds

All films were pre-dried in a desiccator for 48 h prior to testing. The solubility of the composite films was tested in water at different pH values. Specimens (4 x 2 cm) were placed in 50 mL of 0.1 M acetate (pH 4.0), phosphate (pH 7.2), or Tris-HCl (pH 8.5) buffer solutions for 1, 24 and 48 h. The rest of the specimens were removed, and the amounts of pectin and protein from the films that had solubilized into the different solutions were measured by the uronic acid assay (Blumenkrantz & Asboe-Hansen, 1973) and Bradford assay (Bradford, 1976), respectively. The HT/DHPG released in distilled water was determined by HPLC according to a previously published method (Rodríguez et al., 2007).

2.7. Preparation of meat samples

Fresh beef meat was purchased from a local market. The beef samples were obtained from topside muscle containing about 4.3 g of fat/100 g muscle. The beef meat was divided in pieces of 7 g (2 x 2 x 3 cm) and packaged with commercial film, with film without antioxidant or film with different concentrations of HT and DHPG and stored at 4 °C for 6-7 days. One sample was packed for each trial day.

2.8. Thiobarbituric acid reactive substances (TBARS) assay

Lipid oxidation was measured by the 2-thiobarbituric acid extraction method of Witte, Krause, & Bailey (1970) with modifications. Briefly, 1 g of meat sample was mixed with 3 mL of trichloroacetic acid (TCA) (75 g/L) and 10 µl of t-butyl-4-hydroxyanisole and homogenized using a homogenizer (Ultra-Turrax®T50 Basic, IKA, Germany) at a speed of 24,000 rpm for 2 min in an ice bath. The resultant slurry was centrifuged at 5900 g for 5 min and filtered through a 0.45 µm membrane. To a glass tube containing 1 mL of thiobarbituric acid (2.88 g/L), 1 mL of filtrate was added. The tubes were closed and heated at 80° C in a water bath for 90 min. TBARS values were calculated using the absorbance of each sample at 532 nm and a standard curve was constructed using 1,1,3,3-tetraethoxypropane (1–5 mg/L). TBARS values were expressed as mg malonaldehyde/kg meat (MDA mg/kg meat). All measures were carried out in triplicate.

2.9. Statistical analysis

Results were expressed as mean values $\pm$ standard deviations. STATGRAPHICS® plus software was used for statistical analysis. Comparisons amongst samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value of 0.05 was considered significant.

193 3. Results and discussion

194 3.1. Formation of thiobarbituric acid reactive substances (TBARS)

195 The pectin-gelatin based films, with and without beeswax added, containing natural
196 antioxidant (HT or DHPG) were evaluated for their effectiveness as a preservative coating
197 against oxidative changes to beef meat during a storage period of 6–7 days at 4° C. Lipid
198 oxidation of raw beef meat was monitored by measuring the formation of thiobarbituric acid
199 and reactive substances (TBARS), the secondary products of lipid oxidation, such as
200 malonaldehyde (MDA), which forms a pink color upon reaction with thiobarbituric acid.

201 The presence of a significant amount of hydrophilic plasticizer, such as glycerol,
202 incorporated into the film to impart adequate flexibility, showed a significant interference
203 with thiobarbituric acid, whose reaction produced a red color at the concentration (0.5 g/g of
204 polymer) utilized in our initial film preparation studies (Bermúdez-Oria et al., 2017). We
205 also assayed the formation of film with other plasticizer agents such as sorbitol, which
206 showed even greater interference than glycerol (Figure 1a). To the best of our knowledge,
207 this is the first time that plasticizer interference with thiobarbituric acid has been reported.

208 Since the film was very hygroscopic, it was not easy to remove the film completely from the
209 meat, a high moisture product, to make the measurement and we found that the
210 glycerol/sorbitol could migrate into the food with ease. As a result of this interference, we
211 observed an increase in TBARS values, which would mask the antioxidant effect of the
212 compounds and could explain certain apparently inconsistent results from other studies
213 (Coşkun, Çalikoğlu, Emiroğlu, & Candoğan, 2014)(Oussalah, Caillet, Salmiéri, Saucier, &
214 Lacroix, 2004). Thus, the corresponding absorbance of the plasticizer was subtracted in all
215 the determinations to avoid this interference. Two portions of the pectin-gelatin film (1/8
216 and 1/16) with and without beeswax incorporated (Figure 1b), corresponding with two

concentrations of glycerol and sorbitol (mg/ml of TCA) as plasticizer, were assayed using the TBARS method to assign a reference value to be subtracted. The data revealed that a higher concentration of plasticizer in the film formulation produced a greater difference in absorbance: sorbitol+beeswax > sorbitol ~ glycerol+ beeswax > glycerol. However, at low plasticizer concentration, there were practically no differences between glycerol and sorbitol or the addition of beeswax.

Next, we evaluated the influence of the antioxidant doses 0.1 and 0.5 g HT or DHPG/100 g of polymer, corresponding to 100 or 500 g of antioxidant/kg of meat, on the TBARS values of raw beef. Antioxidant incorporation into the edible films was effective against lipid oxidation (Figure 2). The pectin-gelatin control film without antioxidant revealed that the TBARS values increased significantly ($p < 0.05$) during the meat's storage. A similar result was observed for the commercial polypropylene film, with a somewhat smaller increase. TBARS values of the antioxidant film-covered beef were stable during the first three days of storage followed by a significant increase ($p < 0.05$) of lipid oxidation at day 6 at both doses assayed. Nevertheless, there was an improved lipid stabilization of 68 % for HT (0.5%) and of 59% for DHPG (0.5%) compared to the control sample film without antioxidant. Moreover, the effectiveness of HT on TBARS in beef meat was more important than DHPG. This result is in contrast to a previous study, which showed that DHPG was a more effective antioxidant than HT (Rodríguez-Gutiérrez et al., 2012). A film containing a mixture of HT and DHPG did not improve the reduction of TBARS compared to film with the individual antioxidants (data not shown). These results showed that the incorporation of natural antioxidants HT and DHPG in the pectin-gelatin film improved the protection of meat samples against lipid oxidation. The direct application of these compounds alone, as dip solution at the same concentration that in film, reduced the lipid oxidation only until 3 days.

The protective effect of antioxidants incorporated into a second film of pectin-gelatin with beeswax added for improved moisture transfer retardation was evaluated by the determination of TBARS (Figure 3). The meat wrapped with commercial film of low density polyethylene without antioxidant added showed an increase in TBARS formation between 24 and 48 hours storage, reaching a maximum of 1.6 mg malonaldehyde/kg of meat. A significant reduction in values was then observed from day 4 to the end of the 7-day storage period. A similar evolution was observed for the control pectin-gelatin based film sample without antioxidants, reaching a maximum of 1.9 mg malonaldehyde/kg of meat at day 4 of storage. HT and DHPG incorporation significantly ($p < 0.05$) reduced the formation of TBARS compared with the control film. No significant change in the TBARS levels of HT-coated beef meat were detected during the whole storage period; 0.5 g of HT/100g of polymer film totally suppressed lipid oxidation even after 7 days of storage. The only significant difference ($p < 0.05$) was observed between HT and DHPG on day 4 of storage. This fact confirmed the result of the first experiment that HT is a more effective antioxidant than DHPG for protecting beef meat from lipid oxidation.

The results of this study show that all the active films tested exhibited a protective effect on the lipid oxidation of beef. Also, HT showed an even greater antioxidant capacity when incorporated in film containing beeswax.

3.2. Film characterization

Our initial study (Bermúdez-Oria et al., 2017) on a composite edible film based on pectin and fish skin protein, with glycerol added as a plasticizer and HT or DHPG as active agents, showed that the film had adequate physical properties including a marked mechanical resistance and a good barrier to oxygen permeability ($11.2 \text{ cm}^3 \mu\text{m}/\text{m}^2 \text{ d kPa}$). However, the composite film did not reduce moisture loss and thus it was necessary to

incorporate hydrophobic substances such as beeswax to assess the efficacy of the film as a barrier to water. However, the results obtained still showed a poor permeability for water, and no significant differences were found between the film with or without beeswax. Nevertheless, the addition of beeswax improved the oxygen permeability, reducing this to a value of $8.92 \pm 1.11 \text{ cm}^3 \mu\text{m}^2 \text{ d kPa}$, a significant difference compared to the film without beeswax.

In this new composite film prepared with beeswax and HT or DHPG, we studied the release of film components (pectin and protein) and the bioactive compounds. The release of pectin and protein was measured after 1, 24 and 48 hours of incubation at different pHs (4, 7.2 and 8.5) (Figure 4). There were no significant differences between the release of pectin at the different pHs. For the film with beeswax, a slight decrease was observed in the release of solubilized pectin with respect to the film without beeswax (Bermúdez-Oria et al., 2017). Film without beeswax had a maximum release of pectin after 24 h incubation. While the film without beeswax had lower, pectin solubility at pH 4 and almost 100% of uronic acid was released at pH 7.2 following 48 h incubation. The amount of solubilized protein decreased significantly as pectin was released. The solubility of protein at pH 4 was practically null, which is in agreement with the formation of an electrostatic complex at a pH close to pectin's pKa (3.55–4.10) and protein's pI (4.8–5.2) (Liu, Fishman, & Hicks, 2007).

The release of phenolic compounds, HT and DHPG, from the two films with and without beeswax was also evaluated after a 60 min incubation in distilled water (Figure 5). The two film formulations showed a peak of phenol solubilization by 10 mins, with the complete release of HT at 10 min and 80% release of DHPG, with % release values for both remaining constant until 60 min. Therefore, the active migration of antioxidant compounds from the film to the meat, a high moisture product, should be quick and easy. However, it was not easy to remove the film completely from the meat to determine the amount of active

compound into the product, although it can be assumed that the active compounds pass to the meat in approximately 10 minutes.

The availability and stability of the bioactive compounds in the films were evaluated by the HPLC determination of HT and DHPG concentration present in the film during storage for up to 8 weeks in a desiccator. For this experiment, a film containing a mixture of HT and DHPG with and without beeswax was tested. The amounts of HT and DHPG remained constant in both films during 8 weeks of storage (results not shown). These results were consistent in part with those obtained with the DPPH[•] method (Table 1). However, curiously, the antiradical activity increased ($p < 0.05$) with the time of storage, from 5.62 and 6.25 mmol Trolox/g of antioxidant mixture in the film at week 0 to 13.7 and 12.9 mmol Trolox/g antioxidant at week 8 for films with and without beeswax, respectively (Table 1). Maillard reaction between gelatin and pectin, or its degradation products (Wegener, Bornik, & Kroh, 2015), or between gelatin and glycerol (Zhou, Guo, Liu, Liu, & Labuza, 2013) during the storage stage at room temperature could explain this increase of antiradical activity by the formation of antioxidant activity of Maillard reaction products (Phisut, & Jiraporn, 2013).

4. Conclusions

The bioactive edible film made of pectin and fish gelatin containing the natural antioxidants, HT or DHPG, was effective at delaying the lipid oxidation of raw beef meat during refrigerated storage. The use of these compounds, with several beneficial effect in health (ant inflammatory, anticancer, protector cardiovascular, prevention of diabetes, etc) is a good strategy to replace synthetic preservatives in meat. Also, in previous studies, this film exhibited adequate mechanical and oxygen barrier properties (Bermúdez-Oria et al., 2017). This study demonstrated that the addition of beeswax to the film formulation improved the

oxygen barrier property and enhanced the oxidative stability of beef relative to the film control, without natural antioxidant, during storage at 4° C. The best protective effect was obtained for film containing HT and beeswax, which reduced lipid oxidation by 100% during 7 days, possibly by the combined effect of the film acting as an oxygen barrier and the antioxidant protection of HT. The presence of plasticizer (glycerol and sorbitol) in the film could mask the antioxidant effects of the compounds by interference with the TBARS values, thus all data were corrected for this interference. As these preliminary results are promising, additional research will be required to improve the formulation to optimize the film's moisture content as well as the sensory properties of meat treated with edible film containing phenolic compounds present in olive fruit. Therefore, to get an edible active film with these antioxidants had a double benefits, a positive effect on the oxidation stability of meat fat and beneficial effect in the human organism.

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Declaration of interest

None.

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Figure Legends

Figure 1. Effect of plasticizer (mg/mL) on the absorbance value of TBARS method. (a) Influence of plasticizer incorporated in the edible pectin-gelatin film, with and without beeswax added, on the TBARS absorbance value. (b) Portions of 1/16 and 1/8 of a film with 3 g of polymer containing 1.5g of glycerol or sorbitol were solubilized in 3 mL of trichloroacetic acid.

Figure 2. Thiobarbituric acid reaction substances (TBARS) (mg malonaldehyde (MDA)/kg meat) in beef samples coated with edible pectin-gelatin film containing two concentrations of hydroxytyrosol (HT) or 3,4-dihydroxyphenylglycol (DHPG), compared to meat protected by film without antioxidants (control) and meat protected with a commercial polyethylene film, during storage at 4° C for 1 to 6 days. All studies were carried out in duplicate. Means for each storage period marked with * and # are statistically different ($p < 0.05$) with respect to commercial film and control film, respectively.

Figure 3. Thiobarbituric acid reaction substances (TBARS) (mg MDA/kg meat) in beef samples with edible pectin-gelatin film containing beeswax and hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) as active compounds, a control of meat with film without antioxidants, and a meat with a film commercial of polyethylene, during storage at 4 °C for 7 days. All studies were carried out in duplicate. Means for each storage period

marked with *, # are statistically different ($p < 0.05$) with respect to commercial film and control film, respectively.

Figure 4. Release (%) of pectin (a) and protein (b) from pectin-skin fish protein composite film with beeswax added at different pHs (4, 7.2, and 8.5) after 1, 24, and 48 h incubation at room temperature. Results are expressed as mean values and the bars indicate standard deviation.

Figure 5. Release (%) of hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) from pectin-skin fish protein composite film with and without beeswax in distilled water for 60 min. Results are expressed as mean values and the bars indicate standard deviation.

Table 1. Antiradical activity (DPPH[•]) of the film containing a mixture of HT and DHPG during storage in a desiccator during 8 weeks.

	mmolTrolox/ g of theoretical mixture HT + DHPG in film			
	Week 0	Week 1	Week3	Week 8
Film (pectin-fish gelatin + glycerol)	6.25 ± 0.026 ^a	8.71 ± 0.143 ^b	9.64 ± 0.791 ^b	12.9 ± 0.589 ^c
Film (pectin-fish gelatin + glycerol + beeswax)	5.62 ± 0.525 ^a	7.48 ± 0.892 ^a	8.51 ± 1.691 ^a	13.7 ± 0.861 ^b

Values are expressed as millimoles of Trolox per g of theoretical mixture of HT + DHPG present in the film. The radical scavenging capacity of HT + DHPG by the DPPH[•] method was 6.13 ± 0.10 mmolTrolox /g of HT+DHPG. Values are the means of a duplicate assay. Means bearing the same letter are not significantly different at the 5% level.

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