JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Physicochemical Properties of Natural Phenolics from Grapes and Olive Oil Byproducts and Their Antioxidant Activity in Frozen Horse Mackerel Fillets

MANUEL PAZOS,*,‡ ANA ALONSO,‡ JUAN FERNÁNDEZ-BOLAÑOS,§ JOSEP L. TORRES,† AND ISABEL MEDINA‡

Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain, Instituto de Investigaciones Químicas y Ambientales de Barcelona, IIQAB-CSIC, Jordi Girona 18-26, 08034-Barcelona, Spain, and Instituto de la Grasa (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain

The reducing and chelating capacities and the affinity for the incorporation into the fish muscle of grape procyanidins, hydroxytyrosol, and propyl gallate were studied together with their antioxidant activity in frozen horse mackerel (*Trauchurus trauchurus*) fillets. Fillets were supplemented with phenolic antioxidants by (a) spraying an aqueous phenolic solution, (b) glazing with an aqueous phenolic solution, and (c) a previous washing of fillets with water plus spraying an aqueous phenolic solution. The effect of washing on the endogenous pro-oxidant/antioxidant balance of the fillets was also determined. All phenolic compounds were effective delaying lipid oxidation in the fish fillets. The order of antioxidant efficiency in spraying and glazing was propyl gallate > hydroxytyrosol > procyanidins, which was similar to the reducing power of these phenolics, but did not show any correlation with their chelating capacity and their affinity to the fish muscle. Washing the fillets with water prior to spraying phenols increased synergistically the antioxidant activity of grape procyanidins and changed the relative antioxidant efficiency to propyl gallate ≈ procyanidins > hydroxytyrosol. This synergism may be a result of a better distribution of the procyanidins onto the fillet surface because of the residual water that remained on the fillets surface after washing.

KEYWORDS: Frozen fish fillets; oxidation; grape procyanidins; hydroxytyrosol; washing; glazing; synergism

INTRODUCTION

Fatty fish is a well-known nutritive food with a high concentration of healthy n-3 polyunsatured fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), which have shown potential benefits to human health, particularly preventing cardiovascular diseases (*1*, *2*). However, the storage and processing of this seafood are limited due to the liability of its lipids to develop lipid oxidation and therefore off-flavors associated to rancidity (*3*, *4*).

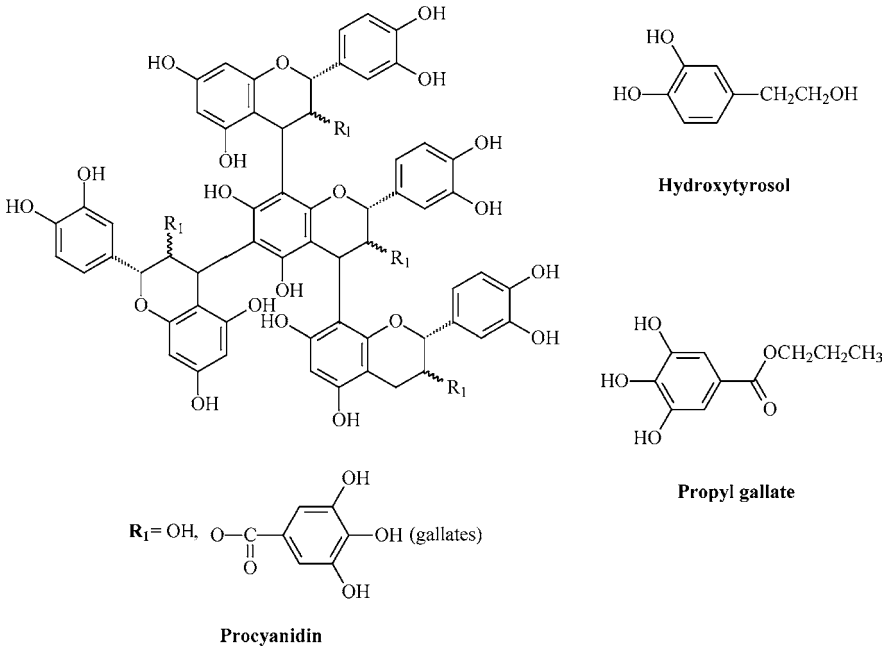
The high susceptibility of fatty fish to suffer oxidation is mainly associated with the simultaneous presence of high amounts of unsaturated lipids and potential activators of lipid oxidation, such as heme pigments and metallic traces (*5*, *6*). Rinsing with water or washing has been employed in chilled and frozen fish for removing heme proteins and metallic traces (*7*, *8*). However, endogenous hydrophilic antioxidants can be lost in these treatments leading to a higher susceptibility of the muscle tissue (*8*). Therefore, the balance between endogenous pro-oxidants and antioxidants must be carefully controlled. Fish tissues contain endogenous antioxidants that are able to stabilize their high content of unsaturated lipids in vivo. In post mortem conditions, the endogenous antioxidants are consumed sequentially (*4*, *9*), and some studies have related the loss of endogenous antioxidants with the development of oxidative rancidity (*10*−*12*).

In recent years, the addition of natural bioactive compounds obtained from plants and vegetables has been employed in an attempt to improve the oxidative stability in fish muscle. Tea catechins (*13*, *14*) and rosemary (*15*), olive oil (*16*), and ginger extracts (*17*) have successfully inhibited rancidity of different seafood. However, the lower effectiveness of natural antioxidants compared to synthetic antioxidants and the requirement of low cost production are important limitations for the practical use of many natural compounds as antioxidant additives in foods. In previous papers, we have reported a high antioxidant activity for hydroxytyrosol and grape procyanidins phenolics obtained from byproducts of olive oil and wine industry, in fish oil emulsions, and minced fish muscle (*18*, *19*). Additionally,

* To whom correspondence should be addressed [e-mail: mpazos@iim.csic.es; telephone: +34 986 231930; fax: +34 986 292762].
‡ Instituto de Investigaciones Marinas.
† Instituto de Investigaciones Químicas y Ambientales de Barcelona.
§ Instituto de la Grasa.

10.1021/jf0518296 CCC: $33.50
Published on Web 12/27/2005

Scheme 1. Structures of Phenolic Compounds Applied on Horse Mackerel Fillets



the potential action of grape procyanidins and hydroxytyrosol on health (*20*, *21*) is attractive for the formulation of functional foods.

The antioxidant activity in food depends not only on the chemical reactivity of the antioxidant (e.g., free radical scavenging and chelation) but also on factors such as the physical location, interactions with other food components, and environmental conditions (e.g., pH, temperature) (*22*). Since there are several factors influencing the antioxidant activity together with the complexity of muscle based food systems, the mechanism of action of added antioxidants continues to be unclear. The elucidation of the antioxidant mechanisms should provide a faster identification of effective antioxidants in muscle tissues and an optimization of their antioxidant effectiveness.

This work is aimed to advance the knowledge of the antioxidant mechanism of natural phenolics, grape procyanidins, and hydroxytyrosol in frozen fish fillets. The antioxidant activity of a synthetic antioxidant, propyl gallate, was also evaluated. The reducing and chelating capacities of phenolics and their affinity to link to the muscle were studied together with their antioxidant activity in the frozen fish fillets. Phenolic antioxidants were supplemented by (a) spraying the aqueous phenolic solution, (b) glazing with the aqueous phenolic solution, and (c) a previous washing step in water plus spraying the aqueous phenolic solution. This last procedure attempts to combine the removing of pro-oxidants substances, such as hemoglobin and metallic traces, with a later application of the phenolic compounds. Changes in the endogenous pro-oxidant/antioxidant balance as a result of washing fillets were also determined. The antioxidant activity of the phenolics was evaluated by means of the peroxide and TBARS values, sensory analysis, and their ability to maintain the levels of endogenous α-tocopherol and total glutathione.

MATERIALS AND METHODS

Materials. A total of 60 kg of fresh Atlantic horse mackerel (*Trachurus trachurus*), 180−200 fish, were supplied by a local market. Grape procyanidins were isolated from grape (*Vitis vinifera*) pomace by application of Toyopearl chromatography, a size-exclusion chromatography in Toyopearl, on the total phenolic fraction soluble in ethanol and ethyl acetate, as described by Torres et al. (*23*). Grape procyanidins employed contained a mixture of oligomers of catechins with mean polymerization and galloylation degrees of 2.7 units and 25%, respectively. **Scheme 1** shows the chemical structure of a procyanidin with four units, indicating the common positions for the esterification with the gallate group. Hydroxytyrosol (**Scheme 1**) was kindly provided by the Instituto de la Grasa (CSIC, Sevilla). Propyl gallate, ferrozine, bovine hemoglobin, $FeCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$ were supplied by Sigma (St. Louis, MO). 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) was obtained from Fluka (New-Ulm, Swizerland). All chemicals and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

Reducing Power of the Phenolic Compounds. The FRAP (ferric reducing/antioxidant power) method, which measures the reducing capacity as the ability for reducing Fe(III) to Fe(II), was used by adaptation of Benzie and Strain's procedure (*24*). The FRAP reagent was prepared daily by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride, in the ratio 10:1:1, respectively. TPTZ solution was prepared in 40 mM HCl. A total of 1.5 mL of FRAP reagent was incubated for 10 min at 37 °C. Then, 150 μL of water and 50 μL of phenolic solution (4−200 mg/L) were added, and the absorbance was measured at 593 nm after 4 min. The standard curve was built with ferrous chloride. The number of donated electrons was calculated from the slopes of the lineal adjustments between the phenolic concentration and the FRAP activity.

Chelating Activity of the Phenolic Compounds. The capacity of the phenols for chelating ferrous iron was determined using an adaptation of Kolayli et al.'s (*25*) procedure. A total of 0.2 mL of phenolic solution (4−400 mg/L) was mixed with 1.2 mL of 0.12 M KCl, 5 mM L-histidine solution (pH 6.8), and 0.2 mL of 0.2 mM ferrous chloride. Then, 0.4 mL of 1 mM ferrozine was added, and the samples were incubated at room temperature for 10 min. Then, the absorbance at 560 nm was measured, and the chelating capacity was calculated according to the following equation:

$$\text{chelating capacity (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100$$

where A_0 represents the absorbance of the blank without phenolic compounds, A_1 represents the absorbance of the sample with phenolic compound, and A_2 is the absorbance of the blank composed by phenolic compound and the ferrous iron, which removes the interferences generated by phenol−Fe(II) complex. EDTA was employed as standard.

Affinity of the Phenolics for the Fish Muscle. A total of 1 g of horse mackerel minced light muscle was mixed with 1 mL of an aqueous phenolic solution (approximately 1 mg/mL) by vigorously vortexing for 1 min. The samples were centrifuged, and the phenolic concentration was determined in the aqueous phase by the Folin−Ciocalteu method (*26*). To avoid interferences with fish proteins, they were removed before phenolic quantification by precipitation with freeze saturated ammonium sulfate solution. Affinity of phenolics for the fish muscle was calculated by the difference between the phenolic concentration before and after the addition of fish muscle. The samples were prepared by duplicate.

Fillets Supplemented with Phenolics. Grape procyanidins, hydroxytyrosol, and propyl gallate were applied on horse mackerel skin-on fillets (20−25 g) using three different procedures: spraying, glazing, and washing plus spraying.

Spraying. A total of 0.5 mL of an aqueous phenolic solution (5 mg/mL) was sprayed on the flesh side of each fillet. Then, fillets were held during 15−30 min to allow the diffusion of the phenolic solution in the flesh before freezing.

Glazing. Unhanded fillets were previously frozen at −80 °C. After 6 h of frozen storage, 1.25 mL of a chilled aqueous phenolic solution (2 mg/mL) were sprayed on the frozen fillet for forming a thin glazing layer. Then, the fillets were immediately frozen at −80 °C for 6 h.

Washing Plus Spraying. Fillets were washed in a container with distilled water for 1 min. The ratio fillet/water was 1:5.3 (w/w). Then, the fillets were held in an inclined surface for 1 min in order to eliminate the water excess, and they were sprayed with 0.5 mL of an aqueous phenolic solution (5 mg/mL). They were held during 15−30 min to allow the diffusion of the phenolic solution in the flesh before freezing.

The final concentration of phenolics in the fillets was 100 ppm (mg/kg muscle). The fillets supplemented with phenolics were compared with control fillets not subjected to any handle and with control fillets of each treatment, which were prepared replacing the phenolic solution by deionized distillate water.

The fillets were introduced into plastic bags and were initially kept at −80 °C for 6 h to obtain a faster freezing and then were stored at −10 °C. Three fillets for each antioxidant procedure were taken at several sampling times and were thawed at room temperature 1 h prior to lipid oxidation analysis. Lipid oxidation was monitored in the light muscle by the methods described below.

Sensory Analysis. A total of four panelists trained in descriptive analysis of fishy off-flavors sniffed the whole fillets that were used for chemical determinations. The fillets were placed in separate sterile polystyrene Petri dishes and put on ice. Panelists concentrated in detecting the rancid odors (*7*).

Removal of Heme Pigments from the Fillets During the Washing Step. The amount of heme pigments removed from the fillets was directly measured in the washing-water. The heme pigments were quantified according to Brown (*27*). A total of 1 mg of sodium dithionate was added to 2.0 mL of washing-water, and subsequently, carbon monoxide gas was bubbled for 20 s. Then, the sample was scanned from 460 to 400 nm (Soret region) in a Beckman Coulter DU 640 spectrophotometer (Beckman Instruments Inc, Palo Alto, CA). The absorbance was recorded, and the difference between the absorbance at the peak (420 nm) and the absorbance at the valley (440 nm) was calculated. A standard curve was built with hemoglobin (Sigma) from bovine blood. The initial levels of heme pigments in the light muscle were determined by a previous extraction of heme pigments from untreated fillets as described Richards and Hultin (*5*) and subsequent quantification as described above.

Determination of Total Glutathione. Glutathione was extracted from fillets using a modification of Petillo et al.'s (*4*) procedure. A total of 2 g of muscle was homogenized with 10 mL of chilled 5% 5-sulfosalicylid acid. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were quantified together as total glutathione using the method described by Griffith (*28*). Reduced glutathione (Sigma) was used as standard.

Determination of α-Tocopherol. α-Tocopherol was extracted by an adaptation of Burton et al.'s (*29*) procedure as described by Pazos et al. (*12*). α-Tocopherol was analyzed by HPLC according to Cabrini et al. (*30*).

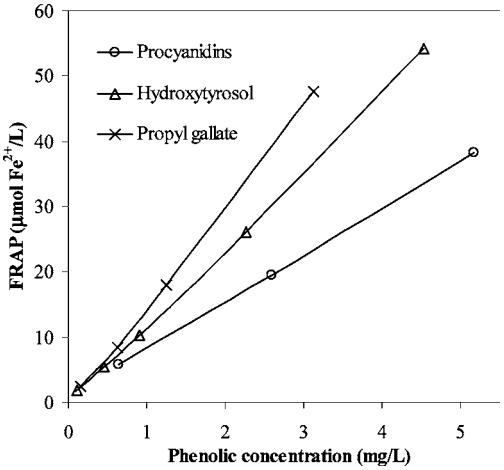


Figure 1. Reducing capacity of the phenolic compounds evaluated by the FRAP method.

Lipid Extraction. Lipids were extracted from fish muscle according to Bligh and Dyer (*31*). Lipid content was determined gravimetrically and expressed on wet weight basis (*32*).

Peroxide Value. Peroxide value was determined by the ferric thiocyanate method (*33*) and expressed as milliequivalents of oxygen/kg lipid.

Thiobarbituric Acid Reactive Substances (TBARS) Analyses. TBARS analyses (μmol malonaldehyde (MDA)/kg muscle) were determined according to Vyncke (*34*).

Synergism Evaluation. Synergistic effects of washing fillets (W) and spraying phenolics (S) on the oxidative stability of the fillets were calculated on the basis of the induction periods (IP) of peroxides and TBARS values (*22*):

$$\text{synergism (\%)} = 100 \times [(IP_{W+S}) - (IP_W + IP_S)/(IP_W + IP_S)]$$

Induction periods were calculated as the time (in weeks) required for a sudden change of the oxidation rate by the method of tangents in the two parts of the kinetic curve (*22*, *35*). Synergism in the inhibition of rancid odors was calculated considering, in the equation shown above, the first moment (in weeks) when the rancid odors were detected. Synergism in the preservation of α-tocopherol and total glutathione was evaluated considering the time in which the levels of these endogenous antioxidants decreased up to 50% of the initial values (approximately 60 μg α-tocopherol/g lipid and 15 μg total glutathione/g muscle).

Statistical Analysis. The analyses were performed at least in duplicate, and the results were expressed as the mean ± standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) (*36*), and the means were compared by a least squares difference method (*37*).

RESULTS

Reducing and Chelating Capacity of the Phenolic Compounds. One possible antioxidant mechanism of phenolics could be through electronic donation from phenolic hydroxyl groups to the free radicals involved in lipid oxidation. The reducing capacity measures the ability of the compounds for donating electrons (**Figure 1**). Hydroxytyrosol showed higher reducing power than grape procyanidins, and both natural phenols were less efficient reducing agents than propyl gallate. Propyl gallate was able to donate 15.4, hydroxytyrosol 11.9, and procyanidins 7.2 μmol of electrons per milligram of compound, respectively.

Phenolic compounds could also delay lipid oxidation via inactivation of metallic promotion of lipid oxidation by chelating metals. **Figure 2** shows the capacity of the phenols for chelating ferrous iron, which is the most pro-oxidant redox state of iron. Grape procyanidins and hydroxytyrosol showed similar chelating

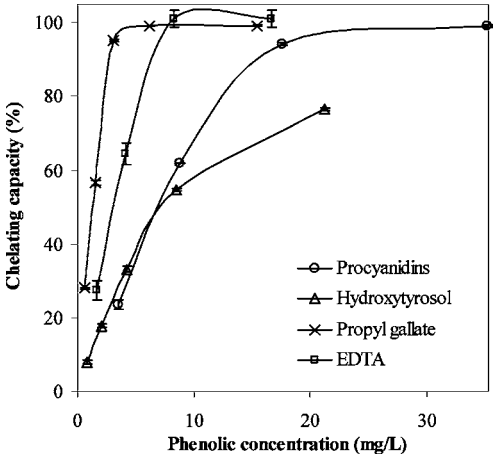


Figure 2. Chelating capacity of the phenolic compounds for the ferrous iron evaluated by the ferrozine method.

Table 1. Storage Time Expressed in Weeks at Which Rancid Odors Were Detected in Frozen Horse Mackerel Fillets[a]

treatment	rancid odors detection (weeks)
control	3
S control	3
S Proc	13
S Hyd	17
S PG	21
G control	5
G Proc	10
G Hyd	13
G PG	17
W+S control	3
W+S Proc	not detected after 21 weeks
W+S Hyd	21
W+S PG	not detected after 21 weeks

[a] Abbreviations defined as in Figure 3.

properties at lower phenolic concentrations while an increment of phenolic concentration produced higher effectiveness of grape procyanidins than hydroxytyrosol. Propyl gallate was significantly the phenolic compound with the highest chelating capacity.

Affinity of the Phenolics for the Fish Muscle. Grape procyanidins and propyl gallate showed an elevate affinity for the fish muscle. These phenolics were totally transferred from the aqueous solution to the fish muscle. Hydroxytyrosol showed the lowest affinity for the fish muscle, given that only a 28.7 ± 1.1% of the hydroxytyrosol was relocated in the muscle.

Endogenous Pro-oxidant/Antioxidant Balance of Washed Horse Mackerel Fillets. The washing of fillets removed 0.50 ± 0.02 μmol heme pigments per kilogram of muscle, expressed on hemoglobin basis. Total levels of glutathione of unwashed and washed fillets were 24.7 ± 0.7 and 24.3 ± 1.0 μg/g muscle, respectively. Therefore, glutathione was not significantly removed from fillets during washing. These results suggest that the washing step slightly reduced the endogenous pro-oxidants/antioxidants ratio of the fish fillets.

Antioxidant Activity of the Phenolic Compounds in Frozen Horse Mackerel Fillets. The light muscle of horse mackerel contained 2.0 ± 1.0% of total fatty content and 7.6 ± 2.1 μmol per kilogram of muscle of content in heme pigments, expressed on hemoglobin basis. The development of rancid odors was delayed in fillets supplemented with grape procyanidins and hydroxytyrosol (**Table 1**). The controls (without phenolics) showed a faster generation of rancid odors than the corresponding fillets with phenolics. By spraying or glazing, the efficiency for preserving the sensorial scores was propyl gallate (PG) > hydroxytyrosol > procyanidins. However, washing plus spraying phenolics significantly improved the antioxidant effect of procyanidins, which was better than that of hydroxytyrosol. The fillets treated with procyanidins or propyl gallate were preserved without rancid odors during 21 weeks of storage at −10 °C, while rancid odors were detected at week 21 in the fillets treated with hydroxytyrosol. Sensory scores also indicated that washing plus spraying fillets was the most effective treatment for the inhibition of rancid odors (**Table 1**). Moreover, spraying phenolics was a better treatment for the preservation of sensory attributes than glazing.

According with the sensory analysis, the addition of procyanidins and hydroxytyrosol delayed the generation of peroxides (**Figure 3**) and TBARS values (**Figure 4**). The induction periods of peroxides and TBARS formation demonstrated that the order of activity was propyl gallate > hydroxytyrosol > grape procyanidins ($p < 0.05$), in both spraying and glazing treatments. With the washing plus spraying procedure, the order of inhibitory activity was propyl gallate ≈ grape procyanidins > hydroxytyrosol. As regards to the suitability of the treatments for applying phenolics, the kinetics of peroxides and TBARS values indicated the following relative order of antioxidant efficiency: washing + spraying > spraying > glazing (**Figure 3** and **Figure 4**).

Figure 5 shows the α-tocopherol concentration after storage. The supplementation of the fillets with 100 ppm of the phenolic compounds was effective for delaying the loss of α-tocopherol. With the spraying and glazing treatments, the efficiency on the inhibition of α-tocopherol loss was propyl gallate > hydroxytyrosol > procyanidins. Again, procyanidins were more active than hydroxytyrosol in the washed−sprayed fillets. Additionally, the application of procyanidins or hydroxytyrosol with the washing plus spraying treatment was more effective for the α-tocopherol preservation than the spraying or glazing procedures. The three applications using propyl gallate showed similar behaviors in the preservation of α-tocopherol after 17 weeks.

The phenolics also inhibited the loss of total glutathione (**Figure 6**). Spraying procyanidins and glazing hydroxytyrosol were the less effective phenolic treatments delaying the loss of glutathione ($p < 0.05$), and there was no significant difference among the fillets subjected to the other treatments ($p > 0.05$).

A relevant aspect was the huge enhancement of antioxidant effectiveness of grape procyanidins when they were applied on washed fillets. In fact, the combination of a washing step plus spraying procyanidins showed a synergistic effect on the oxidative stability of the fillets (**Figure 7**). This synergism on the inhibition of the generation of peroxides and TBARS and on the preservation of α-tocopherol reached 60%. The calculated synergism on the inhibition of the generation of rancid odors and on the preservation of glutathione was over 30%. Still, the synergism for the inhibition of rancid odors could be higher since they were not detected in the fillets subjected to washing plus spraying with procyanidins after 21 weeks of frozen storage. There was an improvement in the antioxidant activity of hydroxytyrosol and propyl gallate when they were applied on fillets previously washed, but this improvement was not synergistic (**Figure 7**).

DISCUSSION

Frozen storage efficiently restrains microbial growth, but lipid oxidation is not sufficiently inhibited. In this experience, rancid odors were detected in the unhanded horse mackerel fillets after

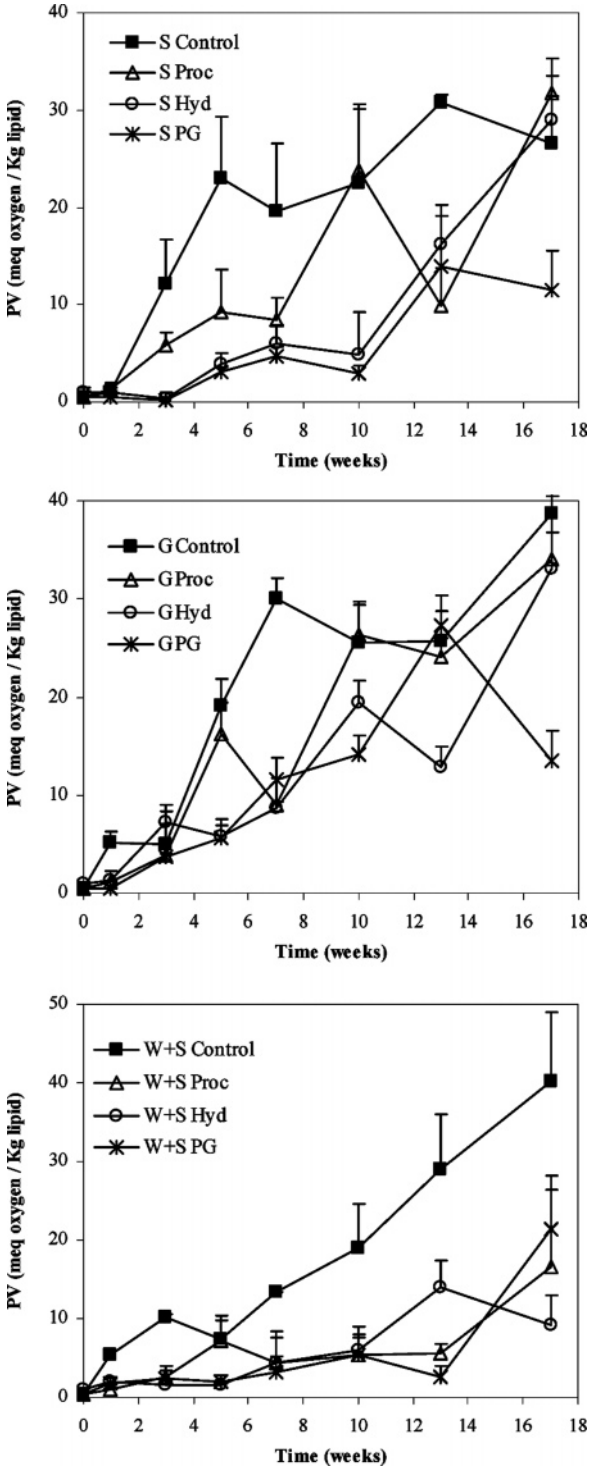


Figure 3. Peroxide values in horse mackerel fillets during frozen storage at −10 °C. Fillets were subjected to spraying (S), glazing (G), and washing plus spraying (W+S) and supplemented with procyanidins (Proc), hydroxytyrosol (Hyd), and propyl gallate (PG).

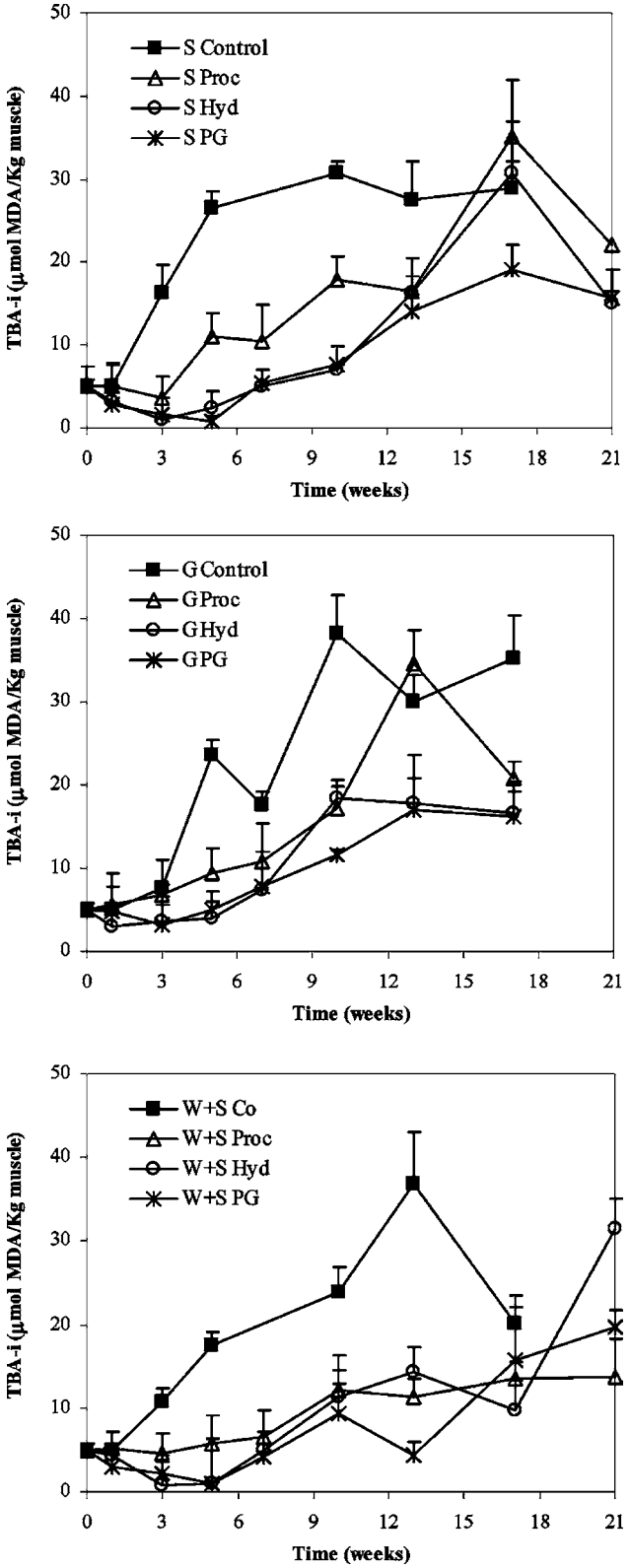


Figure 4. TBARS values in horse mackerel fillets during frozen storage at −10 °C. Abbreviations defined as in Figure 3.

3 weeks of storage at −10 °C. This high susceptibility of the horse mackerel fillets for lipid oxidation was significantly decreased by the supplementation of grape procyanidins or hydroxytyrosol. The two procedures employed for applying these phenols, the direct application by spraying (spraying) or their incorporation in the glazing layer (glazing), were able to include efficiently the phenolics into the fish fillets and consequently there was an inhibition of lipid oxidation. The enhancement of oxidative stability of frozen bonito fillets by glazing with tea extracts have been also reported previously (*38*). However, the addition of the phenols by spraying was more effective than their incorporation in the glazing layer. It is probably due to a better adsorption and penetration of the phenolic compounds in the surface of unfrozen fillets than in the frozen ones.

The decrease of the endogenous pro-oxidant/antioxidant ratio in the washed fillets was not enough for delaying the generation of rancid odors (**Table 1**). However, the washing of fillets

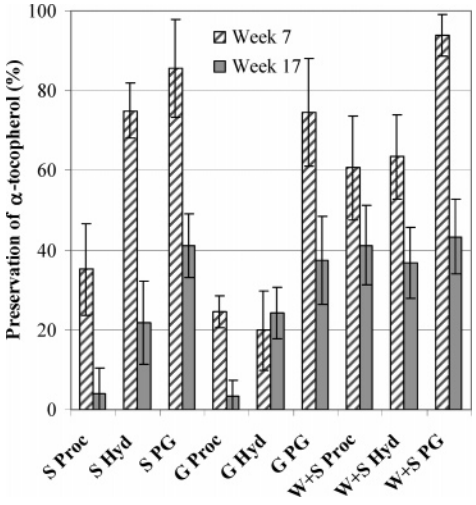


Figure 5. Preservation of initial endogenous α-tocopherol in horse mackerel fillets after 7 and 17 weeks of storage at –10 °C. Abbreviations defined as in Figure 3.

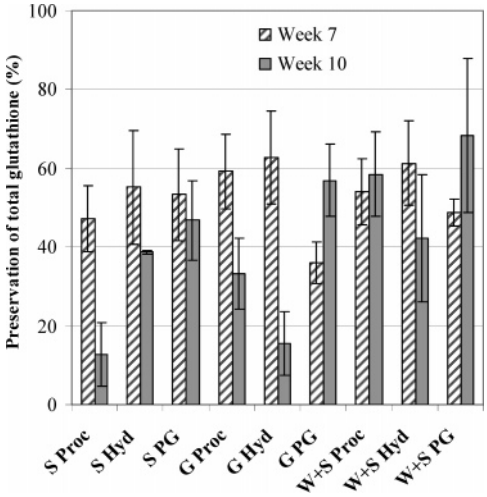


Figure 6. Preservation of initial endogenous total glutathione in horse mackerel fillets after 7 and 10 weeks of storage at –10 °C. Abbreviations defined as in Figure 3.

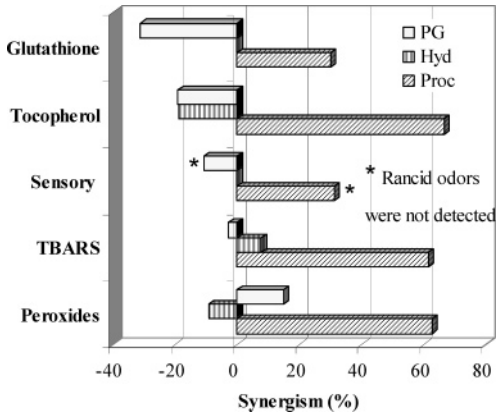


Figure 7. Synergistic effect of combining a previous washing step with spraying of procyacindins, hydroxytyrosol, or propyl gallate, on the preservation of endogenous α-tocopherol, total glutathione, and the inhibition of rancid odors, peroxide values, and TBARS values in horse mackerel fillets during frozen storage at –10 °C.

followed by spraying phenols improved significantly the oxidative stability of the fish compared to fillets only sprayed with phenols, especially in the case of grape procyanidins (**Figure 7**). The synergism found between the washing step and the addition of grape procyanidins could be related to a better distribution and penetration of the procyanidins on the fillet as a result of the residual water that remains on the fillet surface after washing. As a consequence of our washing procedure, 0.3–0.4 g of water was incorporated onto the fillets, based on the gain of fillet weight after washing.

The higher water solubility of grape procyanidins compared to that of propyl gallate (*19*) and the lower mobility of the grape procyanidins than that of hydroxytyrosol, since procyanidins are more strongly incorporated into the fish muscle compared to hydroxytyrosol, could explain this synergistic effect of combining washing with the spraying of procyanidins on the inhibition of lipid oxidation.

The antioxidant activity of the phenolic compounds can be attributed to the reducing capacity, the chelation of free iron, and/or the regeneration of some endogenous antioxidants, such as the α-tocopherol (*18*, *39*, *40*). The antioxidant efficiency in fillets in which the treatment only comprises the addition of phenolic compounds (spraying and glazing fillets), was propyl gallate > hydroxytyrosol > procyanidins, and it matches with the reducing activity measured with the FRAP method (**Figure 1**). Propyl gallate showed the highest chelating activity, and grape procyanidins showed better chelating abilities than hydroxytyrosol at the higher concentrations of phenolics (**Figure 2**). Therefore, these data suggest that the antioxidant activity of phenolics in muscle is more influenced by the properties for donating electrons than by their chelating properties.

The distribution of the antioxidants in the food system can also hugely affect their effectiveness. A selective incorporation of antioxidants into the oxidation-sensitive sites should improve its antioxidant efficiency. The membranes, where phospholipids are localized, are suggested as the more susceptible location for developing lipid oxidation in tissues (*41*, *42*). This consideration is based on their high unsaturated fatty acid content, their large surface area, and the presence of cytosol, which contains catalytic amounts of pro-oxidant compounds, such as metallic traces and enzymes. Our results on the partition of the phenolics between fish muscle and water showed that grape procyanidins and propyl gallate are greatly incorporated onto fish muscle. This fact can be explained because of the presence of gallate groups in the phenolic structure increasing phenol–protein interactions and their incorporation into the membranes (*43*). Hydroxytyrosol was less incorporated onto fish muscle according to its weak bonds to food proteins (*44*) and its much lower affinity to the membranes than grape procyanidins and propyl gallate (data not shown). These results demonstrated that there was not a direct relation between the affinity of phenolics for membranes and the antioxidant efficiency found in horse mackerel fillets in the spraying and glazing procedures: propyl gallate > hydroxytyrosol > grape procyanidins. This finding could be motivated by the difficultness of phenols to reach the membranes in the whole fillet, and phenol–protein interactions could play an important role in this last issue. Recent works have also shown the importance of the antioxidant carrier solvent which can affect the distribution of lipid-soluble antioxidants between membranes and the triacylglycerol fraction of muscle tissue and therefore influence oxidative stability (*45*, *46*). More research is needed for optimizing the effective incorporation of phenolic compounds into fatty components of tissues, particularly in membranes.

In conclusion, grape procyanidins and hydroxytyrosol have been successfully employed as antioxidants in frozen horse mackerel fillets. A simple spraying of the phenolic compounds

on the fillets showed high antioxidant efficiency, in particular using hydroxytyrosol. However, a previous washing of fillets improved the antioxidant effectiveness of phenols, especially of grape procyanidins that showed an activity similar to the synthetic propyl gallate, keeping the frozen fillets free of rancid odors for more than 21 weeks at −10 °C. The results also suggest that the reducing capacity of these phenols is more important for the antioxidant efficiency in frozen fish fillets than their chelating capacity and their affinity to be incorporated into the muscle. Moreover, the antioxidant potency of a given compound can be optimized by improving its distribution into the tissue. This work demonstrated that, for the entire knowledge of the antioxidant mechanism involved in complex food systems or tissues supplemented with additives, physicochemical aspects must be studied together with the inhibitory efficiency achieved.

LITERATURE CITED

(1) Bang, H. O.; Dyerberg, J.; Nielsen, A. B. Plasma lipid and lipoprotein pattern in Greenlandic west-coast Eskimos. *Lancet* **1971**, *1*, 1143−1146.

(2) Dyerberg, J.; Bang, H. O.; Stoffersen, E.; Moncada, S.; Vane, J. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis. *Lancet* **1978**, *2*, 117−119.

(3) Hsieh, R.; Kinsella, J. Oxidation of polyunsaturated fatty acids: Mechanisms, products and inhibition with emphasis on fish. *Adv. Food Res. Nutr. Res.* **1989**, *33*, 233−241.

(4) Petillo, D.; Hultin, H. O.; Krzynowek, J.; Autio, W. R. Kinetics of antioxidant loss in mackerel light and dark muscle. *J. Agric. Food Chem.* **1998**, *46*, 4128−4137.

(5) Richards, M. P.; Hultin, H. O. Contribution of blood and blood components to lipid oxidation in fish muscle. *J. Agric. Food Chem.* **2002**, *50*, 555−564.

(6) Undeland, I.; Hultin, H. O.; Richards, M. P. Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *J. Agric. Food Chem.* **2002**, *50*, 6847−6853.

(7) Richards, M. P.; Kelleher S. D.; Hultin, H. O. Effect of washing with or without antioxidants on quality retention of mackerel fillets during refrigerated and frozen storage. *J. Agric. Food Chem.* **1998**, *46*, 4363−4371.

(8) Undeland, I.; Ekstrand, B.; Lingnert, H. Lipid oxidation in minced herring (Clupea harengus) during frozen storage. Effect of washing and precooking. *J. Agric. Food Chem.* **1998**, *46*, 2319−2328.

(9) Undeland, I.; Hall, G.; Lingnert, H. Lipid oxidation in fillets of herring (Clupea harengus) during ice storage. *J. Agric. Food Chem.* **1999**, *47*, 524−532.

(10) Erickson, M. C. Compositional parameters and their relationship to oxidative stability of channel catfish. *J. Agric. Food Chem.* **1993**, *41*, 1213−1218.

(11) Jia, T.-d.; Kelleher, S. D.; Hultin, H. O.; Petillo, D.; Maney, R.; Krzynowek, J. Comparison of quality loss and changes in the glutathione antioxidant system in stored mackerel and bluefish muscle. *J. Agric. Food Chem.* **1996**, *44*, 1195−1201.

(12) Pazos, M.; González, M. J.; Gallardo, J. M.; Torres, J. L.; Medina, I. Preservation of the endogenous antioxidant system of fish muscle by grape polyphenols during frozen storage. *Eur. Food Res. Technol.* **2005**, *220*, 514−519.

(13) He, Y.; Shahidi, F. Antioxidant activity of green tea and its catechins in a fish meat model system. *J. Agric. Food Chem.* **1997**, *45*, 4262−4266.

(14) Tang, S. Z.; Sheehan, D.; Buckley, D. J.; Morrissey, P. A.; Kerry, J. P. Anti-oxidant activity of added tea catechins on lipid oxidation of raw minced red meat, poultry and fish muscle. *Int. J. Food Sci. Technol.* **2001**, *36*, 685−692.

(15) Vareltzis, K.; Koufidis, D.; Gavriilidou, E.; Papavergou, E.; Vasiliadou, S. Effectiveness of a natural Rosemary (Rosmarinus officinalis) extract on the stability of filleted and minced fish during frozen storage. *Food Res. Technol.* **1997**, *205*, 93−96.

(16) Medina, I.; Satue-Gracia, M. T.; German, J. B.; Frankel, E. N. Comparison of natural polyphenol antioxidants from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. *J. Agric. Food Chem.* **1999**, *47*, 4873−4879.

(17) Fagbenro, O.; Jauncey, K. Chemical and nutritional quality of fermented fish silage containing potato extracts, formalin or ginger extracts. *Food Chem.* **1994**, *50*, 383−388.

(18) Pazos, M.; Sánchez, L.; Medina, I. α-Tocopherol oxidation in fish muscle during chilling and frozen storage. *J. Agric. Food Chem.* **2005**, *53*, 4000−4005.

(19) Pazos, M.; Gallardo, J. M.; Torres, J. L.; Medina, I. Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chem.* **2005**, *92*, 547−557.

(20) Matito, C.; Matorakou, F.; Centelles, J. J.; Torres, J. L.; Cascante, M. Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7. *Eur. J. Nutr.* **2003**, *42*, 43−49.

(21) Fabiani, R.; De Bartolomeo, A.; Rosignoli, P.; Servili, M.; Montedoro, G. F.; Morozzi, G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G(1) cell cycle arrest and apoptosis. *Eur. J. Cancer Prev.* **2002**, *11*, 351−358.

(22) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 1998.

(23) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, C.; Sort, X.; Bobet, R. Valorization of grape (Vitis vinifera) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548−7555.

(24) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(25) Kolayli, S.; Ocak, M.; Kucuk, M.; Abbasoglu, R. Does caffeine bind to metal ions? *Food Chem.* **2004**, *84*, 383−388.

(26) Singleton, V. L.; Rossi, J. A. J. Colorimetry of total phenolics with phosphomolybdic-phosphotungtic acid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144−158.

(27) Brown, W. D. Chromatography of myoglobin on diethylaminoethyl cellulose columnas. *J. Biol. Chem.* **1961**, *236*, 2238−2240.

(28) Griffith, O. W. Determination of glutathione and glutathione disultide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **1980**, *106*, 207−212.

(29) Burton, G. W.; Webb, A.; Ingold, K. U. A mild, rapid, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* **1985**, *20*, 29−39.

(30) Cabrini, L.; Stefanelli, C.; Fiorentini, D.; Landi, L. Ubiquinol prevents α-tocopherol consumption during liposome peroxidation. *Biochem. Int.* **1991**, *23*, 743−750.

(31) Bligh, E. G.; Dyer, W. J. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911−917.

(32) Herbes, S.; Allen, C. Lipid quantification of freshwater invertebrates: method modification for microquantitation. *Can. J. Fish Aquat. Sci.* **1983**, *40*, 1315−1317.

(33) Chapman, R. A.; Mackay, K. The estimation of peroxides in fats and oils by the ferric thiocyanate method. *J. Am. Oil Chem. Soc.* **1949**, *26*, 360−363.

(34) Vyncke, W. Direct determination of the thiobarbituric acid value in trichloracetic acid extracts of fish as a measure of oxidative rancidity. *Fette Wiss Technol.* **1970**, *72*, 1084−1087.

(35) Alaiz, M.; Hidalgo, F. J.; Zamora, Z. Comparative antioxidant activity of Maillard- and oxidized lipid-damage bovine serum albumin. *J. Agric. Food Chem.* **1997**, *45*, 3250−3254.

(36) Sokal, R.; Rohlf, F. *Biometry*; Freeman and Co.: San Francisco, CA, 1981.

(37) Statsoft. *Statistica for Macintosh.* Statsoft and its Licensors: Tulsa, OK, 1994.

(38) Lin, C.-C.; Lin, C.-S. Enhancement of the storage quality of frozen bonito fillets by glazing with tea extracts. *Food Control* **2005**, *16*, 169−175.

(39) Zhu, Q. Y.; Huang, Y.; Tsang, D.; Chen, Z. Y. Regeneration of α-tocopherol in human low-density lipoprotein by green tea catechin. *J. Agric. Food Chem.* **1999**, *47*, 2020−2025.

(40) Zhou, B.; Wu, L.-M.; Yang, L.; Liu, Z.-L. Evidence for α-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Biol. Med.* **2005**, *38*, 78−84.

(41) Gandemer, G. Lipids and meat quality: lipolysis, oxidation, Maillard reaction and flavour. *Sci. Aliments* **1999**, *19*, 439−458.

(42) Igene, J. O.; Pearson, A. M. Role of phospholipids and triglycerides in warmed-over flavor development in meat model systems. *J. Food Sci.* **1979**, *44*, 1285−1290.

(43) Caturla, N.; Vera-Samper, E.; Villalain, J.; Mateo, C. R.; Micol, V. The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biol. Med.* **2003**, *34*, 648−662.

(44) Pripp, A. H.; Vreeker, R.; Van Duynhoven, J. Binding of olive oil phenolics to food proteins. *J. Sci. Food Agric.* **2005**, *85*, 354−362.

(45) Raghavan, S.; Hultin, H. O. Model system for testing the efficacy of antioxidants in muscle foods. *J. Agric. Food Chem.* **2005**, *53*, 4572−4577.

(46) Raghavan, S.; Hultin, H. O. Effect of carrier solvent polarity on selective incorporation of exogenous delta-tocopherol into muscle membranes. *J. Muscle Foods* **2005**, *16*, 117−125.

Received for review July 27, 2005. Revised manuscript received November 24, 2005. Accepted November 28, 2005. The authors acknowledge financial support from Xunta de Galicia (PGIDIT02AL40201PR) and Spanish Ministry of Science and Technology (PPQ2003-06602-C04-03 and -01 and Ph.D. grant for Manuel Pazos).

JF0518296