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Galloylated Polyphenols as Inhibitors of Hemoglobin-Catalyzed Lipid Oxidation in Fish Muscle

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ABSTRACT: The influence of galloyl residues on the antioxidant mechanism of polyphenols to prevent hemoglobin-promoted lipid oxidation was investigated by using polyphenolic fractions with different degrees of galloylation: nongalloylated structures from pine bark (IVP), medium-galloylated from grape pomace (IVG), and high-galloylated from witch hazel bark (IVH). Hemoglobin (Hb) from the pelagic fish horse mackerel (*Trachurus trachurus*) was employed as a Hb standard. In vitro experiments showed an important increase in the deoxygenation and autoxidation of horse mackerel Hb at acidic pH values. All polyphenolic fractions significantly reduced the redox stability of Hb in buffer solutions, showing a greater deoxygenation and methemoglobin (metHb) formation in the presence of IVH, followed in decreasing order by IVG and IVP. However, galloylated polyphenols (IVH and IVG) were efficient to inhibit the oxidation of the oxygenated Hb (OxyHb) and the formation of lipid oxidation products in chilled washed fish muscle. This antioxidant activity of galloylated proanthocyanidins showed a positive relationship with the phenolic concentration. Polyphenols devoid of galloyl groups (IVP) were less active to prevent either Hb oxidation or lipid oxidation in fish muscle. The results draw attention to the potential role of galloyl residues to lessen Hb-catalyzed lipid oxidation in muscle and to maintain Hb in reduced and oxygenated states, which exhibit lower pro-oxidant activity as compared to the metHb and deoxyHb species.

KEYWORDS: Lipid oxidation, hemoglobin, methemoglobin, fish muscle, natural proanthocyanidins, galloylation

INTRODUCTION

Pelagic fish muscle is more susceptible to lipid oxidation than other foods due to the critical coexistence of highly oxidizable polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid and docosahexaenoic acid,[1] and substances with a strong ability to initiate lipid oxidation, such as redox-active metals and hemeproteins.[2] Lipid oxidation leads to the rapid development of rancidity and potential toxic products, causing final rejection by consumers and low utilization for human food applications. For that reason, fishery and fish-processing industries are demanding effective treatments to retard lipid oxidation in pelagic fish muscle.

The incorporation of natural polyphenols during the processing or storage of seafood has emerged as one of the most common strategies to prevent lipid oxidation. Polyphenolic compounds provide important advantages as antioxidant food ingredients: (i) They can be employed at relatively low concentrations due to their high antioxidant activity, (ii) they are abundant in low-cost raw materials like agro-forestal byproducts, and (iii) some of them possess functional character in the prevention of human diseases. Among the potential pathways involved in the antioxidant activity of phenols, radical-scavenging and metal-chelating abilities have been thoroughly investigated, and comprehensive information about the structural factors implicated in such antioxidant mechanisms is available.[3,4] The ability of several phenolics to establish redox cycles with endogenous antioxidants in low-density lipoproteins[5,6] and fish muscle[7] is also well documented. Structure−activity relationships to regenerate endogenous α-tocopherol have been reported for monomeric and oligomeric catechins,[8,9] benzoic-derived acids,[10] and cinnamic-derived acids,[7] being in part linked to their capacity to donate electrons/hydrogen atoms to α-tocopheroxyl radicals. Endogenous ascorbic acid also seems able to regenerate exogenous caffeic acid in fish muscle.[7] These cooperative redox interactions have been related with synergistic antioxidant effects of phenolics.[11] Phenolic compounds have also demonstrated potential in deactivating the pro-oxidant activity of hemeproteins as Hb/myoglobin,[12,13] but the factors that regulate this inactivation are essentially unknown.

Several investigations point out deactivation of the pro-oxidant activity of Hb as a decisive factor to prevent deleterious lipid oxidation in pelagic fish. Published information and data obtained in our laboratory reveal that representative pelagic species such as mackerel and herring contain typically from 3 to 12 μmol of Hb per kg of muscle, in contrast with a lean fish as cod that possesses 0.2 μmol of Hb per kg.[2,14] The pro-oxidant activity of Hb is concentration-dependent, detecting an extensive promotion of lipid oxidation for the Hb levels found in pelagic species.[2] Moreover, fish Hb is significantly more active promoting lipid oxidation than those from terrestrial animals.[15] Recent investigations have evidenced an intimate relationship between the pro-oxidant ability of fish Hb and its vulnerability to be oxidized to metHb[16,17] and that certain exogenous antioxidants can be successfully used to maintain Hb in its reduced state in fish muscle.[18] In previous studies, we have demonstrated that galloylation (content of esterified galloyl groups) influences decisive physicochemical parameters involved in the antioxidant mechanisms of polyphenols (electron-donating capacity, ferrous-chelating ability, tocopherol-regenerating

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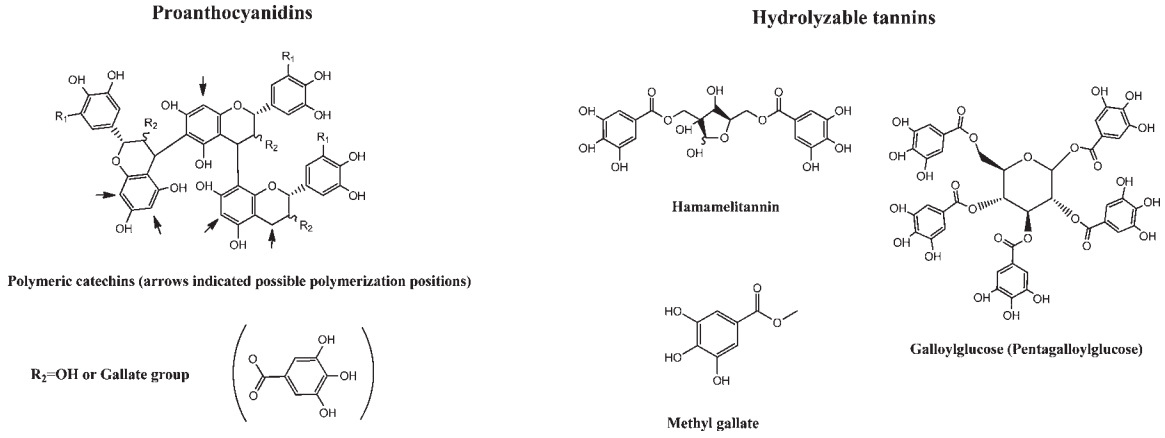

Figure 1. Chemical structures of proanthocyanidins and hydrolyzable tannins.

activity, and lipophility), modulating in the last instance their antioxidant activity depending on the food system.[9,19] Galloylated polyphenols have also been successfully employed to retard Hb-catalyzed oxidation,[13,20] although the effect of galloyl groups on the redox stability of Hb and on its pro-oxidant activity has not been fully clarified.

The aim of the present study was progress in knowledge of the antioxidant mechanism of natural polyphenols, particularly by studying the inhibitory effect of gallate groups on fish Hb-mediated lipid oxidation. For this purpose, polyphenolic extracts with different galloylation from pine bark, grape pomace, and witch hazel bark were selected. The pelagic fish species horse mackerel (*Trachurus trachurus*) was chosen as a source of Hb, and its redox stability was compared with that from a commercial lean fish species such as hake (*Merlucius merlucius*). In vitro experiments were first performed to investigate differences in the capacity of non-, medium- and high-galloylated polyphenols to maintain Hb in a ferrous-reduced state. Then, polyphenols were tested as inhibitors of Hb-catalyzed oxidation in washed fish mince, and such antioxidant activity was related with the effect of the polyphenols on the redox stability of Hb. Washed fish muscle preserves the fundamental structure of fish muscle but contains very low levels of hydrophilic pro-oxidants that can be added controllably.[14]

MATERIALS AND METHODS

Materials and Chemicals. Fresh Atlantic horse mackerel (*T. trachurus*) and hake (*M. merlucius*) were acquired in a local market and presented an extra quality of freshness.[21] Bovine Hb, sodium heparin, sodium chloride, tris[hydroxymethyl]aminomethane (Tris), sodium dithionate, dibasic sodium phosphate ($Na_2HPO_4 \cdot 2H_2O$), thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetraethoxypropane (TEP), butylated hydroxytoluene (BHT), $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, streptomycin sulfate, L-histidine, and potassium chloride (KCl) were purchased from Sigma (Steinheim, Germany). Carbon monoxide was provided by Air Liquide (Porriño, Spain). All chemicals and solvents used were either analytical or high-performance liquid chromatography (HPLC) grade (Merck, Darmstadt, Germany).

Isolation of Polyphenolic Extracts. The polyphenolic fractions from pine (*Pinus pinaster*) bark, grape (*Vitis vinifera*) pomace, and witch hazel (*Hamamelis virginiana*) bark, labeled as IVP, IVG, and IVH, respectively, were obtained by fractionation of a crude polyphenolic extract soluble in both ethyl acetate and water according to Torres and co-workers.[22−24] Briefly, fractions IVP, IVG, and IVH were isolated by applying size exclusion chromatography on Toyopearl resin to the corresponding crude extract. Mean degrees of polymerization and galloylation of proanthocyanidins were estimated by HPLC analysis after depolymerization with cysteamine.[25] IVG and IVP were mainly composed by proanthocyanidins with similar polymerization degrees (2.7−2.9 catechin units/molecule), but they differed in the percentage of galloylation. IVP and IVG contained, respectively, 0.0 and 0.25 mols of galloyl groups per mol of molecule (Figure 1). The witch hazel fraction, IVH, was composed of 20% proanthocyanidins and 80% hydrolyzable tannins, fundamentally hamamelitannin, methyl gallate, and galloyl glucoses with 5−10 galloyl moieties (Figure 1).[26] Such a composition rich in galloylated hydrolizable tannins rendered a high galloylation level superior than 1 galloyl group per molecule.

Preparation of Fish Hb. Fish blood was taken from the caudal vein of the different fish species, in rigor mortis state, after the tail was cut off. Blood was collected with a transfer glass pipet rinsed with 150 mM NaCl and sodium heparin solution (30 units/mL), and it was immediately mixed with 1 volume of the saline sodium heparin solution. Hemolysate was prepared by following the modification by Richards and Hultin[27] of the procedure of Fyhn et al.[28] Hb was stored at − 80 °C and was thawed just before use.

Quantification of Fish Hb. Hb levels were determined according to Brown.[29] Briefly, the hemolysate was diluted 100 times in 50 mM Tris, pH 8.6, and 3 mL of this solution was transferred to a cuvette. Then, approximately 1 mg of sodium dithionite was added and mixed. The mixture was bubbled with carbon monoxide gas for 20 s and was immediately scanned from 440 to 400 nm against a blank using a spectrophotometer model Beckman DU 640 (Beckman Instruments, Inc., Palo Alto, CA). The peak at 418 nm was recorded. Standard curves were plotted using bovine Hb standard.

Measuring the Relative Oxygenation and Autoxidation of Hb. Hemolysate of the different species was diluted until an Hb concentration of 10 μM in 0.12 M potassium chloride and 5 mM histidine buffer was reached. Polyphenolic extracts were incorporated at 18.5 μg/mL. The ratio between the concentrations of Hb and polyphenols was approximately 34:1 (w/w), which is similar to that found in pelagic muscle with high Hb content (13.5 μmol Hb/kg) and supplemented with 25 μg/g of phenolic concentration. Changes in spectra from 360 to 640 nm were determined in the presence or absence of polyphenolic fractions using a spectrophotometer model Beckman DU 640 (Beckman Instruments, Inc.). Hb oxygenation was estimated as the difference

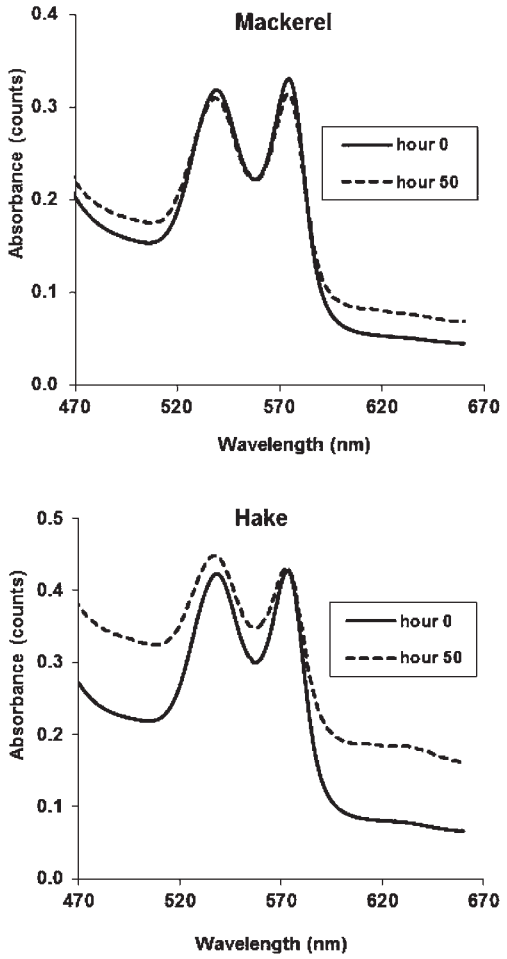

Figure 2. UV−vis spectra of Hb from horse mackerel and hake at time 0 and after 50 h of storage to 4 °C. Hemeproteins were dissolved in 0.12 M potassium chloride and 5 mM histidine buffer solutions (pH 6.8) to a final concentration of 10 μM.

between the peak absorbance at 576 nm and the "valley" at 560 nm.[30] The relative rate and extent of Hb autoxidation were determined from the formula proposed by Winterbourn[31] that estimates the concentration of metHb in micromolar (on Hb basis) considering absorbances at 576 and 630 nm:

$$[\text{MetHb}] = \left(\frac{279A_{630} - 3A_{576}}{4}\right)$$

Preparation of Washed Fish Muscle. Washed muscle from Atlantic horse mackerel (*T. trachurus*) was prepared according to Richards and Hultin.[27] Briefly, light muscle was obtained from fresh individuals of horse mackerel, and after three washes with distilled water, the muscle was finally homogenized with a buffer 50 mM sodium phosphate, 0.12 M potassium chloride, and 5 mM histidine, pH 6.8. Finally, washed fish muscle was frozen at −80 °C for less than 1 week. The muscle was thawed for 30 min in a sealed plastic bag under running cold water. Then, muscle was supplemented with streptomycin sulfate (200 μg/g) to inhibit microbial growth, and 15 μmol/kg fish of horse mackerel Hb was added as lipid oxidation initiator. After the addition of the different antioxidants, portions of 8 g were placed into 50 mL Erlenmeyer flasks and stored at 4 °C on ice. Controls were samples without polyphenolic supplementation. Triplicate samples were taken at different sampling times.

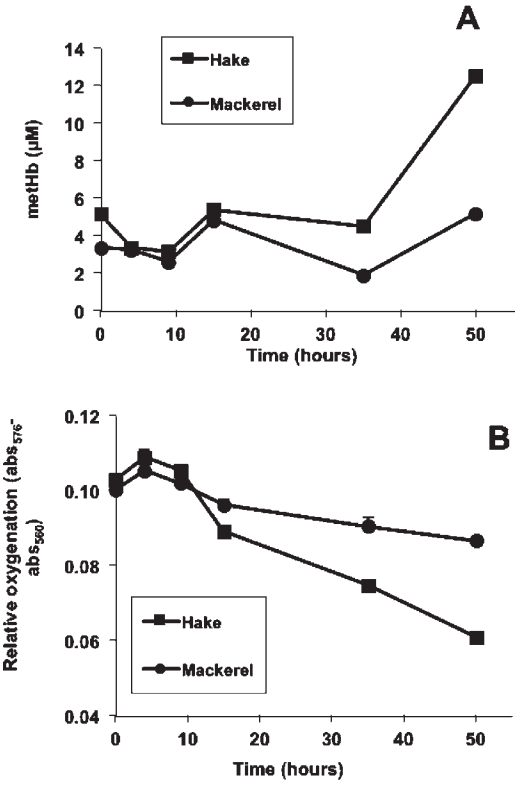

Figure 3. Kinetics for the formation of metHb (A) and relative deoxygenation (B) during storage of Hb solutions from horse mackerel and hake. Hb solutions (10 μM) were prepared in 0.12 M potassium chloride and 5 mM histidine buffer (pH 6.8) and stored at 4 °C.

Lipid oxidation was monitored by means of peroxide value (PV), thiobarbituric acid reactive substances index (TBARS), and sensory analysis. Induction periods of oxidation were calculated as the time (in days) required for a sudden change in the rate of the oxidation by the method of tangents to the two parts of the kinetic curve.[32] The oxidative stability of Hb was investigated by following the loss of the colorimetric parameter redness (a^*) in the washed fish muscle.

Peroxide Value (PV). Lipid peroxides contained in washed fish muscle were determined according to the adaptation by Buege and Aust[33] of the ferric thiocyanate method suggested by Chapman and McKay.[34] Results were expressed as milliequivalents of oxygen per kg of lipid.

TBARS. The TBARS index was determined according to McDonald and Hultin.[35] The standard curve was constructed with TEP that renders malondialdehyde (MDA) in aqueous solution. Data were expressed in mg MDA/kg muscle.

Loss of Redness. Changes in the colorimetric parameter redness (a^*) of the washed fish muscle samples were measured using a colorimeter (Minolta Chroma Meter CR-200, Minolta Corp., Osaka, Japan). Samples were introduced in transparent plastic bags, and analyses were carried out by pressing the probe against the container surface. Measurements were carried out in different locations of the sample, and an average value was used in further calculations.

Sensory Analysis. Sensory analysis was evaluated by an expert panel formed by four trained specialists in descriptive analysis of fishy off-flavors, in a room designed for the purpose, after placing the samples for 10 min at room temperature. The fish muscle contained in each Erlenmeyer flask was placed in separate sterile polystyrene Petri dishes and put on a tray of ice. The odor of fish muscle was classified based on the intensity of the rancid off-flavors: fresh, no rancid, incipient rancid, and rancid.

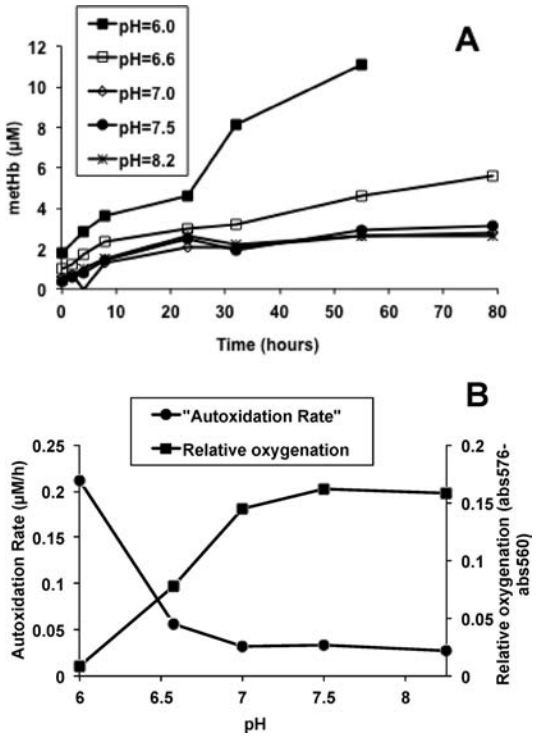

Figure 4. Effect of pH on the formation of metHb (A), autoxidation rate, and initial relative oxygenation (B) of horse mackerel Hb. Hb samples were prepared in 0.12 M potassium chloride and 5 mM histidine buffer solutions at a final concentration of 10 μM.

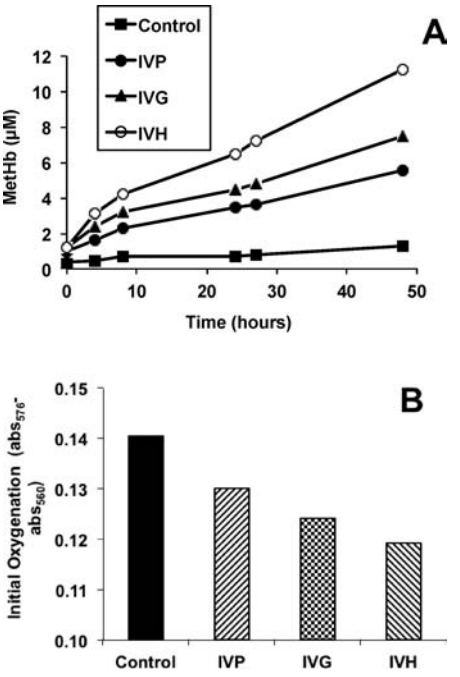

Figure 5. Influence of different polyphenolic fractions differing in galloylation on the formation of metHb (A) and the relative initial oxygenation (B) of horse mackerel Hb. Experiments were carried out in buffer solutions (pH 6.8) stored at 4 °C. Final concentrations of Hb and antioxidant were 10 μM and 18.5 μg/mL, respectively.

Statistical Analysis. Analyses were realized by triplicate, and the data were compared by one-way analysis of variance (ANOVA). The means were compared by a least-squares difference method with Statistica 6.0 program (Statsoft, Tulsa, Oklahoma).

RESULTS AND DISCUSSION

Oxidative Stability of Atlantic Horse Mackerel Hb. Previous studies have revealed important differences in the pro-oxidant capacity of Hb depending on the fish species, finding a direct relationship between the pro-oxidant activity and their vulnerability to be oxidized to Fe(III)-metHb.[16,17] Additionally, the resistance to either spontaneous or forced oxidation to metHb has been related with its state of oxygenation. The loss of the oxygen molecule coordinated to the heme group increments the accessibility of oxidants to the iron atom.[17,27] The following in vitro experiments were focused on characterizing the level of oxygenation and the rate of spontaneous metHb generation (autoxidation) for horse mackerel Hb. The redox stability of horse mackerel Hb was then compared to that from hake Hb, a commercial lean fish species.

Visible spectra showed higher redox stability for horse mackerel Hb as compared to hake Hb (Figure 2). Hake Hb exhibited an extensive increment of absorbance at 630 nm, a wavelength at which metHb has a strong absorption,[31] after 50 h of incubation at pH 6.8. Horse mackerel Hb showed a lower increment of absorbance at 630 nm. The kinetics of metHb formation also evidenced a much faster autoxidation for hake as compared to horse mackerel Hb. After 35 h of incubation at pH 6.8, the concentration of metHb for hake and horse mackerel was found to be 4.5 and 1.8 μM, respectively, values that represent the 45 and 18% of total Hb (Figure 3A). This tendency found for the autoxidation rates is in agreement with the rapid deoxygenation for hake Hb and the minor redox stability of deoxygenated Hbs (Figure 3B). Our data show similarity with previous studies that reported faster autoxidation rates for Hb from Atlantic pollock, a gadidae fish species such as hake, in comparison to horse mackerel Hb.[17] Other pelagic fish species like herring and mackerel showed similar or lower autoxidation rates than cod and pollock.[30,36] Taking into consideration the direct connection between the redox instability of Hb and its pro-oxidant activity, the present results point out that the extensive lipid oxidation developed in pelagic fish is more due to the abundant presence of Hb in its flesh (pelagic fish contain about 15−50-fold more Hb as compared to lean fish) than to a distinctive high pro-oxidative capacity of Hb from pelagic species.

The oxygenation state of fish Hb is commonly pH-dependent as consequence of the so-called Bohr effect that describes the diminution of Hb oxygenation with increasing H^+ concentration.[37] Considering that muscle tissues undergo naturally postmortem pH reduction due to the anaerobic glycolitic metabolism,[38] the Bohr effect could increment the proportion of deoxygenated Hb in postmortem fish muscle, compromising Hb redox stability. To evaluate the contribution of the pH reduction to the deoxygenation and autoxidation rate of horse mackerel Hb, in vitro experiments were performed in the range of pH values found in postmortem fish muscle (6.0−8.2).[30] The results indicated the fastest kinetics for the formation of metHb at pH 6.0, increasing the metHb concentration in an almost linear manner to 10 μM, which represents the 100% of Hb, after 55 h of incubation (Figure 4A). The Hb redox instability at pH 6.0 was also evident by the formation of a red precipitate, which could be the low spin

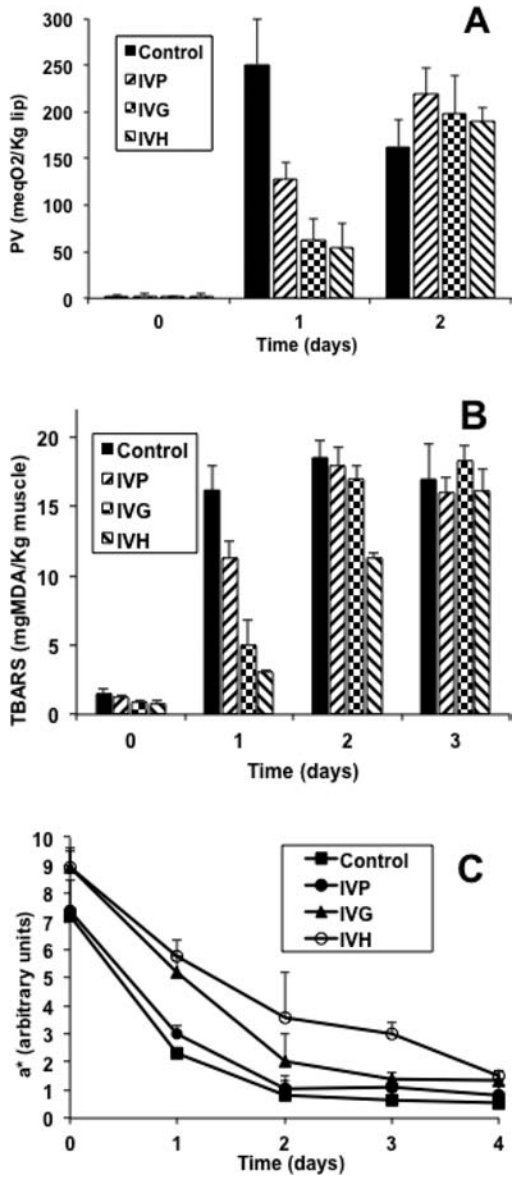

Figure 6. Formation of lipid hydroperoxides (A) and aldehydes (B) and evolution of colorimetric parameter a^* (C) during the storage of washed fish muscle supplemented with the polyphenolic fractions IVP, IVG, and IVH. Lipid oxidation was initiated with 15 μmol/kg of horse mackerel Hb, and fractions were added to a final concentration of 50 μg/g (w/w).

iron(III)-hemichrome, the product of the disturbance of the globin structure of the metHb.[39] An important generation of metHb was also observed at pH 6.6, reaching a metHb concentration of 5 μM (equivalent to the 50% of total Hb) after 55 h (Figure 4A). The formation of metHb was significantly slowed down at pH 7.0 or higher, with the behavior of horse mackerel Hb not significantly different ($p > 0.05$) at those elevated pH values. Figure 4B shows clearly the inverse relation between the initial Hb oxygenation and the autoxidation rates, which were estimated from the slopes of the corresponding pseudo linear kinetics of metHb formation. Hb exhibited much lower Hb oxygenation at pH 6.0, followed in increasing order by pH 6.6 < pH 7.0 ≈ pH 7.5 ≈ pH 8.2. Accordingly, the autoxidation rate was more elevated at pH 6.0 than pH 6.6, while the range of pH 7.0−8.2 provided the slowest generation of metHb. These results

Table 1. Inhibition Percentages[a] on the Formation of Lipid Hydroperoxides and TBARS Obtained by Supplementing Washed Fish Muscle with Nongalloylated Pine (IVP), Medium-Galloylated Grape (IVG), and High-Galloylated Witch Hazel (IVH) Polyphenols (Means ± SDs)[b]

	inhibition percentage (%)		
	peroxide values	TBARS	
	day 1	day 1	day 2
IVP	48.9 ± 2.7 a	30.2 ± 7.1 a	3.0 ± 6.4 a
IVG	75.4 ± 7.3 b	68.8 ± 11.1 b	8.6 ± 4.8 a
IVH	78.2 ± 9.2 b	80.9 ± 0.1 b	39.0 ± 2.8 b

[a] % Inhibition = $[(C - S)/C] \times 100$, where C = amount of oxidation product formed in control and S = amount of oxidation product formed in muscle supplemented with polyphenols. [b] Values in each column with the same letter were not significantly different ($p < 0.05$).

indicate that autoxidation of horse mackerel is an important source of metHb at a pH below 7.0, while the formation of metHb at higher pH should be essentially explained by a forced-oxidative mechanism. Previous research has reported an efficient formation of metHb from the interaction of ferrous-Hb with lipid oxidation byproduct as lipid hydroperoxides and aldehydes.[17,36]

Influence of Polyphenolic Extracts Differing in Galloylation on the Redox Stability of Horse Mackerel Hb. The effect of phenolics on the stability of fish Hb and the potential role of the esterified galloyl groups attached to the polyphenolic structure were determined. Control Hb without polyphenolic supplementation exhibited the slowest autoxidation rates (Figure 5A), according to its strongest oxygenation state (Figure 5B). Phenolics were significantly active in the promotion of metHb, finding the fastest rates in the presence of IVH, followed in decreasing order by IVG > IVP (Figure 5A). This tendency highlights a direct relation between the content in galloyl residues and the hastening of metHb formation. Previous investigations have proven an increment of the electron-donating capacities with galloylation.[19,24] Although phenolics donate electrons to electron-deficient compounds as free radicals, strong reducing polyphenols like those hosting pyrogalloyl structures as (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) are able to form superoxide radical from molecular oxygen.[40] Because galloyl residues incorporate pyrogalloyl structures, the formation of superoxide radicals could explain the direct relation found in vitro between the content in galloyl residues and the pro-oxidant activity on Hb. Our results suggest that both galloylated/nongalloylated polyphenols are not able to preserve Hb in the reduced ferrous state by direct reduction of metHb; however, galloylated polyphenols exhibited an important antioxidant activity to protect red bloods cells in the presence of oxidative free radicals.[24,41] Because important proportions of metHb can be generated in muscle tissues from the interaction of Hb with the lipid oxidation products, as free radicals and aldehydes, the inhibitory action of polyphenols on the formation of these lipid oxidation products should contribute positively, increasing the oxidative stability of Hb.

Effect of Polyphenolic Extracts Differing in Galloylation To Prevent Hb-Mediated Lipid Oxidation in Washed Fish Muscle. The antioxidant efficacy of the polyphenolic extracts was tested at 50 μg/g (w/w) in washed horse mackerel muscle, a matrix that has the structure of fish muscle, that is, with myofibril proteins and membranes, but essentially free of hydrophilic pro-oxidants

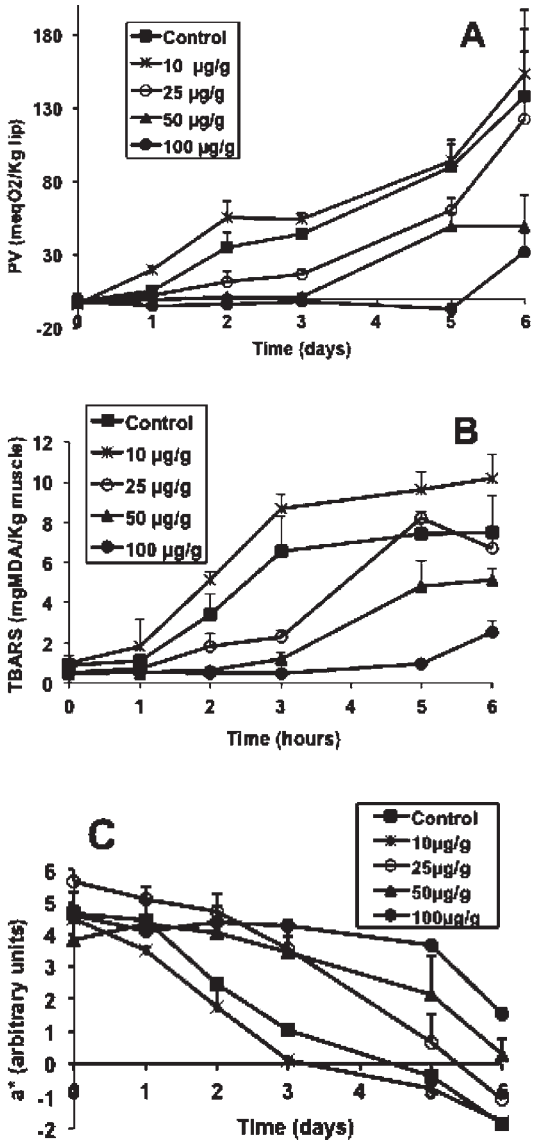

Figure 7. Formation of lipid hydroperoxides (A) and aldehydes (B) and evolution of colorimetric parameter a^* (C) during the storage of washed fish muscle supplemented with different concentrations of IVG. Lipid oxidation was initiated by the addition of 15 μmol/kg of horse mackerel Hb.

as Hb.[14] Lipid oxidation was initiated by the addition of 15 μmol/kg of horse mackerel Hb.

The formation of lipid peroxides showed a significant increment between days 0 and 1 for all systems. Fish muscle nonsupplemented with polyphenols (control samples) exhibited the highest amount of peroxides after 1 day of chilling storage (Figure 6A). All polyphenolic fractions were effective for inhibiting the formation of peroxides, but significant differences were found in their inhibitory activity: IVH ≈ IVG > IVP. Accordingly, fraction IVP from pine showed a lower percentage of inhibition for the formation of peroxides after 1 day (49%), in comparison with samples supplemented with grape (75%) and witch hazel (78%) fractions (Table 1). The TBARS index also showed an inferior efficiency of the nongalloylated fraction from pine bark to inhibit Hb-promoted lipid oxidation as compared to the galloylated polyphenols from grape and witch hazel (Figure 6B). After 1 day, inhibition percentages for the generation of TBARS were lower for the pine

Table 2. Inhibition Percentages[a] on the Formation of Lipid Hydroperoxides and TBARS Obtained by Supplementing Washed Fish Muscle with Different Concentrations of Fraction IVG (Means ± SDs)[b]

concentration (μg/g)	day 3	day 5	day 6
		PV (%)	
10	−23.5 ± 14.9 a	−4.3 ± 12.6 a	−11.4 ± 26.5 a
25	61.4 ± 8.7 b	33.5 ± 7.0 b	11.0 ± 21.6 a
50	96.1 ± 4.4 c	45.3 ± 5.3 b	64.3 ± 7.6 b
100	103.2 ± 6.4 c	107.4 ± 5.5 c	76.8 ± 12.5 b
		TBARS (%)	
10	−31.7 ± 2.6 a	−30.1 ± 7.7 a	−31.9 ± 7.7 a
25	65.1 ± 8.4 b	−14.3 ± 2.6 a	12.5 ± 4.8 b
50	82.5 ± 2.7 c	34.8 ± 13.5 b	30.3 ± 6.7 c
100	92.5 ± 1.8 d	87.0 ± 1.8 c	67.0 ± 5.8 d

[a] % Inhibition = [(C−S)/C] X 100 where C = amount of oxidation product formed in control and S = amount of oxidation product formed in muscle supplemented with polyphenols. [b] Values in each column with the same letter were not significantly different ($p < 0.05$).

fraction (30%), followed by the grape polyphenols (69%) and witch hazel polyphenols (81%) (Table 1). The most galloylated fraction (IVH) was also the most effective for preventing the formation of aldehydes at day 2. The measurement of the colorimetric parameter a^* (redness) showed differences in the effectiveness of polyphenolic fractions for preserving the oxidative stability of Hb (Figure 6C). Higher values of a^* are associated with the bright red ferrous-Hb, and the conversion to the brownish oxidized Hb species like metHb is implicated in the redness decay.[42] Nongalloylated polyphenols from pine (IVP) were not effective to retard the loss of redness as compared to the control muscle; therefore, this fraction was not able to inhibit the formation of metHb in fish muscle. Conversely, an improvement of the redox stability of Hb in fish muscle supplemented with IVG and IVH was observed, since they maintained a significantly ($p < 0.05$) higher redness at day 1. Polyphenolic fractions from grape (IVG) and witch hazel (IVH) showed a similar ability to retard the redness decay (Figure 6C).

These results must not be interpreted as a direct stabilization of the reduced Hb by galloylated polyphenols. In vitro studies showed that polyphenols do not have the capacity to inhibit Hb autoxidation, and conversely, galloylated fractions (IVH and IVG) accelerated extensively the oxidation of Hb to metHb. Therefore, the high preservation of Hb in reduced state by supplementing fish muscle with galloylated polyphenols differs with the Hb instability detected in in vitro studies in which no lipid oxidation subtracts were present. In a recent paper, Maestre et al.[17] demonstrated an extensive formation of metHb in the presence of lipid oxidation products such as hydroperoxides or volatile aldehydes. Consequently, the strong ability of IVG and IVH to inhibit the formation of peroxides (PV) and aldehydes (TBARS) in fish muscle could explain their protective effect against Hb oxidation. The results also indicated that the presence of galloyl groups flavors the ability of polyphenols to inhibit Hb-catalyzed lipid oxidation in washed fish muscle, although the high-galloylated IVH did not improve significantly the antioxidant activity of the medium-galloylated IVG. Similar conclusions have been previously obtained

Table 3. Sensory Analysis of Washed Fish Muscle Supplemented with Different Concentrations of IVG during Chilling Storage[a]

system	day 0	day 1	day 2	day 3	day 5	day 6
control	fresh odor	fresh odor	rancid odor	rancid odor	rancid odor	rancid odor
10 μg/g	fresh odor	fresh odor	incipient rancid odor	rancid odor	rancid odor	rancid odor
25 μg/g	fresh odor	fresh odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor
50 μg/g	fresh odor	fresh odor	no rancid odor	no rancid odor	rancid odor	rancid odor
100 μg/g	fresh odor	fresh odor	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor

[a] Lipid oxidation was initiated by addition of 15 μmol/kg of horse mackerel Hb.

in fish oil emulsion and pelagic fish muscle supplemented with polyphenolic fractions differing in galloylation.[19,43]

Another experiment was carried out with different supplemented amounts of IVG (10−100 μg/g) to establish how the ratio Hb/polyphenol influences Hb redox stability and development of Hb-catalyzed lipid oxidation. Fraction IVG was used for this experiment since it showed a similar efficacy as fraction IVH, but the production cost was lower in comparison to that from witch hazel. The maximum level of IVG was chosen by reference of the legal limit in the European law for the synthetic antioxidant BHT.[18] Figure 7A,B show the evolution of the peroxides and TBARS indexes for the different systems. The incorporation of the polyphenolic fraction to a final concentration of 10 ppm was not able to decrease the formation of peroxides and aldehydes, since the levels of these lipid oxidation products were similar to controls and even higher (Figure 7A,B). In the remaining systems, the antioxidant efficacy to prevent Hb-promoted lipid oxidation was proportional to the added amount of IVG, showing samples supplemented with 100 ppm, the higher induction periods (5 days) and inhibition percentages (Table 2). The sensory analysis of rancid off-flavors showed the same antioxidant efficiency (Table 3). Rancid odors were detected in both control and 10 ppm-supplemented muscle at day 2. The supplementation with 100 ppm provided the highest inhibition of rancidity; rancid odors were evident after 6 days. A similar tendency was observed by monitoring the colorimetric parameter a^* (Figure 7C); therefore, the capacity of IVG to preserve Hb in reduced state showed a direct concentration dependence: 100 > 50 > 25 > 10 ppm. The loss of redness was not retarded for the incorporation of a concentration as low as 10 ppm. However, higher concentrations of grape polyphenols were able to decrease the rate of Hb oxidation, especially in the range 50−100 ppm (Figure 7C).

In summary, the addition of the different polyphenolic fractions extracted from pine bark, grape pomace, and hamamelis bark to pure horse mackerel Hb aqueous solutions increased the oxidation rate of the hemeprotein, showing an apparent pro-oxidant activity. On the contrary, colorimetric measurements showed an important stabilization of oxyHb in washed fish mince supplemented with galloylated polyphenols. Such stabilization is accompanied by a reduction on the formation of lipid oxidation products derived from fish muscle. The results stress the role of galloyl residues to inhibit the formation of lipid oxidation products that actively catalyze the formation of metHb.[17] This effect provides a major stabilization of fish Hb and, as a final result, reduces the incidence of Hb-promoted lipid oxidation in muscle. However, an insignificant difference was observed in the activity of medium- and high-galloylated polyphenols. The antioxidant activity of galloylated proanthocyanidins was concentration dependent and was especially efficient in the range of 50−100 ppm. The present research provides useful information on the rational design of new antioxidant ingredients from natural sources directed to prevent lipid oxidation in pelagic fish species and in other foodstuffs in which oxidation is essentially initiated by hemeproteins.

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