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

Jacobo Iglesias*1, Manuel Pazos1, Rodrigo Maestre1, Josep L. Torres2 and Isabel
Medina1

1 Instituto de Investigaciones Marinas del CSIC, Eduardo Cabello 6, E-36208 Vigo,
Spain.

2 Instituto de Química Avanzada de Catalunya (IQAC) CSIC, Jordi Girona 18-26,
08034 Barcelona, Spain

*Author to whom correspondence should be addressed [e-mail: jacobo@iim.csic.es;
telephone +34 986 231930; fax +34 986 292762].
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 degree of galloylation: non-galloylated 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 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 (methHb) 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 state, which exhibits lower pro-oxidant activity 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 (PUFA), particularly eicosapentaenoic acid and docosahexaenoic acid (1), and substances with strong ability to initiate lipid oxidation, such as redox-active metals and hemeproteins (2). Lipid oxidation leads to 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 seems also 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 activity and lipophilicity), modulating in last instance their antioxidant activity depending of 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 present study was aimed to progress in the 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 (Trauchurus trauchurus) was
chosen as source of Hb, and its redox stability was compared with that from a
commercial lean fish species as hake (Merluccius merluccius). In vitro experiments were
firstly performed to investigate differences in the capacity of non-, medium- and high-
galloylated polyphenols to maintain Hb in 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
containing very low levels of hydrophilic pro-oxidants that can be added controllably
(14).

MATERIALS AND METHODS.

Materials and Chemicals. Fresh Atlantic horse mackerel (Trauchurus trauchurus)
and hake (Merluccius merluccius) 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
(Na₂HPO₄.2H₂O), thiobarbituric acid, trichloroacetic acid, 1,1,3,3-tetraethoxypropane
(TEP), butylated hydroxytoluene (BHT), FeCl₂.4H₂O, FeCl₃.6H₂O, 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 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 coworkers (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 moles of galloyl groups per mol of molecule (Figure 1). Witch hazel fraction, IVH, was composed by 20% of proanthocyanidins and 80% of hydrolyzable tannins, fundamentally hamamelitannin, methyl gallate, and galloyl glucose with 5–10 galloyl moieties (Figure 1) (26). Such composition rich in galloylated hydrolyzable 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 pipette 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 used.

**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 were 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. 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., Palo Alto, CA). Hb oxygenation was estimated as the difference between the peak absorbance at 576 nm and the “valley” at 560 nm (30). The relative rate and extent of Hb autoxidation was 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 (\textit{Trachurus trachurus}) was prepared according to Richards and Hultin (27). Briefly, light muscle was obtained from fresh individuals of horse mackerel, and after 3 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 during 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 ºC on ice. Controls were samples without polyphenolic supplementation. Triplicate samples were taken at different sampling times. 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.

**Thiobarbituric Acid Reactive Substances (TBARS).** TBARS index was determined according to McDonald and Hultin (35). The standard curve was constructed with 1,1,3,3-tetraethoxypropane (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 during 10 minutes 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.

**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 compared to hake Hb (Figure 2). Hake Hb exhibited an extensive increment of absorbance at 630 nm, wavelength at which metHb has a strong absorption (31), after 50 h of incubation at pH 6.8. Horse mackerel Hb showed lower increment of absorbance at 630 nm. The kinetics of metHb formation also evidenced a much faster autoxidation for hake compared to horse mackerel Hb. After 35h 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 specie 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 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 post-mortem pH reduction due to the anaerobic glycolitic metabolism (38), the Bohr effect could increment the proportion of deoxygenated Hb in post-mortem 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 found in post-mortem fish muscle (6.0-8.2) (30). The results indicated a fastest kinetic 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). Hb redox instability at pH 6.0 was also evident by the formation of a red precipitate, which could be the low spin iron(III)-hemichrome, 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 hours (Figure 4A).

The formation of metHb was significantly slowed down at pH 7.0 or higher, being the behaviour of horse mackerel Hb not significant different (p>0.05) at those elevated pH values. Figure 4B shows clearly the inverse relation between the initial Hb oxygenation and autoxidation rates, which were estimated from the slopes of the corresponding pseudo linear kinetics of metHb formation. Hb exhibited much lowest 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 indicate that autoxidation of horse mackerel is an important source of metHb at 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 by-products 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 was determined. Control Hb without polyphenolic supplementation exhibited the
slowest autoxidation rates (Figure 5A), accordingly 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). Since 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 are suggesting that both galloylated/non-galloylated 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). Since 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, i.e., with myofibril proteins and membranes,
but essentially free of hydrophilic pro-oxidants 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 non-supplemented 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). TBARS index showed also an inferior efficiency of the non-galloylated fraction from pine bark to inhibit Hb-promoted lipid oxidation 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 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 to the bright red ferrous-Hb, and the conversion to the brownish oxidized Hb species like metHb is implicated in the redness decay (42). Non-galloylated polyphenols from pine (IVP) were not effective to retard the loss of redness compared to the control muscle, and therefore, this fraction was not able to inhibit the formation of metHb in fish muscle. Conversely, it was observed an improvement of the redox stability of Hb in fish muscle supplemented with IVG and IVH, since they maintained a significant (p < 0.05) higher
redness at day 1. Polyphenolic fractions from grape (IVG) and witch hazel (IVH) showed 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 have not 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 to the Hb instability detected in vitro studies in which no lipid oxidation substracts were present. In a recent paper, Maestre et al. (17) demonstrated an extensive formation of metHb in presence of lipid oxidation products 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 favor 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 in fish oil emulsion and pelagic fish muscle supplemented with polyphenolic fractions differing in galloylation (19, 43).

Other experiment was carried out with different supplemented amounts of IVG (10-100 µg/g) in order 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). Figures 7A and 7B 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 (Figures 7A and 7B). 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-favors 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. Similar tendency
was observed by monitoring the colorimetric parameter $a^*$ (Figure 7C), and therefore,
the capacity of IVG to preserve Hb in reduced state showed a direct concentration-
dependence: 100 ppm > 50 ppm > 25 ppm > 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, not significant difference was observed in the activity of medium- and high-galloylated polyphenols. The antioxidant activity of galloylated proanthocyanidins was concentration dependent, being especially efficient in the range of 50-100 ppm. The present research provides useful information to 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.

Acknowledgements

This work was performed within the research project AGL2009-12374-C03-01. The financing and doctoral grant to R. M. by the Spanish Ministry of Science and Innovation are gratefully acknowledged. The authors acknowledge Consejo Superior de Investigaciones Científicas (CSIC) and Xunta de Galicia for PhD grant to J. I. and the postdoctoral contract “Eduardo Parga Pondal” to M. P, respectively.

LITERATURE CITED


Figure captions

Figure 1. Chemical structures of proanthocyanidins and hydrolysable tannins.

Figure 2. UV-Vis spectra of Hb from horse mackerel and hake at time 0 and after 50 hours 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.

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 to 4 °C.

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 to 4 °C. Final concentrations of Hb and antioxidant were 10 µM and 18.5 µg/mL, respectively.

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).
**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 addition of 15 µmol/Kg of horse mackerel Hb.
Figure 1

Proanthocyanidins

Hydrolyzable tannins

Polymeric catechins (arrows indicated possible polymerization positions)

R₂OH or Gallate group

Fractons IVG and IVH contain gallate groups in the proanthocyanidinic structure. They are absent in fraction IVP.
Figure 2

**Mackerel**

**Hake**
Figure 3

A

B

[Graphs showing data for Hake and Mackerel over time (hours) for two different metrics: one for methylation (µM) and another for relative oxygenation (obs-obs_0)/(obs_0)].
Figure 4

A

![Graph showing the effect of pH on the formation of metHb (µM) over time (hours).](image)

B

![Graph showing the relationship between pH and autoxidation rate (µmol/h) and relative oxygenation.](image)
Figure 5

A

B

<table>
<thead>
<tr>
<th>Control</th>
<th>IVP</th>
<th>IVG</th>
<th>IVH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.14</td>
<td>0.13</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Figure 6

A

PV (mg CHO/kg lip)

Control
IVP
IVG
IVH

Time (days)

B

TBARS (mg MD/ Kg muscle)

Control
IVP
IVG
IVH

Time (days)

C

α (arbitrary unit)

Control
IVP
IVG
IVH

Time (days)
Figure 7

A

![Graph A showing PV (meqO2/Kg lip) vs. Time (days)]

B

![Graph B showing TBARS (mgMDA/Kg muscle) vs. Time (hours)]

C

![Graph C showing a* (arbitrary units) vs. Time (days)]
Tables

Table 1. Inhibition percentages\(^1\) on the formation of lipid hydroperoxides and TBARS obtained by supplementing washed fish muscle with non-galloylated pine (IVP), medium-galloylated grape (IVG) and high-galloylated witch hazel (IVH) polyphenols (mean±sd)\(^2\).

<table>
<thead>
<tr>
<th></th>
<th>Peroxide Values</th>
<th>TBARS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 1</td>
</tr>
<tr>
<td>IVP</td>
<td>48.9 ± 2.7(^a)</td>
<td>30.2 ± 7.1(^a)</td>
</tr>
<tr>
<td>IVG</td>
<td>75.4 ± 7.3(^b)</td>
<td>68.8 ± 11.1(^b)</td>
</tr>
<tr>
<td>IVH</td>
<td>78.2 ± 9.2(^b)</td>
<td>80.9 ± 0.1(^b)</td>
</tr>
</tbody>
</table>

\(^1\) % 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.  
\(^2\) Values in each column with the same superscript letter were not significantly different (p < 0.05).
Table 2. Inhibition percentages\(^1\) on the formation of lipid hydroperoxides and TBARS obtained by supplementing washed fish muscle with different concentrations of fraction IVG (mean±sd)\(^2\).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>PV (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 6</td>
</tr>
<tr>
<td>10 µg/g</td>
<td>-23.5 ± 14.9(^a)</td>
<td>-4.3 ± 12.6(^a)</td>
<td>-11.4 ± 26.5(^a)</td>
</tr>
<tr>
<td>25 µg/g</td>
<td>61.4 ± 8.7(^b)</td>
<td>33.5 ± 7.0(^b)</td>
<td>11.0 ± 21.6(^a)</td>
</tr>
<tr>
<td>50 µg/g</td>
<td>96.1 ± 4.4(^c)</td>
<td>45.3 ± 5.3(^b)</td>
<td>64.3 ± 7.6(^b)</td>
</tr>
<tr>
<td>100 µg/g</td>
<td>103.2 ± 6.4(^c)</td>
<td>107.4 ± 5.5(^c)</td>
<td>76.8 ± 12.5(^b)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration</th>
<th>TBARS (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td>Day 5</td>
<td>Day 6</td>
</tr>
<tr>
<td>10 µg/g</td>
<td>-31.7 ± 2.6(^a)</td>
<td>-30.1 ± 7.7(^a)</td>
<td>-31.9 ± 7.7(^a)</td>
</tr>
<tr>
<td>25 µg/g</td>
<td>65.1 ± 8.4(^b)</td>
<td>-14.3 ± 2.6(^a)</td>
<td>12.5 ± 4.8(^b)</td>
</tr>
<tr>
<td>50 µg/g</td>
<td>82.5 ± 2.7(^c)</td>
<td>34.8 ± 13.5(^b)</td>
<td>30.3 ± 6.7(^c)</td>
</tr>
<tr>
<td>100 µg/g</td>
<td>92.5 ± 1.8(^d)</td>
<td>87.0 ± 1.8(^c)</td>
<td>67.0 ± 5.8(^d)</td>
</tr>
</tbody>
</table>

\(^1\) % Inhibition = \([(\text{C-S})/\text{C}] \times 100\) where \(\text{C}\) = amount of oxidation product formed in control and \(\text{S}\) = amount of oxidation product formed in muscle supplemented with polyphenols. \(^2\) Values in each column with the same superscript letter were not significantly different (\(p < 0.05\)).
Table 3. Sensory analysis of washed fish muscle supplemented with different concentrations of IVG during chilling storage. Lipid oxidation was initiated by addition of 15 µmol/Kg of horse mackerel Hb.

<table>
<thead>
<tr>
<th>System</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>fresh odor</td>
<td>fresh odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
</tr>
<tr>
<td>10 µg/g</td>
<td>fresh odor</td>
<td>fresh odor</td>
<td>incipient rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
</tr>
<tr>
<td>25 µg/g</td>
<td>fresh odor</td>
<td>fresh odor</td>
<td>no rancid odor</td>
<td>incipient rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
</tr>
<tr>
<td>50 µg/g</td>
<td>fresh odor</td>
<td>fresh odor</td>
<td>no rancid odor</td>
<td>no rancid odor</td>
<td>rancid odor</td>
<td>rancid odor</td>
</tr>
<tr>
<td>100 µg/g</td>
<td>fresh odor</td>
<td>fresh odor</td>
<td>no rancid odor</td>
<td>no rancid odor</td>
<td>no rancid odor</td>
<td>incipient rancid odor</td>
</tr>
</tbody>
</table>