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Inhibition of Hemoglobin- and Iron-Promoted Oxidation in Fish Microsomes by Natural Phenolics

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Natural phenolic antioxidants have been tested in hake (*Merluccious merluccious*) microsomes as inhibitors of lipid oxidation promoted by fish muscle prooxidants: hemoglobin (Hb), enzymatic NADH-iron and nonenzymatic ascorbate-iron. The phenolics selected were as follows: (a) a grape phenolic extract (**OW**), (b) a fraction (**IV**) with isolated grape procyanidins with a medium-low degree of polymerization and galloylation percentage, (c) hydroxytyrosol obtained from olive oil byproducts, and (d) a synthetic phenolic antioxidant, propyl gallate. All compounds delayed lipid oxidation activated by Hb, enzymatic NADH-iron, and nonenzymatic ascorbate-iron, excluding hydroxytyrosol that was not an effective antioxidant on oxidation promoted by nonenzymatic iron. The relative antioxidant efficiency was independent of the prooxidant system, **IV** > propyl gallate > **OW** > hydroxytyrosol, and showed a positive correlation with their incorporation into microsomes ($p < 0.05$). The reducing capacity or ability for donating electrons and the chelating properties may also contribute to the antioxidant activity of phenolics, although these factors were less decisive than their affinity for incorporating into the microsomes. Conversely, the inhibition of Hb oxidation by phenolics and their polarity did not seem to play an important role on antioxidant mechanism. These results stressed the importance of incorporating the exogenous antioxidants into the membranes where are located key substances for fish lipid oxidation (highly unsaturated phospholipids, iron-reducing enzymes, and endogenous α-tocopherol).

KEYWORDS: Fish; hemoglobin; enzymatic iron; nonenzymatic iron; lipid oxidation; microsomes; grape polyphenols; hydroxytyrosol

INTRODUCTION

Deterioration in flavor, color, texture, nutritive value, and the production of toxic compounds, as a consequence of lipid oxidation, is a major cause of quality loss in fish muscle (*1*). Iron-containing compounds, low molecular weight iron (LMW-iron) complexes and heme proteins, have been suggested to be the most important endogenous promoters of lipid oxidation in fish muscle (*2*, *3*). Since iron can be released from heme proteins during storage, it is difficult to establish the contributions of heme proteins and LMW-iron on lipid oxidation.

The term low molecular weight iron is used instead of free iron because iron seems to be associated with small metabolites, as ATP, ADP, and amino acids, due to the particularly low solubility of ferric iron at physiological pH. LMW-iron is ordinarily present as the inactive ferric state, but its reduction by enzymatic and nonenzymatic pathways can generate the extremely prooxidant ferrous iron (*3*). Endoplasmic and sarcoplasmic reticula possess an enzymatic system that reduces ferric iron to ferrous iron utilizing electrons from NAD(P)H (*4*−*6*). In fish muscle, this iron-reducing enzymatic system has a strong preference for NADH over NADPH (*6*). Superoxide and ascorbate are suggested to be the two major reducing agents involved in nonenzymatic iron reduction. At the concentrations normally present in fish muscle, 60−100 mM, ascorbate is an effective reducing agent of ferric iron (*7*).

Several studies have shown the importance of heme proteins in the development of lipid oxidation in fish muscle and tissues. Richards and Hultin (*8*) found that a loss of 33−45% of hemoglobin (Hb) content by bleeding enhanced the oxidative stability of mackerel light muscle and whole trout muscle during chilling. In trout fillets, bleeding was also effective in reducing lipid oxidation during frozen storage (*9*). Additionally to the hemoglobin concentration, other factors that include the type of hemoglobin, postmortem pH, plasma volume, and erythrocyte integrity seem to play an important role on the blood-mediated lipid oxidation (*10*). Moreover, different fish hemoglobins have activated lipid oxidation in systems rich in membrane lipids, such as microsomes (*11*) and washed muscle systems (*12*−*15*).

There is some evidence that membrane lipids are primary substrates of lipid oxidation (*16*). This could be attributed to

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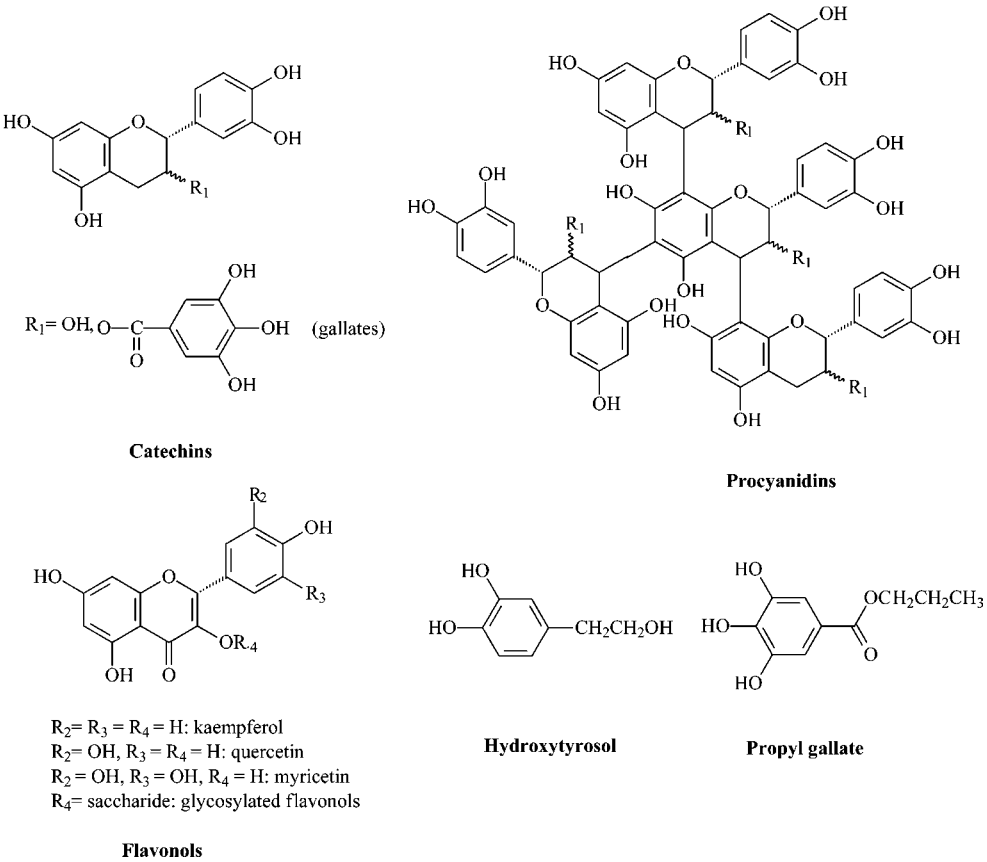

Figure 1. Molecular structures of monomeric catechins, oligomeric catechins (procyanidins), flavonols, hydroxytyrosol, and propyl gallate.

the high degree of phospholipid unsaturation, the close contact of membranes with catalysts of lipid oxidation located in the aqueous phase of muscle cells, and their large surface area (*17*). The carrying out of lipid oxidation studies in microsomes is attractive because they supply not only the lipid substrate but also the iron-reducing enzymatic system and the endogenous α-tocopherol.

The objective of our study was to examine the effect of some phenolic antioxidants on oxidation mediated by Hb or LMW-iron in a fish microsomal system. Two phenolic extracts obtained from grape pomace, one of them composed by several monomeric and polymeric phenolics (**OW**) and the other contained isolated procyanidins (**IV**), and hydroxytyrosol obtained from olive oil byproducts, were compared with a synthetic antioxidant, propyl gallate. The inhibitory efficiency of the phenolics was related to their physicochemical properties: polarity, affinity for being incorporated into the microsomes, reducing capacity, chelating activity, and inhibitory activity against the hemoglobin autoxidation. This work attempts to contribute to a better knowledge of the antioxidant mechanism attributed to the phenolic compounds in muscle tissues.

MATERIALS AND METHODS

Materials and Chemicals. Hake (*Merluccious merluccious* (*M. merlucciousi*) and horse mackerel (*Trachurus trachurus* (*T. trachurus*)) were acquired fresh from a local market and were immediately iced and filleted.

The total extract (**OW**) was obtained from grape (*Vitis vinifera* (*V. vinifera*)) pomace following the procedure by Torres and Bobet (*18*). **OW** contained monomeric catechins (6%), oligomeric catechins (or procyanidins) (92%), and in less proportion (2%) flavonols, mainly glycosylated (**Figure 1**). The mean degree of polymerization and galloylation percentage of **OW** were 1.7 units and 15%, respectively. Isolated procyanidins (**IV**) were obtained by application of size-exclusion chromatography to **OW**, as described by Torres et al. (*19*). Fraction **IV** was composed by a mixture of grape procyanidins with a mean degree of polymerization and galloylation percentage of 2.7 units and 25%, respectively. The mean molecular weight for **OW** and **IV**, calculated by thioacidolysis with cysteamine (*20*), was 552 and 880, respectively. Hydroxytyrosol was kindly provided by the Instituto de la Grasa (CSIC, Sevilla).

Carbon monoxide gas was provided by Air Liquide (Porriño, Spain). Propyl gallate, ferrozine, bovine hemoglobin, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) buffer, sodium heparin, tris-[hydroxymethyl]aminomethane (Tris), bovine serum albumin, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane, $FeCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, ascorbic acid, adenosine 5′-diphosphate (ADP), and reduced β-nicotinamide adenine dinucleotide (β-NADH) 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). Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA)

Preparation of Fish Hemoglobin. Fish blood was taken from the caudal vein after the tail of horse mackerel individuals was cut off in rigor state. 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 approximately 1 volume of the saline sodium heparin solution. Hemolysate was then prepared according to Fyhn et al. (*21*) and modifications by Richards and Hultin (*22*). Hemolysate was stored at −80 °C and was thawed just before it was used.

Quantification of Hemoglobin. Hemoglobin levels were quantified according to Brown (*23*). Briefly, hemolysate diluted in 50 mM Tris, pH 8.6, was mixed with around 1 mg of sodium dithionite. Diluted hemolysate was bubbled with carbon monoxide gas for 20 s. The sample was then scanned from 400 to 440 nm against a blank that contained only buffer. The peak absorbance was recorded, and absorbance at the peak minus the absorbance at the valley was related to the concentration of hemoglobin. A standard curve was built with hemoglobin from bovine blood.

Isolation of Fish Microsomes. Microsomes were isolated from hake white muscle by modification of MacDonald and Hultin's procedure (*4*), as described by Pazos et al. (*11*). Briefly, minced hake white muscle was homogenized with 4 volumes of chilled 0.1 M HEPES buffer (pH 7.5) for 30 s. The homogenate was adjusted to pH 7.5 and centrifuged at 10000*g* for 20 min at 5−10 °C. Then, the supernatant was centrifuged at 130000*g* for 30 min at 5−10 °C. The resulting sediment was suspended in chilled 0.12 M KCl and 5 mM histidine (pH 6.8) buffer with a Potter homogenizer and stored at −80 °C for no longer than 1 week.

Oxidation of Microsomes. The isolated microsomes were suspended in 0.12 M potassium chloride and 5 mM histidine (pH 6.8) up to a final concentration of 0.7 mg/mL membrane protein. Oxidation promoted by Hb was initiated by addition of fish Hb up to a final concentration of 5 μM. The membranes oxidized by enzymatic iron contained 20 μM Fe(III), 100 μM ADP, and 100 μM NADH, while NADH was substituted by 100 μM ascorbate in microsomes oxidized by nonenzymatic iron. The phenolic antioxidants were added up to a concentration of 2 and 5 μM in the systems activated by Hb and LMW-iron, respectively. Such phenolic concentrations were previously optimized for each system, to avoid long-term experiments and degradations associated with microbial growth. Oxidation was initiated by addition of Hb or iron to microsomes. Incubation was carried out in 25 mL Erlenmeyer flasks in a shaking water bath at 20 °C. Incubation times covered between 3 and 28 h, depending on the prooxidant system.

The pH was adjusted to 6.8 before oxidation was initiated, and it was just rechecked after the oxidation was activated. In the controls, the phenolic compounds were replaced by water. Oxidation was followed by measurement of peroxides and TBARS values. Each experiment was performed at least twice, and each sample was replicated.

Determination of Protein Content. Protein content was determined by the method of Markwell et al. (*24*). Bovine serum albumin was used as standard.

Peroxide Value. Peroxide value (PV) was measured using the adaptation of the ferric thiocyanate method (*25*) by Buege and Aust (*26*). PV was expressed in milliequivalents (mequiv) of oxygen per kilograms of microsomal protein.

Thiobarbituric Acid Reactive Substances Analyses. TBARS was determined according to the method of McDonald and Hultin (*4*). 1,1,3,3-Tetraethoxypropane was used as standard. The data are expressed in terms of nanomoles of malonaldehyde (MDA) per milligram of microsomal protein.

Study of the Incorporation of Phenolics into Fish Microsomes. Phenolic compounds were added to isolated microsomes suspended in 0.12 M potassium chloride and 5 mM histidine (pH 6.8 and 0.7 mg/mL membrane protein). The phenolic compounds were incubated with microsomes in darkness and at room temperature during 30 min. Then, the aqueous phase and microsomes were separated by centrifugation at 130000*g* and 4 °C for 30 min. Phenolics were determined by the Folin−Ciocalteu method (*27*) in the aqueous phase after a previous elimination of protein interferences with saturated freeze ammonium sulfate. Samples without phenolic compounds were used as blanks. The proportion of phenolics incorporated into microsomes was calculated as the difference between the total phenolic concentration and the phenolic concentration found in the aqueous phase after the phenol−microsomes interaction.

Effect of Phenolic Compounds on Hemoglobin Autoxidation. Horse mackerel Hb (10 μM) and phenolic compounds (4 μM) were incubated in 0.12 M potassium chloride and 5 mM histidine buffer (pH 6.8) at 20 °C. Hb was incubated without phenols in the controls. Hb autoxidation lessens the absorbance peak at 576 nm (*13*) and was determined monitoring the difference of absorbance between 576 and 560 nm.

Polarity of Phenolic Compounds. The polarity of phenolics was determined by their partition between an aqueous and an oily phase according to Pazos et al. (*28*).

Reducing Power of the Phenolic Compounds. The FRAP (ferric reducing/antioxidant power) method was used by adaptation of the procedure of Benzie and Strain (*29*). 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 1.5 mL aliquot of FRAP reagent were 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 phenolic concentration and FRAP activity.

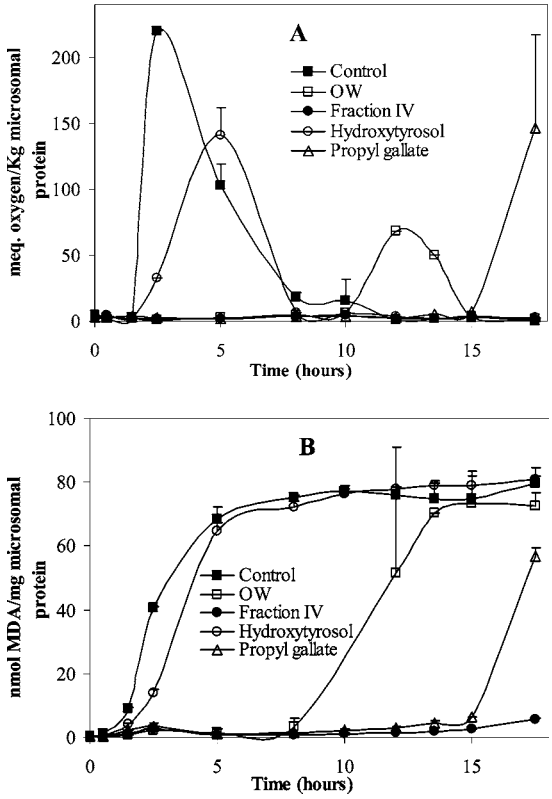

Figure 2. Effect of phenolic compounds on the generation of peroxides (**A**) and TBARS (**B**) in microsomal systems activated by 5 μM of hemoglobin. The phenolics were tested at a concentration of 2 μM. Error bars represent standard deviation.

Chelating Capacity of the Phenolic Compounds. The capacity of the phenols for chelating ferrous iron was determined according to the procedure of Kolayli et al. (*30*) and the adaptation by Pazos et al. (*31*). The chelating activity was expressed as the percentage of ferrous iron (20 μM) chelated by 10 μM phenolic compound.

Statistical Analysis. All experiments were replicated at least twice, and analyses were performed in duplicate. Data were shown as mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method (*32*). Correlations between the propagation rates of lipid oxidation products (peroxides and TBARS) and the physicochemical properties of phenolics were determined by Pearson coefficients. The rates of oxidation product formation were established by calculating the quotient between the highest level of oxidation product generated and the time necessary for reaching this value. Statistical analyses were performed with the software Statistica 6.0 (*33*).

RESULTS

Effectiveness of Phenolics for Inhibiting Oxidation in Fish Microsomes. Figure 2A shows the kinetics of formation/decomposition of peroxides in fish microsomes activated by 5 μM horse mackerel Hb. Peroxide formation was significantly faster in control microsomes than in those supplemented with 2 μM phenolic compounds ($p < 0.05$). The induction periods of peroxide formation also indicated significant differences in the antioxidant effect of each phenolic compound ($p < 0.05$).

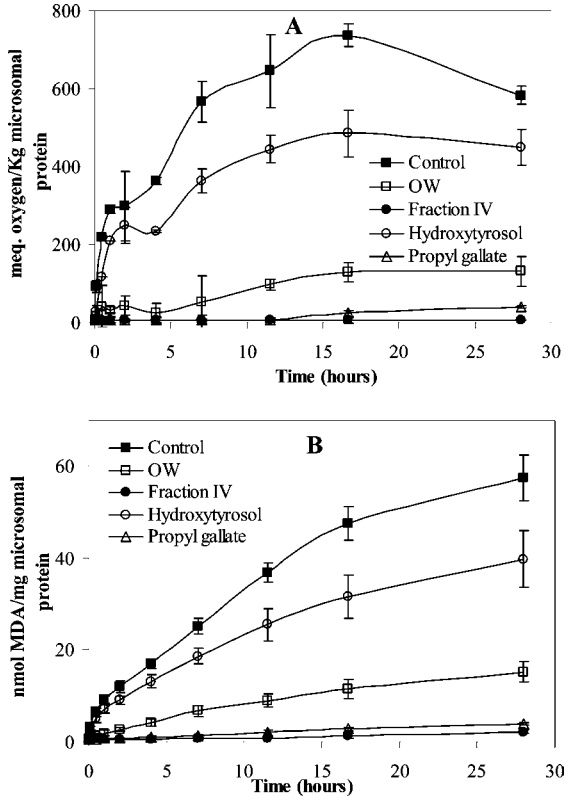

Figure 3. Effect of phenolic compounds on the generation of peroxides (**A**) and TBARS (**B**) in microsomal systems activated by enzymatic NADH-iron: 20 μM Fe(III), 100 μM ADP, and 100 μM NADH. The phenolics were tested at a concentration of 5 μM. Error bars represent standard deviation.

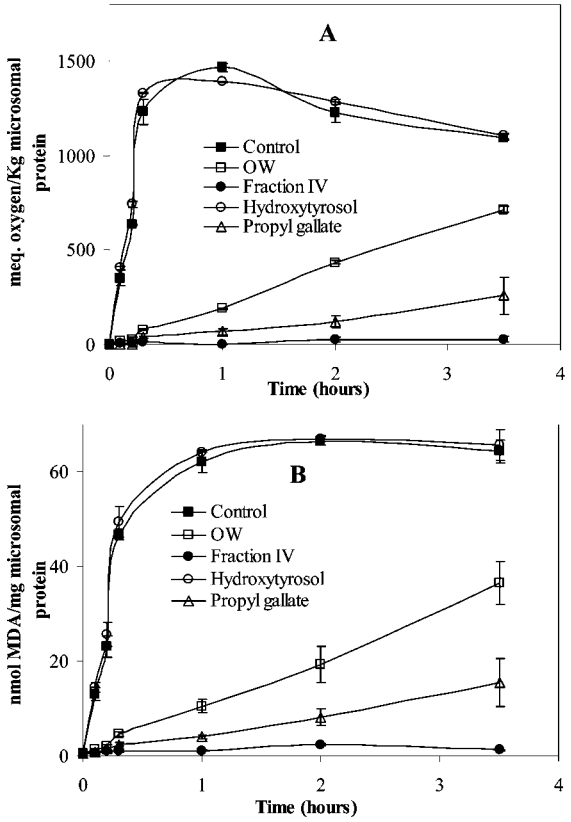

Figure 4. Effect of phenolic compounds on the generation of peroxides (**A**) and TBARS (**B**) in microsomal systems activated by nonenzymatic ascorbate-iron: 20 μM Fe(III), 100 μM ADP, and 100 μM ascorbate. The phenolics were tested at a concentration of 5 μM. Error bars represent standard deviation.

Fraction **IV**, composed of grape procyanidins with medium-low polymerization and galloylation degrees, showed the highest inhibitory effect on the peroxides, followed in decreasing order by propyl gallate > **OW** ≫ hydroxytyrosol (**Figure 2A**). This antioxidant order was confirmed by the TBARS values (**Figure 2B**). A remarkable fact was that fraction **IV** could inhibit the generation of peroxides and TBARS during the whole experiment. As a general rule, phenolic compounds were more active at delaying the induction periods of TBARS than their formation during the propagation of lipid oxidation. These results demonstrated that all phenolic compounds, tested at a concentration of 2 μM, delayed effectively the lipid oxidation activated by fish Hb in microsomes.

During the oxidation activated by Hb, the induction periods of peroxides and TBARS were similar (**Figure 2A**,**B**). However, the levels of TBARS were maintained almost constant after their highest values were reached, while peroxides disappeared completely after their formation.

All phenolic compounds supplemented at 5 μM were also active at inhibiting the lipid oxidation promoted by the enzymatic NADH-iron in fish microsomes (**Figure 3**). Phenolic compounds delayed the formation of both peroxides and TBARS and reduced their maximum values. According to the results obtained with Hb as catalyst, hydroxytyrosol was the compound that showed less antioxidant effectiveness, followed in increasing order of efficiency by **OW** < propyl gallate < fraction **IV**.

With regard to the antioxidant efficiency during the oxidation activated by nonenzymatic ascorbate-iron, all phenolics except hydroxytyrosol were very effective at delaying lipid oxidation (**Figure 4**). According to the above microsomes oxidized by Hb or enzymatic NADH-Fe, the antioxidant efficiency was fraction **IV** > propyl gallate > **OW**.

Table 1. Affinity for Incorporation into the Microsomes, Reducing Power, and Chelating Activityof Phenolic Compounds[a]

phenolic compd	incorporation into the membranes (%)	reducing power (mol of donated electrons/(mol of phenolic compd))	chelating activity[b] (%)
OW	45.4 ± 3.3[b]	3.3	34.8 ± 0.5[b]
IV	70.6 ± 1.4[d]	6.0	61.7 ± 0.0[d]
hydroxytyrosol	6.4 ± 0.7[a]	1.7	17.7 ± 0.5[a]
propyl gallate	52.1 ± 1.7[c]	3.2	56.6 ± 0.7[c]

[a] Values in each column with the same letter were not significantly different ($p > 0.05$). [b] Percentage of chelated ferrous iron by 10 μM of phenolic compound.

Relationship between Physicochemical Properties of Phenolics and Their Antioxidant Activity in Microsomes. The phenolic compounds tested in this work have different polarities. Partitioning coefficients between oil and water were as follows: **OW**, 0.11 ± 0.01; **IV**, 0.11 ± 0.02; hydroxytyrosol, 0.61 ± 0.02; propyl gallate, 0.85 ± 0.01. Therefore the polarity decreases in the following order: **OW** = fraction **IV** ≫ hydroxytyrosol ≫ propyl gallate.

The studies focused on the incorporation of phenolic compounds into fish microsomes revealed that over 71% of procyadinins **IV** were located within the membranes (**Table 1**) against 52% of propyl gallate and 45% of **OW**. Hydroxytyrosol had notably lower affinity for the membranes than for the aqueous phase, keeping only over 6% in microsomes. Therefore, the phenolics showed the following lessening affinity for the membranes: fraction **IV** > propyl gallate > **OW** ≫ hydroxytyrosol.

Table 2. Correlations between Physicochemical Properties of Phenolic Compounds (Affinity for Membranes, Reducing, and Chelating Capacities) and the Rates of Peroxides and TBARS Generation in the Microsomal Systems Supplemented with Phenolics[a]

	peroxide value			TBARS		
	hemoglobin	NADH-Fe	ascorbate-Fe	hemoglobin	NADH-Fe	ascorbate-Fe
affinity for membranes	–0.97*	–0.93	–0.93	–1.00*	–0.97*	–0.96*
reducing power	–0.86	–0.71	–0.71	–0.88	–0.80	–0.76
chelating activity	–0.84	–0.85	–0.85	–0.95*	–0.97*	–0.89

[a] Asterisks indicate significant correlation with $p < 0.05$.

Regarding the reducing capacity, grape procyanidins **IV** were clearly the highest reductor by donating over 6 electrons per phenolic molecule (**Table 1**). Grape extract **OW** and propyl gallate donated over 3 electrons per molecule and hydroxytyrosol less than 2 electrons. Therefore, all phenolic hydroxyls of propyl gallate and hydroxytyrosol donated 1 electron each (**Figure 1**). **OW** and **IV** provided more than the 2 electrons per molecule donated by monomeric catechins (data obtained in our laboratory). Therefore, the reducing capacity decreased in this order: fraction **IV** > propyl gallate ≈ **OW** > hydroxytyrosol.

Fraction **IV** showed also the highest chelating capacity (**Table 1**). Tested at phenolic concentration of 10 μM and iron concentration of 20 μM, procyanidins of fraction **IV** complexed over 62% of ferrous iron, against 57, 35, and 18% reached for propyl gallate, **OW**, and hydroxytyrosol, respectively. So, the chelating capacity decreased in the following order: fraction **IV** > propyl gallate > **OW** > hydroxytyrosol.

Table 2 shows the correlations between the physicochemical properties of the phenolic compounds and their antioxidant effect in microsomes oxidized by Hb, enzymatic NADH-iron, and nonenzymatic ascorbate-iron. The reducing and chelating capacities and the affinity for being incorporated into the microsomes were related to the rates of peroxides and TBARS generation in the presence of phenolics. The formation of TBARS gave better correlations than that of peroxides, possibly as a consequence of the decomposition of peroxides by Hb and ferrous iron (**Table 2**). The affinity of the phenolic compounds for the membranes showed a high significant correlation ($p < 0.05$) with the inhibition of TBARS generation in all prooxidant systems tested. Also chelating capacity and the inhibition of TBARS showed significant correlations in microsomes activated by Hb and enzymatic NADH-iron. The reducing power and the antioxidant effectiveness exhibited good correlation, although this relationship was not significant ($p > 0.05$).

Autoxidation of Hb. Phenolic compounds were not effective at inhibiting the autoxidation of Hb, when they were tested at the same molar ratio Hb:phenolic (5:2) that was used in the microsomal systems. The difference of absorbance 576−560 nm were similar in control Hb (with absence of phenolic compounds) and in Hb with phenolics, after 4 h of incubation (**Table 3**). After 19 h, control Hb and Hb with phenolics showed similar autoxidation rates, excluding Hb supplemented with propyl gallate, which significantly activated Hb autoxidation showing a lower difference of absorbance 576−560 nm than controls. Therefore, the mechanism of phenolics for inhibiting lipid oxidation promoted by Hb does not seem to be related to a direct effect of phenolics on Hb autoxidation, at least with the experimental conditions employed.

DISCUSSION

The results of this study revealed that natural phenolic antioxidants such as grape procyanidins and hydroxytyrosol can inhibit lipid oxidation in fish microsomes, which are the sites where oxidation is probably initiated (*16*). According to Raghavan and Hultin (*34*), the location of an antioxidant where oxidation occurs should be an important aspect in determining its antioxidant efficiency. The results of this study emphasized that grape procyanidins, especially those with a medium-low degree of polymerization and galloylation, protect effectively the sensitive fish membrane lipids against oxidation promoted by the most significant prooxidant components of fish muscle: Hb, enzymatic NADH-iron, and nonenzymatic ascorbate-iron. In previous works, catechins and procyanidins have demonstrated antioxidant properties against the oxidation activated by free radicals in microsomes and other systems rich in phospholipids (*35*−*37*). Hydroxytyrosol was also effective at delaying lipid oxidation promoted by Hb and enzymatic NADH-iron, but it did not show antioxidant activity in the microsomes activated by nonenzymatic ascorbate-iron (**Figure 4**). However, hydroxytyrosol was able to retard oxidation initiated by ascorbate-iron when it was supplemented at higher concentrations (20 μM) (data not shown).

Table 3. Effect of Phenolic Compounds (4 μM) on Hemoglobin (10 μM) Autooxidation in 0.12 M Potassium Chloride and 5 mM Histidine Buffer at pH 6.8 and 20 °C [a]

	difference of absorbance 576–560 nm		
sample	0 h	4 h	19 h
control	0.117 ± 0.001^a	0.111 ± 0.002^a	0.076 ± 0.002^b
OW	0.117 ± 0.002^a	0.112 ± 0.000^a	0.077 ± 0.001^b
IV	0.117 ± 0.002^a	0.112 ± 0.000^a	0.079 ± 0.006^b
hydroxytyrosol	0.116 ± 0.001^a	0.112 ± 0.000^a	0.077 ± 0.001^b
propyl gallate	0.117 ± 0.001^a	0.111 ± 0.000^a	0.069 ± 0.003^a

[a] Values in each column with the same letter were not significantly different ($p > 0.05$).

The relative antioxidant activity of phenolics, tested at the same molar concentration, was similar independently of the lipid oxidation promoter: procyanidins **IV** > propyl gallate > **OW** > hydroxytyrosol. This finding could imply that the antioxidant mechanism of these phenolics in microsomes is governed by the same factors independently of the promoters of oxidation. In enzymatic NADH-iron and nonenzymatic ascorbate-iron systems, the activation of oxidation depends basically on the ferric reducing activity to ferrous iron. Ferrous iron can promote lipid oxidation by the following (*1*): (i) formation of reactive hydroxyl radicals in Fenton reactions, (ii) breakdown of preformed lipid peroxides to generate peroxyl and alkoxyl radicals, and (iii) generation of H_2O_2 consumed in the Fenton reaction. Several mechanisms could explain the inhibitory activity of phenolics against LMW-iron-promoted oxidation: (i) scavenging peroxyl and alkoxyl radicals by donating electrons, (ii) scavenging hydrogen peroxide (*38*, *39*), and (iii) chelating ferrous iron. The results of this research showed that the antioxidant effectiveness of the phenolics against the oxidation activated by ferrous iron was more correlated with their ferrous

chelating capacity than with their reducing power (**Table 2**). These results contrasted with previous studies in rat microsomes, which suggested that hydrogen/electron donating properties play a major role on the inhibition of lipid oxidation promoted by NADPH-iron (*40*, *41*) and ascorbate-iron (*41*, *42*). The different composition of rat and fish microsomes, and a possible cooperative effect between the affinity of phenolics for the membranes and their chelating properties may be responsible of the role of chelating activities of phenolics on their antioxidant activity.

Hb-decomposed peroxides extensively and generated chain-propagation radicals (**Figure 2A**). These results are in agreement with other research, which indicated that the breakdown of peroxides is faster with Hb than with ferrous iron (*43*). Another important prooxidant pathway is the formation of hypervalent ferryl Hb radicals by autoxidation, which can abstract H atoms of a polyunsaturated fatty acid (*44*). Previous works indicated that protein binding can be related to deactivation of hypervalent ferril proteins in the presence of cinnamic acid derivatives such as chlorogenic acid (*45*). These prooxidant pathways of Hb do not seem to explain the significant role of the chelating capacity of phenolics on their antioxidant properties in microsomes activated by Hb (**Table 2**). Recent studies performed on the antioxidant activity of a fish aqueous extract (*46*) have also highlighted the chelating capacity as the major factor responsible for the inhibition of lipid oxidation in a model system of cod phospholipids oxidized by Hb. Clearly, more research is needed for clarifying the inhibitory activity of phenolics compounds on lipid oxidation promoted by Hb.

The incorporation of phenolic compounds into fish microsomes was the most decisive factor for the antioxidant efficiency of phenolics (**Table 2**). The presence in the membranes of chain-propagating lipid peroxyl radicals and ferrous-generating enzymatic systems can explain the importance of the phenolic introduction into the membranes for a more effective inhibition of the lipid oxidation. The location of endogenous α-tocopherol in the membranes should also enhance the positive effect of phenolics, since it plays an important role in the endogenous antioxidant system of tissues and can be regenerated by catechins and other phenolics (*36*, *47*, *48*). Moreover phenolics, particularly galloylated catechins, could affect the membrane configuration by forming more compact structures that restrain the access of prooxidants (*35*). The results obtained did not demostrate a direct relationship between the affinity of phenolics for the fish membranes and their polarity, but the incorporation into the membranes could be explained by interactions between phenolics and phospholipid headgroups (*37*), or between phenolics and membrane proteins (*49*). Galloylated procyanidins have hydrophobic cores with hydrophilic hydroxyl groups and may expose both or any of the two regions to establish hydrophobic and/or hydrophilic interactions, depending on the environment (*19*).

In a previous study performed in intact fish fillets (*31*), the reducing capacity of phenolic compounds and their antioxidant activities were highly correlated, while the affinity of phenolics for fish muscle or microsomes did not show correlation with their antioxidant activities. These exogenous antioxidants may have difficulty reaching the membranes in the intact muscle. Recent studies have pointed out the behavior of the antioxidant carrier solvent for selectively introducing the antioxidant into the membranes (*34*, *50*). Studies about the distribution of antioxidants in the muscle components and their effective incorporation into membranes are needed for a complete understanding of the activity of additive antioxidants in muscle and food. The results of the present work also corroborated the importance of distributing bioactive compounds within the membranes for preventing loss of quality and nutritional value associated with the oxidative stress of fish muscle. Moreover, this research underlines the inhibition of both hemoglobin and Fe-promoted oxidation as antioxidant mechanisms of grape procyanidins, hydroxytyrosol, and propyl gallate in muscle tissues.

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