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Structure−Activity Relationships of Polyphenols To Prevent Lipid Oxidation in Pelagic Fish Muscle

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The influence of polymerization (number of monomers) and galloylation (content of esterified gallates) of oligomeric catechins (proanthocyanidins) on their effectiveness to prevent lipid oxidation in pelagic fish muscle was evaluated. Non-galloylated oligomers of catechin with diverse mean polymerization (1.9−3.4 monomeric units) were extracted from pine (*Pinus pinaster*) bark. Homologous fractions with galloylation ranging from 0.25 to <1 gallate group per molecule were obtained from grape (*Vitis vinifera*) and witch hazel (*Hamamelis virginiana*). The results showed the convenience of proanthocyanidins with medium size (2−3 monomeric units) and low galloylation degree (0.15−0.25 gallate group/molecule) to inhibit lipid oxidation in pelagic fish muscle. These optimal structural characteristics of proanthocyanidins were similar to those lately reported in fish oil-in-water emulsions using phosphatidylcholine as emulsifier. This finding suggests that the antioxidant behavior of polyphenols in muscle-based foods can be mimicked in emulsions prepared with phospholipids as emulsifier agents. The present data give relevant information to achieve an optimum use of polyphenols in pelagic fish muscle.

KEYWORDS: Lipid oxidation; fish muscle; proanthocyanidins; polymerization; galloylation

INTRODUCTION

Lipid oxidation is an important cause of quality loss and shelf life shortening in muscle-based foods, sensory attributes (odor, flavor, texture, and color), nutritional value (vitamins, PUFA, etc.), and even safety being compromised by the propagation of lipid oxidation during processing and storage (*1*). The incorporation of natural antioxidants is an emerging procedure to avoid oxidative deterioration in food systems. The food industry is demanding alternative antioxidant treatments to those that traditionally employed synthetic additives, in order to avoid the potential toxic effects of some synthetic antioxidants (*2*). Phenolic compounds have garnered important interest for use as food additives because they are largely distributed in vegetables, fruits, and agro-forestry byproducts, and most of them possess strong antioxidant abilities (*3*). Moreover, the dietary consumption of phenolics has been related with beneficial effects on the prevention of cardiovascular diseases, diabetes, and cancer (*4*), and therefore, their application adds functionality and added value to the food system. Among phenolic compounds, the family of flavan-3-ols is extensively distributed in nature (*3*). Flavan-3-ols are nonplanar by virtue of their saturated C3 carbon, ranging from the simple monomers (+)-catechin and its isomer (−)-epicatechin to complex structures including oligomeric and polymeric proanthocyanidins. Catechins can be hydroxylated to form gallocatechins and also undergo esterification with gallic acid to originate catechin gallates.

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Previous investigations have demonstrated that the antioxidant properties of proanthocyanidins are largely modulated by the polymerization degree (number of polyphenolic units) and galloylation (content of esterified gallate groups) in several in vitro assays (*5*, *6*), low-density lipoproteins (*7*), corn oil-in-water emulsion (*6*), and bulk fish oil and fish oil-in-water emulsions (*8*). Although the main mechanisms behind the ability to delay the lipid oxidation process are related to the capacity to inactivate free radicals or metals (*9*) and regenerate endogenous antioxidants (*10*), antioxidant effectiveness in foods depends significantly on other factors such as the effective location in active oxidation sites. Proanthocyanidins have surfactant-like character to establish hydrophobic or hydrophilic interactions depending on the environment, which favor their accumulation in water−oil interfaces (*11*). A recent study has reported differences in the optimal polymerization and galloylation degrees of proanthocyanidins to prevent lipid oxidation in fish oil-in-water emulsions and bulk fish oil (*8*).

The control of lipid oxidation in pelagic fish muscle is a complex challenge as a consequence of the simultaneous coexistence of long-chain n-3 polyunsaturated fatty acids (PUFA), principally eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), and the elevated amounts of catalysts of lipid oxidation, such as heme proteins and redox active metals (*12*). EPA and DHA are characterized by an extremely high oxidative instability (*13*), and fish hemoglobins are stronger promoters of lipid oxidation in comparison with those from terrestrial animals (*14*). Accordingly, minced mackerel muscle developed faster lipid oxidation than mince from beef, duck, ostrich, pork, and chicken (*15*). Although several studies

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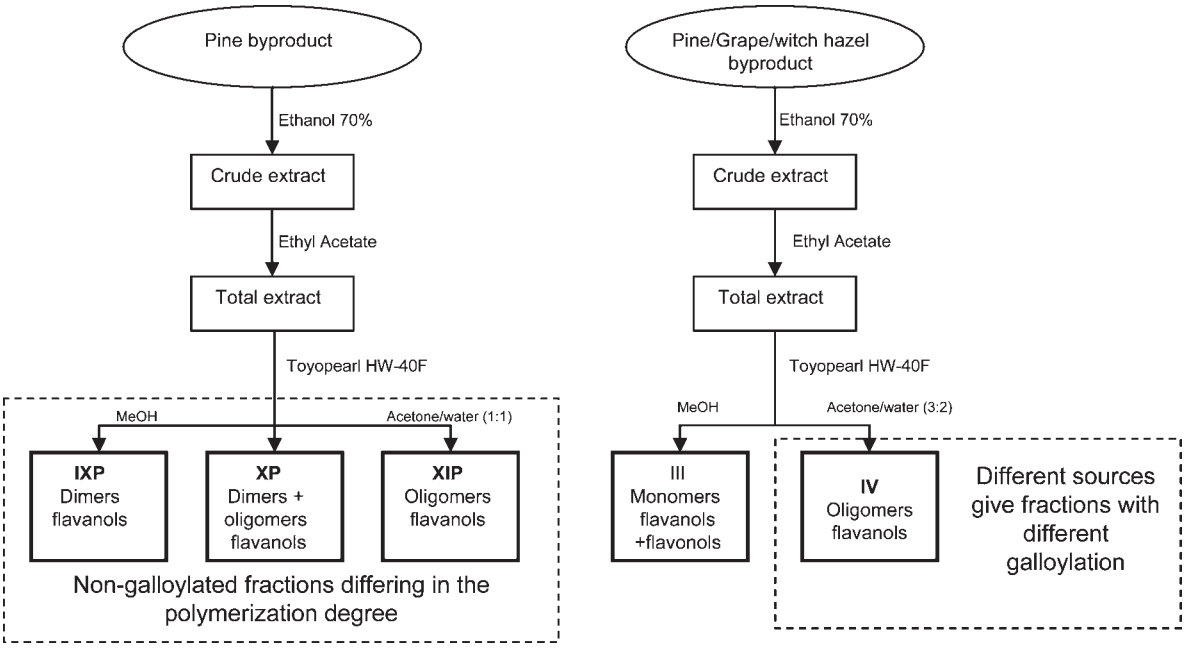

Figure 1. Scheme of preparation of the different fractions from pine, grape, and witch hazel residues.

have demonstrated the ability of catechins and proanthocyanidins to retard lipid oxidation in pelagic fish muscle (*11*, *16*) or in model systems based on minced washed fish muscle supplemented with fish hemoglobin (*17*, *18*), there is no comprehensive knowledge about how the structural diversity of flavan-3-ols modulates their antioxidant activity in fish muscle.

The objective of the present work was to investigate the influence of the number of monomeric units (polymerization) and content of esterified galloyl groups (galloylation) of polyphenols on their efficiency to prevent lipid oxidation in the highly oxidizable pelagic fish muscle. Polyphenols of different natural byproducts (pine bark, grape pomace, and witch hazel bark), or mixtures of them, were selected with the purpose of obtaining homologous fractions differing in polymerization and galloylation. To search for more convenient natural sources to minimize production cost, an alternative fraction purified from a commercial grape seed extract rich in proanthocyanidins was also evaluated.

MATERIALS AND METHODS

Chemicals. Fresh horse mackerel (*Trachurus trachurus*) was acquired from a local market, and light muscle was immediately isolated and minced. Trichloroacetic acid, 1,1,3,3-tetraethoxypropane, ferrozine, $FeCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$ were obtained from Sigma (St. Louis, MO). Thiobarbituric acid was purchased from Merck (Darmstadt, Germany). 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) was obtained from Fluka (New-Ulm, Switzerland). All chemicals were of analytical grade, and the water was purified using a Milli-Q system (Millipore, Billerica, MA).

Polyphenolic Fractions. Fractions from pine (*Pinus pinaster*) bark, grape (*Vitis vinifera*) pomace, and witch hazel (*Hamamelis virginiana*) bark were obtained as previously described by Torres et al. (*6*, *19*, *20*). Briefly, the fractions from pine, IXP, XP, and XIP, were fractionated by applying size exclusion chromatography on Toyopearl resin to the polyphenols soluble in both ethyl acetate and water (**Figure 1**). The estimation of the averaged molecular weight, mean degree of polymerization, and galloylation was performed by HPLC analysis after depolymerization with cysteamine (*21*), being summarized in **Table 1**. Fractions IXP, XP, and XIP provided non-galloylated proanthocyanidins with different numbers of catechin monomers. Homologous fractions IV from pine (IVP), grape (IVG), and witch hazel (IVH) were also obtained by Toyopearl chromatography. IVP and IVG differed essentially in the content of gallate groups esterified because both fractions contain oligomers with similar size (2.7−2.9 monomers), but IVP was lacking galloyl groups and IVG had a low galloylation degree (0.25 mol of gallate/mol of polyphenol) (**Table 1**). IVH was composed by 80% of hydrolyzable tannins, mainly galloyl glucose with 5−10 galloyl moieties, hamamelitannin, and methyl gallate, and 20% of proanthocyanidins with mean polymerization and galloylation of 1.6 units and 0.23 mol of gallate/mol of polyphenol (*22*) (**Figure 2**). The hydrolyzable tannins confer elevated galloylation (>1 mol of gallate/mol polyphenol) to the witch hazel fraction. IVMix was prepared by mixing non-galloylated pine polyphenols and highly galloylated witch hazel polyphenols in a ratio of 1:1 to constitute a fraction with an intermediate galloylation between IVG and IVH (**Table 1**).

Table 1. Polymerization and Galloylation of the Polyphenolic Fractions

	polymerization (mean number of catechin monomers)[a]	galloylation (mol of gallate/mol of polyphenol)[b]
IXP	1.9	0.00
XP	2.2	0.00
XIP	3.4	0.00
IVP	2.9	0.00
IVG	2.7	0.25
IVH[c]	1.6	>1
IVMix	2.3	>0.5
IVGseed	2.4	0.15

[a] Mean degree of polymerization of the proanthocyanidic fraction. [b] Galloylation degree sums the contribution of both condensed and hydrolyzable tannins. [c] *Hamamelis* fraction was composed by 80% of hydrolyzable tannins.

An alternative polyphenolic fraction, IVGseed, was prepared by applying directly size exclusion chromatography on Toyopearl resin to a commercial grape seed extract (Le Grandonnenque, Cruviers-Lascours, France) that contained 40% of proanthocyanidins. IVGseed contained proanthocyanidins with averaged molecular weight of 760 g/mol, main polymerization of 2.4 monomeric units, and galloylation of 0.15 gallate group per molecule.

Electron-Donating Capacity. The ferric reducing/antioxidant power (FRAP) method was used to estimate the electron-donating capacity of polyphenols. The procedure was adapted from that described by Benzie and Strain (*23*). Briefly, 1.5 mL of daily prepared FRAP reagent (containing acetate buffer (pH 3.6), TPTZ, and ferric chloride) was mixed with 150 μL of different concentrations of polyphenols in water (30−250 μM). The absorbance was measured at 593 nm after 4 min of incubation at room temperature. The number of donated electrons was

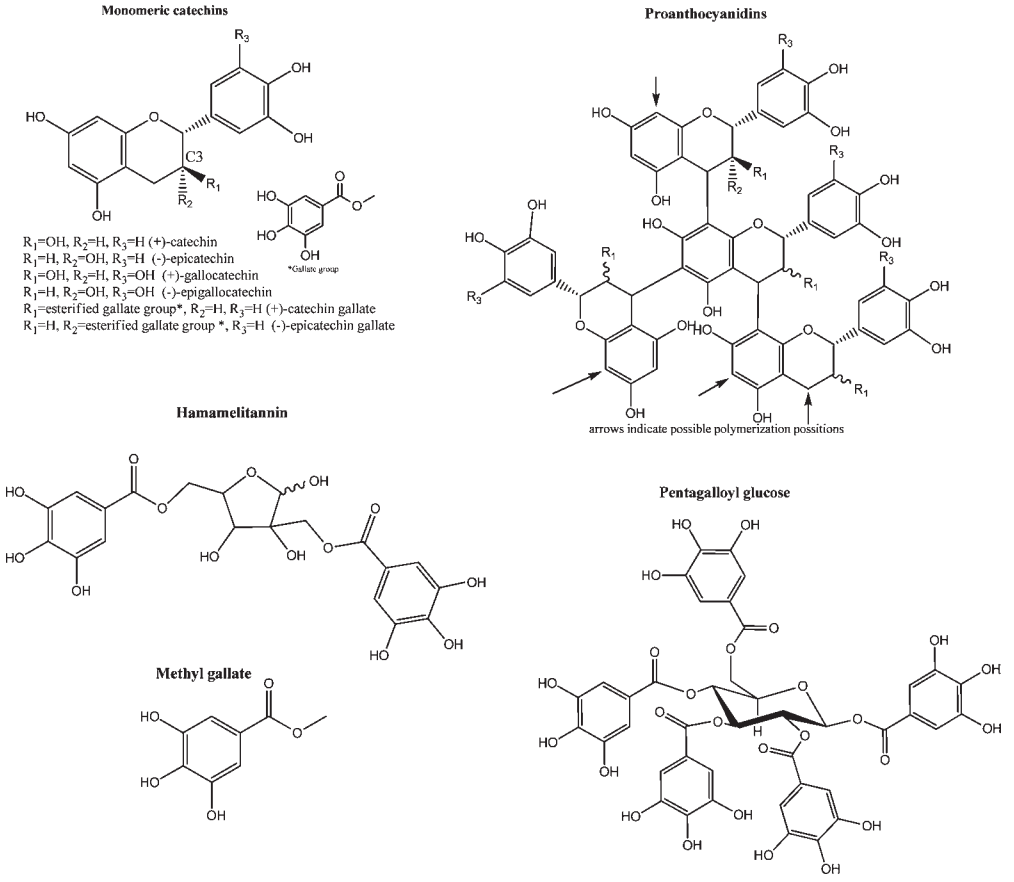

Figure 2. Chemical structures of polyphenols present in the polyphenolic fractions.

calculated from the slopes of the lineal adjustments between the FRAP activity and the polyphenolic concentration.

Ferrous-Chelating Activity. The capacity of polyphenols for chelating ferrous iron was determined using an adaptation of Kolayli et al.'s procedure (*24*). Briefly, 0.2 mL of an aqueous polyphenolic solution was mixed 0.2 mL of 0.2 mM ferrous chloride and 1.2 mL of a buffer solution, pH 6.8, containing 0.12 M KCl and 5 mM L-histidine. Then, 0.4 mL of 1 mM ferrozine was added, and the samples were incubated at room temperature for 10 min. The absorbance was measured at 560 nm, and the chelating capacity was expressed as the percentage of ferrous iron chelated by 0.2 mM polyphenolic compound.

Preparation of Minced Fish Muscle. For each experiment 15/20 different individuals of fresh Atlantic horse mackerel (*T. trachurus*), 8−10 kg, were deboned and eviscerated. White muscle was separated and minced to obtain a muscle homogenate. Polyphenols were incorporated into the fish muscle as powder, and portions of 10 g of fish muscle were placed into screw-capped 50 mL Erlenmeyer flasks and stored chilled at 4 °C. Lipid oxidation was monitored by sensory analysis and the formation of different lipid oxidation byproducts: lipid peroxides (peroxide value), thiobarbituric acid reactive substances (TBARS), and volatiles.

Preparation of Fish Fillets. For each experiment, skin-on fillets (25−30 g) were obtained from 10−12 kg of fresh horse mackerel (*T. trachurus*). Fish fillets were washed in a container with distilled water, as previously described (*25*). Briefly, the ratio fillet/water was 1:5 (w/w). Then, the excess of water was removed by holding the fillets on an inclined surface, and they were sprayed with approximately 0.5 mL of a polyphenolic water solution. Before freezing, fillets were held during 15−30 min to allow the diffusion of polyphenols in the flesh. The final concentration of polyphenols in the fillets was 100 ppm (mg/kg of muscle). The polyphenolic solution was replaced by deionized distillate water in control fillets. Fillets were introduced into plastic bags and kept at −18 °C. Three fillets of each antioxidant procedure were taken at several sampling times and were thawed at room temperature for 1 h prior to lipid oxidation analysis. The development of lipid oxidation was evaluated by sensory analysis and monitoring the formation of lipid peroxides, TBARS, and volatiles.

Evaluation of the Antioxidant Effectiveness. The antioxidant effectiveness of the different fractions was evaluated by comparing induction periods and inhibition percentages for the formation of the lipid oxidation products, lipid peroxides, and volatiles responsible of rancidity. Induction periods were calculated as the time (in days or months) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve (*9*). The method of tangents calculates the induction period from the intersection points between the tangent to the initiation phase and the tangent to the propagation phase of the kinetic of oxidation. The inhibition percentages of lipid oxidation were calculated during the propagation period of oxidation by using the formula proposed by Frankel (*9*)

$$\frac{C-S}{C}\times 100$$

where C represents the amount of the corresponding lipid oxidation product in the control sample without antioxidant and S the concentration of the lipid oxidation product in the fish muscle supplemented with polyphenols.

Lipid Extraction. Lipids were extracted from fish muscle according to the method of Bligh and Dyer (*26*). Lipid content was determined gravimetrically and expressed on a wet weight basis (*27*).

Peroxide Value. Peroxide value was determined according to the ferric thiocyanate method (*28*) and expressed as milliequivalents of oxygen per kilogram of lipid.

Thiobarbituric Acid Reactive Substances (TBARS) Analyses. TBARS (mg of malonaldehyde (MDA)/kg of muscle) were determined by using Vyncke's procedure (*29*). 1,1,3,3-Tetraethoxypropane was used as standard.

Volatile Analysis. Volatile compounds associated with the lipid oxidation of fish muscle were concentrated with a procedure of headspace solid-phase microextraction (HS-SPME) and determined using gas chromatography in combination with mass spectrometry (GC-MS), as previously described by Iglesias et al. (*30*). Briefly, 6 mL of a saline extract of

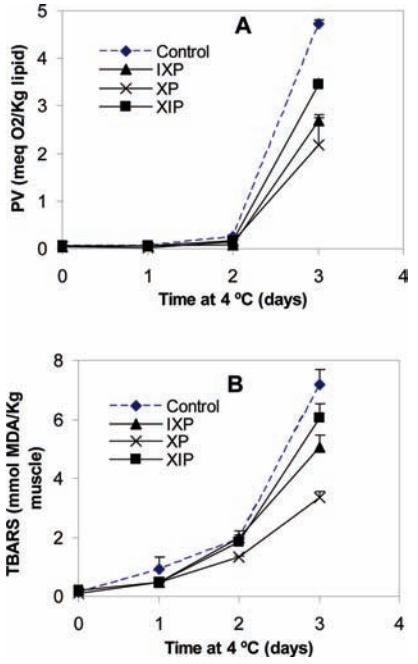

Figure 3. Comparative formation of lipid peroxides (**A**) and TBARS (**B**) in refrigerated horse mackerel mince supplemented with 50 ppm of different pine fractions (IXP, XP, and XIP) differing in mean degree of polymerization.

the fish muscle was incubated on a CAR/PDMS fiber (75 μm Carboxen/polydimethylsiloxane coating, Supelco, Bellefonte, PA) during 30 min at 60 °C under magnetic stirring. GC-MS analysis was performed in a Thermo Finnigan ThermoQuest (San Jose, CA) gas chromatograph equipped with a split/splitless injector and coupled with a Trace quadrupole mass detector (Thermo Finnigan ThermoQuest). The volatiles were desorbed in the GC injection port for 10 min at 300 °C, according to technical recommendations, and quantified by MS in the selected ion monitoring mode by using 3-methyl-3-buten-1-ol as internal standard.

Sensory Analysis. Sensory analysis was evaluated by an expert panel formed by four specialists in distinguishing fishy off-flavors. Approximately 10 g of muscle was placed in separate sterile polystyrene Petri dishes and placed at room temperature during 10 min before analysis. Panelists were concentrated in detecting rancid odors.

Statistical Analysis. The experiments were performed twice, and data are reported as the mean ± standard deviation of three replicates. The data were analyzed by one-way analysis of variance (ANOVA) and the least-squares difference method. Statistical analyses were performed with the software Statistica 6.0 (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Effect of Polymerization on the Antioxidant Efficacy of Polyphenols. The influence of the number of catechin monomers on the capacity to prevent lipid oxidation in fish muscle was investigated by using different polyphenolic fractions from pine bark. Pine bark has been previously shown as a good source of non-galloylated proanthocyanidins (*6*). The null content of the pine polyphenols in esterified gallate moieties is relevant because galloylation has been shown to modulate important physicochemical properties involved in the antioxidant activity of polyphenols, such as membrane affinity (*31*), electron-donating capacity (*8*), chelating capacity (*8*), and tocopherol-regenerating capacity (*32*).

Polyphenolic fractions were tested in minced horse mackerel muscle with a 3.7 ± 0.2% of fat content. The pine bark fractions evaluated, IXP, XP, and XIP, possessed mean degrees of polymerization of 1.9, 2.2, and 3.4, respectively (**Table 1**). The results evidenced the ability of all pine bark fractions (50 ppm) to reduce the generation rate of lipid oxidation byproducts; however, the induction periods for the formation of lipid oxidation byproducts were not increased by the presence of the polyphenolic fractions (**Figure 3**). The fraction more polymerized (XIP) was significantly less effective in preventing the formation of either lipid peroxides or TBARS, and therefore, muscle supplemented with XIP exhibited the highest values of these lipid oxidation byproducts at the third day. Fraction XP was significantly the most effective in retarding the formation of TBARS and also showed a strong activity inhibiting peroxides, which was comparable to that found for IXP. Samples with XP reached inhibition percentages for the formation of peroxides and TBARS of approximately 53% (day 3), whereas with XIP the inhibition percentages ranged from 15 to 26% and with IXP ranged from 29 to 43% (**Table 2**). Therefore, the inhibition percentages evidenced that polymerization degrees between 2 and 3 monomers were adequate to be employed as antioxidants in fish muscle. The data were in agreement with previous research employing low-galloylated grape proanthocyanidins that showed higher antioxidant activity in frozen fish muscle for an intermediate polymerization of 2.7 units (*11*). Moreover, this effect of polymerization was similar to that found in a recently published study performed in emulsified fish oil with phosphatidylcholine, in which catechin oligomers with 2−3 monomeric units showed an optimum antioxidant activity (*8*).

Table 2. Inhibition Percentages on the Formation of Peroxide Value (PV) and Thiobarbituric Acid Reactive Substances (TBARS) in Fish Muscle Supplemented with 50 ppm (Milligrams per Kilogram of Muscle) of the Pine Bark Fractions IXP, XP, and XIP[a]

	PV	TBARS
control	0.0 ± 1.8 a	0.0 ± 7.5 a
IXP	43.1 ± 2.9 c	29.6 ± 6.0 b
XP	53.9 ± 12.1 c	53.4 ± 3.2 c
XIP	26.8 ± 2.6 b	15.5 ± 6.8 b

[a] Percentages of inhibition were calculated after 3 days of refrigerated storage. Values with the same letter in the same column are not significantly different ($p > 0.05$).

On the basis of previous investigations that evidenced a positive contribution of polymerization on the capacity of oligomeric polyphenols to chelate ferrous ions, or to donate hydrogen atoms or electrons (*8*), a most advantageous antioxidant activity would be expected for the highest polymerized fraction, XIP, and an intermediate activity for the medium-sized polyphenols of fraction XP. Therefore, our results suggest that the strongest antioxidant activity of medium-sized proanthocyanidins in fish muscle is regulated by ferrous-chelating and reducing properties.

Effect of Galloylation on the Antioxidant Activity of Polyphenols. The influence of galloyl moieties on the antioxidant capacity was investigated by comparing the activity of homologous polyphenolic fractions obtained from pine bark, grape pomace, and witch hazel bark, or mixtures of them (**Table 1**). Galloyl moieties were absent in the polyphenols from pine bark (IVP), and the grape extract (IVG) exhibited a low-medium galloylation degree, 0.25 gallate per polyphenolic unit. Polyphenols from witch hazel (IVH) contained high galloylation (> 1 gallate per molecule) due to the important content in hammamelitanin (2 gallates per molecules) and hydrolyzable tannins with 5−10 galloyl residues. An intermediate galloylation (> 0.5 gallate/molecule) was achieved in the composite fraction IVMix, in which pine and witch hazel polyphenols were mixed in ratio 1:1 (IVMix).

The antioxidant activity was evaluated in refrigerated minced horse mackerel muscle supplemented with polyphenolic fractions at 50 ppm. The lipid content of fish muscle was 1.3 ± 0.15%. The results revealed that all polyphenolic fractions were able to prevent lipid oxidation by either increasing induction periods for the formation of lipid oxidation byproducts or reducing their

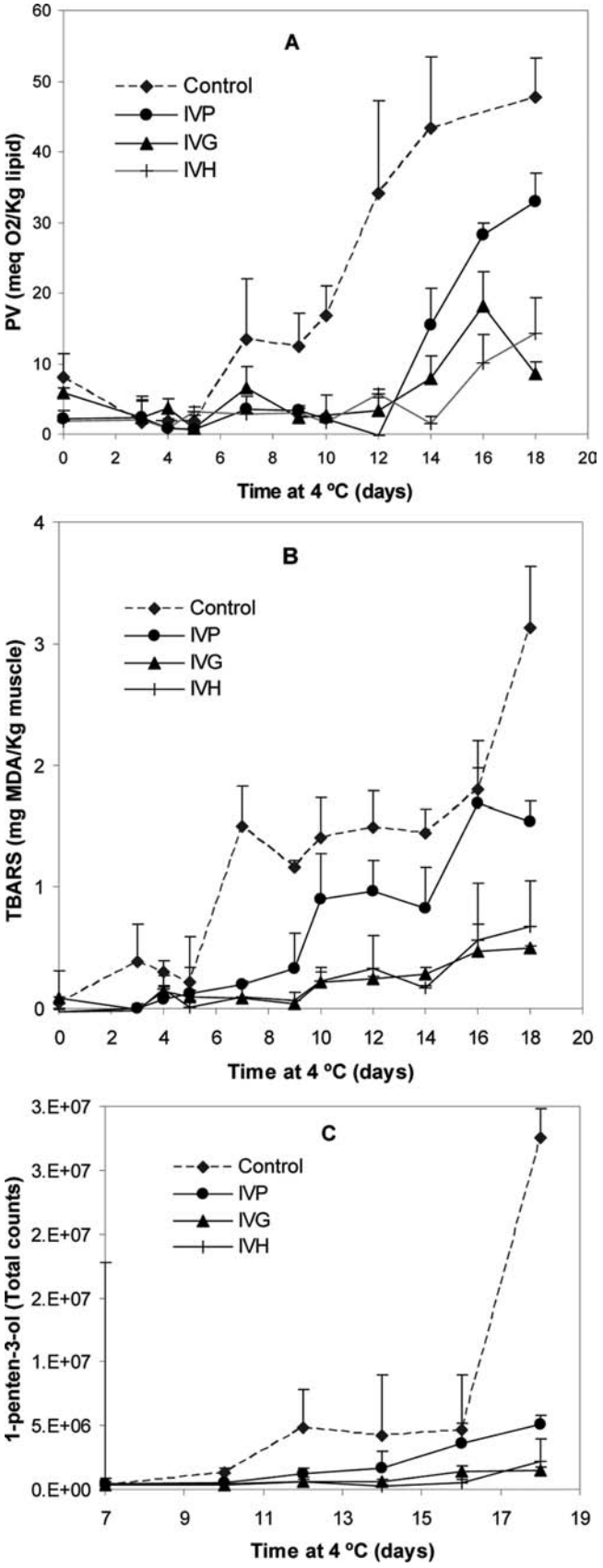

Figure 4. Comparative formation of lipid peroxides (**A**), TBARS (**B**), and 1-penten-3-ol (**C**) in refrigerated horse mackerel mince supplemented with 50 ppm homologues fractions from pine bark (IVP), grape pomace (IVG), and witch hazel bark (IVH), which provide non-galloylated, low-galloylated, and high-galloylated polyphenols respectively.

Table 3. Inhibition Percentages on the Formation of Peroxide Value (PV), Thiobarbituric Acid Reactive Substances (TBARS), and 1-Penten-3-ol in Fish Muscle Supplemented with 50 ppm (Milligrams per Kilogram of Muscle) of the Pine Bark Fraction IVP and the Homologous Fractions from Grape (IVG) and Witch Hazel Bark (IVH)[a]

	PV	TBARS	1-penten-3-ol
control	0.0 ± 7.8 a	0.0 ± 10.9 a	0.0 ± 5.0 a
IVP	30.7 ± 8.8 b	50.8 ± 5.2 b	81.4 ± 2.6 b
IVG	82.1 ± 3.8 c	84.1 ± 0.5 c	94.5 ± 0.9 c
IVH	73.2 ± 16.3 c	79.4 ± 11.9 c	92.2 ± 6.4 c

[a] Percentages of inhibition were calculated after 18 days of chilled storage. Values with the same letter in the same column are not significantly different ($p > 0.05$).

generation rate during the propagation step of lipid oxidation (**Figure 4**). The formation of peroxides showed induction periods of 5 days for controls not supplemented with polyphenols, whereas samples with pine, grape, and witch hazel extracts retarded the formation of hydroperoxides by 7−9 days (**Figure 4A**). In accordance with the lower fat content, the induction periods were significantly higher than those found in the previous experiments in which the influence of polymerization was studied. The comparison of fractions indicated less efficiency of non-galloylated polyphenols from pine bark to prevent the generation of peroxides. The inhibition percentage for the formation of peroxides reached values of 30.7% for IVP, those for IVG and IVH being not significantly different with values ranging from 73.2 to 82.1% (**Table 3**). The secondary oxidation byproducts measured by TBARS were also effectively retarded by the polyphenolic extracts. Again, non-galloylated pine polyphenols IVP were less effective in inhibiting TBARS (**Figure 4B**). IVG with low galloylation and IVH with high galloylation exhibited similar capacities to prevent the formation of TBARS. After 18 days of storage at 4 °C, fish muscle with IVP, IVG, and IVH exhibited inhibition percentages of 50.8, 84.1, and 73.2%, respectively (**Table 3**). A similar tendency was observed for the formation of 1-penten-3-ol, which has been previously described as a marker of rancid odors (*30*). IVP was less effective in inhibiting the generation of 1-penten-3-ol, IVG and IVH showing the strongest capacity to delay the formation of that volatile (**Figure 4C**). The inhibition percentage for the formation of 1-penten-3-ol was lower for IVP (81.4%) at day 18, followed by IVG (94.5%) and IVH (92.2%), which did not show significant differences. These results demonstrated that the presence of esterified galloyl residues enhances the ability of polyphenols to inhibit lipid oxidation in pelagic fish muscle, although fractions IVG, with low galloylation (0.25 gallate/molecule), and IVH, with high galloylation (> 1 gallate/molecule), displayed similar antioxidant effectiveness.

To test the influence of intermediate galloylations between that of IVG and IVH, the antioxidant capacity of a combined fraction (IVMix) obtained by mixing pine and witch hazel polyphenols was also evaluated. Galloylation in the fraction IVMix was half of that found in IVH (**Table 1**). Considering the high commercial interest for stabilizing frozen fish products, this fraction was evaluated in frozen horse mackerel fillets even though previous arrays of experiments have shown the utility of chilled tests to predict the antioxidant behavior in frozen fish (*33*). Fish fillets were supplemented with 100 ppm polyphenols by spraying those in water solution after fillets had been washed with water. The combination of washing with water plus spraying polyphenols has been previously used to enhance the antioxidant capacity of grape polyphenolics in fish fillets (*25*). IVMix was efficient in inhibiting the formation of lipid peroxides, such activity being not significantly different from that found for IVG (**Figure 5A**) Both fractions were also active in delaying the formation of TBARS

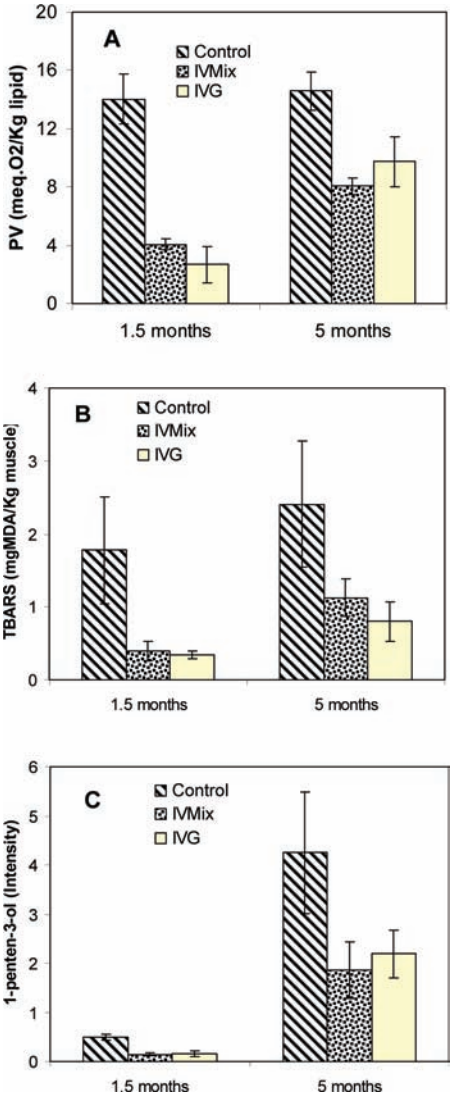

Figure 5. Formation of lipid peroxides (**A**), TBARS (**B**), and 1-penten-3-ol (**C**) in frozen horse mackerel fillets supplemented with 100 ppm of the fraction obtained from grape pomance (IVG) and the composite fraction obtained by mixing pine and witch hazel polyphenols in a ratio of 1:1 (IVMix). IVG and IVMix contained low (0.25 gallate groups/molecule) and medium (>0.5 gallate groups/molecule) galloylation.

and 1-penten-3-ol during the entire period of frozen storage, 5 months (**Figures 5B**,**C**). Significant differences were not found between IVMix and IVG for the inhibition of TBARS and 1-penten-3-ol. The sensory analysis detected rancid odors in control fillets at 1.5 months, whereas rancidity was observed in the fillets enriched with IVMix or IVG after 2.5 months. These results and those from the previous experiments evidenced that the antioxidant activity of proanthocyanidins in pelagic fish muscle is not enhanced by increasing the galloylation degree of the grape fraction (0.25 gallate/molecule) to higher values such as intermediate (> 0.5 gallate/molecule) or elevated galloylation (> 1 gallate/molecule). A similar effect of galloylation has been previously described in fish oil emulsions with phosphatidylcholine as emulsifying agent (*8*). In this study, the evaluation of a large collection of polyphenolic fractions revealed that proanthocyanidins with low−medium polymerization and galloylation are optimal for preventing oxidation in this emulsion. This finding highly suggests that emulsions prepared with phospholipids as emulsifying agents can mimic the behavior of polyphenols in muscle-based foods considering the structural similarities of both

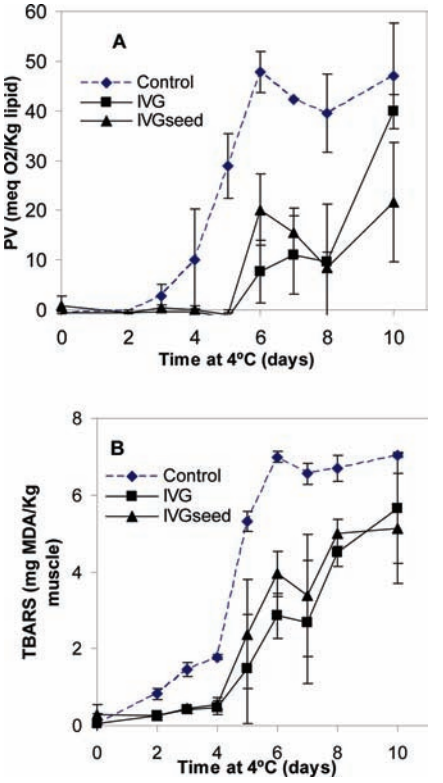

Figure 6. Comparative formation of lipid peroxides (**A**) and TBARS (**B**) in refrigerated horse mackerel mince supplemented with 10 ppm of the fraction obtained from grape pomance (IVG) and the homologous low-cost fraction obtained from a commercial grape seed extract (IVGseed).

systems. Biological membranes are sensitive oxidative sites for oxidation in muscle tissues because they are composed by phospholipids highly unsaturated and surrounded by cytosolic medium, which contains high proportions of redox active metals (*13*). Phospholipids display similar disposition in cellular membranes and oil-in-water emulsions, locating the negatively charged phosphate group (polar head) to the external face and the apolar tails to the inner part. This may explain the similarities found for the antioxidant activity of proanthocyanidins in fish muscle and fish oil emulsified by phospholipids. However, less polymerized and galloylated proanthocyandins were more active in preventing oxidation in a monophasic system such as bulk fish oil (*8*, *11*). The higher hydrophilic character of the less polymerized and galloylated polyphenols (*8*), together with the polar paradox that establishes higher antioxidant activity in bulk oil for those more hydrophilic (*9*), can reasonably explain the poor antioxidant behavior of large-sized galloylated polyphenols in bulk fish oil.

Previous investigations indicated the strongest ability of highly galloylated polyphenols from witch hazel to recycle endogenous α-tocopherol from α-tocopheroxyl radical in comparison to less galloylated polyphenols from pine bark and grape (*32*). Galloylation especially favored the capacity to regenerate α-tocopherol in heterogeneous systems of sodium docecyl sulfate (SDS) micelles, which imitate structurally cellular membranes. The presence of galloyl groups also enhanced the iron-chelating activity and electron-donating capacity of polyphenols (*8*). Additionally, galloylation increased the incorporation of polyphenols into membranes (*31*), well-known sensitive oxidative sites in muscle tissues. Therefore, all in vitro properties would suggest a much elevated antioxidant ability of the highly galloylated witch hazel polyphenols. We hypothesize that the strong interactions shown

by galloylated polyphenols with proteins (*34*) may explain in part the minor activity expected of the most galloylated ones. Because the protein content of fish muscle is relatively high (18−22%), the establishment of protein−polyphenol bindings should limit the accessibility of the most galloylated to the active sites of oxidation (membranes and aqueous interfaces).

The preparation of effective polyphenols from grape pomace is a quite costly and time-consuming procedure. For this reason, we propose to employ a commercial grape seed extract rich in proanthocyanidins as an alternative polyphenolic source. The direct application of size exclusion chromatography on the commercial grape seed extract provided proanthocyanidins with low−medium polymerization (2.4 monomeric units) and galloylation (0.15 galloyl unit/molecule), labeled IVGseed. In vitro tests showed similar abilities of IVGseed and IVG in donating electrons and chelating ferrous ions, two well-established antioxidant routes of polyphenols. IVGseed was able to donate approximately 6.0 electrons/molecule and to chelate about 83% of the ferrous ions in equimolar concentration, these values being analogues to those previously reported for IVG (*8*, *11*). The antioxidant activity of the low-cost fraction IVGseed was investigated in chilled pelagic fish mince at two different concentrations (10 and 100 ppm). The results showed that fish muscle supplemented with the lowest polyphenolic concentration increased the induction periods for the formation lipid peroxides. Control samples not supplemented with polyphenols exhibited induction periods of 3 days for the formation of peroxides, whereas muscle supplemented with IVGseed showed induction periods 3 days longer (**Figure 6A**). IVGseed provided a similar ability in inhibiting lipid peroxides as fraction IVG. Both fractions were found to be active to the same extent in inhibiting TBARS (**Figure 6B**). Accordingly, sensory analysis detected incipient rancid odors after 5 days in muscle supplemented with IVG or IVGseed and after 4 days in control samples. At the highest concentration, 100 ppm, the effectiveness displayed for IVGseed and IVG in retarding lipid oxidation in chilled fish muscle was also similar (data not shown).

In summary, the present investigation has demonstrated that proanthocyanidins with medium size (2−3 monomeric units) and low galloylation degree (0.15−0.25 gallate group/molecule) are convenient to inhibit lipid oxidation in pelagic fish muscle. Higher polymerization was found to reduce activity, whereas the increment of galloylation originated the same efficiency. This tendency showed similarities with that found previously in fish oil emulsions with phosphatidylcholine as emulsifier (*8*), suggesting that such emulsion systems can be employed as simple models to evaluate antioxidant activity in muscle-based foods. Polyphenols with adequate polymerization and galloylation employed in pelagic fish muscle can be also prepared from commercial grape seed extracts, which reduces notably the production cost. The present results add relevant information to optimize the application of proanthocyanidins as antioxidants in pelagic fish muscle and also to find satisfactory natural sources to produce effective polyphenols at competitive cost.

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