

Contribution of Galloylation and Polymerization to the Antioxidant Activity of Polyphenols in Fish Lipid Systems

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Polyphenolic fractions extracted from pine (*Pinus pinaster*) bark, grape (*Vitis vinifera*) pomace, and witch hazel (*Hamamelis virginiana*) bark were selected for investigating the influence of the number of phenolic units, polymerization, and the content of esterified galloyl residues (galloylation) on their efficacy for inhibiting lipid oxidation in fish lipid enriched foodstuffs. Experiments carried out with nongalloylated pine bark fractions with different polymerization degrees demonstrated that the number of catechin residues per molecule modulates their reducing and chelating properties in solution. In real food systems such as bulk fish oil and fish oil-in-water emulsions, the efficacy against lipid oxidation was highly dependent on the physical location of the antioxidant at the oxidative sensitive sites. The lowest polymerized fractions were the most efficient in bulk fish oil samples, whereas proanthocyanidins with an intermediate polymerization degree showed the highest activity in fish oil-in-water emulsions. Galloylation did not influence the antioxidant effectiveness of proanthocyanidins in bulk fish oils. The presence of galloyl groups favored the antioxidant activity of the polyphenols in emulsions, although results indicated that a high degree of galloylation did not improve significantly the activity found with medium galloylated proanthocyanidins. The results obtained in this research provide useful information about the relationship between structure and antioxidant activity in order to design antioxidant additives with application in fish oil-enriched functional foods.

KEYWORDS: Lipid oxidation; proanthocyanidins; polymerization; galloylation; fish oil; fish oil emulsion

INTRODUCTION

Seafood is very susceptible to suffer lipid oxidation, which leads to the development of rancidity and the reduction of shelf life and nutritive value. The delay of this process is important for the entire production and commercialization chain of this type of foodstuff, and the addition of antioxidant substances is one of the most widely used strategies for preventing or retarding lipid deterioration (1). The adverse toxicological reports of many synthetic compounds and consumer interest in natural food additives have caused in recent years an enormous demand for natural bioactive substances with food antioxidant activity.

Polyphenolic extracts from natural sources are very interesting additives because of their natural origin and efficiency in inhibiting lipid oxidation processes. Mechanistically, the antioxidant behavior of phenolics has been attributed to their free radical scavenging activity (2, 3), capacity to protect or recycle endogenous antioxidants (4, 5), and ability to chelate redox-active metals (6). Among polyphenolics, the family of flavan-3-ols, together with their related oligomers, proanthocyanidins (Figure 1), is extensively distributed in nature (7). The importance of the polymerization degree (number of polyphenolic units) and the percentage of galloylation (content of esterified galloyl groups) in their antioxidant activity has been evidenced in several in vitro assays (8, 9) in low-density lipoproteins (10) or human colon cancer cells (11). Oligomeric procyanidins (roughly two to seven residues) have been considered to be more

efficient than monomers, but high degrees of polymerization have been also associated with mucosal irritability and astringency effects. The increment of hydroxylic groups in the pyrogallol moiety provides more hydrogen atoms or electrons than the catechol group and affects the redox potential and the metal chelation capacity of polyphenols (1). Variation of polymerization and galloylation can also affect the polarity of polyphenols, influencing their location on the oxidation sensitive sites of foods in which lipids can be found in different compartments.

The use of polyphenols from agricultural and forestry byproducts provides additional advantages of waste recycling and low cost of the raw material. Pine (*Pinus pinaster*) bark, grape (*Vitis vinifera*) pomace, and witch hazel (*Hamamelis virginiana*) bark are residues from the wood and wine industries that contain a great variety of phenolic substances (11, 12). In recent studies, the extraction, fractionation, and characterization of the polyphenolic content of these residues showed a high content of proanthocyanidins with different polymerization degrees. Grape fractions presented gallate esters in their structure (Figure 1), whereas no galloylation was detected in the pine extracts (11). Witch hazel extracts showed a low content of oligomeric proanthocyanidins but a high content of hydrolyzable tannins based on gallate esters (Figure 1) (13).

The aim of this investigation was to study the influence of polymerization and galloylation on the activity of polyphenols for inhibiting oxidation of fish lipid systems. For this purpose, polyphenolic fractions extracted from pine bark, grape pomace, and witch hazel bark, with different compositions, were tested as antioxidants in bulk oil and oil-in-water emulsions. Results were

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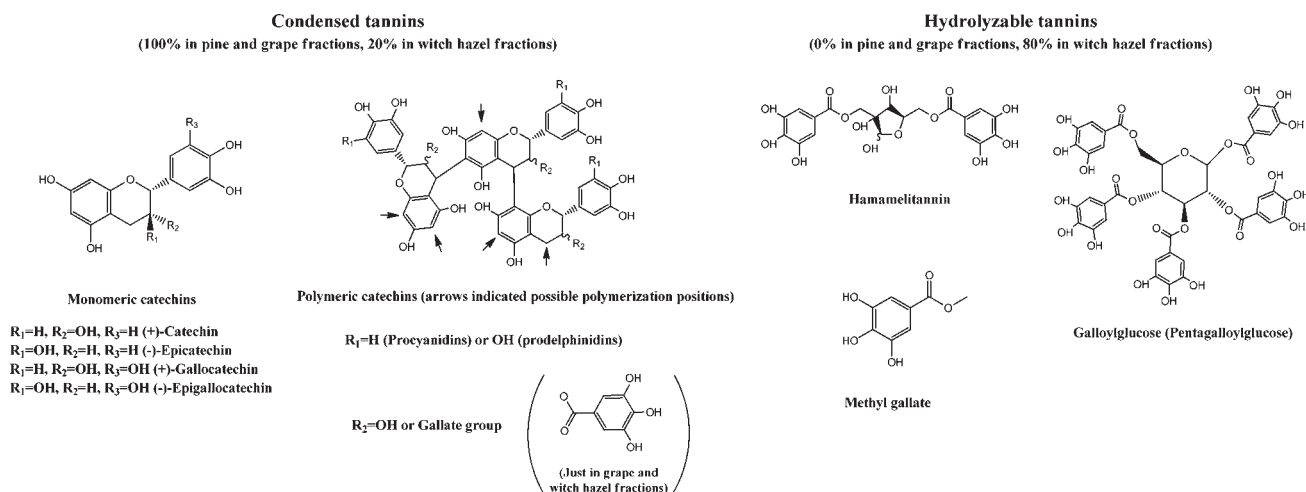


Figure 1. Structures of hydrolyzable and condensed tannins.

explained by considering the structural moieties of the phenolic compounds that have a significant influence on their reducing and chelating activities and polarity.

MATERIALS AND METHODS

Materials. High-quality fish liver oil from cod (*Gadus morhua*) was purchased from Fluka (New-Ulm, Switzerland). Cod liver oil was flavorless and odorless and had a peroxide value of 0.5 mequiv of oxygen/kg. Soybean lecithin (40% L- α -phosphatidylcholine; Sigma, St. Louis, MO) was used as emulsifying agent. Ferrozine, $FeCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$ were also supplied by Sigma. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was obtained from Fluka. All chemicals and solvents used were of either analytical or HPLC grade (Ridel-Haën, Seelze, Germany).

Polyphenolic Fractions. Phenolic fractions from pine (*P. pinaster*) bark (IP, IIP, IIIP, IVP, VP, VIP, VIIP, IXP, XP, and XIP), grape (*V. vinifera*) pomace (IVG, VIG, and VIIIIG), and witch hazel (*H. virginiana*) bark (IVH, VIH, and VIIIH) were prepared by fractionation of polyphenolic extracts soluble in both ethyl acetate and water according to the methods of Torres and Bobet (14) and Touriño et al. (11, 12) following the general procedure shown in Scheme 1. Briefly, the crude extract contained mainly flavanol (catechin) monomers, flavanol oligomers (proanthocyanidins), and monomeric glycosylated flavonols. It was then separated into a set of fractions differing in composition and proanthocyanidin structure (Scheme 1; Figure 1; Table 1). The RP-HPLC step fractionated the components on the basis of their hydrophobic interactions with the stationary phase, which is not always related to the size of the flavonoids. Chromatography on Toyopearl separated the components mainly by size: monomers from oligomers. The average molecular weight and composition (mean degree of polymerization, mDP, and percentage of galloylation) (Table 1) were estimated by HPLC analysis after depolymerization with cysteamine (13). Homologous fractions extracted from pine bark and grape pomace are mainly composed by proanthocyanidins with similar polymerization degrees, but they differed in the percentage of galloylation, because grape fractions showed medium galloylation and esterified galloyl groups were absent in pine polyphenols (Table 1). Fractions II and III from pine and grape also contained monomeric glycosylated flavonols. *H. virginiana* fractions were composed by >80% of hydrolyzable tannins and 20% of proanthocyanidins (15), fundamentally hamamelitannin, methyl gallate, and galloyl glucoses with 2–10 galloyl moieties (Figure 1). Such composition leads to a high galloylation level $pf > 100\%$ because hydrolyzable tannins contain several galloyl residues per molecule in their structure (Figure 1). A more detailed description of the composition of each fraction has been previously reported (11, 12, 14).

Reducing Power of the Phenolic Compounds. The ferric reducing antioxidant power (FRAP) method was used by adaptation of the procedure of Benzie and Strain (16). 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. FRAP reagent (1.5 mL) was incubated for 10 min

at 37 °C. Then, 150 μ L of water and 50 μ L of phenolic solution (0.2–4 mg/L) were added, and the absorbance was measured at 593 nm after 4 min. The standard curve was built with ferrous chloride. FRAP values were calculated for increasing molar concentrations of each antioxidant, and a linear relation was obtained. The slope provided the number of moles of ferric chloride that was reduced by 1 mol of antioxidant. Because only one electron is required to reduce each Fe^{3+} atom, the obtained value provided the number of electrons donated for each antioxidant molecule.

Chelating Activity of the Antioxidants. The capacity of the polyphenolic fractions for chelating ferrous iron was determined using an adaptation of the Kolayli et al. procedure (17). A total of 0.2 mL of polyphenolic solution was mixed with 1.2 mL of 0.12 M KCl, 5 mM L-histidine solution (pH 6.8), and 0.2 mL of 0.2 mM ferrous chloride. Then, 0.4 mL of 1 mM ferrozine was added, and the samples were incubated at room temperature for 10 min. The absorbance was measured at 560 nm, and the chelating capacity was expressed as the percentage of ferrous iron chelated by 0.2 mM phenolic compound.

Polarity of the Antioxidants. The polarity of phenolics was determined by their partition between aqueous and oily phases according to the methods of Huang et al. (18) and Pazos et al. (19). Briefly, 1 mL of fish oil and 1 mL of water containing antioxidants were well mixed and centrifuged. The phenolic content in the aqueous phase before and after mixing was quantified by the Folin–Ciocalteu method (20), and the amount of antioxidant in the oily phase was calculated as the difference between the total amount of antioxidant in water before mixing and the amount after mixing with oil. Data were shown as the percentage of the antioxidant in the oily phase and calculated as $(W_O/W_T) \times 100$, where W_O is the amount of antioxidant in the oily phase and W_T is the total amount of antioxidant.

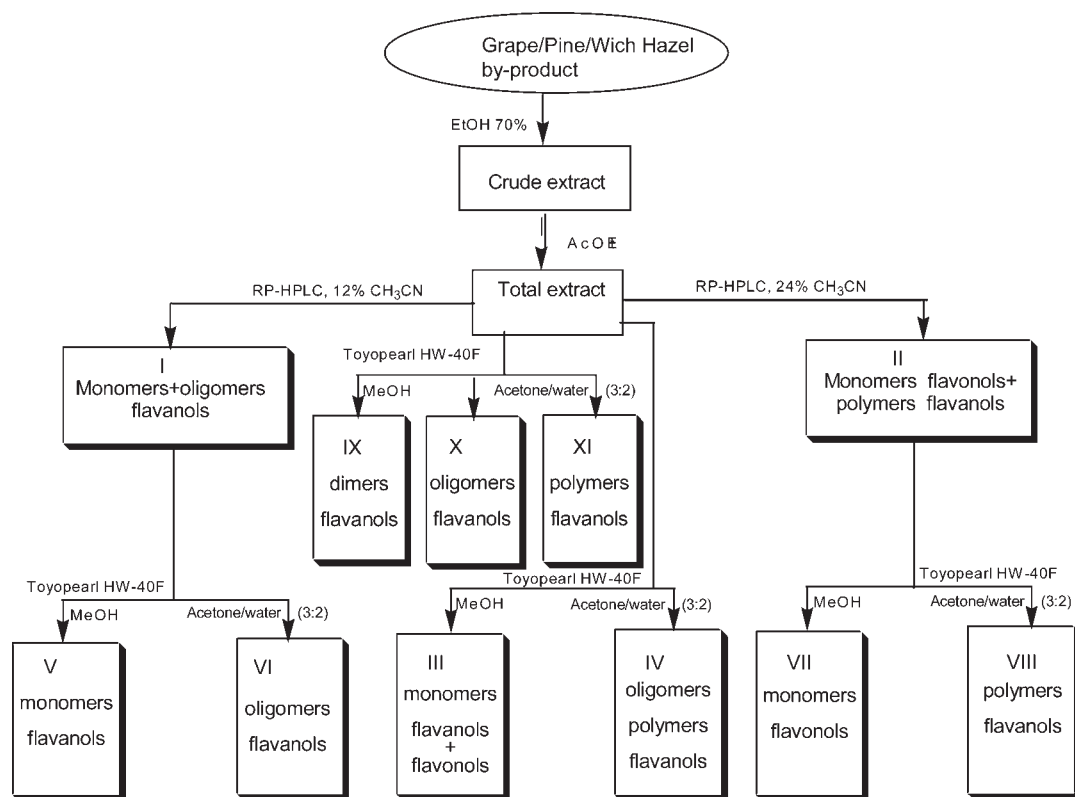
Oxidation of Fish Oil and Fish Oil-in-Water Emulsions. Samples of fish oils (5 g) were introduced into 50 mL Erlenmeyer flasks, and the polyphenolic fractions were added at a concentration of 100 μ g/g. For each system, triplicate samples were prepared and subjected to oxidation at 40 °C. Oxidative stability was followed by measuring conjugated diene and triene hydroperoxides in duplicate. Oil-in-water emulsions, containing 1% lecithin and 10% fish oil, were prepared as previously described (18). Briefly, lecithin and fish oil were homogenized with distilled water and emulsified by sonicating for a total of 10 min in an ice bath (Selecta, Barcelona, Spain). Five milliliters of emulsion was introduced into 50 mL Erlenmeyer flasks, and the different antioxidants were added at 100 μ g/g. For each system, triplicate samples were prepared and subjected to incubation at 35 °C. Lipid oxidation was followed by the measurement of conjugated diene hydroperoxides and the measurement of fluorescence compounds or the formation of volatiles. Triplicate samples were taken at different sampling times. Induction oxidation periods were calculated as the value in days corresponding to the intersection point of the tangents in the initiation and propagation steps of the oxidation curve.

Conjugated Diene and Triene Hydroperoxides. Conjugated dienes and trienes, expressed in millimoles of hydroperoxide per kilogram of oil, were determined by using the method reported by Huang et al. (21). Briefly, about 100 mg of fish oil samples and emulsion samples was

Table 1. Composition and Characterization of the Different Fractions

fraction	mDP ^a	galloylation ^b (mol of gallate/mol of polyphenol)	reducing capacity (no. of donated electrons/mol)	chelating ability (%)	partition coefficient (% in oil phase)
IP	1.9	0.00	3.3 ± 0.2	49.4 ± 0.0	10.6 ± 0.6
IIP	2.9	0.00	5.6 ± 0.0	70.0 ± 0.4	42.0 ± 1.1
IIIP	1.0	0.00	3.1 ± 0.1	33.1 ± 0.2	17.7 ± 1.6
IVP	2.9	0.00	5.6 ± 0.0	77.4 ± 0.5	18.5 ± 3.0
VP	1.0	0.00	2.0 ± 0.2	15.6 ± 0.3	8.1 ± 2.0
VIP	2.7	0.00	5.9 ± 0.0	75.5 ± 0.1	9.8 ± 1.5
VIIIP	3.0	0.00	5.4 ± 0.2	82.6 ± 0.7	16.4 ± 0.4
IXP	1.9	0.00	4.4 ± 0.1	44.1 ± 0.1	6.1 ± 0.4
XP	2.2	0.00	4.0 ± 0.0	58.1 ± 0.5	8.1 ± 0.2
XIP	3.4	0.00	6.1 ± 0.2	88.3 ± 0.1	18.5 ± 2.2
IVG	2.7	0.25	5.8 ± 0.1	94.2 ± 0.3	24.7 ± 2.0
VIG	2.4	0.16	6.0 ± 0.0	87.0 ± 0.4	10.1 ± 1.4
VIIIG	3.4	0.34	6.3 ± 0.1	95.5 ± 0.1	27.0 ± 1.5
IVH ^b	1.6	>1 ^c	6.1 ± 0.0	92.8 ± 3.6	29.9 ± 2.5
VIH ^b	2.6	>1 ^c	7.0 ± 0.2	86.2 ± 1.4	24.3 ± 2.4
VIIIH ^b	1.1	>1 ^c	8.0 ± 0.3	93.4 ± 3.3	41.3 ± 4.1

^a Mean degree of polymerization of the proanthocyanidinic fraction. ^b *Hamamelis* fractions were composed by >80% of hydrolyzable tannins. ^c Galloylation degree adds up the contributions of both condensed and hydrolyzable tannins.

Scheme 1. Preparation of the Different Fractions from Pine, Grape, and Witch Hazel Residues

dissolved in hexane and ethanol, respectively. Absorbance was measured at 234 and 268 nm for determining the conjugated diene and triene hydroperoxides respectively (Perkin-Elmer UV-vis spectrophotometer).

Measurement of Fluorescence Compounds. One hundred milligrams of emulsion samples was dissolved in ethanol. Fluorescence was measured at 345/416 and 393/463 nm (Perkin-Elmer LS 3B) and standardized with a quinine sulfate solution (1 µg/mL in 0.05 M H₂SO₄) according to the procedure described by Pazos et al. (22).

Volatile Analysis in Fish Oil-in-Water Emulsions. Volatiles were extracted from fish oil-in-water emulsions by headspace solid-phase microextraction (HS-SPME) and analyzed by gas chromatography-mass spectrometry according to the method of Iglesias et al. (23). Briefly, 0.5 mL of emulsion was introduced into a HS-SPME vial fitted with a silicone septum and exposed to a CAR-PDMS fiber (75 µm Carboxen/polydimethylsiloxane coating, Supelco, Bellefonte, PA) during 30 min at 60 °C.

The fiber was then removed from the vial and inserted into the GC injection port for desorption during 10 min to 300 °C. Determination of volatiles was performed by the method of internal standard using 3-methyl-3-buten-1-ol. 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).

Sensory Analysis. Sensory analysis was evaluated by an expert panel formed by four specialists trained in descriptive analysis of fishy off-flavors, in a room designed for the purpose. Samples were placed at room temperature during 10 min before analysis. Panelists concentrated on detecting rancidity odors (24).

Statistical Analysis. Analyses were performed in triplicate, and the data were compared by one-way analysis of variance (ANOVA) and the least-squares difference method (25). The means were compared by

the least-squares difference method with the Statistica 6.0 program (Statsoft, Tulsa, OK).

RESULTS

Several fractions extracted from pine bark were chosen for studying the relationship between antioxidant activity and polymerization degree in fish lipid systems (Table 1). Pine fractions with different polymerizations constitute an excellent model for evaluating this factor because they are composed by oligomeric catechins of different sizes devoid of gallate esters (11). Grape proanthocyanidins have a low–medium galloylation percentage; therefore, the presence of galloylated residues in the proanthocyanidin moiety was addressed by comparing the activity found in some homologous fractions extracted from pine bark and grape pomace (IVG, VIG, and VIIG, Table 1). Finally, the effect of the galloylation was assured by testing fractions from witch hazel (IVH, VIH and VIIIH, Table 1) having a high content of galloyl groups.

Relationship between Polyphenolic Structure and Physicochemical Characteristics. The capacity to donate electrons (reducing capacity) of the different proanthocyanidins was estimated through the FRAP assay. Values obtained for the nongalloylated fractions (pine fractions) indicated that the number of donated electrons per molecule was increased with the number of catechin monomers. According to this, the maximum value of this parameter (6.1) was attributed to the highest polymerized fraction, XIP, with mDP = 3.4 (Table 1). Data were submitted to statistical analysis, and a significant correlation between polymerization and the number of donated electrons was then detected ($R^2 = 0.91$).

The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ions and, therefore, measure the ability of the different proanthocyanidins to inactivate one of the most important metallic catalysts of lipid oxidation. The plots of iron-chelating capacity as a function of IIP, IIP, IVP, VP, and VIIP concentration are shown in Figure 2A. Clear differences on the chelating behavior were observed depending on the size of the proanthocyanidin. Table 1 illustrates such differences showing the percentage of ferrous iron chelated by 0.2 mM phenolic compound. In agreement with data obtained for the reducing ability, the results obtained for the different pine fractions showed a direct relationship between this capacity and the size of the proanthocyanidin. A significant correlation between size and chelating ability was also found ($R^2 = 0.92$).

The effect of galloylation on the *in vitro* reducing and chelating properties was checked by comparing homologous fractions extracted from pine bark and grape pomace. Table 1 shows the capacity to donate electrons and chelating ability for some grape fractions (IVG, VIG, and VIIG), in order to compare their values with those corresponding from pine. With regard to the reducing ability, data indicated that grape fractions showed higher capacity to donate electrons than their corresponding nongalloylated fractions, especially in the case of the most galloylated grape fraction, VIIG. Additionally, the highly galloylated fractions from witch hazel, IVH, VIH, and VIIIH, showed a clearly higher reducing ability than medium-galloylated grape fractions.

Comparison of the nongalloylated fractions IVP, VIP, and VIIP and their galloylated counterparts from grape pomace demonstrated a positive influence of the esterified galloyl groups on the chelating ability (Figure 2B). Fractions IVG, VIG, and VIIG showed very similar values of this property (94.2, 87.0, and 95.5), which were higher than those of their homologous fractions extracted from pine bark (77.4, 75.5, and 82.6). The highly galloylated fractions, IVH, VIH, and VIIIH, exhibited abilities to chelate ferrous ions similar to those of their equivalent medium-galloylated grape fractions (92.8, 86.2, and 93.4).

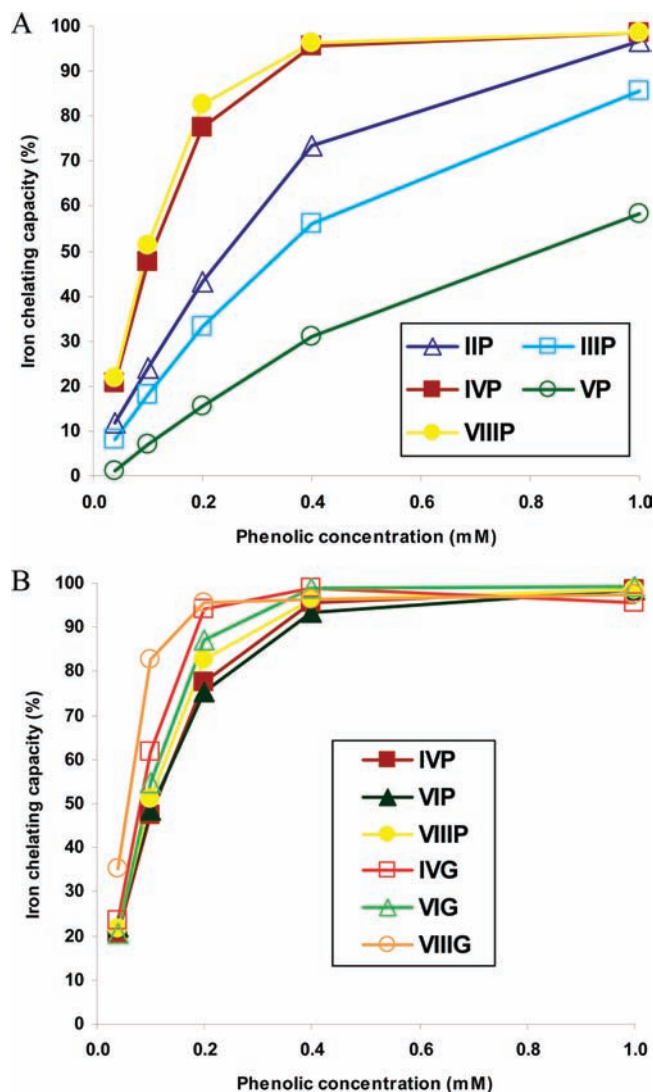


Figure 2. Chelating capacity of the different pine fractions (A) and comparison between pine and grape homologues (B).

One important aspect in the antioxidant efficiency of proanthocyanidins in lipid systems is related to their location at the oxidative sensitive sites, which is highly dependent on their solubility in lipid and water. Table 1 shows the partition coefficients between fish oil and water for every fraction tested in this study. Pine fractions IVP, VP, VIP, VIIP, IXP, XP, and XIP, with a similar composition (monomers and oligomers of flavanols), showed an increase in the oily solubility related to the growing number of phenolic residues (Table 1). The increment of the number of phenolic monomers holding apolar aromatic rings could explain this observation. With regard to galloylation, grape galloylated fractions showed a higher distribution in the oily phase compared to their pine homologous. The increment in the number of apolar aromatic rings forming part of the gallate group could explain this finding. Finally, fractions IVH, VIH, and VIIIH from witch hazel bark showed the highest distribution in the oily phase. Consequently, polymerization and galloylation confer apolar character to the polyphenols, favoring their partition to oily phases.

Influence of Polymerization on the Antioxidant Activity of Polyphenols in Bulk Fish Oil and Fish Oil-in-Water Emulsions. Figure 3 illustrates the effect of polymerization on the generation of conjugated dienes and trienes in bulk fish oils. Fish oils were supplemented with IXP (polymerization = 1.9), XP (polymerization = 2.2), and XIP (polymerization = 3.4). Data obtained indicated

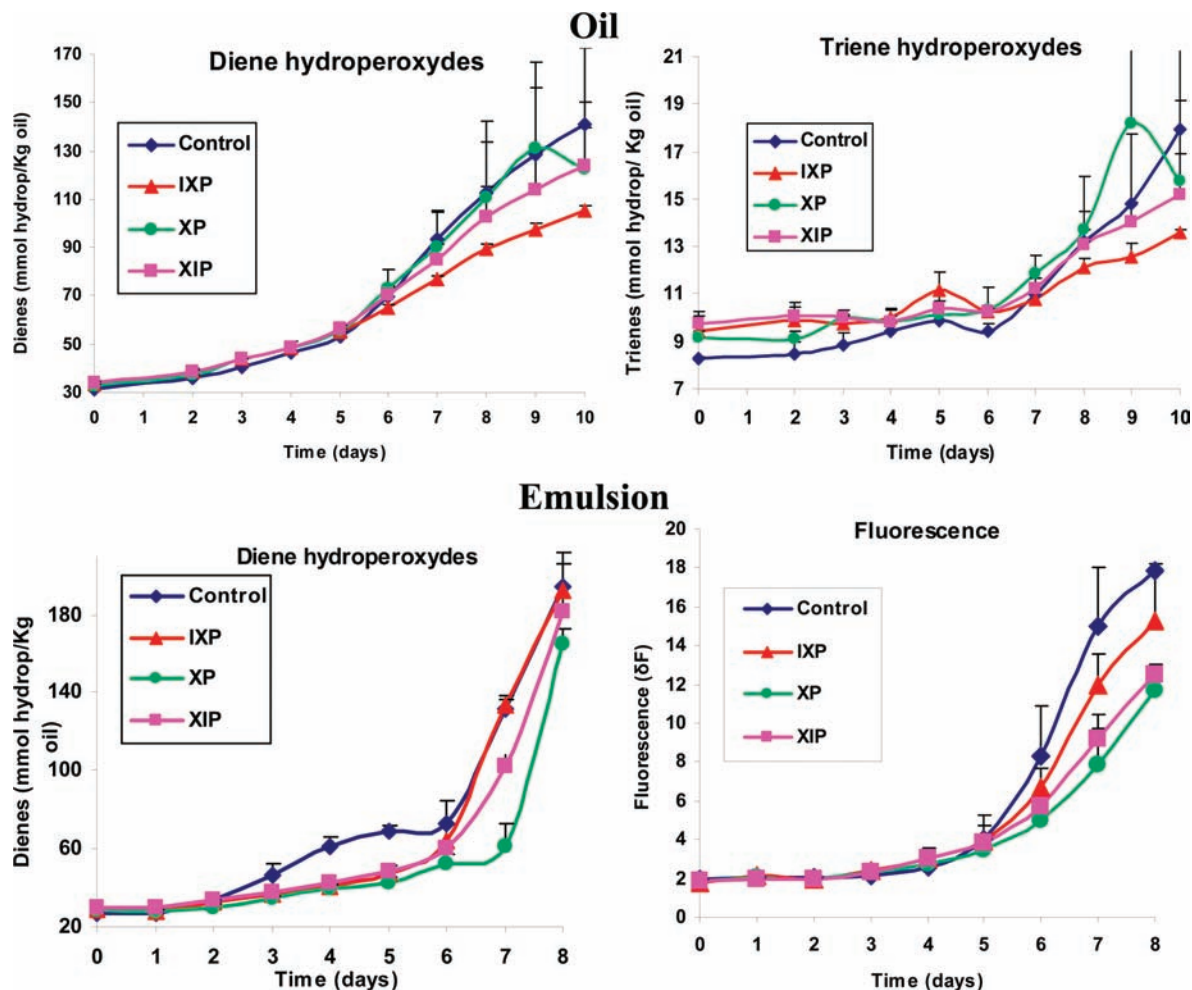


Figure 3. Formation of conjugated diene and triene hydroperoxides in fish oil and fish oil-in-water emulsions supplemented with pine procyanidinic fractions with different polymerization degrees.

that the rate of oxidation and the amount of oxidation products formed was lower in IXP samples. Pine fractions with higher polymerization degrees were not active for inhibiting lipid oxidation in fish oils. These results were in agreement with sensory detection of off-flavors (Table 2). Incipient rancid odors were detected in control oils and oil samples supplemented with fractions XP and XIP by the sixth day of storage and in samples with fraction IXP by the seventh day. Table 3 illustrates the percentage of inhibition on the formation of the lipid oxidation products in bulk oils supplemented with different pine fractions. Only fraction IXP, with the lower polymerization degree, showed a significant inhibition of the formation of lipid oxidation products.

The addition of 100 $\mu\text{g/g}$ of pine fractions to fish oil-in-water emulsions significantly inhibited the development of oxidation (Figure 3). Comparison among fractions IXP, XP, and XIP illustrates the significant effect of polymerization. Table 4 exemplifies such an effect expressed as the percentage of inhibition of the formation of lipid oxidation products, conjugated hydroperoxides, and fluorescent compounds. Low antioxidant activity was detected for the less polymerized fraction IXP. Fraction XP, with an intermediate polymerization degree, inhibited the formation of conjugated dienes and trienes and fluorescence compounds by 53, 38, and 54%, respectively, after 7 days of storage. In the same period, fraction XIP also inhibited the formation of conjugated dienes and trienes and fluorescent compounds (22, 25, and 43%, respectively) but with lower activity than fraction XP. Fractions IXP and VIIIIP also showed lower values of inhibition than those corresponding to

XP. Therefore, an optimum polymerization degree seems to occur around mDP values of 2.2.

Results obtained in both experiments did not evidence a positive trend between the polymerization degree of the proanthocyanidins and their antioxidant activity in both fish oil and fish oil emulsions. The less polymerized proanthocyanidins were more efficient for preventing lipid oxidation in bulk fish oil samples, whereas medium–high-polymerized fractions were the most active in delaying lipid oxidation in fish oil emulsions.

Influence of Galloylation on the Antioxidant Activity of Polyphenols in Bulk Fish Oil and Fish Oil-in-Water Emulsions. Comparison between the effectiveness for inhibiting lipid oxidation of bulk fish oils of grape and pine homologous fractions demonstrated that galloylation did not improve the antioxidant effect of proanthocyanidins (Figure 4; Table 3). The values of inhibition for the formation of conjugated dienes and trienes showed a lack of efficiency of any grape galloylated proanthocyanidins for depleting lipid oxidation. The presence of galloylated residues did not improve the null antioxidant effectiveness detected for pine fractions having polymerization degrees of >2 . Fraction IXG (mDP = 2.0) did not show any antioxidant activity because the inhibition values obtained in the formation of lipid oxidation products were very low (Table 3). Furthermore, galloylation seems to induce prooxidant effects on bulk oils as can be observed from the negative values given in Table 3. Figure 4 illustrates the data comparing fraction IXP versus IVG and fraction VIIIIP versus VIIIIG. The data demonstrated the absence of antioxidant

Table 2. Development of Rancid Odors during Storage of Fish Oil and Fish Oil Enriched Real Samples Supplemented with Different Proanthocyanidinic Fractions

		polymerization					
		day 3	day 4	day 5	day 6	day 7	day 8
oil	control	no rancid odor	incipient rancid odor	incipient rancid odor	rancid odor	rancid odor	rancid odor
	IXP	no rancid odor	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	incipient rancid odor
	XP	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor
	XIP	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	incipient rancid odor	rancid odor
emulsion	control	incipient rancid odor	rancid odor	rancid odor	rancid odor	rancid odor	rancid odor
	IXP	no rancid odor	incipient rancid odor	rancid odor	rancid odor	rancid odor	rancid odor
	XP	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor	rancid odor
	XIP	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor	rancid odor
		galloylation					
		day 3	day 4	day 5	day 6	day 7	day 8
oil	control	no rancid odor		no rancid odor	rancid odor	rancid odor	rancid odor
	IVP	no rancid odor		no rancid odor	rancid odor	rancid odor	rancid odor
	IVG	no rancid odor		no rancid odor	rancid odor	rancid odor	rancid odor
	VIIIP	no rancid odor		no rancid odor	rancid odor	rancid odor	rancid odor
	VIIIG	no rancid odor		incipient rancid odor	rancid odor	rancid odor	rancid odor
emulsion	Control	incipient rancid odor	rancid odor	rancid odor	rancid odor	rancid odor	rancid odor
	IVP	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor
	IVG	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor
	VIIIP	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor	rancid odor
	VIIIG	no rancid odor	no rancid odor	no rancid odor	incipient rancid odor	rancid odor	rancid odor

Table 3. Inhibition^a of the Formation of Lipid Oxidation Products in Bulk Fish Oils by Homologous Fractions with Different Polymerization Degrees Extracted from Pine Bark and Grape Pomace (Mean \pm SD; Day 8)^b

fraction	fish oil			
	conjugated dienes (%)		conjugated trienes (%)	
	pine	grape	pine	grape
IV	1.2 \pm 9.5 a	-9.5 \pm 14.2 b	-1.7 \pm 1.2 a	-9.9 \pm 10.3 b
VIII	0.2 \pm 5.8 a	-37.0 \pm 5.1 a	-2.1 \pm 1.6 a	-34.1 \pm 4.0 a
IX	20.3 \pm 1.4 b	3.8 \pm 11.7 b	7.9 \pm 2.9 b	0.6 \pm 6.5 b
X	2.0 \pm 3.2 a	6.9 \pm 4.1 b	-3.9 \pm 0.8 a	-3.8 \pm 2.5 b
XI	8.8 \pm 2.8 a	10.3 \pm 8.2 b	0.9 \pm 5.0 a	-2.1 \pm 0.6 b

^a % inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed in control and S = oxidation product formed in sample. ^b Values in each column with the same letter are not significantly different ($p < 0.05$).

activity for any proanthocyanidin fraction evaluated in this experiment. Conversely, a significant prooxidant activity was observed in samples supplemented with the galloylated fractions. Sensory analysis confirmed these results as rancid off-odors were detected in all samples after 6 days of storage (Table 2). Therefore, the presence of galloyl residues in the grape fractions was not able to enhance the antioxidant activity of proanthocyanidins in bulk fish oils.

The presence of galloyl groups significantly favored the antioxidant behavior of proanthocyanidins in fish oil-in-water emulsions (Table 4). Grape galloylated fractions showed higher inhibition values than their corresponding pine nongalloylated fractions. Figure 4 summarizes the data comparing fraction IVP versus IVG and fraction VIIIP versus VIIIG. Control samples showed shorter induction periods of formation of both conjugated dienes (3 days) and fluorescent compounds (4 days) than the samples supplemented with proanthocyanidins. Emulsions supplemented with fraction VIIIP exhibited a significant increment of conjugated dienes and fluorescent compounds by the fifth and sixth days of storage, respectively, whereas the induction periods in the remainder samples were over 6 and 7 days. These induction periods were in accordance with the sensory detection

of rancid off-flavors (Table 2). The amount of conjugated dienes and fluorescent compounds formed was significantly lower in the presence of galloylated fractions from grape, IVG and VIIIG, than in the case of their nongalloylated counterparts from pine. Therefore, the galloylated fractions were more effective in inhibiting oxidation in fish oil-in-water emulsions than their counterparts without galloyl residues. It is interesting to note that fraction VIIIG, having the higher galloylation degree of 0.34 (Table 1), was generally the grape fraction with lower antioxidant effectiveness in fish oil emulsions.

The antioxidant activity of the low–medium-galloylated IVG fraction was compared with the highly galloylated fraction IVH, extracted from witch hazel (Figure 5; Table 5). Rancid off-flavors were detected by the fourth day in the control samples, whereas in the remaining ones off-odors were not perceived until the fifth day of storage. Incipient rancidity was detected in IVG and IVH by the sixth day. The analysis of lipid oxidation products confirmed these results. The formation of conjugated dienes and volatiles indicated very similar induction periods for all samples, but the generation rate of these lipid oxidation products and the amount formed was found to be significantly different for each sample (Figure 5). Fraction IVG was the most effective and inhibited significantly the formation of conjugated dienes and volatiles (Table 5). Fraction IVH showed an intermediate antioxidant inhibition with values lower than those of IVG. Consequently, the presence of galloyl residues favors the antioxidant activity of the polyphenols in emulsions, although the antioxidant performance of polyphenols with low–medium galloylation was not apparently improved by highly galloylated polyphenols. These results confirm the previous finding described in the above experiments related to fraction VIIIG.

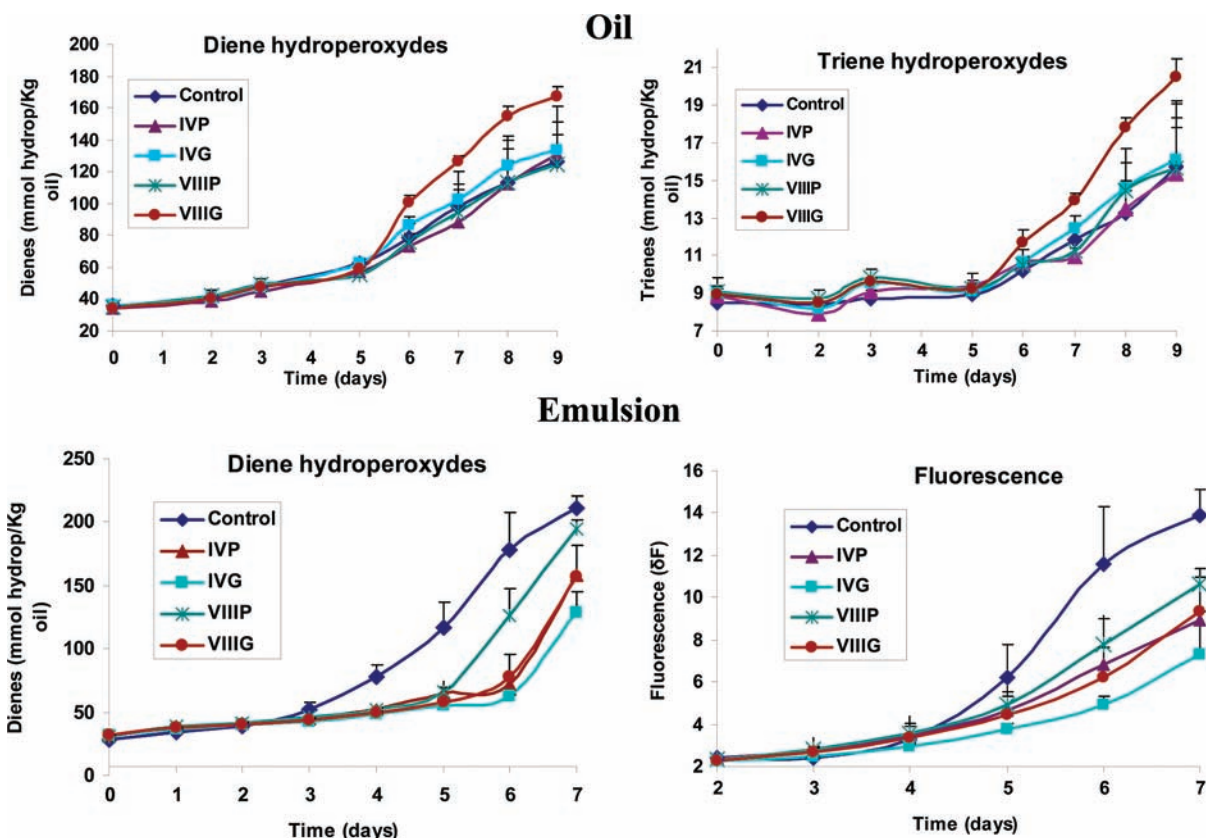
DISCUSSION

The present study revealed a direct relationship between the size of polyphenols and an improvement in their reducing and iron-chelating properties. In vitro experiments carried out with pine fractions demonstrated that the most polymerized proanthocyanidins provide the major reducing and chelating capacities

Table 4. Inhibition^a of the Formation of Lipid Oxidation Products in Fish Oil-in-Water Emulsions by Homologous Fractions with Different Polymerization Degrees Extracted from Pine Bark and Grape Pomace (Mean \pm SD; Day 7)^b

fraction	fish oil emulsion					
	conjugated dienes (%)		conjugated trienes (%)		fluorescent compounds (%)	
	pine	grape	pine	grape	pine	grape
IV	29.1 \pm 3.8 c	45.3 \pm 7.7 c	33.2 \pm 12.1 b	62.3 \pm 2.8 c	35.6 \pm 0.1 b	47.7 \pm 2.1 b
VIII	9.0 \pm 3.1 b	29.4 \pm 6.5 a	25.4 \pm 2.7 b	47.0 \pm 5.7 a	32.7 \pm 1.6 b	23.5 \pm 0.7 a
IX	-1.6 \pm 2.2 a	40.2 \pm 1.8 b	3.8 \pm 2.6 a	39.5 \pm 0.5 b	20.6 \pm 11.1 a	39.8 \pm 12.8 b
X	53.3 \pm 8.7 c	54.0 \pm 6.4 c	38.4 \pm 8.3 c	48.5 \pm 5.3 d	54.1 \pm 6.1 d	58.8 \pm 11.8 c
XI	22.3 \pm 4.1 b	57.9 \pm 4.8 bc	24.7 \pm 3.9 b	49.4 \pm 3.8 cd	43.5 \pm 1.2 c	61.9 \pm 13.7 c

^a% inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed in control and S = oxidation product formed in sample. ^bValues in each column with the same letter are not significantly different ($p < 0.05$).

**Figure 4.** Comparative formation of lipid oxidation products in fish oil and fish oil-in-water emulsions supplemented with pine and grape procyanidins.

determined through the FRAP and ferrous-chelating assays. The strong capacity of highly polymerized polyphenols to donate electrons and chelate ferrous can be attributed to the presence of more hydroxyl groups contained in their structure (1). Our results reinforce those reported by Touriño et al. (11), who found a slight positive correlation between the degree of polymerization and the antiradical scavenging properties against DPPH radical. Steric impediments resulting from the incorporation of additional monomers and the relatively high volume of the DPPH radical may restrict the accessibility of these radicals to the active hydroxyl groups of highly polymerized polyphenols. This fact seems to limit the application of the DPPH assay to low-medium-polymerized polyphenols. Steric interactions could also explain the lack of correlation between the electronic transference of the organic tri(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTN) radical and the size of proanthocyanidins (11). The assays to evaluate reducing (FRAP) and metal chelation capacities (ferrous-ferrozine) probably enclosed lower steric impediments because they are based on the electronic donation or chelation of ferrous ions, with relatively small size.

Despite the improvement observed for the in vitro reducing and chelating capacities, with the number of phenolic residues, such a direct relationship was not observed in bulk fish oils or in fish oil in-water emulsions. The efficacy of the pine proanthocyanidins in bulk fish oil was negatively related to the polymerization degree. This result is in agreement with a preliminary study in which galloylated monomers extracted from grape pomace were more efficient than oligomers for inhibiting oxidation of bulk fish oils (22). According to the polar paradox hypothesis, hydrophilic antioxidants are more effective in bulk oil systems because they are positioned at the oil-air interface where lipids are exposed to oxygen (1). Polymerization diminishes the hydrophilicity of the proanthocyanidins, and as a consequence, the most polymerized are more diluted in the oily phase and their antioxidant activity in bulk fish oil is weak. These results are in agreement with previous investigations that showed a decrease of the antioxidant activity in bulk fish oil by increasing the size of the alkyl chain of hydroxytyrosol fatty acid esters and, therefore, increasing lipophilicity (26).

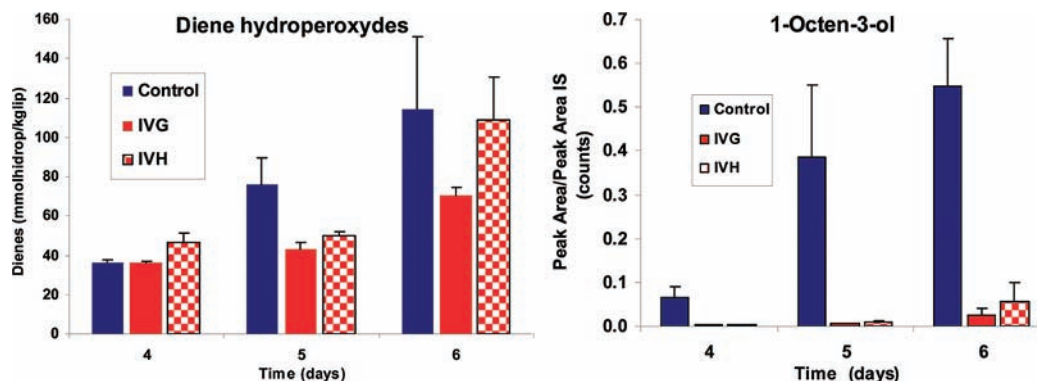


Figure 5. Comparative formation of lipid oxidation products in fish oil-in-water emulsions supplemented with grape and witch hazel phenolics.

Table 5. Inhibition^a of the Formation of Lipid Oxidation Products on Fish Oil-in-Water Emulsions by the IV Fractions Extracted from Grape Pomace and Witch Hazel Bark (Mean \pm SD)^b

	conjugated dienes (%)		1-octen-3-ol (%)	
	day 5	day 6	day 5	day 6
IVG	42.8 \pm 4.0 b	38.6 \pm 4.1 b	92.2 \pm 0.6 b	42.8 \pm 4.0 b
IVH	33.8 \pm 2.1 a	19.8 \pm 8.1 a	89.4 \pm 1.4 a	33.8 \pm 2.1 a

^a% inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed in control and S = oxidation product formed in sample. ^b Values in each column with the same letter are not significantly different ($p < 0.01$).

A different tendency was observed when pine fractions were tested in fish oil-in-water emulsions. Maximum inhibition was achieved with an intermediate number of polyphenolic residues, situated between 2 and 3, according to previous results suggested by Pazos et al. (22). The potential of phenolics to behave as effective antioxidants in lipid biphasic matrixes appears to be also dictated by their location at the lipid interfaces, where their efficacy is higher. Medina et al. (26) have revealed that the presence of esterified aliphatic chains with low–medium length (2–10 carbons) enhances the antioxidant activity of hydroxytyrosol by favoring its location in the water–oil interfaces. Proanthocyanidins are a special case with regard to their situation in biphasic systems, because they might be able to promote both hydrophobic and hydrophilic interactions due to their amphipathic structure with polar hydroxyl groups and lipophilic aromatic rings. Consequently, they can act as a surfactant, and such a property favors their location in the water–oil interface. De Gaulejac et al. (27) have suggested that the largest proanthocyanidins possess a reduced accessibility for the –OH groups, which could diminish their tendency to be located in the lipid–water interface.

Galloylation seems to play an important role in the antioxidant efficacy of the polyphenolic fractions in fish oil-in-water emulsions. Galloylated fractions from grape and witch hazel bark showed higher antioxidant activity than their nongalloylated counterparts extracted from pine bark. The positive influence of the galloylation on the antioxidant activity is consistent with the fact that the pyrogallol moiety provides additional hydroxyl groups with radical scavenging and chelating abilities (22). Accordingly, the polyphenolic fractions with elevated galloylation exhibited strong electron donation power and chelating capacity (Table 1). The π – π stacking arrangement between the aromatic gallate and the catechol B ring could cause an additional stabilization of the respective phenoxyl radical by delocalization of the unpaired electron between these π orbitals as has been previously demonstrated by Freitas et al. (28).

With regard to the location at the oxidative sensitive sites of the emulsion, the “polar paradox” establishes that the lower polarity

of the galloylated proanthocyanidins (Table 1) facilitates their location in the lipid–water interfaces, improving their antioxidant efficacy (1). In the case of bulk fish oils, an increase of galloyl residues, increasing solubility in the oily phase, results in decreased antioxidant activity.

The efficacy of fraction IVH was slightly lower than that of IVG when both were compared in fish oil-in-water emulsions. The high content in hydrolyzable tannins with esterified gallate groups in their structure, as hamamelitannin or different galloylated glucoses (15), provides to these fractions an important content of pyrogallol moieties (Figure 1). Therefore, our results reveal that a medium–low galloylation confers optimum antioxidant activity to the polyphenols in fish oil emulsions, which is not improved by increasing the content of galloylated residues.

The present investigation describes how the reducing, iron chelating, and lipophilic properties of the polyphenols are modulated by structural factors such as polymerization and galloylation. As a consequence, the impact of both parameters on the antioxidant efficacy was different when they were added to bulk fish oil or fish oil enriched emulsions. The results here exposed provide useful insights into the polyphenolic structure–activity relationship. Therefore, this study could help in the rational design of antioxidants to obtain a final additive with optimal polymerization and galloylation degrees and a convenient cost.

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