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Incorporation and Interaction of Grape Seed Extract in Membranes and Relation with Efficacy in Muscle Foods

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The interaction and location of phenolic antioxidants in model membranes has been related with their effectiveness for inhibiting lipid oxidation of fish mince with the aim to identify mechanisms involved in the antioxidant effectiveness in muscle foods. For such scope, the effect of grape seed extract and its main components, catechin, epicatechin and procyanidin B_2 to be located and induce changes in phospholipid model membranes was studied by different biophysical techniques and related to their antioxidant efficiency. Grape seed extract showed the highest inhibition of oxidation in chilled minced fish muscle. Antioxidant in- vitro capacities were also studied but they did not show a clear relationship with the antioxidant efficiency found in fish muscle. The phospholipid/water partition coefficients and fluorescence quenching studies showed that procyanidin B_2 was located in a more internal location than monomeric catechin and epicatechin within the phospholipid palisade. Grape seed extract showed strongest effect compared to its main components in the increase of the lipid order at the DMPC fluid phase by fluorescence anisotropy measurements. Grape seed extract also promoted a dehydration effect in DMPC membranes at the phospholipid/water interface and resistance to solubilization by nonionic detergents in DMPC membranes. The presence of molecular linkages, probably by hydrogen bonding, is proposed between procyanidins (or some galloylated catechins) and the polar head groups of the phospholipids to account for the dehydration effect at the phospholipid/water interface and membrane-stabilizing effects. These effects may be directly related to the higher efficacy of grape seed extract to inhibit lipid oxidation in fish muscle, probably by hindering radical propagation.

KEYWORDS: Catechins; procyanidin B_2; grape extract; lipid oxidation; membranes; phospholipid vesicles

INTRODUCTION

Fish are rich in essential ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentanoic acid (20:5 ω-3) and docosahexanoic acid (22:6 ω-3). These acids have shown potential benefits for human health (*1*, *2*). However, the presence of these unsaturated lipids and large amounts of heme pigments and metallic traces on fish muscle and tissues contributes to the susceptibility of fish lipids to suffer oxidation. Once the oxidative reaction has been initiated, the hydroperoxides formed are converted to free radicals and finally to the volatiles that are the responsible for the development of rancid off-flavors. The oxidation products formed react with other muscle components as proteins, amino acids, or vitamins, leading to changes in color or texture (*3*). All these reactions lead to a loss of the organoleptic and nutritional attributes and limits the storage and processing of fish species, especially those having high PUFA concentrations. Fish rancidity cannot be totally avoided but different procedures have been proposed to delay the oxidative process and inhibit the apparition of the first hydroperoxides or volatiles. Washing with water or the use of brine immersion or glazing can retard lipid oxidation in fatty fish(*4*) . The use of slurry ice, vacuum packaging, or the application of oxygen absorbers have been proposed by the fish product industry to control lipid oxidation (*5*−*7*).

In the last years, the use of natural antioxidants becomes an emergent methodology for controlling lipid oxidation and its deleterious repercussions (*8*−*10*). Nowadays, the addition of natural antioxidant extracts to foods is a common practice in industry. The use of polyphenols coming from agricultural and forestry byproduct provides excellent extracts that fulfill this objective. Grape flavonoids have shown to act as powerful antioxidants by scavenging free radicals and terminating oxidative reactions (*11*). Different authors have successfully tested grape seed and pomace extracts as food ingredients in different lipid systems and foodstuffs as vegetable and fish oils, beef, turkey, and chicken meat or fish muscle (*9*, *12*−*14*). The efficiency found in these studies was highly dependent on the type of food muscle system. Some of these studies have demonstrated the ability of the grape seed extracts for inhibiting oxidation during long-term food storage. In addition, Branan et al (*15*) have

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demonstrated that the antioxidant activity of grape seed extracts does not seem to be affected by changes in the physicochemical conditions of food as variations in the moisture content or pH during storage. These works have also shown different grades of efficacy depending on the physical state of the lipids or oils. Some polar grape extracts were not very effective for bulk fish oils but were effective for minced fish muscle (*9*). The antioxidant action can progress through different routes that include, among others, the capacity for chelating metals, the redox capacity of the antioxidant and the free radicals scavenging ability. It is well established that the in vitro measures of these antioxidant properties do not always reflect the activity found in real foods (*16*). The physical interactions and the incorporation of the antioxidant into the oxidative sensitive sites of the system seem to be significant parameters involved in the antioxidant efficacy as well (*17*).

The high oxidative susceptibility of fish muscle has been traditionally attributed to the total lipid content (*18*). However, there is some evidence that phospholipids (PL), which give structure and fluidity to membranes, are the primary lipid substrate for lipid oxidation in muscle food, whereas triglyacylglycerols play a minor role (*19*). This could be attributed to their PL high degree of unsaturation, the close contact of membranes with catalysts of lipid oxidation located in the aqueous phase of muscle cells, and their large surface area (*20*). PUFAs are highly concentrated in PL, which content is more or less constant in fish muscle (0.5−1.0%). Few studies published during the last years have tried to explain the antioxidant mechanism of action of polyphenols based on their localization and affinity for lipid bilayers (*21*−*23*). Plant phenolics and tocopherol have demonstrated to perturb the phase behavior of the lipid bilayers and have a fluidifying effect that could favor the known antioxidant capability and scavenging characteristics of these compounds (*24*) Gallate catechins have also demonstrated a high affinity for lipid bilayers resulting in a perturbation of the membrane structure (*22*). Additionally recent works have stressed the interactions of catechins with lipid bilayers of cell membranes establishing different mechanisms of interaction and as consequence different biological in vivo activities (*25*). Phenolic antioxidants having the highest affinity for membranes have also resulted the most effective antioxidants for inhibiting oxidation of fish microsomes (*26*).

Considering the above-mentioned, the aim of this work was to evaluate the relation of the interaction and location of antioxidants in model membranes with their effectiveness for inhibiting lipid oxidation of minced fish muscle. For such scope, different antioxidants as grape seed extract and its main components (catechin, epicatechin, and procyanidin B_2), which show differences in their molecular structure and therefore in their physicochemical interactions with membranes, were selected. In addition, the scavenging, reductant, and chelating capacities of these components were also determined to check the main parameters influencing the antioxidant efficacy found in the fish minced muscle model. The localization, affinity, and effects of the antioxidants selected on the lipid order and hydration state of membranes were studied by fluorescence spectroscopy. Then, these effects were related to the antioxidant effectiveness of these compounds to explain their inhibitory effect on fish lipid oxidation. Finally, the antioxidant in vitro capacity of the grape seed extract and its main components was determined by their reducing ability and chelating and radical scavenging capacities. This study attempts to contribute to a better knowledge of the antioxidant mechanism attributed to natural phenolics in muscle tissues.

MATERIALS AND METHODS

Reagents. Synthetic lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Stock solutions were prepared in chloroform/methanol (1:1) and stored at −20 °C. EDTA, (−)−epicatechin, (+)−catechin, procyanidin B_2, $FeCl_2 \cdot 4H_2O$, sodium acetate trihydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric suphate, streptomycin sulfate salt, ferrozine, $FeCl_3 \cdot 6H_2O$, L-histidine and potassium chloride (KCl), ammonium heptamolybdate tetrahydrate, perchloric acid potassium hydrogen phosphate, L-ascorbic acid, HEPES, and sodium chloride (NaCl) were purchased from Sigma (Steinheim, Germany). The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5 hexatriene (TMA-DPH), spin labels 5-doxyl-stearic acid (5-NS) and 16-doxyl-stearic acid (16-NS), and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were obtained from Molecular Probes (Eugene, OR). Stock solutions of DPH probes and Laurdan were prepared in *N*,*N*′-dimethyl formamide (Merck KgaA, Darmstadt, Germany). 2,4,6-Tri-(2-pyridyl)-s-triazine was obtained from Fluka (New-Ulm, Switzerland). All solvents and chemicals used were either analytical or high-performance liquid chromatography grade (Ridel-Haën, Seelze, Germany).

Grape Proanthocyanidins. A grape fraction rich in oligomeric catechins (proanthocyanidins) was prepared by fractionation of a commercial grape seed extract (Le Grandonnenque, Cruviers-Lascours, France) that contained 40% of proanthocyanidins. Fractionation was performed by size exclusion chromatography based on Toyopearl resin as previously described in Torres et al. (*27*). Briefly, the commercial extract containing mainly flavanol (catechin) monomers, flavanol oligomers (procyanidins), and monomeric-glycosylated flavonols was separated by chromatography on Toyopearl. A set of fractions differing in size, monomers from oligomers, was obtained. Each grape fraction was characterized in terms of mean molecular weight, mean polymerization degree, and percentage of galloylation (presence of esters with galloyl moieties) by depolymerization with cysteamine according with Torres and Lozano (*28*). An individual fraction having mean molecular weight of 760 g/mol, polymerization degree of 2.4 units, and galloylation percentage of 15% was selected. The major components expressed in molar percentage were catechin (29.56%), of epicatechin (29.09%) and procyanidin B_2 (7.38%).

Phospholipid Vesicle Preparation. Aliquots containing the appropriate amount of lipid in chloroform/methanol (1:1, v/v) and antioxidants were placed in a test tube, the solvents removed by evaporation under a stream of O_2-free nitrogen, and finally traces of solvents were eliminated under vacuum for >3 h. The lipid films were resuspended in buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM EDTA) and incubated either 25 or 10 °C above to gel-to-liquid crystalline phase transition temperature (Tm) with intermittent vortexing for 30 min to hydrate the samples and obtain multilamellar vesicles (MLVs). The samples were frozen and thawed five times to ensure complete homogenization and maximization of antioxidants/lipid contact with occasional vortexing. Large unilamellar vesicles (LUVs) were prepared by the extrusion method (*29*), using polycarbonate filters with a pore size of 0.2 μm (Nucleopore Corp, Cambrigde, CA). The phospholipid concentration was measured by the method of Böttcher et al. (*30*).

Determination of the Partition Coefficient. The phospholipid/water partition coefficient, which accounts for the percentage of antioxidant molecules bound to membranes, was measured using the Nernst partition coefficient described by (*31*)

$$K_P = \frac{\frac{n_l}{V_l}}{\frac{n_w}{V_w}}$$

where n_i stands for moles of compound in phase i and V_i for volume of phase i; the phase was either aqueous (i = w) or lipidic (i = L). The quantitation of K_P was determined from the intrinsic fluorescence intensity increase of the compound upon the incorporation into LUVs composed of DMPC as compared to that in the aqueous phase, according to (*32*)

$$\Delta I = \frac{\Delta I_{max}[L]}{1/K_P\gamma + [L]}$$

Fluorescence Quenching Experiments. Differential quenching data using 5-NS and 16-NS were analyzed by the Stern−Volmer plot of I_0/I versus $[Q]_L$, where I_0 and I stand for the fluorescence intensities of the catechins in the absence and in the presence of the quencher, respectively,

and $[Q]_L$ is the quencher concentration in the phospholipid phase given by (*4*)

$$[Q]_L = \frac{K_{PQ} V_T}{V_w + V_L K_{PQ}} [Q]_T$$

where

$$K_{PQ} = \frac{[Q]_L}{[Q]_W}$$

is the partition coefficient of the quencher between the phospholipid phase and the aqueous phase, $[Q]_T$ is the concentration of the quencher in the total volume V_T ($V_T = V_L + V_W$), and V_L and V_W are, respectively, the volume of the lipid and aqueous phases. For 5-NS and 16-NS, K_{PQ} in the fluid phase is 89 000 and 9730, respectively (*33*). Experiments were carried out in LUVs of DMPC containing different catechins at 30 °C at lipid-saturating conditions of the resulting curves of the partition experiments, by adding aliquots from a 1 mM solution containing either the 16-NS or 5-NS spin probes in ethanol to the vesicle suspensions. Measurements were taken immediately after preparation.

The hydrophilic quencher acrylamide was used to study the presence of catechin molecules accessible to the aqueous phase and to confirm the possible location of catechins in phospholipid model membranes. LUVs composed of DMPC were incubated with the appropriate amounts of catechins for 30 min at 30 °C before the quenching experiments. Then, quenching of catechins was performed in the absence and in the presence of DMPC vesicles, at lipid-saturating conditions, by adding acrylamide from a 4 M acrylamide/water solution to the catechin/vesicles or catechin/buffer suspensions.

Differential Scanning Calorimetry. The preparation of the samples for the calorimetric measurements was performed as previously described for MLVs vesicles. Afterward, the samples were centrifuged and the pellets containing lipid vesicles were transferred to 50 μL DSC aluminum and hermetically sealed pans and subjected to DSC analysis in a differential scanning calorimeter Pyris 6 DSC (Perkin-Elmer Instruments, Shelton, CT). The heat capacity was obtained raising the temperature at a constant rate of 1 °C/min over the range from 8 to 37 °C. A series of three consecutive scans of the same sample were performed to ensure scan-to-scan reproducibility. The second scan was used for transition calculations. After thermal measurements, the phospholipid content of the sample was determined(*34*). For thermal analysis, data aquisition was performed using the Pyris Software, version 4.0 (Perkin-Elmer Instruments LLC). Microcal Origin software (Microcal Software Inc., Northampton, MA) was used for data analysis.

Steady-State Fluorescence Anisotropy Measurements. The changes in the phospholipids order of model membranes were measured by using two probes located at different depths into the membrane, TMA-DPH and DPH. DMPC model membranes containing different catechins or procyanidin-B_2 concentrations were prepared as previously described for MLVs vesicles. To minimize the scattering of the samples, the phospholipid concentration was kept at 0.5 mM. Aliquots of TMA-DPH or DPH in *N*,*N*′dimethylformamide were added into the lipid dispersion to obtain a probe/lipid molar ratio of 1/500. Different times of incubation were used for the spin probes according to their location. Samples were incubated for 15 or 90 min at 30 °C when TMA-DPH or DPH were used, respectively. All fluorescence studies were carried using 5 × 5 mm quartz cuvettes in a final volume of 400 μL.

The steady state fluorescence anisotropy, ⟨r⟩, of TMA-DPH or DPH was measured with an automated polarization accessory using a Varian Cary Eclipse fluorescence spectrometer, coupled to a Peltier device (Varian) for automatic temperature change. The vertically and horizontally polarized emission intensities, elicited by vertically polarized excitation, were corrected for background scattering by subtracting the corresponding polarized intensities of a phospholipid preparation lacking probes. The G-factor, accounting for differential polarization sensitivity, was determined by measuring the polarized components of the fluorescence of the probe with horizontally polarized excitation ($G = I_{HV}/I_{HH}$). Samples were excited at 360 nm (slit width, 5 nm) and fluorescence emission was recorded at 430 nm (slit width, 5 nm). The steady-state anisotropy was defined by the equation

$$\langle r \rangle = \frac{(I_{VV} - G I_{VH})}{(I_{VV} + 2G I_{VH})}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively.

Laurdan Polarization. The steady-state generalized polarization of the fluorescent probe Laurdan has specific values for the gel and liquid crystalline phases of phospholipids and it is independent of the phospholipid polar head, acyl chain composition, or pH ranging from 4 to 10 (*35*). This parameter relies on the spectral shifts that this probe undergo depending on the chromophores surrounding polarity and solvent dynamics (*35*). Excitation generalized polarization (GP_{ex}) values are given by

$$GP_{ex} = (I_{440} - I_{480})/(I_{440} + I_{480})$$

where I_{440} and I_{480} are the fluorescence intensities at 440 and 480 nm, respectively, after excitation at 380 nm.

For these experiments DMPC multilamellar vesicles (MLVs) model membranes containing different molar ratios of the antioxidant compounds were used. Aliquots of Laurdan were directly added into the lipid dispersion to obtain probe/lipid molar ratio of 1:500. Samples with probes were incubated for 15 min above the gel-to-liquid crystalline phase transition temperature (30 °C) and measurements were taken immediately after. GP_{ex} measurements were recorded at temperatures below and above the phospholipid temperature transition at 15 and 37 °C.

Light Scattering Measurements. MLVs composed of DMPC and containing grape seed extract or pure compounds were prepared. For measuring the membrane solubilization capacity upon detergent addition, the optical density of a 0.95 mM phospholipid dispersion with or without antioxidant was registered at 400 nm using the same buffer as described in the DSC experiments at 25 °C (POLAR star Galaxy, BMG). After the addition of Triton X-100 to a final concentration of 0.25% (w/v), the optical density was measured with continuous mixing.

Reducing Power of the Phenolic Compounds. FRAP (Ferric Reducing/Antioxidant Power) method was used by adaptation of the procedure of Benzie and Strain (*36*) adapted by Pazos et al (*24*). The FRAP reagent was prepared daily by mixing acetate buffer 300 mM (pH 3.6), TPTZ 10 mM, and ferric chloride 20 mM in the ratio 10:1:1, respectively. TPTZ solution was prepared in HCl 40 mM. A sample of 1.5 mL of FRAP reagent was incubated for 10 min at 37 °C. Then, 150 μL of water and 50 μL 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. The results were expressed as micromoles Fe reduced per micromoles antioxidant.

Chelating Capacity of the Phenolic Compounds. The capacity of the phenols for chelating ferrous iron was determined using an adaptation of the procedure by Kolayli et al. (*37*) . A total of 0.2 mL of phenolic solution (4−400 mg/L) 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. Then, the absorbance at 560 nm was measured, and the chelating capacity was calculated according to the following equation

$$\text{Chelating } (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100$$

where A_0 represents the absorbance of the blank without phenolic compounds, A_1 represents the absorbance of the sample with phenolic compound, and A_2 is the absorbance of the blank composed by phenolic compound and the ferrous iron, which removes the interferences generated by phenol-Fe(II) complex. EDTA was employed as a standard.

Determination of Scavenging Capacity (DPPH Radical Scavenging Method). The antioxidant activity of the different polyphenols used was measured in terms of hydrogen-donating or radical-scavenging ability using the stable radical DPPH (*38*) according to the procedure by Von Gadow et al. (*39*). For each antioxidant, different concentrations were tested (expressed as the number of moles of antioxidant/mol DPPH). Antioxidant solution in methanol (0.1 mL) was added to 3.9 mL of a 6×10^{-5} mol/L methanol DPPH e solution. The decrease in absorbance was determined at 515 nm at 15 min. The exact initial DPPH* concentration (C_{DPPH}) in the reaction medium was calculated from a calibration curve with the equation $Abs_{515nm} = 12\,509 \times (C_{DPPH}) - 2.58 \times 10^{-3}$ as determined by linear regression.

Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%.

Chilled Minced Mackerel. Fish were purchased in a local market. Fish was deboned and eviscerated and the white muscle with no skin was separated. The muscle was chopped with a grinder machine to get ground fish muscle. Streptomycine sulfate (200 ppm) was added for inhibiting microbial growth. The different antioxidant compounds were added as a powder at a concentration of 65.7 μmol/kg of minced muscle. Control samples were prepared with no addition of antioxidants. Portions of 6 g of minced muscle were stored at 4 °C. Triplicate samples were taken at different sampling times.

Lipid Extraction. Lipid was extracted from fish muscle according to Bligh and Dyer (*40*) and quantified gravimetrically (*41*).

Peroxide Value. The peroxide value of fish muscle was determined by the ferric thiocyanate method (*42*) and was expressed as mequiv oxygen/kg lipid. Analyses were performed in triplicate.

Thiobarbituric Acid Reactive Substances (TBARS). The thiobarbituric acid index (mg malondialdehyde/kg muscle) was determined according to Vyncke (*43*). Analyses were performed in triplicate.

Sensory Analysis. Sensory analysis was evaluated by an expert panel formed by four trained specialists in descriptive analysis of fishy off-flavors in a room designed for the purpose. The group formed by two women (thirty-five years old) and two men (sixty and thirty-six years old) trained for 10 years in descriptive analysis of fishy off-flavors sniffed the raw samples that were used for chemical determinations. Approximately 10 g of muscle was placed in separate sterile polystyrene Petri dishes and placed at room temperature during 10 min before analysis. Panelists concentrated on detecting rancidity/painty odors using a hedonic scale from 8 to 1, where 8 was the aroma of absolutely fresh and 1 was a putrid aroma (*4*). The odor scores were 8, fresh seaweedy; 7, low odor; 6, stale, earthy; 5, sour, fishy, rotting orange; 4, slight paint; 3, moderate paint; 2, strong paint; and 1, putrid/ammonia.

Statistical Analysis. Each sample type (antioxidant) was replicated in two independent storage experiments ($n = 2$) using different batches of mackerel. The samples were analyzed in triplicate. Assays were carried out on single bags for each formulation. An average value of the replicate analyses was used in calculations of sample variation and significance testing. One-way analysis of variance (ANOVA) was done as a function of antioxidant and storage time (*44*). Then, per each treatment one-way ANOVA has been performed as a function of time, and per each storage time one way ANOVA was done as a function of treatment. Values are presented as means ± standard deviations (SD). Statistical analyses were performed with the software Statistica 6.0.

RESULTS

Partition Coefficients of Pure Compounds in Model Membranes. Emission spectra of pure compounds were much more intense in methanol and in membranes than in water most likely due to its higher quantum yield in these solvents. The fact that the compounds studied showed stronger fluorescence emission properties in methanol solutions, which environment shows similar polarity to the membrane surroundings, facilitated the study of the interaction of these compounds with model membranes. The measurement of the phospholipid/water partition coefficient value (K_P) in phospholipid membranes of DMPC showed that procyanidin B_2 had the highest partition coefficient with a value of $(2.490 \pm 0.370) \times 10^3$ followed by epicatechin with a K_P value of $(9.60 \pm 1.10) \times 10^2$ and catechin with a value of $(5.70 \pm 0.90) \times 10^2$. This result indicates that procyanidin B_2, and probably other dimeric procyanidins, present stronger interaction with phospholipid membranes than their respective monomeric forms.

Localization of Procyanidins and Monomeric Catechins in Model Membranes. Fluorescence-Quenching Experiments. The penetration of the different compounds into the lipid bilayer was determined by monitoring the relative quenching of the fluorescence of the molecules incorporated into the fluid phase of DMPC by the lipophilic spin probes 5-NS and 16-NS. These spin probes are fatty acids differing in the position of a nitroxide group into the molecule. The nitroxide group is located at 5C for the spin probe 5-NS and is located at 16C for spin probe 16-NS, respectively. The intrinsic fluorescence of the different compounds decreased upon the addition of the nitroxide quenchers. The Stern−Volmer plots (**Figure 1**) show how the compounds were quenched by the two probes located at different depths in the bilayer, that is, 5-NS and 16-NS. The results show that quenching of catechin (**Figure 1A**) and epicatechin (**Figure 1B**) was quite similar by the two spin probes regardless of the position of the nitroxide group, showing a low level of quenching in both cases. In contrast, procyanidin B_2 fluorescence was quenched in a higher degree by 5-NS spin probe compared to 16-NS (**Figure 1C**).

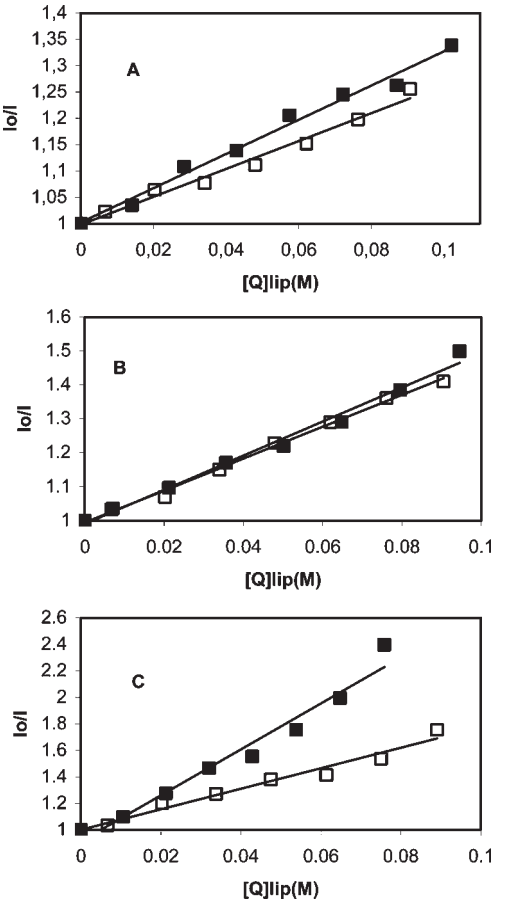

Figure 1. Stern−Volmer quenching plots of (**A**) catechin, (**B**) epicatechin, and (**C**) procyanidin B_2 by 5-NS (■) and 16-NS (□) incorporated into lipid vesicles of DMPC at 30 °C.

The Stern−Volmer constants were obtained from the respective plot slopes. The ratio of the Stern−Volmer constants for probes 5-NS and 16-NS ($K_{SV(5\text{-}NS)}/K_{SV(16\text{-}NS)}$) indicates the relative quenching between 5-NS and 16-NS probes. This ratio was found to be approximately 1 for catechin and epicatechin (**Figure 1A**,**B**) and 2.24 for dimer-B_2 (**Figure 1C**). These results indicate a superficial location for catechin and epicatechin since both compounds were poorly quenched by the two spin probes. Procyanidin B_2 was efficiently quenched by 5-NS probe that reveals a position of its fluorophore group very close to the C5 region.

To confirm the position of the antioxidant compounds into the lipid bilayer, the hydrophilic quencher acrylamide was used. Acrylamide can be used as a quencher to determine fluorophores located at superficial positions or outside the membrane. It can provoke a decrease in the fluorescence intensity of the antioxidant determining the relative position of the antioxidant, inside or outside the membrane. **Figure 2A−C** shows the acrylamide quenching plots for

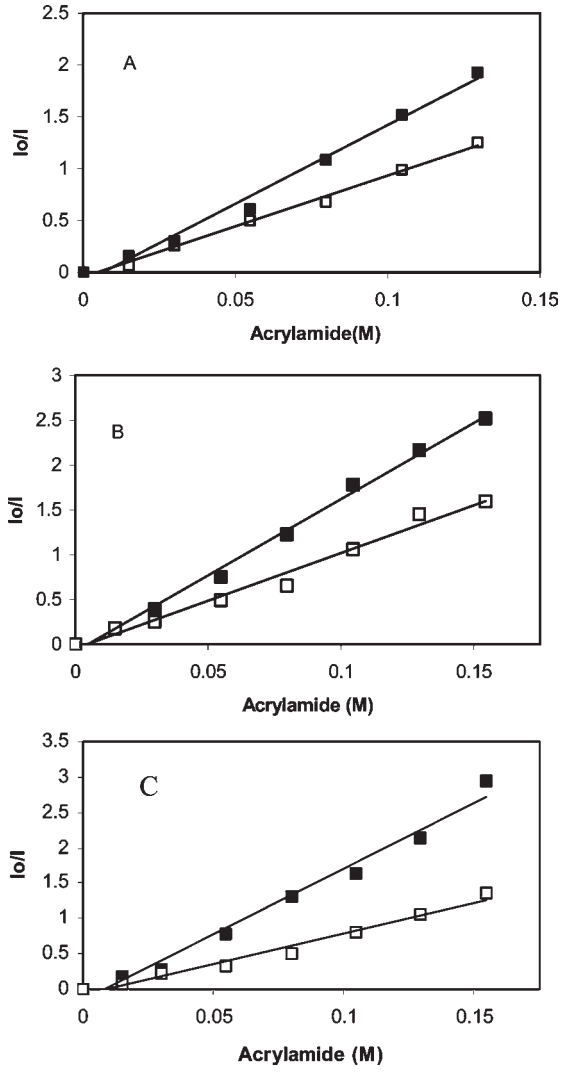

Figure 2. Stern−Volmer quenching plots of (**A**) catechin, (**B**) epicatechin, and (**C**) procyanidin B_2 by acrylamide in buffer (■) or incorporated into lipid vesicles of DMPC (□) at 30 °C.

catechin, epicatechin, and procyanidin B_2, respectively. The Stern−Volmer constants were used to confirm the location of the antioxidant compounds. The value of the ratio $K_{SV\ LIPID\ VESICLES}/K_{SV\ BUFFER}$ for procyanidin-B_2 was 2.14, while for catechin and epicatechin the values were close to 1.5. These results indicate that procyanidin B_2 was much more efficiently quenched by acrylamide when it was dissolved in a buffer than when it was in presence of DMPC vesicles. Such results evidenced a major proportion of procyanidin B_2 inside the membrane where it cannot be quenched by acrylamide than catechin and epicatechin.

Effect of the Monomeric Catechins and Procyanidin on the Thermotropic Behavior of Model Membranes Composed of Phosphatidylcholine. The thermotropic effects of the pure compounds and the grape seed extract in phospholipid membranes were studied by DSC. The phospholipid DMPC was used to test the effect of increasing amounts of the antioxidant compounds on the main gel-to-liquid crystalline phase transition.

The incorporation of increasing amounts of catechin and epicatechin, showed a similar effect on the thermotropic behavior of DMPC vesicles, that is, the elimination of the pretransition at low percentages (2%) and gradual broadening and shifting of the main transition to lower temperatures when the concentration was increased up to 10 molar % (**Figure 3A**,**B**). Procyanidin B_2 had a weaker effect on the broadening and shifting of the main transition of DMPC than that observed for the monomeric catechins (**Figure 3C**). The addition of grape seed extract to DMPC vesicles (**Figure 3D**) showed a similar effect to that observed for catechin and epicatechin. Considering the lower concentrations of procyanidin B_2 in the grape seed extract compared with those of catechin and epicatechin, it can be assumed that the contribution of the procyanidin B_2 to the thermotropic effect of grape seed extract in PC membranes was low.

Lipid/Water Interface Hydration Evaluation through Laurdan Fluorescence Measurements. It has been shown that Laurdan fluorescence spectrum is sensitive to the polarity or hydration state of the lipid/water interface as well as to water molecules dynamics surrounding Laurdan's chromophore (*45*, *46*). Laurdan molecule is strongly anchored in the hydrophobic core of the bilayer due to hydrophobic interactions between its lauric acid tail and the lipid alkyl tails while its fluorescence moiety is located at the glycerol level of the phospholipid head groups. The spectral changes in the emission spectra of Laurdan can be characterized by the generalized emission polarization value (GPex). It has been shown that the GPex value decreased when water penetration into the bilayer increases and this is due to the red shift of Laurdan fluorescence spectrum caused by dipole−dipole interactions and reorientation of available water molecules in the vicinity of the Laurdan probe in the bilayer.

Figure 4 shows the value of GPex determined in phospholipid dispersion containing increasing concentrations of the antioxidant compounds (extract and pure compounds). A slight increase of GPex values was observed when DMPC dispersion contained increasing concentrations of procyanidin B_2 or catechin in the liquid crystalline state at 37 °C. Epicatechin had a weaker effect in increasing the GPex values at high concentrations (20 mol %), but grape seed extract showed the strongest effect. This result reveals that grape seed extract promotes a less polar or more motion-restrictive environment of the Laurdan fluorophore. In contrast, no significant changes were observed for the Laurdan GPex values at temperatures below the gel-to-liquid crystalline phase transition, that is 17 °C, when the antioxidant compounds were added to DMPC lipid dispersions (data not shown).

Steady-State Fluorescence Anisotropy. The effect of pure compounds and grape seed extract in the lipid order of the lipid bilayer was investigated by measuring the steady-state fluorescence anisotropy. Two different probes, TMA-DPH, which is localized in a superficial position of the lipid bilayer, and DPH, which is more internally localized in the membrane, were employed. Changes of the anisotropy values (⟨r⟩) are indicative of variations in the lipid order or packing level of the phospholipid acyl chains. The increase of ⟨r⟩ value by the incorporation of some compounds to the membrane may be due to a decrease of fluorescence probes (TMA-DPH or DPH) incorporated in DMPC vesicles. Such fluorescence decrease is related to a minor rotation of the probe incorporated in DMPC vesicles produced by a decrease of the lipid fluidity. **Figure 5** shows the steady-state fluorescence anisotropy for the DPH probe in DMPC membranes at the fluid state when increasing amounts of catechin, epicatechin, procyanidin B_2, or grape seed extract were included. Only grape seed extract promoted an increase of the ⟨r⟩ value when incorporated at high concentrations. No changes in the anisotropy values were observed when TMA-DPH was used in DMPC dispersions containing the antioxidant compounds (data no shown).

Light Scattering. The results obtained using acrylamide quenching and Laurdan fluorescence suggested that catechin monomers and procyanidin B_2 might be located close to the phospholipid/water interface or in the upper half of the phospholipid bilayer. This location would promote changes in the hydration state or packing of the phospholipid/water interface.

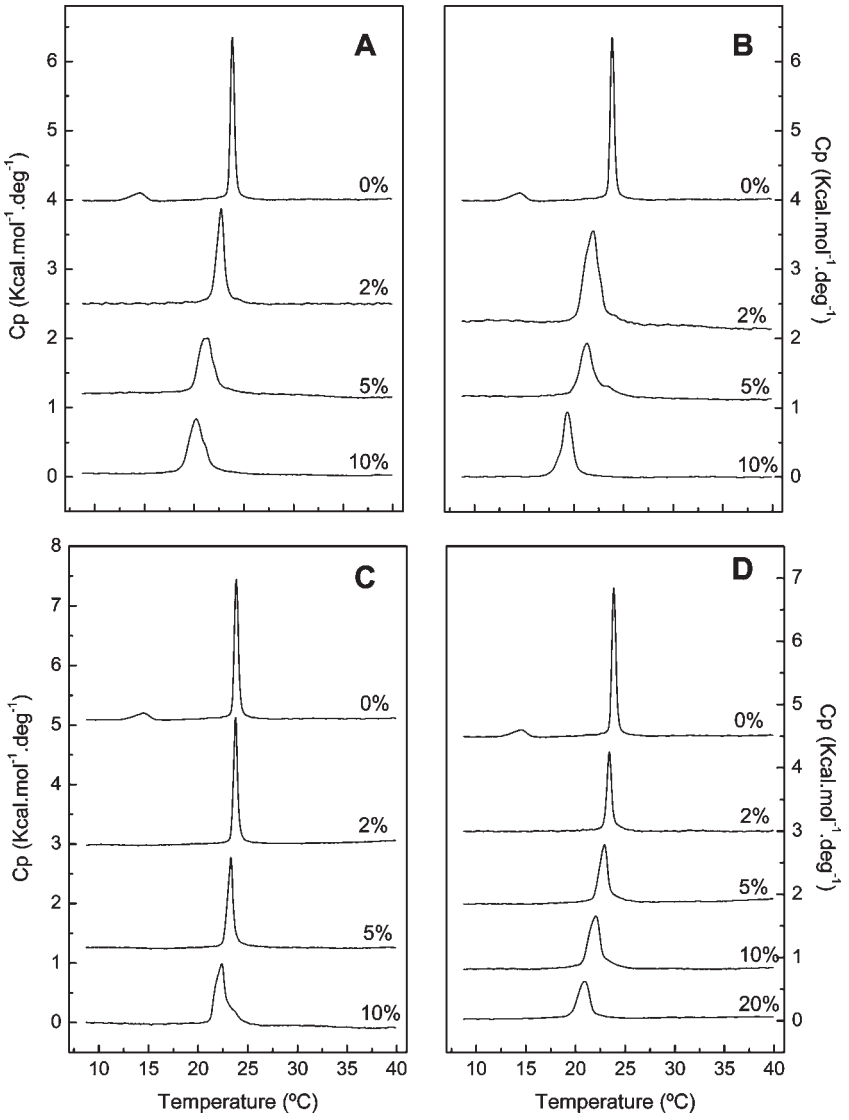

Figure 3. Differential scanning calorimetry heating-scan thermograms for mixtures of catechin-DMPC (**A**), epicatechin-DMPC (**B**), procyanidin B_2-DMPC (**C**), and grape seed extract-DMPC (**D**). Molar percentages of the compounds included in phospholipid dispersions are indicated on the curves.

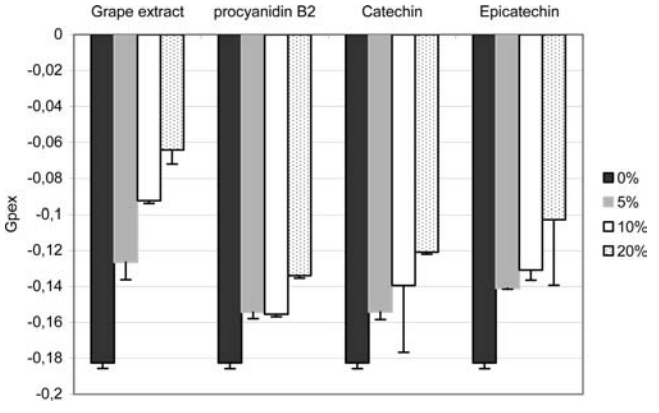

Figure 4. GPex values of Laurdan incorporated into DMPC vesicles in the liquid crystalline phase and containing increasing amounts of grape seed extract, procyanidin B_2, catechin, and epicatechin.

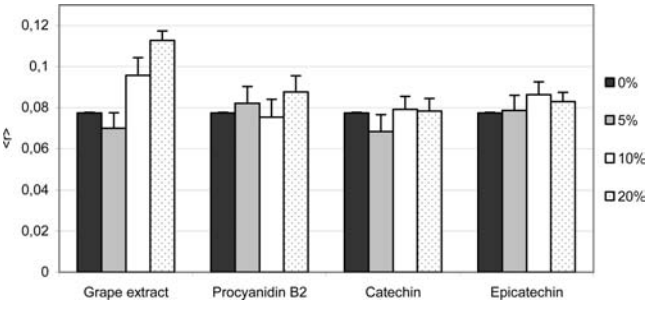

Figure 5. Steady-state fluorescence anisotropy of DPH probe incorporated in DMPC vesicles at 40 °C, containing increasing amounts of grape seed extract, procyanidin B_2, catechin, and epicatechin.

To test if the presence of procyanidin B_2 promoted a different packing or hydration effect on this region, solubilization experiments using a nonionic detergent such as Triton X-100 were carried out. The determination of the insolubility of lipid bilayers in nonionic detergents like Triton X-100 is a useful method to detect the presence of tight-lipid-packing structures that avoid the solubilization of biological membranes (*47*). It has been previously shown that some galloylated catechins promote highly ordered structures, which protect membranes from detergent solubilization (*21*). Then, the capacity of the catechin monomers and procyanidin B_2 to promote resistance to solubilization was compared through the decrease of the light scattering of vesicles'

suspension containing the antioxidant compounds at 25 °C (**Figure 6**). The optical density of DMPC vesicles dropped rapidly meaning that the vesicles were solubilized upon the addition of Triton X-100. DMPC vesicles containing either catechin or epicatechin at 30 mol % showed similar solubilization patterns than that of DMPC vesicles. In contrast, when procyanidin B2 was incorporated into DMPC vesicles at 30 mol %, the decay of the optical density was much slower than that one observed for the catechins-containing DPMC vesicles (**Figure 6**). When grape seed extract was added to DMPC multilamellar vesicles, the optical density was maintained after the TX-100 addition throughout the time of the measurement.

Comparative in Vitro Antioxidant Capacities of Catechin, Epicatechin, Procyanidin B_2, and Grape Seed Extract. Table 1 shows the results related to the ferric reducing antioxidant power measured by FRAP method, antiradical capacity through DPPH method, and metal-chelating capacity. The FRAP assay is based on the measurement of the ability of the substances to reduce Fe^{3+} to Fe^{2+}. The results were expressed as micromoles Fe^{3+} per micromoles of antioxidant compound. Procyanidin B_2 showed the highest power in the reduction reaction of Fe^{3+} to Fe^{2+}. The order of reduction power found was procyanidin B_2 > grape seed extract > epicatechin > catechin.

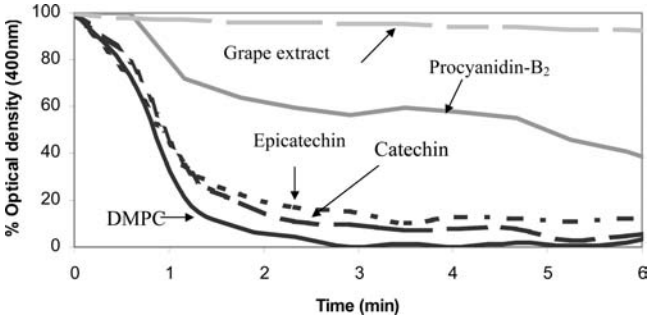

Figure 6. Triton X-100-induced solubilization of MLVs composed of pure phospholipid and mixtures of phospholipid containing 30 mol % of catechin, epicatechin, procyanidin B_2, or grape seed extract. Lipid concentration was 0.95 mM and Triton X-100 concentration was 0.25% (w/v). Curves are the average of three independent experiments.

Table 1. Values of FRAP (Micromoles of Fe^{+3} Reduced Per Micromoles of Antioxidant), Antiradical Capacity (Expressed As Micromolar Needed to Decrease by DPPH 50%), and Chelating Capacity (Calculated at 40 μM)

	reducing capacity	scavenging capacity (EC_{50})	chelating capacity
catechin	1.98 ± 0.08	383.5 ± 29.9	88.67 ± 2.33
epicatechin	2.39 ± 0.16	324.5 ± 27.1	84.80 ± 2.68
procyanidin B_2	7.05 ± 0.30	148.0 ± 27.1	99.22 ± 2.52
grape seed extract	5.77 ± 0.43	153.5 ± 15.3	99.65 ± 1.91

The variation on the concentration of DPPH (stable free radical) was measured to estimate the concentration of antioxidant compound needed to decrease by 50% the absorbance of DPPH (EC_{50}) (**Table 1**). The antiradical capacity order of the antioxidant compounds according to this assay was grape seed extract > procyanidin B_2 > epicatechin > catechin.

The ferrous ions chelating capacity of the different antioxidant compounds were measured and compared to the chelating ability of ethylene diamine tetraacetic acid (EDTA) (**Table 1**). The results showed that grape seed extract and procyanidin B_2 exhibited the highest chelating capacity followed by catechin and epicatechin, which showed the lowest efficiency to chelate metals.

Oxidative Stability of Fish Muscle Supplemented with Grape Seed Extract and Pure Compounds. The capacity of the catechin monomers, procyanidin B_2, and grape seed extract to inhibit lipid peroxidation in fish muscle was followed by the formation of hydroperoxides and TBARS after addition of these antioxidant agents to minced fish muscle (**Table 2**). When all the antioxidant agents were supplemented at 65.8 μmol/kg to chilled minced fish muscle, a significant preservation of the lipids from peroxidation was denoted. The results demonstrated the highest antioxidant efficiency of grape seed extract with no significant increment of oxidation products during the whole experiment. Fish muscle containing catechin or epicatechin showed significant differences in their antioxidant efficacy compared to the control by the fifth day, showing epicatechin higher inhibition capacity of lipid oxidation than catechin. Procyanidin B_2 shows intermediate antioxidant effectiveness compared to the monomers and the grape seed extract. Therefore, the order of the antioxidant capacity for the different antioxidant compounds was grape seed extract > procyanidin B_2 > epicatechin ~ catechin. These results were in accordance with the sensory detection of rancid off-flavors that were not detected in samples supplemented with the grape seed extract during the whole experiment.

DISCUSSION

In the present study, the antioxidant capacity of grape seed extract and its main components to inhibit oxidation in fish muscle was studied stressing their interaction and effects on phospholipid membranes. The final aim was to explain the mechanism involved in the antioxidant efficiency of these compounds in muscle foods. There is evidence to suggest that phospholipids are the primary

Table 2. Values of Hydroperoxide and TBARS during Chilled Storage of Minced Fish Muscle Supplemented with Differents Antioxidants (Mean ± Standard Deviation of Experiments Performed in Triplicate)[a]

	Peroxide Values (mequiv O_2/kg lipid)				
day	control	grape extract	procyanidin-B_2	catechin	epicatechin
0	10.88 ± 2.23 b	4.15 ± 0.18 a	5.82 ± 4.02 ab	5.17 ± 2.48 ab	5.71 ± 1.61 a
2	88.39 ± 16.40 c	7.94 ± 1.17 a	15.28 ± 4.50 b	21.86 ± 12.18 b	12.90 ± 1.16 b
3	90.44 ± 9.60 d	4.37 ± 3.15 a	9.05 ± 1.99 a	31.28 ± 8.48 c	18.59 ± 3.16 b
5	73.99 ± 19.16 d	6.80 ± 0.59 a	19.16 ± 2.36 b	44.40 ± 7.28 c	24.66 ± 5.51 b
	TBARS (mg MDA/kg muscle)				
0	0.83 ± 0.24 ab	0.51 ± 0.08 a	0.55 ± 0.05 a	0.64 ± 0.01 b	0.66 ± 0.23 ab
2	7.65 ± 0.30 d	1.03 ± 0.08 a	1.58 ± 0.12 b	2.42 ± 0.33 c	2.42 ± 0.31 c
3	7.82 ± 0.24 d	1.35 ± 0.47 a	1.73 ± 0.27 a	4.01 ± 0.80 c	2.82 ± 0.54 b
5	8.01 ± 0.23 d	1.56 ± 0.56 a	2.74 ± 0.21 b	5.08 ± 0.69 c	4.22 ± 0.50 c

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

substrates of fish lipid oxidation (*48*) and the location and interaction with membranes can play a significant role in the antioxidant activity of polyphenols (*21*). In addition, chemical properties of antioxidants have shown a high correlation with their antioxidant efficacy found in fish muscle, in particular the reductant capacity (*49*).

The results obtained in this work for the in vitro antioxidant test do not show a clear relationship with the effectiveness found in minced fish muscle. There was a significant dependence of the in vitro values with the number of monomers and hydroxyl groups per molecule. According to that, FRAP measurements indicated that the amount of micromole of Fe^{3+} reduced by procyanidin B_2 or grape seed extract was higher than that corresponding to the monomers. Catechin and epicatechin showed a similar value of Fe^{3+} micromole reduced per micromole of antioxidant, but the value of epicatechin was significantly greater (**Table 1**). The cis-hydroxyl group on C-3 of the C-ring is more effective than the trans configuration in donating electrons making epicatechin a stronger reducing agent (*50*). Flavonoids with adjacent dihydroxy substituents on the B-ring have been shown to be effective in radical scavenging (*51*). Accordingly, the results showed lower values of EC_{50} in the DPPH assay for procyanidin B_2 and grape seed extract compared with the monomers. This result may be related to the higher antioxidant capacity of dimeric and oligomeric B-type procyanidins toward peroxyl radicals than that of catechins. Such a fact might be attributed to the presence of interflavonoid linkage, which increases the electron delocalization capacity of the phenyl radical (*52*). The DPPH scavenging capacity depends also on the number of o-hydroxyl groups per molecule.

Forming stable coordination complexes with prooxidant metals inhibits effectively the metal-catalyzed initiation of free radical oxidation and the decomposition of hydroperoxides. The results of this study show a greater capacity of procyanidin B_2 and grape seed extract to chelate Fe^{2+}. The capacity of antioxidants for chelating metals is strongly dependent on the number of o-hydroxylic groups (*53*). This could explain the higher chelating capacity of procyanidin B_2 and grape seed extract, since these compounds have a higher number of phenolic residues.

The experiment with minced mackerel muscle showed different antioxidant effectiveness depending on the antioxidant compounds employed. The grape seed extract, having a polymerization degree of 2.4 and a galloylation degree of 15%, was the most efficient followed by procyanidin B_2 and then epicatechin and catechin. Several authors have pointed out the difficulty of getting conclusions drawn from the antioxidative capacity assays. As it has been discussed above, procyanidin B_2 and grape extract showed similar chelating and scavenging antioxidant activities and they differed in the FRAP values, which was higher for procyanidin B_2. However, the grape seed extract showed significant higher activity than that of procyanidin B_2 for inhibiting oxidation in the fish minced muscle. Therefore, it seems evident that not only the chemical characteristics are important in the antioxidant mechanism. The incorporation of phenolic compounds and grape extracts into fish microsomes has been previously suggested as one decisive factor for the antioxidant efficiency of phenolics (*26*). Considering the significant effectiveness found for grape extracts in different muscle foods (*9*, *12*−*14*), partition and interaction of their components into the oxidative sensitive sites of muscle may then play a decisive role.

The partition coefficient determined by using the intrinsic fluorescence of catechins and procyanidin B_2 showed that procyanidin B_2 showed stronger affinity for DMPC membranes than catechin monomers. The polymeric character of procyanidin B_2 compared with catechin and epicatechin could explain its higher partition coefficient compared to the monomers through hydrogen-bond formation with some groups of the lipid/water interface. Some authors have demonstrated that catechins with a higher number of hydroxyl groups are able to form a higher number of H-bonds between the compound and the bilayer surface (*25*). These results were compatible with those values obtained with octanol/water partition or RP-HPLC (*22*, *54*).

The results derived from the experiments of membrane localization using spin probes showed a superficial localization of catechin and epicatechin and a more internal situation of procyanidin B_2 probably placed near to the phospholipid/water interface. The superficial localization of catechin and epicatechin has been also reported (*21*, *54*). Quenching experiments using acrylamide confirmed that procyanidin B_2 was less accessible to the quencher in the presence of phospholipid membranes.

DSC experiments showed that the addition of the different compounds studied did not produce drastic changes in the gel-to-liquid crystalline phase transition of DMPC. Nonetheless, the pretransition was abolished at low concentrations of the compounds and the main phase transition was broadened when higher concentrations were utilized. This effect would indicate that the monomeric catechins and procyanidin B_2 would somehow weaken the hydrophobic interactions between phospholipid acyl chains on DMPC and decrease the cooperativity of the transition phase process. A similar arrangement has been postulated for several phenolic compounds such as triclosan (*55*) and the diterpenoids totarol (*56*) and abietic acid (*57*).

The spectroscopic properties of the Laurdan probe were used to examine the polarity and dynamics of the lipid−water interface in model membranes containing catechin monomers or procyanidin B_2. GPex values of Laurdan enhanced as the amount of the compounds was increased in fluid DMPC vesicles. Grape seed extract promoted the strongest increase in the GPex values. An increase in the GPex value can be interpreted as a decrease in water-free movement or hydration at the phospholipid interface of the bilayer due to dipolar relaxation processes of the water molecules surrounding Laurdan chromophore, which indicates less accessibility of water-soluble substances to the surface of the membrane (*58*, *59*). A similar effect has been observed in phospholipid bilayers containing cholesterol (*46*) or rosemary diterpenes (*60*). Then, we postulate that some components present in grape seed extract would promote a dehydration effect in the phospholipid water interface. A decrease of the water molecules movement due to a packing effect at the polar head groups should be discarded, since no effect was observed in the behavior of the TMA-DPH anisotropy probe, as observed for bilayers containing neoglycolipids (*61*).

The steady-state fluorescence anisotropy experiments only showed a decrease of the membrane fluidity when grape seed extract was included in DMPC vesicles containing the DPH probe, indicating that some components of grape seed extract are situated an internal zone of the lipid bilayer. Others authors (*21*) have reported that galloylated catechins induced a decrease of membrane fluidity. Therefore, the presence of galloyl groups in the grape seed extract might be the reason of the membrane-rigidifying effects observed at the core of the bilayer.

The results of Triton X-100 solubilization experiments showed less accessibility of the nonionic detergent into the phospholipid bilayer for grape seed extract and procyanidin B_2. Hence, the presence of tight-lipid-packing structures in the surface of the phospholipid bilayer for procyanidin B_2 and grape seed extract could be suggested. However, since a decrease of the fluidity in the superficial zone of phospholipid bilayer was not observed by the studies performed with the TMA-DPH probe, the reason of the decrease of membrane solubility in the presence of Triton may

be associated with significant bonds between the phospholipid head groups and procyanidin B_2 or other major compounds of grape seed extract. Verstraeten et al. (*62*) have reported that dimers of catechins produced a more efficient protection of bilayer from Triton X-100 than monomers, indicating that water-soluble substances are less accessible to the hydrophobic region of membrane. The formation of H-bonds between flavonoids and the polar headgroup of lipids at the water−lipid interface of membranes has been suggested for explaining this finding (*60*). Sirk et al. (*25*) also observed a greater number of H-bonds between some compounds having higher number of hydroxyl groups and the phospholipid bilayer.

Considering the above exposed, it seems evident that there is a correlation among the effectiveness of the antioxidant compounds in fish muscle and the partition and location of these compounds in membranes, their effects on phospholipid order, and interaction with the membrane surface. Therefore, the results obtained through this work stress that the physical interactions of the antioxidants with the oxidative sensitive sites of the muscle food might play also a decisive role on the kinetics of lipid oxidation.

In conclusion, the antioxidant efficacy observed for procyanidin B_2, grape seed extract, and monomeric catechins for inhibiting lipid oxidation of fish muscle can be assigned to the following reasons:

(I) The less hydration of the lipid/water interface observed by the increase of GPex values. This could reduce the contact of prooxidants compounds as Fe^{2+} or hemoglobin and the penetration of free radicals into the phospholipid membrane. The formation of highly ordered structures probably decreases the accessibility of free radicals into hydrocarbon chain of PUFAs that can initiate or propagate the lipid oxidation.

(II) The localization of procyanidin B_2 and grape seed extract compounds into lipid bilayers, probably by H-bond interaction with polar head groups, could inhibit free radical propagation through the formation of rigid structures. This localization of the compound close to the active points of oxidation would help to inhibit lipid oxidation more efficiently considering their chemical antioxidant properties.

(III) The greater capacity of the grape seed extract and procyanidin B_2 for chelating metals, donating electrons, and scavenging free radicals contribute to explain the higher antioxidant capacity found in fish muscle against catechin and epicatechin.

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