DOI:10.1021/jf900437m

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Galloylated Polyphenols Efficiently Reduce α-Tocopherol Radicals in a Phospholipid Model System Composed of Sodium Dodecyl Sulfate (SDS) Micelles

Manuel Pazos,*,† Josep Lluís Torres,§ Mogens L. Andersen,# Leif H. Skibsted,# and Isabel Medina†

†Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain, §Instituto de Química Avanzada de Catalunya, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain, and #Food Chemistry, Department of Food Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

The ability of several polyphenolic fractions from grape (*Vitis vinifera*) pomace, pine (*Pinus pinaster*) bark, and witch hazel (*Hammamelis virginiana*) bark to repair α-tocopherol (α-TOH) through reduction of the α-tocopheroxyl radical was investigated in a homogeneous hexane system and a phospholipid-like system based on SDS micelles. These natural polyphenols were compared with pure related phenolics (epicatechin, gallic acid, epigallocatechin gallate, quercetin, and rutin) and ascorbic acid, which is a substance with a well-recognized capacity for regenerating α-TOH. α-Tocopheroxyl radicals were monitored and quantified by electron spin resonance (ESR) spectroscopy in the absence and presence of phenolics. Polyphenols from grape and pine bark were essentially catechin monomers and proanthocyanidins differing in the content of galloyl residues; those from pine bark had a negligible degree of galloylation. Polyphenolic fractions from witch hazel bark were composed of approximately 80% hydrolyzable tannins rich in galloyl moieties, together with a smaller amount of catechin monomers and proanthocyanidins. In the homogeneous hexane system, polyphenols from grape and pine bark exhibited similar activities, reducing the α-tocopheroxyl radicals by over 27−40%, whereas phenols from witch hazel were more highly effective, reducing 80% of α-TOH. In contrast, pine bark polyphenols were found to be significantly less active than the grape fractions in SDS micelles, reducing 30 and 70% of α-tocopheroxyl radicals, respectively. Polyphenolic fractions from witch hazel were also able to reduce the highest amount of α-TOH in SDS-micelles. The reducing capacity on α-tocopheroxyl radical of polyphenolic fractions was found to be pH-dependent and more effective at higher pH in the range of pH studied (5.8−7.8). These results stress the potential role of polyphenols, in particular those rich in galloyl groups, to maintain intact endogenous α-TOH in biological membranes through reduction of α-tocopheroxyl radicals.

KEYWORDS: α-Tocopherol regeneration; polyphenols; grape; pine bark; witch hazel bark; antioxidant synergism

INTRODUCTION

Polyphenols are ubiquitous compounds in fruits and vegetables and are associated with beneficial dietary effects, including the prevention of cancer and cardiovascular diseases (*1*, *2*). The antioxidant activity, taken in a broad sense, is believed to be responsible for both biological and food-preservative activities of polyphenols. Polyphenolic compounds have been applied successfully as food additives to preserve the quality of meat and fish by inhibiting lipid peroxidation that is a crucial factor limiting the shelf life of muscle-based foods during storage and processing (*3*−*5*). The antioxidant activity of polyphenols in biological tissues is currently being associated with their capacity to scavenge free radicals (*6*), chelate active redox metals (*7*, *8*), and protect the endogenous antioxidant systems (*9*−*11*). The free radical-scavenging and metal-chelating properties are the best understood antioxidant mechanisms for polyphenolic compounds. The phenolic structure provides the ability to scavenge reactive free radicals by donating an electron or hydrogen atom and to stabilize the phenoxyl radical formed by delocalizing the unpaired electron. The deactivation of catalytic transition metal ions by metal chelation is mainly linked to the presence of at least two *o*-hydroxyl groups at the phenolic ring (*12*).

Several investigations have suggested that α-tocopherol (or vitamin E), a principal lipophilic component of cellular

*Author to whom correspondence should be addressed (e-mail mpazos@iim.csic.es; telephone +34 986 231930; fax +34 986 292762).

 Published on Web 05/07/2009

membranes, is an important antioxidant that stops the propagation of oxidative stress in muscle tissues, and its reduction below critical levels leads to lipid oxidation (*13*, *14*). It is well-documented that α-tocopherol (α-TOH) is preserved by ascorbic acid, a hydrophilic component of the endogenous antioxidant system of tissues, through the reduction of α-tocopheroxyl radical formed during the antioxidant activity of α-TOH (*15*). Such cooperative interaction seems to be responsible for the antioxidant synergism observed when α-TOH and ascorbic acid are used in combination (*12*). Earlier studies have also suggested a regenerative capacity of the phenolic caffeic acid on α-TOH during low-density lipoprotein (LDL) peroxidation, whereas caffeic acid appears to be regenerated by ascorbic acid (*16*, *17*). Similar redox interactions have been recently proposed in fish muscle because the redox stability of endogenous α-TOH was increased by caffeic acid addition, and the resulting endogenous ascorbic acid was rapidly consumed in the presence of caffeic acid (*18*). Other investigations have demonstrated the potential of green tea catechins to regenerate α-TOH in homogeneous solutions (*19*, *20*), sodium dodecyl sulfate (SDS) micelles (*21*), and human LDL (*22*). Myricetin, quercetin, and gallic acid have also exhibited certain abilities to regenerate α-TOH (*23*, *24*). Despite all of the recent progress, more research is required to distinguish which structural factors of polyphenols are relevant for their regenerative activity on α-TOH.

Previous investigations in fish muscle have revealed the effectiveness of grape proanthocyanidins to either delay the lipid oxidation progress or protect endogenous α-TOH against oxidative degradation (*10*, *25*, *26*). Moreover, polyphenols obtained from grape pomace, pine bark, and witch hazel bark have been suggested as active antioxidants in cell assays (*27*, *28*). The present investigation aimed to evaluate the potential impact of these polyphenolic compounds to protect α-TOH from oxidation and, also, to establish which of the structural factors of polyphenols are the most important for α-TOH regeneration via reduction of α-tocopheroxyl radicals. A collection of polyphenolic fractions from grape (*Vitis vinifera*) pomace, pine (*Pinus pinaster*) bark, and witch hazel (*Hammamelis virginiana*) bark, differing in the content of hydrolyzable tannins and galloylated oligomeric catechins (**Figure 1**), was analyzed to characterize their ability to reduce α-tocopheroxyl radicals. Electron spin resonance (ESR) spectroscopy was employed to quantify α-tocopheroxyl radicals. Polyphenolic fractions were also compared with pure related phenolics (epicatechin, gallic acid, epigallocatechin gallate, quercetin, and rutin) and ascorbic acid, a substance with a well-recognized capacity for regenerating α-TOH. The experiments have been conducted in both a homogeneous hexane system and a heterogeneous phospholipid-like system based on SDS micelles.

MATERIALS AND METHODS

Chemicals. DL-*all-rac*-α-Tocopherol, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), SDS, gallic acid, epigallocatechin gallate (EGCG) quercetin, rutin, and ascorbic acid were purchased from Sigma (Steinheim, Germany). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) (98%) and epicatechin were obtained from Fluka (Buchs, Swizerland). All chemicals were of analytical grade, and the water was purified using a Milli-Q system (Millipore, Billerica, MA).

Grape Fractions. The starting material was the byproduct from pressing Parellada white grapes (*V. vinifera*) and consisted of skins, seeds, and a small amount of stems. This byproduct was collected in the month of October during the 2004 harvest. A polyphenolic mixture of mainly monomeric and oligomeric catechins (proanthocyanidins) soluble in both ethyl acetate and water (OWG) was prepared by extraction with water/ethanol (*3*, *7*) following the procedure described by Torres and Bobet (*29*). Fractions IVG, VIIIG, and XIG were obtained by fractionation of OWG

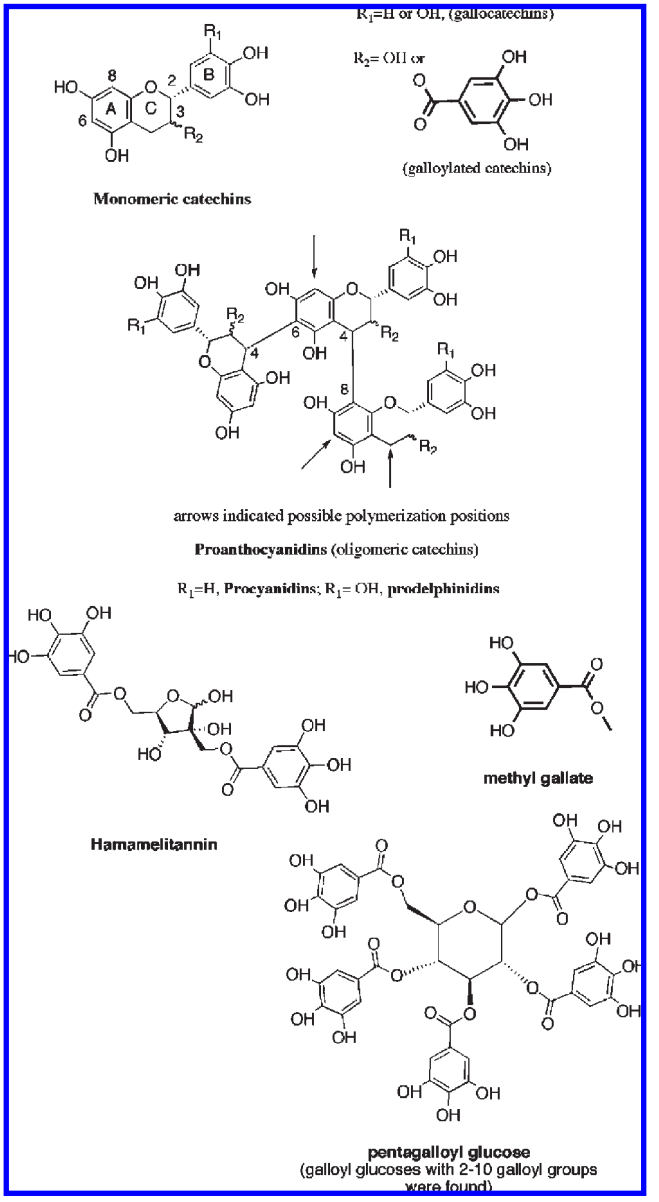

Figure 1. Structures of proanthocyanidins and hydrolyzable tannins found in pine, grape, and witch hazel fractions.

as previously described by Torres et al. (*30*). Grape fractions containing proanthocyanidins with different degrees of polymerization (mDP) and percentages of galloylation (presence of esters with galloyl moieties) as previously described (*30*) are summarized in **Table 1**. The degree of polymerization and percentage of galloylation were evaluated chromatographically after depolymerization with cysteamine according to the method of Torres and Lozano (*31*). Structures of polyphenolic compounds found in the fractions are shown in **Figure 1**.

Pine Bark Fractions. The polyphenolic fractions IP and IIP were prepared by fractionation on preparative RP-HPLC of a total extract OWP from pine (*P. pinaster*) bark, which was obtained essentially as described for grape pomace (*30*), with some modifications (*32*). Fractions IP and IIP contained different combinations of catechin monomers, mainly (+)-catechin, and other flavonoid monomers, mainly taxifolin, and oligomeric catechins. Galloylated catechins were not detected in any of the fractions of pine bark (**Table 1**).

Witch Hazel Bark Fractions. Following a procedure modified from the previously described for grape pomace, a polyphenolic extract soluble in both ethyl acetate and water (OWH) was obtained from witch hazel bark (*28*). Briefly, a sugar free mixture was obtained by extraction with water/acetone (*3*, *7*). After the acetone was evaporated, the lipid soluble material was removed with hexane, and the resulting aqueous phase was

Table 1. Mean Degree of Polymerization (mDP) and Galloylation of the Oligomeric Catechins (Proanthocyanidins) That Compose Grape and Pine Bark Fractions

fraction	mDP	galloylation (mol of gallate/mol of polyphenol)
OWG	1.7	0.15
IVG	2.7	0.25
VIIIG	3.4	0.34
XIG	3.7	0.31
IP	1.9	0
IIP	2.9	0

extracted with ethyl acetate. The fraction IVH was isolated by purification of OWH by size exclusion chromatography on Toyopearl HW-40, as the analogous grape fraction IVG. Witch hazel fractions contained flavanol (catechin) monomers, proanthocyanidins, hydrolyzable tannins such as hamamelitannin, methyl gallate, and galloyl glucoses with 2−10 galloyl moieties. The hydrolyzable tannins accounted for >80% of the polyphenolic content and had a much richer content of galloyl units than the proanthocyanidin fraction. The proanthocyanidins in the OWH and IVH fractions have mean degrees of polymerization (mDP) of 1.2 and 1.6 units, respectively, and galloylation ratios of 0.11 and 0.23 galloyl unit per proanthocyanidin unit (*28*). The degree of polymerization and percentage of galloylation were characterized as above-described for grape fractions.

ESR Experiments To Evaluate the Capacity of Polyphenols To Regenerate α-TOH via Reduction of α-Tocopheroxyl Radical. The ability of polyphenols to restore α-TOH from the α-tocopheroxyl radical was evaluated in two different environments, a micellar system of SDS as a model for phosphylipid membranes and in a homogeneous solution in hexane. α-Tocopheroxyl radical was generated by the chemical reaction of α-TOH in molar excess with DPPH and subsequently quantified by ESR spectroscopy.

The conditions for the experiments carried out in the hexane medium were described in a previous paper (*24*). Briefly, α-tocopheroxyl radicals were produced directly in an ESR quartz capillary tube with an internal diameter of 4.2 mm (Wilmad, Buena, NJ) by mixing α-TOH and DPPH radical in a N_2-saturated hexane solution. Polyphenolic fractions at identical mass concentration were introduced in ethanolic solution 20 s after mixing α-TOH and DPPH radical, when the reaction between α-TOH and DPPH radical was completed as shown previously (*24*). Polyphenolic solutions were substituted by absolute ethanol in control samples. The final concentrations of α-TOH, DPPH radical, and polyphenols were on the order of 2.0 mM, 0.013 mM, and 0.033 mg/mL, respectively. The reaction mixture was homogenized by bubbling N_2 for 40 s. ESR spectra were recorded at room temperature after 1 min of DPPH addition on a JEOL Jes-FR30 ESR spectrometer (JEOL Ltd., Tokyo, Japan) with the following settings: microwave power, 4 mW; sweep width, 50 G; sweep time, 2 min; modulation amplitude, 3.2 G; time constant, 0.3 s.

SDS micelles were essentially prepared as previously described (*18*). Briefly, a micellar solution containing 200 mM SDS and dispersed α-TOH was prepared in 50 mM phosphate buffer, with pH 6.8. SDS micelles were also prepared in phosphate buffer, pH 7.8 and 5.8, in the experiments in which the pH effect was studied. α-Tocopheroxyl radical was generated by mixing 1.8 mL of N_2-saturated SDS micellar solution with 0.1 mL of DPPH radical in ethanol under magnetic stirring. After 20 s, polyphenolic compounds were incorporated in water solution to the micellar system and substituted by water in controls. Final concentrations of α-TOH and DPPH radical were 1.8 and 0.025 mM, respectively. Polyphenols were employed in a concentration of 0.025 mg/mL, except for witch hazel polyphenols in the pH-dependence experiments when the phenolic concentration was 10 times diluted. SDS micellar solution was pumped into an ESR quartz capillary tube with an internal diameter of 0.75 mm (Wilmad), and ESR spectra were recorded at room temperature after 1 min of DPPH addition on a JEOL Jes-FR30 ESR spectrometer. ESR parameters were as mentioned above for experiments in hexane.

The ratio between peak-to-peak amplitude of α-tocopheroxyl radical signal and Mn(II) marker attached to the cavity of the spectrometer was used as a relative signal intensity of α-tocopheroxyl radical, as previously described (*24*). The number of moles of α-tocopheroxyl radical reduced per mole of polyphenolic compound was estimated by the slopes of the linear regressions between the amount of reduced α-tocopheroxyl radical and the polyphenolic concentration. The concentration of α-tocopheroxyl radical was calculated by relating the total double-integrated area of the α-tocopheroxyl radical signal to the total signal area found for a known concentration of TEMPO radical. The area of signals was integrated by using Bruker WinEPR software, whereas the simulation and fitting of the ESR spectra were performed using the PEST WinSIM program.

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

RESULTS AND DISCUSSION

Capacity of Polyphenolic Fractions To Reduce α-Tocopheroxyl Radicals in Homogenous Hexane Medium. The α-tocopheroxyl radical is produced during the antioxidant action of α-TOH by one-electron oxidation (*12*). In our experiments, α-tocopheroxyl radical was first generated in hexane solvent by chemical reaction between DPPH and α-TOH. Polyphenols were supplemented to the samples at the same mass concentration once DPPH radical had been fully consumed, and their effect on α-tocopheroxyl radical was monitored using ESR spectroscopy. The results showed the effectiveness of the polyphenolic fractions to decrease the levels of α-tocopheroxyl radicals in comparison with control samples without polyphenols (**Figure 2A**). The reduction of α-tocopheroxyl radicals in the presence of polyphenols is attributed to the capacity of some phenolic compounds to donate electrons/hydrogen atoms to the α-tocopheroxyl radical and, therefore, to regain the antioxidant activity of α-TOH. Previous investigations have reported a reducing effect on α-tocopheroxyl radicals for phenolic compounds, such as epicatechin, epigallocatechin gallate, quercetin, and caffeic acid, in homogeneous systems of ethanol, 2-propanol/water, and hexane (*18*, *20*, *24*).

The polyphenols obtained from grape and pine bark did not show significant differences in their capacity to regenerate α-TOH ($p < 0.05$), and they were able to reduce the levels of α-tocopheroxyl radicals by over 27−40%, in comparison with control samples without polyphenols (**Figure 2A**). The polyphenolic fractions from witch hazel reduced the levels of α-tocopheroxyl radicals approximately 80% and, therefore, witch hazel polyphenols showed the strongest recycling of α-TOH among the phenolic compounds studied. The proanthocyanidins in the witch hazel fractions represent <20% of the total polyphenolic content and are characterized by a similar medium degree of polymerization (1.2−1.6 units of monomer) and galloylation (0.11−0.23 mol of galloyl group/mol of polyphenol) compared with those found in OW from grape. Consequently, the high capacity of witch hazel fractions to reduce α-tocopheroxyl radicals should be attributed to the important proportion of hydrolyzable tannins with elevated content of galloyl groups (galloylation range = 1−10 galloyl units per polyphenolic unit) that constituted >80% of total polyphenolic content. Nongalloylated proanthocyanidins from pine bark exhibited a similar capacity to reduce α-tocopheroxyl radicals as the grape proanthocyanidins with a galloylation ratio ranging from 0.15 to 0.34 galloyl unit per proanthocyanidin unit. It is well-established that the presence of a galloyl moiety will reduce the bond dissociation enthalpy (BDE) of the O−H bond, in effect facilitating H-atom transfer to reactive free radicals (*33*). In fact, we have previously found a positive correlation between the reduction of α-tocopheroxyl radical and the BDEs of the OH bond for phenolics in homogeneous hexane medium (*24*). Our results appear to indicate that the differences in galloylation ratio between pine bark and grape fractions, which were as much as

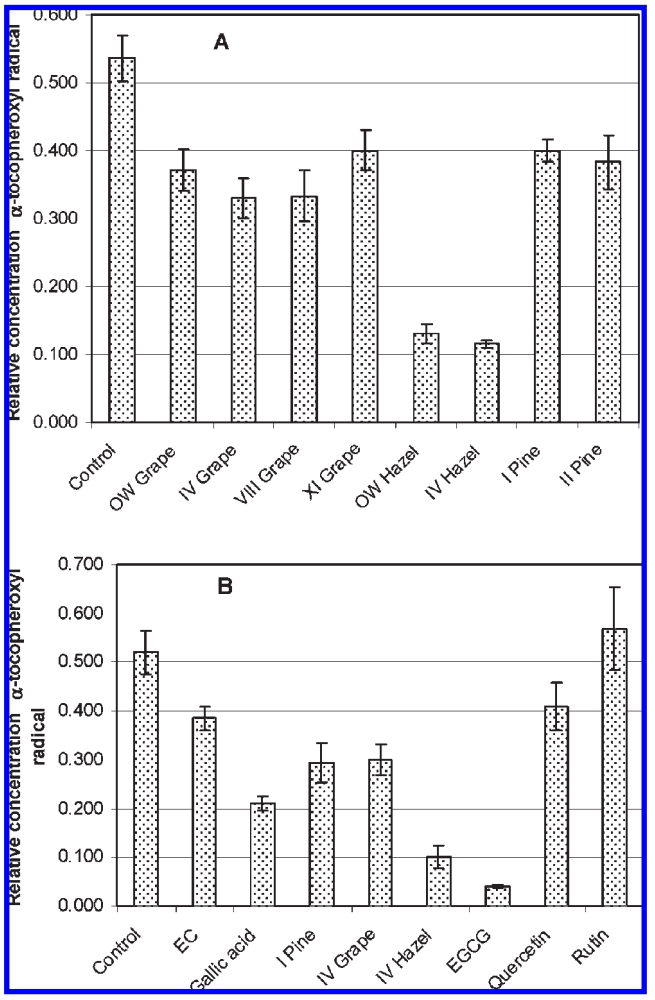

Figure 2. Effect of polyphenolic fractions extracted from pine bark, grape, and witch hazel bark (**A**) on α-tocopheroxyl radical in a homogeneous hexane medium. The activities of representative pine (IP), grape (IVG), and witch hazel (IVH) fractions were also compared with pure phenolic compounds, epicatechin (EC), gallic acid, epigallocatechin gallate (EGCG), quercetin, and rutin (**B**). Polyphenolic fractions and pure phenolics were tested at the same concentration based on weight (0.033 mg/mL).

0.34 galloyl unit/proanthocyanidin unit, are not sufficient to alter significantly the BDE of the O−H bond. This observation is in concordance with previous investigations that reported similar antioxidant properties for pine bark and grape polyphenolic fractions (*27*), whereas witch hazel polyphenols exhibited more elevated antioxidant activity (*28*). The content of galloyl residues has also demonstrated a positive correlation with the antioxidant capacity determined with either the TEAC (*34*) or DPPH assay (*35*) and the reaction rate constants with hydroxyl (*36*) and superoxide radicals (*37*). Additionally, the radical-scavenging activity in galloylated polyphenols appears to be favored considering their capacity to undergo oxidative degradations through coupling reactions (or nucleophilic addition) that retain the number of hydroxyl groups responsible for their antioxidant capacity (*38*).

Representative polyphenolic fractions from pine bark, grape, and witch hazel bark were also compared with other related pure phenolic compounds such as epicatechin (EC), gallic acid, EGCG, quercetin, and rutin. EC, the monomer of nongalloylated proanthocyanidins, was found to be as effective as quercetin in reducing α-tocopheroxyl radicals, whereas the glycoside of quercetin, rutin, did not show significant activity (**Figure 2B**). The lower activity of rutin should be explained by the high contribution of the sugar fraction to the weight of rutin (approximately half of its weight is due to the sugar) and the poor radical-scavenging ability assigned to sugars. The polyphenolic fractions from pine, IP, and grape, IVG, exhibited more elevated regenerative activities than EC and lower activity than gallic acid on a weight basis. EGCG was found to be more efficient in repairing α-TOH than fraction IV from witch hazel (**Figure 2B**). In summary, the relative capacity to reduce α-tocopheroxyl radicals in a homogeneous system in hexane was found to be EGCG > IV witch hazel > gallic acid > I pine ≈ IV grape > EC ≈ quercetin > rutin (no effect). Previous investigations in hexane medium have reported that EGCG is approximately 150 times more effective than EC; however, the former was approximately 14 times less active than ascorbyl palmitate, a lipophilic analogous of ascorbic acid (*24*).

Capacity of Polyphenolic Fractions To Reduce α-Tocopheroxyl Radicals in SDS Micelles. Polyphenolic fractions were evaluated in a SDS micellar system with the aim of understanding their ability to repair endogenous α-TOH in the biological membranes, where α-TOH is localized in muscle tissues. SDS micelles can to some extent mimic membranes considering the structural similarities of SDS micelles and phospholipids. Both systems possess a negatively charged polar head (a sulfate group in SDS) oriented to the external part and a lipophilic tail (a dodecyl group in SDS) situated near the internal part.

The experiments revealed an equivalent activity for the two polyphenolic fractions obtained from pine bark (IP and IIP), with both fractions reducing >30% of the α-tocopheroxyl radical (**Figure 3A**). All grape polyphenolic fractions (OWG, IVG, VIIIG, and XIG) were found to regenerate α-TOH more effectively, as these fractions were capable of repairing >70% of α-tocopheroxyl radical. As found in the homogeneous hexane system, the fractions from witch hazel (OWH and IVH) exhibited the best capacity to repair α-TOH, regenerating approximately 90% of α-tocopheroxyl radical (**Figure 3A**). Therefore, significant differences have not been detected between polyphenolic extracts from the same natural source, and the relative capacity of each polyphenolic source was found to be positively correlated with the content in galloyl moiety: witch hazel bark > grape > pine bark. Previous investigations have revealed that galloylated catechins possess higher phospholipid/water partition coefficients than their homologous nongalloylated forms, probably due to the establishment of strong physical interactions between the galloyl moieties and phospholipids (*39*). He et al. (*40*) suggested that hydrophobic association of galloyl groups of polyphenols and the hydrocarbon chains of phospholipids is responsible for the stronger affinity between galloylated polyphenols and phospholipids. Such physical association should facilitate a neighboring contact between polyphenols and α-TOH, taking the α-TOH location in the phospholipidic bilayers of cell membranes into consideration. Consequently, the regenerative behavior of polyphenolic fractions on α-TOH in the SDS micelles seems to be closely correlated with the galloylation degree and the more elevated affinity of galloylated polyphenols by phospholipids.

Nongalloylated proanthocyanidins from pine bark displayed an activity similar to that of its monomer EC in reducing α-tocopheroxyl radicals (**Figure 3B**). Quercetin showed effectiveness comparable to that of these polyphenols, whereas the glycoside rutin was barely able to regenerate α-TOH at the same weight concentration. EGCG and ascorbic acid exhibited the most effective activity in reducing α-tocopheroxyl radical, followed in decreasing order of efficiency by IV hazel, gallic acid,and IV grape. The high efficiency of EGCG, and in general of galloylated polyphenols, to reduce α-tocopheroxyl radical in

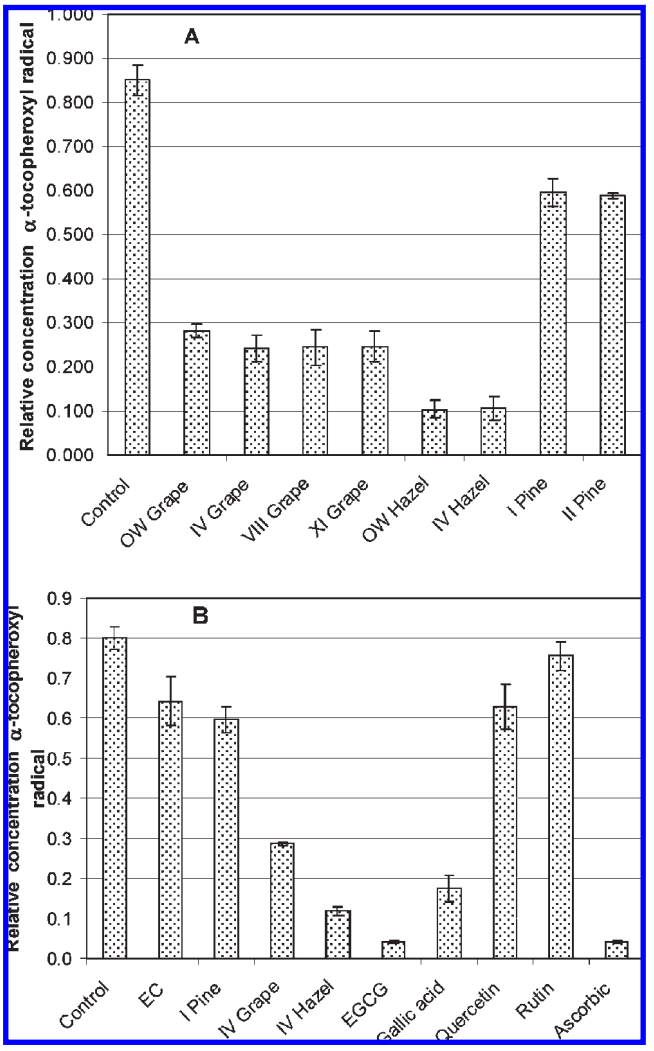

Figure 3. Effect of polyphenolic fractions extracted from pine bark, grape, and witch hazel bark (**A**) on α-tocopheroxyl radical in SDS micelles. The activities of representative pine (IP), grape (IVG), and witch hazel (IVH) fractions were also compared with ascorbic acid and pure phenolic compounds, epicatechin (EC), gallic acid, epigallocatechin gallate (EGCG), quercetin, and rutin (**B**). Polyphenolic fractions and pure phenolics were tested at the same concentration based on weight (0.025 mg/mL).

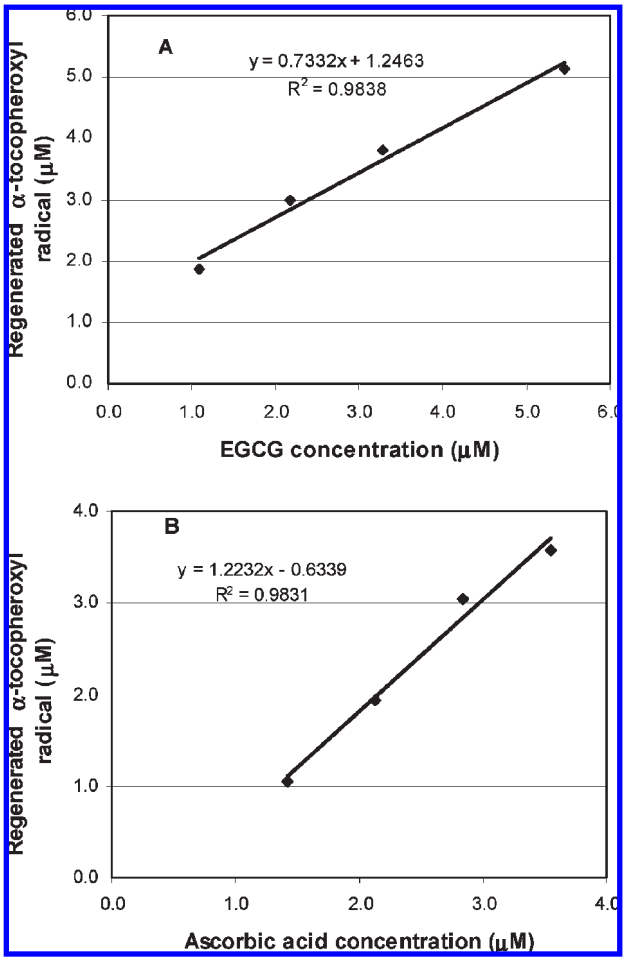

Figure 4. Linear relationship between the amount of α-tocopheroxyl radical regenerated and the molar concentrations of EGCG (**A**) and ascorbic acid (**B**).

Table 2. Moles of α-Tocopheroxyl Radical Reduced per Mole of EGCG or Ascorbic Acid, Determined as the Slope of the Linear Regressions between the Amount of Reduced α-Tocopheroxyl Radical and the Respective Concentrations of EGCG or Ascorbic Acid

	slope (mol of tocopheroxyl radical reduced/mol of compound)	R^2	relative regenerative capacity
EGCG	0.73	0.984	1.00
ascorbic acid	1.22	0.983	1.67

SDS micelles is in accordance with the synergism observed between EGCG and α-TOH when present together either in SDS micelles (*21*) or with linoleic acid in SDS micelles (*41*).

Grape proanthocyanidins have previously shown effectiveness in preserving endogenous α-TOH from oxidation in both LDL (*11*) and fish muscle (*25*, *26*). Such protective action of proanthocyanidins on α-TOH consumption could simply be attributed to a reduction of the reactive free radicals levels as a consequence of the ability of proanthocyanidins as free radical scavenger and transition metal chelator. However, the present investigation has demonstrated a high efficiency of grape proanthocyanidins to reduce α-tocopheroxyl radicals in SDS micelles and, therefore, the regeneration of intact α-TOH seems to be a realistic mechanism for the protection of the endogenous α-TOH in muscle tissues by grape proanthocyanidins.

EGCG versus Ascorbic Acid Regeneration of α-TOH in SDS Micelles. The capacity of EGCG and ascorbic acid to regenerate α-TOH in SDS micelles was evaluated in more detail to estimate the number of moles of α-tocopheroxyl radicals regenerated for each molecule. For the concentration ranges studied, the amount of reduced α-tocopheroxyl radical exhibited a positive linear relationship ($R^2 > 0.983$) with the concentration of EGCG or ascorbic acid present in the medium (**Figure 4**). The slopes of the linear regression lines were used to estimate the number of α-tocopheroxyl radicals regenerated for each EGCG or ascorbic acid molecule (**Table 2**). The results showed that each molecule of EGCG and ascorbic acid was able to repair 0.73 and 1.22 molecules of α-TOH, respectively. Therefore, ascorbic acid was found to be approximately 1.7-fold more effective than EGCG in reducing α-tocopheroxyl radical in SDS micelles at pH 6.8. In a previous investigation in a homogeneous hexane system, it was reported that each molecule of EGCG and ascorbyl palmitate (a lipophilic analogous to the ascorbic acid) was capable of reducing 0.066 and 0.93 molecules of α-tocopheroxyl radical, respectively (*24*). This superior performance of EGCG to reduce α-tocopheroxyl radical in SDS micelles could be partially attributed to the presence of a galloyl group in the EGCG structure and a more active incorporation of galloylated catechins into membranes as discussed previously. The higher incorporation of

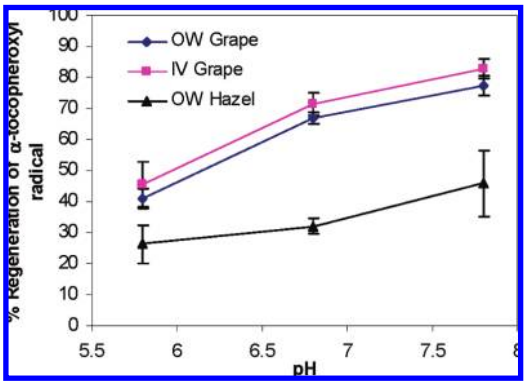

Figure 5. Influence of pH on the tocopherol regeneration reaction by grape and witch hazel fractions in SDS micelles. Grape fractions (OWG and IVG) and witch hazel (IVH) fractions were evaluated, respectively, at concentrations of 0.025 and 0.0025 mg/mL.

EGCG into the membranes should facilitate the redox interaction with α-TOH. Previous investigations have also reported a positive correlation between the phospolipid/water partitions of phenolic compounds and their antioxidant activity (*7*, *39*). Regeneration of > 1 mol/mol for ascorbic acid may indicate a partial two-electron oxidation of ascorbic acid to dehydroascorbic acid during the regeneration.

Influence of pH on the α-TOH-Regeneration Reaction by Polyphenolic Fractions in SDS Micelles. The reducing capacity of polyphenols is linked to their ability to either donate hydrogen atoms or undergo an equivalent two-step reaction consisting of a deprotonation followed by a one-electron transfer (*42*). The two-step reaction is attributed to the existence of dissociable hydroxyl groups, and a pH dependence for the antiradical activity of polyphenols is therefore expected. The behavior of two grape polyphenolic fractions (OWG and IVG) and a witch hazel fraction (OWH) to reduce α-tocopheroxyl radicals in SDS micelles was evaluated at three different pH values (5.8, 6.8, and 7.8). Witch hazel polyphenols were tested at a concentration 10-fold lower due to the higher efficiency. The same tendency was observed for all polyphenolic fractions, and the increment of pH from 5.8 to 7.8 enhanced the activity of polyphenols to reduce α-tocopheroxyl radical (**Figure 5**). This observation is in accordance with previous investigations that found higher reaction rates between pure catechins and α-tocopheroxyl radicals by increasing the anionic character of catechins (*20*). This pH-dependent behavior of polyphenols can be explained considering that catechins are more partially dissociated in neutral−basic media to form the corresponding phenoxide [e.g., the pK_{a1} values of EGCG, EC, and gallic acid are 7.75, 8.64, and 8.73, respectively (*43*)], which more easily undergoes electron transfer oxidation to produce relatively stable phenoxyl radical in alkaline solutions. Therefore, the greater reducing capacity on α-tocopheroxyl radicals observed at increasing pH value for grape and witch hazel fractions appears to be caused by the superior efficiency of the electron transfer processes in neutral−basic media.

In summary, this work draws attention to the potential of natural polyphenols to restore endogenous α-TOH in biological membranes, especially for those rich in galloyl groups. Polyphenols from witch hazel, and to a lesser extent fractions from grape, were found to be more effective in reducing α-tocopheroxyl radicals in a phospholipid-like system composed of SDS micelles, according to their relative galloylation. It is hypothesized that the stronger α-TOH-regenerative capacity assigned to galloylated polyphenols is attributable to the elevated capacity of those to transfer electrons/hydrogen atoms to α-tocopheroxyl radicals, together with the superior incorporation of polyphenols with galloyl groups into phospholipid membranes, where α-TOH is naturally localized.

LITERATURE CITED

(1) Hussain, S. P.; Hofseth, L. J.; Harris, C. C. Radical causes of cancer. *Nat. Rev. Cancer* **2003**, *3*, 276–285.
(2) Barnham, K. J.; Masters, C. L.; Bush, A. I. Neurodegenerative diseases and oxidative stress. *Nat. Rev. Drug Discovery* **2004**, *3*, 205–214.
(3) Pazos, M.; Gallardo, J. M.; Torres, J. L.; Medina, I. Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chem.* **2005**, *92*, 547–557.
(4) Pazos, M.; Alonso, A.; Sanchez, I.; Medina, I. Hydroxytyrosol prevents oxidative deterioration in foodstuffs rich in fish lipids. *J. Agric. Food Chem.* **2008**, *56*, 3334–3340.
(5) Chen-Hsien, L.; Reed, J. D.; Richards, M. P., Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork. *J. Muscle Foods* **2006**, *17* (3), 248−266.
(6) Rice-Evans, C.; Miller, N.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.
(7) Pazos, M.; Lois, S.; Torres, J. L.; Medina, I. Inhibition of hemoglobin- and iron-promoted oxidation in fish microsomes by natural phenolics. *J. Agric. Food Chem.* **2006**, *54*, 4417–4423.
(8) van Acker, S. A. B. E.; van Balen, G. P.; van den Berg, D.-J.; Bast, A.; van der Vijgh, W. J. F. Influence of iron chelation on the antioxidant activity of flavonoids. *Biochem. Pharmacol.* **1998**, *56*, 935–943.
(9) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433–446.
(10) Pazos, M.; González, M. J.; Gallardo, J. M.; Torres, J. L.; Medina, I. Preservation of the endogenous antioxidant system of fish muscle by grape polyphenols during frozen storage. *Eur. Food Res. Technol.* **2005**, *220*, 514–519.
(11) Lourenço, C. F.; Gago, B.; Barbosa, R. M.; De Freitas, V.; Laranjinha, J. LDL isolated from plasma-loaded red wine procyanidins resist lipid oxidation and tocopherol depletion. *J. Agric. Food Chem.* **2008**, *56*, 3798–3804.
(12) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, Scotland, 1998.
(13) Pazos, M.; Sánchez, L.; Medina, I. Alpha-tocopherol oxidation in fish muscle during chilling and frozen storage. *J. Agric. Food Chem.* **2005**, *53*, 4000–4005.
(14) Faustman, C.; Liebler, D. C.; Burr, J. A. Alpha-tocopherol oxidation in beef and in bovine muscle microsomes. *J. Food Lipids* **1999**, *47*, 1396–1399.
(15) Packer, J. E.; Slater, T. F.; Willson, R. L. Direct observation of a free-radical interaction between vitamin-E and vitamin-C. *Nature* **1979**, *278*, 737–738.
(16) Laranjinha, J.; Vieira, O.; Madeira, V.; Almeida, L. Two related phenolic antioxidants with opposite effects on vitamin E content in low density lipoproteins oxidized by ferrylmyoglobin: consumption vs regeneration. *Arch. Biochem. Biophys.* **1995**, *323*, 373–381.
(17) Laranjinha, J.; Cadenas, E. Redox cycles of caffeic acid, α-tocopherol, and ascorbate: implications for protection of low-density lipoproteins against oxidation. *IUBMB Life* **1999**, *48*, 57–65.
(18) Iglesias, J.; Pazos, M.; Andersen, M. L.; Skibsted, L. H.; Medina, I. Caffeic acid as antioxidant in fish muscle: mechanism of synergism with endogenous ascorbic acid and α-tocopherol. *J. Agric. Food Chem.* **2009**, *57*, 675–681.
(19) Jia, Z. S.; Zhou, B.; Yang, L.; Wu, L. M.; Liu, Z. L. Antioxidant synergism of tea polyphenols and α-tocopherol against free radical induced peroxidation of linoleic acid in solution. *J. Chem. Soc., Perkin Trans. 2* **1998**, 911–915.
(20) Mukai, K.; Mitani, S.; Ohara, K.; Nagaoka, S. I. Structure−activity relationship of the tocopherol-regeneration reaction by catechins. *Free Radical Biol. Med.* **2005**, *38*, 1243–1256.

(21) Zhou, B.; Wu, L.-M.; Yang, L.; Liu, Z.-L. Evidence for α-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Biol. Med.* **2005**, *38*, 78–84.
(22) Zhu, Q. Y.; Huang, Y.; Tsang, D.; Chen, Z. Y. Regeneration of α-tocopherol in human low-density lipoprotein by green tea catechin. *J. Agric. Food Chem.* **1999**, *47*, 2020–2025.
(23) Zhu, Q. Y.; Huang, Y.; Tsang, D.; Chen, Z. Y. Interaction between flavonoids and α-tocopherol in human low density. *J. Nutr. Biochem.* **2000**, *11*, 14–21.
(24) Pazos, M.; Andersen, M. L.; Medina, I.; Skibsted, L. H. Efficiency of natural phenolic compounds regenerating α-tocopherol from α-tocopheroxyl radical. *J. Agric. Food Chem.* **2007**, *55*, 3661–3666.
(25) Pazos, M.; Alonso, A.; Fernández-Bolaños, J.; Torres, J. L.; Medina, I. Physicochemical properties of natural phenolics from grapes and olive oil by-products and their antioxidant activity in frozen horse mackerel fillets. *J. Agric. Food Chem.* **2006**, *54*, 366–373.
(26) Medina, I.; Lois, S.; Lizarraga, D.; Pazos, M.; Tourino, S.; Cascante, M.; Torres, J. L. Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *J. Agric. Food Chem.* **2006**, *54*, 3598–3603.
(27) Ugartondo, V.; Mitjans, M.; Tourino, S.; Torres, J. L.; Vinardell, M. P. Comparative antioxidant and cytotoxic effect of procyanidin fractions from grape and pine. *Chem. Res. Toxicol.* **2007**, *20*, 1543–1548.
(28) Touriño, S.; Lizarraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell, M. P.; Julia, L.; Cascante, M.; Torres, J. L. Highly galloylated tannin fractions from witch hazel (*Hamamelis virginiana*) bark: electron transfer capacity, in vitro antioxidant activity, and effects on skin-related cells. *Chem. Res. Toxicol.* **2008**, *21*, 696–704.
(29) Torres, J. L.; Bobet, R. New flavanol derivatives from grape (*Vitis vinifera*) byproducts. Antioxidant aminoethylthio-flavan-3-ol conjugates from a polymeric waste fraction used as a source of flavanols. *J. Agric. Food Chem.* **2001**, *49*, 4627–4634.
(30) Torres, J. L.; Varela, B.; García, M. T.; Carilla, J.; Matito, C.; Centelles, J. J.; Cascante, C.; Sort, X.; Bobet, R. Valorization of grape (*Vitis vinifera*) byproducts. Antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. *J. Agric. Food Chem.* **2002**, *50*, 7548–7555.
(31) Torres, J. L.; Lozano, C. Chromatographic characterization of proanthocyanidins after thiolysis with cysteamine. *Chromatographia* **2001**, *54*, 523–526.
(32) Touriño, S.; Selga, A.; Jimenez, A.; Julia, L.; Lozano, C.; Lizarraga, D. L.; Cascante, M.; Torres, J. L. Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem.* **2005**, *53*, 4728–4735.
(33) Lucarini, M.; Mugnaini, V.; Pedulli, G. F. Bond dissociation enthalpies of polyphenols: the importance of cooperative effects. *J. Org. Chem.* **2002**, *67*, 928–931.
(34) Plumb, G. W.; Pascual-Teresa, S.; C., S.-B.; Cheynier, V.; Williamson, G. Antioxidant propeties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosilation. *Free Radical Res.* **1998**, *29*, 351–358.
(35) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G.-I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56*, 213–222.
(36) Bors, W.; Michel, C. Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies. *Free Radical Biol. Med.* **1999**, *27*, 1413–1426.
(37) Jovanovic, S. V.; Hara, Y.; Steenken, S.; Simic, M. G. Antioxidant potential of gallocatechins. A pulse radiolysis and laser photolysis study. *J. Am. Chem. Soc.* **1995**, *117*, 9881–9888.
(38) Bors, W.; Michel, C.; Stettmaier, K. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch. Biochem. Biophys.* **2000**, *374*, 347–355.
(39) Caturla, N.; Vera-Samper, E.; Villalain, J.; Mateo, C. R.; Micol, V. The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biol. Med.* **2003**, *34*, 648–662.
(40) He, Q.; Shi, B.; Yao, K. Interactions of gallotannins with proteins, amino acids, phospholipids and sugars. *Food Chem.* **2006**, *95*, 250–254.
(41) Zhou, B.; Jia, Z.-S.; Chen, Z.-H.; Yang, L.; Wu, L.-M.; Liu, Z.-L. Synergistic antioxidant effect of green tea polyphenols with a-tocopherol on free radical initiated peroxidation of linoleic acid in micelles. *J. Chem. Soc., Perkin Trans. 2* **2000**, 785–791.
(42) Fang, J. G.; Bo, Z. Structure−activity relationship and mechanism of the tocopherol- regenerating activity of resveratrol and its analogues. *J. Agric. Food Chem.* **2008**, *56*, 11458–11463.
(43) Jovanovic, S. V.; Hara, Y.; Steenken, S.; Simic, M. G. Antioxidant potential of gallocatechins—a pulse-radiolysis and laser photolysis study. *J. Am. Chem. Soc.* **1995**, *117*, 9881–9888.

Received February 7, 2009. Revised Manuscript Received April 27, 2009. This study was supported by the Spanish Ministry of Science (Grants AGL-2006-12210-C03-01 and -02) and the LMC-Centre for Advance Food Studies. The Spanish Ministry of Science is also gratefully acknowledged for a postdoctoral grant to M.P.