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ARTICLE

Structure−Property−Activity Relationship of Phenolic Acids and Derivatives. Protocatechuic Acid Alkyl Esters

Bruno Reis,[†] Marta Martins,[†] Bárbara Barreto,[†] Nuno Milhazes,[‡,§] E. Manuela Garrido,[†,§] Paulo Silva,[†] Jorge Garrido,*[,†,§] and Fernanda Borges*[,§]

[†]CIETI/Departamento de Engenharia Química, Instituto Superior de Engenharia do Porto, IPP, Porto, Portugal, [‡]Instituto Superior de Ciências de Saúde-Norte, Gandra, PRD, Portugal, and [§]CIQUP/Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal

The esterification of hydrophilic phenolic antioxidants is an efficient approach to enhance their solubility in apolar media. Herein, structure−property studies on the antiradical activity of a series of protocatechuic acid alkyl esters have been accomplished. The increase of the lipophilicity was shown to significantly improve the antioxidant activity of protocatechuic esters. Their efficiency as radical scavengers was evaluated using distinctive analytical methods, namely, 2,2-diphenyl-1-picrylhydrazyl (DPPH) UV/visible method, electrochemistry, and differential scanning calorimetry. All the new alkyl protocatechuate antioxidants studied possessed better radical-scavenging capacity than the natural antioxidant protocatechuic acid. This work has shown that the alkyl ester side chain markedly influences the lipophilicity of this type of phenolic system without disturbing the core of the molecule responsible for antioxidant activity. The data on the antioxidant activity obtained using the different analytical methods correlated well with each other and have revealed the interesting antioxidant potential of alkyl esters of protocatechuic acid.

KEYWORDS: Protocatechuic acid; alkyl esters; antioxidant activity; electrochemistry; DSC

INTRODUCTION

Lipid oxidation is an old but still very important topic in food stability and in human health. Many studies have been carried out to search for and to develop antioxidants having a natural origin. The potential of using phenolic acid compounds as natural antioxidants has drawn more attention because of their ubiquitous occurrence in nature. Phenolic acids have been widely investigated as potential models for the development of new primary antioxidants, which can prevent or delay in vitro and/or in vivo oxidation processes (*1*−*3*). The antioxidant activity of dietary phenolic compounds has attracted much attention in relation to their physiological functions, namely, in the prevention of coronary heart disease, cancer, and inflammation (*4*, *5*). Phenolic antioxidants have also been added to food for years to prevent oxidation processes and are widely used today for better food preservation (*1*, *3*).

The relative low solubility of phenolic compounds in apolar media may be seen as a drawback when considering their use as antioxidants for stabilizing unsaturated lipids in formulated complex emulsified systems. Recently, some modifications of these phenolics by the use of reactions that increase lipophilicity have been suggested in order to enhance their hydrophobicity and improve their resulting antioxidant properties by a better location at the lipid/aqueous phase interface where the oxidation occurs (*6*−*8*).

Protocatechuic acid (3,4-dihydroxybenzoic acid) and its esters are *o*-diphenols ubiquitously found in edible plants, vegetables, and fruits. They are known to exhibit potent in vitro antioxidant activities (*9*, *10*). It has been found that protocatechuic acid has preventive effects in carcinogenesis and cardiovascular diseases that are associated with free radicals (*11*−*13*).

The radical-scavenging activity of protocatechuic acid and derivatives toward DPPH was reported in several in vitro studies (*14*−*16*). Despite many studies, important points regarding the structure−activity of protocatechuic acid derivatives are still poorly clarified, especially those concerning the relative antioxidant activity in organic and aqueous environments (*17*). Moreover, studies concerning the relation between lipophilicity and antioxidant efficiency are scarce for this type of compounds.

The present study evaluates the impact of hydrophilic−lipophilic balance on the antioxidant activity of protocatechuic acid derivatives. An insight on the structure−property−activity relationship that underlies the antioxidant activity of protocatechuic esters (**Figure 1**) will be presented. With this aim protocatechuic acid (**1**) and its esters, protocatechuic methyl ester (**2**), protocatechuic ethyl ester (**3**), and protocatechuic propyl ester (**4**) were synthesized, and some biological relevant physicochemical parameters, such as redox potentials (E_p) and partition coefficients (log *P*), were evaluated.

*To whom correspondence should be addressed. For J.G.: (e-mail) jjg@isep.ipp.pt; (address) Departamento de Engenharia Química, Instituto Superior de Engenharia do Porto, IPP, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal; (phone) +351 228 340 500; (fax) +351 228 321 159. For F.B.: (e-mail) fborges@fc.up.pt; (address) Departamento de Química, Faculdade de Ciências, Universidade do Porto, Rua Campo Alegre 687, 4169-007 Porto, Portugal; (phone) +351 220 402 560; (fax) +351 220 402 009.

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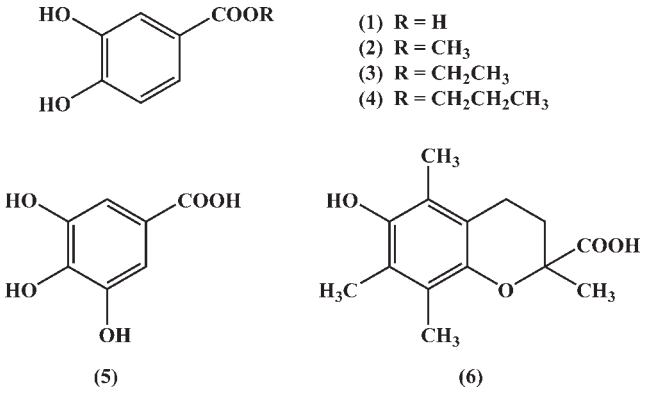

Figure 1. Chemical structures of (**1**) protocatechuic acid, (**2**) protocatechuic methyl ester, (**3**) protocatechuic ethyl ester, (**4**) protocatechuic propyl ester, (**5**) gallic acid, and (**6**) Trolox.

The antioxidant activity of the compounds was evaluated using a total antioxidant capacity radical assay (DPPH$^{\bullet}$, 2,2′-diphenyl-1-picrylhydrazyl radical) and by assessment of its ability to limit the oxidation of linoleic acid, using differential scanning calorimetry (DSC). The antioxidant properties obtained for protocatechuic acid and its esters were compared with those acquired for the reference compounds gallic acid and Trolox. Gallic acid was chosen because of its recognized antioxidant capacity and as representative of natural phenolics and Trolox, a water-soluble analogue of vitamin E, mainly because of its effectiveness in both lipophilic and hydrophilic systems.

MATERIALS AND METHODS

General. Protocatechuic acid, gallic acid, Trolox, DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl), and linoleic acid (LA, *cis*,*cis*-9,12-octadecadienoic acid, 99%) were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). Dimethyl sulfoxide-d_6 (99.8%) and tetramethylsilane (TMS) were obtained from Merck (Lisbon, Portugal). All other reagents and solvents were pro analysis grade and were acquired from Merck and used without additional purification. Deionized water (conductivity of <0.1 μS cm^{-1}) was used throughout all the experiments.

Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 F254 (Merck) with layer thickness of 0.2 mm. For analytical control the following systems were used: ethyl acetate/petroleum ether, ethyl acetate/methanol, chloroform/methanol in different proportions. The spots were visualized under UV detection (254 and 366 nm) and iodine vapor. Normal-phase chromatography was performed using Merck silica gel 60, 0.2−0.5 or 0.040−0.063 mm.

Solvents were evaporated in a Büchi rotavapor (Flawil, Switzerland).

Apparatus. ^{1}H and ^{13}C NMR data were acquired at room temperature on a Brüker AMX 300 spectrometer operating at 300.13 and 75.47 MHz, respectively. Dimethyl sulfoxide-d_6 was used as a solvent. Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference, and coupling constants (J) are given in Hz. Electron impact mass spectrometry (EI-MS) was carried out on a VG AutoSpec instrument. The data are reported as m/z (% of relative intensity of the most important fragments). Melting points were obtained on a Köfler microscope (Reichert Thermovar) and are uncorrected.

Voltammetric studies were performed using an Autolab PGSTAT 12 potentiostat/galvanostat (Eco-Chemie, The Netherlands) and a one-compartment glass electrochemical cell. Voltammetric curves were recorded at room temperature using a three-electrode system. A glassy carbon working electrode (GCE) (d = 2 mm), a platinum wire counter electrode, and an Ag/AgCl saturated KCl reference electrode were used (Metrohm, Switzerland). A Crison pH meter with glass electrode was used for the pH measurements (Crison, Spain).

Spectrophotometric measurements were carried out using a Helios Gamma (Unicam, Portugal) UV/visible spectrophotometer.

A Netzsch DSC 204 calorimeter (Netzsch, Germany) was employed for the thermoxidation stability measurements. The temperature scale was calibrated using In, Bi, Sn, Zn, and KNO_3, and the enthalpy calibration has been carried out to the heat of fusion of the same standards. The oxidation induction temperature (OIT) of pure and spiked linoleic acid was analyzed by heating the samples at 5 K min^{-1} under constant oxygen flow (50 mL/min). A computer-generated plot of heat flow (W/g) versus temperature was used to graphically determine OIT. OIT was determined by the onset of the oxidation process that is characterized by an exothermic peak in the heat flow−temperature plot.

Synthesis. *General Synthetic Procedure*. The alkyl esters of protocatechuic acid were obtained by acid catalyzed esterification following the general procedure: a solution of the phenolic acid (1.0 g, 6.49 mmol) was stirred at room temperature with 75 mL of the corresponding alcohol (methanol, ethanol, or *n*-propanol) containing 2 mL of H_2SO_4 for ∼5 days. The solvent was then partially evaporated under reduced pressure, and the solution was neutralized with aqueous 1 M Na_2CO_3. The mixture was extracted with diethyl ether (3 × 150 mL). The organic phases were combined, washed with water (3 × 100 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was then purified by column chromatography using mixtures of petroleum ether/ethyl ether (7:3 to 5:5) as eluent and recrystallized.

Protocatechuic Methyl Ester (*Methyl 3,4-dihydroxybenzoate*) (***2***). Yield 81%. FTIR υ_{max} (cm^{-1}): 3467, 3263, 1689, 1612, 1448, 1296, 1269, 1240, 1184, 1165, 1095, 764. UV λ_{max} (nm) (log ε): 297 (3.8), 262 (4.0), 221 (4.2), 208 (4.2). ^{1}H NMR δ: 3.76 (3H, s, OC$\underline{H}_3$), 6.80 (1H, d, J = 8.1, H(5)), 7.30 (1H, dd, J = 8.2, 2.1, H(6)), 7.34 (1H, d, J = 2.1, H(2)), 9.64 (2H, br s, O$\underline{H}$). ^{13}C NMR δ: 51.6 O$\underline{C}$H$_3$, 115.3 C(5), 116.2 C(2), 120.5 C(6), 121.8 C(1), 145.1 C(3), 150.4 C(4), 166.2 (C═O). EI-MS m/z (%): 168 (M$^{+\bullet}$, 59), 138 (15), 137 (100), 109 (27), 81 (17). Mp 133−135 °C (diethyl ether/*n*-hexane).

Protocatechuic Ethyl Ester (*Ethyl 3,4-dihydroxybenzoate*) (***3***). Yield 75%. FTIR υ_{max} (cm^{-1}): 3498, 3274, 1682, 1610, 1516, 1369, 1334, 1294, 1236, 1124. UV λ_{max} (nm) (log ε): 296 (3.8), 262 (4.0), 221 (4.2), 208 (4.2). ^{1}H NMR δ: 1.27 (3H, t, J = 7.1, C$\underline{H}_3$), 4.22 (2H, m, OC$\underline{H}_2$), 6.80 (1H, d, J = 8.2, H(5)), 7.30 (1H, dd, J = 8.2, 2.1, H(6)), 7.35 (1H, d, J = 2.1, H(2)), 9.58 (2H, br s, O$\underline{H}$). ^{13}C NMR δ: 14.3 $\underline{C}$H$_3$, 60.0 O$\underline{C}$H$_2$, 115.3 C(5), 116.2 C(2), 120.8 C(6), 121.7 C(1), 145.0 C(3), 150.3 C(4), 165.7 (C═O). EI-MS m/z (%): 182 (M$^{+\bullet}$, 50), 154 (25), 138 (17), 137 (100), 109 (22), 81 (14). Mp 131−133 °C (diethyl ether/*n*-hexane).

Protocatechuic Propyl Ester (*Propyl 3,4-dihydroxybenzoate*) (***4***). Yield 71%. FTIR υ_{max} (cm^{-1}): 3492, 3328, 1684, 1608, 1442, 1292, 1230, 1161, 1099, 984, 771. UV λ_{max} (nm) (log ε): 296 (3.8), 262 (4.0), 221 (4.2), 208 (4.2). ^{1}H NMR δ: 0.94 (3H, t, J = 7.4, C$\underline{H}_3$), 1.67 (2H, m, C$\underline{H}_2$), 4.13 (2H, t, J = 6.6, OC$\underline{H}_2$), 6.80 (1H, d, J = 8.2, H(5)), 7.31 (1H, dd, J = 8.3, 2.1, H(6)), 7.36 (1H, d, J = 2.0, H(2)), 9.57 (2H, br s, O$\underline{H}$). ^{13}C NMR δ: 10.4 $\underline{C}$H$_3$, 21.7 $\underline{C}$H$_2$, 65.5 O$\underline{C}$H$_2$, 115.3 C(5), 116.2 C(2), 120.8 C(6), 121.7 C(1), 145.1 C(3), 150.4 C(4), 165.7 (C═O). EM-IE m/z (%): 196 (M$^{+\bullet}$, 33), 154 (72), 138 (17), 137 (100), 109 (24), 81 (16). Mp 113−115 °C (diethyl ether/*n*-hexane).

Electrochemical Measurements. Stock solutions of protocatechuic acid and its alkyl esters (10 mM) were prepared by dissolving an appropriate amount in ethanol. The voltammetric working solutions were prepared, in the electrochemical cell, by diluting 0.1 mL of the stock solution in 10 mL of supporting electrolyte to get a final concentration of 0.1 mM.

The pH 7.3 supporting electrolyte used in the voltammetric determinations was prepared by dilution to 100 mL of 6.2 mL of 0.2 M dipotassium hydrogen phosphate and 43.8 mL of 0.2 M potassium dihydrogen phosphate.

DSC Measurements. Samples of linoleic acid (2.5 to 3.0 mg) were placed in standard aluminum pans. An appropriate amount of each tested antioxidant was dissolved in methanol to produce a 1 mM solution. Linoleic acid was then spiked, in the aluminum pan, with 10 μL of the antioxidant solution. Small variations in sample size in this weight range had no noticeable effect on the oxidation induction temperature (OIT) measured. Samples were then kept at room temperature for a time period to allow the removal of excess of solvent. Each sample test was run in triplicate.

Radical-Scavenging Activity (DPPH Assay). The radical scavenging activity of protocatechuic acid and its esters was determined using the free radical DPPH$^{\bullet}$ in ethanol (0.25 mM) (*18*). Monitoring of the DPPH$^{\bullet}$ concentration depletion was accomplished by absorbance measurement at 517 nm. Different concentrations were used, expressed as (moles of

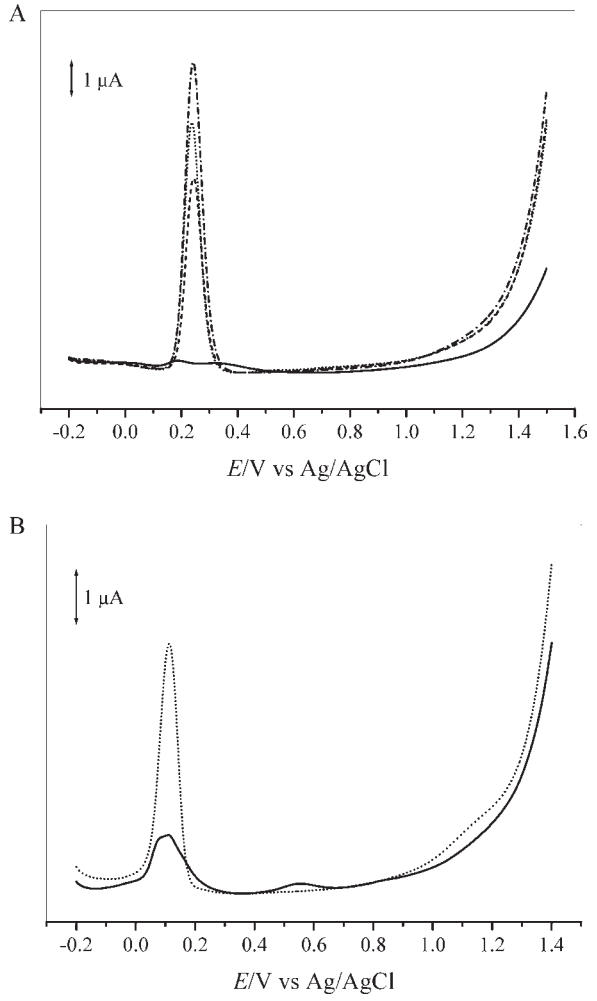

Figure 2. Differential pulse voltammograms of 0.1 mM solutions of (**A**) (—) protocatechuic acid, (- - -) protocatechuic methyl ester, (· · ·) protocatechuic ethyl ester, and (- · -) protocatechuic propyl ester and (**B**) (—) gallic acid and (· · ·) Trolox in physiological pH 7.3 buffer electrolyte. Scan rate was 5 mV s^{-1}.

antioxidant AH)/(mole of DPPH•), and for each one the reaction kinetics was plotted. From these graphs the percentage of [DPPH•] remaining at the steady state was determined. These values were used to determine the efficient concentration (EC_{50}), the amount of antioxidant necessary to decrease the initial [DPPH•] by 50%. Moreover, the antiradical power (ARP), defined as $1/EC_{50}$, the reaction time needed to reach the steady state for EC_{50} (T_{EC50}), and the antiradical efficiency, AE = $(1/EC_{50})$-(T_{EC50}), were also calculated (*19*).

Despite the widespread use of the DPPH• method, the lack of standardization of the results makes it difficult to compare the antioxidant strengths of the different compounds. To establish an appropriate relationship of the antioxidant strength of the new synthesized esters, protocatechuic acid, and reference compounds with results available from literature, the antioxidant activity index (AAI) was also evaluated (*20*).

For comparison, the radical-scavenging activity of gallic acid and Trolox was also tested. All tests were performed in triplicate.

Calculation of Partition Coefficient (log *P*). Calculation of the log *P* values, simulating partitioning of tested compounds in an *n*-octanol/water (1:1, v/v) system, was based on Crippen's fragmentation method (*21*) and was accomplished using the ChemDraw software (ChemDraw Ultra 12.0, Cambridge Soft Corp.).

RESULTS AND DISCUSSION

Chemistry. Protocatechuic acid alkyl ester derivatives (**Figure 1**) were synthesized straightforwardly by Fisher acid catalysis esterification wherein the carboxylic acid is treated with the alkyl alcohol in the presence of a sulfuric acid as catalyst. In this condensation reaction, which proceeds by a nucleophilic acyl substitution mechanism, the equilibrium was influenced by the use of an excess of alcohol. The reaction products were afterward purified by multiple liquid−liquid extraction, column chromatography, and/or crystallization. The reactions led to moderate/high yields of the desired compounds, which were identified by spectroscopic techniques: NMR (^{1}H NMR, ^{13}C NMR), FT-IR, and EI-MS.

Table 1. Redox Potentials (E_p) and Partition Coefficients (log *P*) Obtained for Protocatechuic Acid Its Alkyl Esters and Reference Compounds

compd	E_p (mV)	log *P*
protocatechuic acid (**1**)	188, 317	0.81
protocatechuic methyl ester (**2**)	244	1.08
protocatechuic ethyl ester (**3**)	237	1.41
protocatechuic propyl ester (**4**)	242	1.90
gallic acid (**5**)	110, 544	0.42
Trolox (**6**)	113	4.29

Electrochemical Studies. The antioxidant activity of different species is closely related to their redox properties, and consequently, knowledge of their electrochemical behavior is a very important basis to obtain better explanations of their properties. To contribute to the understanding of the structure−property−activity relationship of hydroxybenzoic acids and to comprehend the role that redox potentials exert on this relation, the study of the electrochemical properties of protocatechuic acid and its synthesized derivatives was done.

Thus, the oxidative behavior of the phenolic compounds was studied, at physiological pH 7.3, by differential pulse and cyclic voltammetry, using a glassy carbon working electrode.

The differential pulse voltammetric study of protocatechuic acid (**1**) (**Figure 1**) revealed the presence of two convolved anodic peaks at physiological pH (**Figure 2A**). The oxidation peaks at E_p = +0.188 V, E'_p = +0.317 V (**Table 1**) are related to the oxidation of the catechol group present in the structure. The results may be interpreted by assuming that the oxidation takes place by electron transfer for both free and adsorbed forms. The free form corresponds to the first peak, E_p, while the strongly adsorbed form, which is consequently stabilized, is oxidized at a more anodic potential, E'_p. The appearance of adsorption peaks, E'_p, has already been described in the literature for electrochemical studies involving cinnamic acids (*22*).

For all the protocatechuic acid alkyl esters (**2**−**4**) (**Figure 1**), only one anodic wave was observed at physiological pH using differential pulse voltammetry (**Figure 2A**). The oxidation waves observed at $E_p \approx +0.24$ V (**Table 1**) for these derivatives are intrinsically related to the oxidation of catechol group present in the molecule's structure. The occurrence of a single voltammetric wave for the alkyl esters derivatives could indicate a higher superimposition of the peaks from both free and adsorbed forms or might indicate a lesser adsorption propensity of these compounds related to the parent acid (*22*).

Cyclic voltammograms were recorded at different sweep rates. The cyclic voltammogram obtained for protocatechuic acid (**1**) shows two convolved anodic peaks corresponding to formation of *o*-quinone (**Figure 3**). On the reverse scan, two reduction peaks were observed. Cyclic voltammograms for the protocatechuic acid alkyl esters (**2**−**4**) show single anodic and cathodic peaks (**Figure 3**). Studies at different scan rates involving the peak current ratio I_{pc}/I_{pa} and the difference between cathodic and anodic peaks potential values (ΔE_p) for the different compounds under study indicate an irreversible electrode process (data not shown).

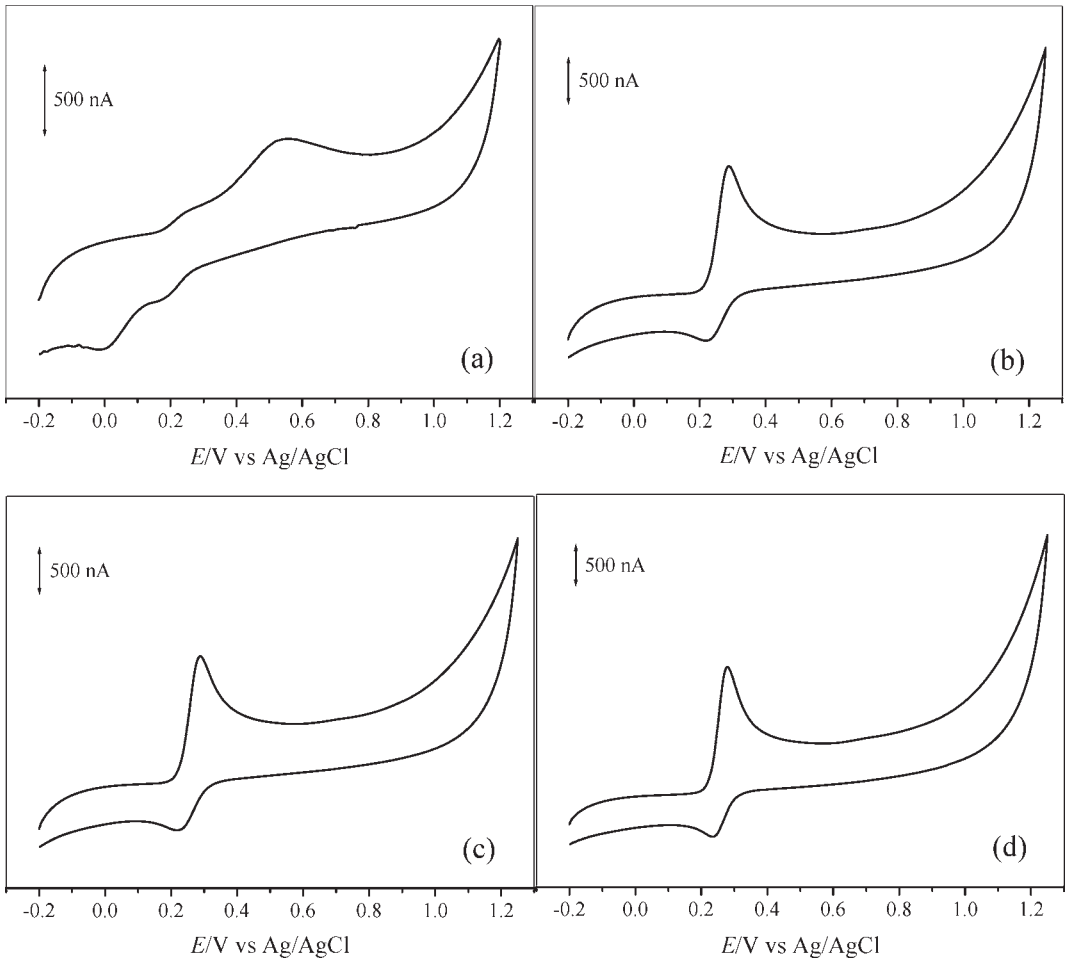

Figure 3. Cyclic voltammograms for 0.1 mM solutions of (**a**) protocatechuic acid, (**b**) protocatechuic methyl ester, (**c**) protocatechuic ethyl ester, and (**d**) protocatechuic propyl ester in physiological pH 7.3 buffer electrolyte. Scan rate was 20 mV s^{-1}.

The gathered results are in agreement with the literature that concerns the oxidative behavior of the lead compound (*23*). Electrochemical studies on the oxidation mechanisms of parent phenolic acids (*22*) have shown that the first oxidation step involves two electrons per molecule which likely corresponds to the formation of a *o*-quinone as the first oxidation product. The formation of a number of coupling products, namely, polymers, is also likely especially for protocatechuic acid because of the presence of a −COOH group with electron-withdrawing character linked to the aromatic ring.

The differential pulse voltammetric study of the reference compounds gallic acid and Trolox (**Figure 1**) revealed the presence of well-defined anodic peaks at physiological pH (**Figure 2B**). The main oxidation waves observed for these compounds occurred at $E_p = +0.113$ V and $E_p = +0.110$ V, respectively (**Table 1**).

The electrochemical data revealed that esterification of protocatechuic acid does not cause significant changes in the oxidation potentials, when compared to the lead compound. The results obtained strongly suggest that the alkyl ester group has a modest effect on the electron density of the catechol ring. Hence, the introduction of an alkyl group and the increase of its length do not significantly influence the energetic of the electron transfer, as can be ascribed from the similarity of the E_p values for these derivatives. However, the introduction of alkyl groups to the carboxylic group of protocatechuic acid produced a raise of protocatechuate radicals, as observed in the increase of the voltammetric signal (**Figure 2**).

From the voltammetric data found it is evident that all the studied compounds investigated present antioxidant activity under the experimental conditions used. Considering that the compounds that exert strong scavenging capabilities are those presenting the lowest reducing potential, it may be concluded that both compounds used as reference must show the highest antioxidant activity. However, the most powerful reducing agents are phenolics with low reduction potential and they can, by autoxidation, exert prooxidant activity (*23*, *24*). The frontier between antioxidative and prooxidative effects is therefore very faint. Consequently, conclusions based exclusively on voltammetric data should be drawn cautiously.

DSC Measurements. Foods containing long-chain polyunsaturated fatty acids are especially labile with respect to oxidation, which causes formation of undesirable flavors and rancid odors, production of potentially toxic compounds, and loss of the health beneficial and essential fatty acids. One industrially acceptable method to overcome the oxidative instability of fatty products and to simultaneously increase nutritional, therapeutic, or food quality preservation is the application of natural or synthetic antioxidants.

Because autoxidation is a process in which heat is released, thermal analysis is a simple and direct analytical method used to follow the reaction course by continuous monitoring of total thermal effect of lipid oxidation occurring in the microcalorimeter vessel. Differential scanning calorimetry (DSC) has been increasingly used in several studies of edible oils autoxidation (*25*).

A simple method to compare the efficiency of several antioxidants and/or stabilizing systems is by determination of the

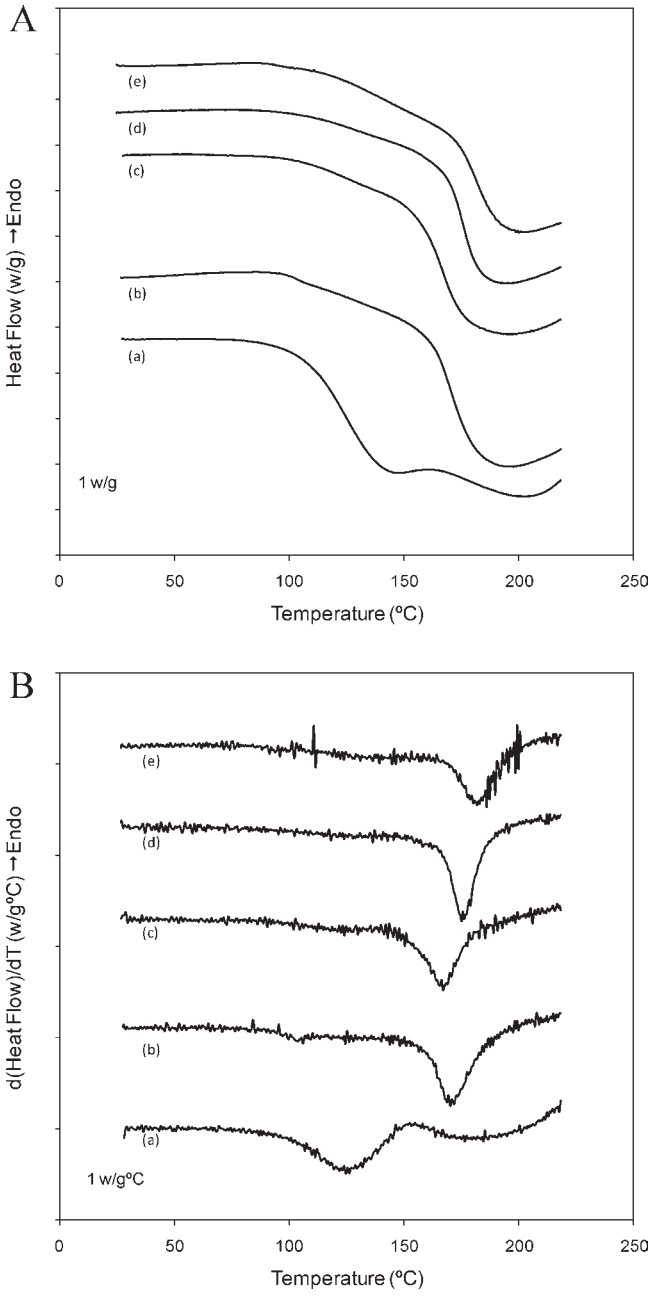

Figure 4. (**A**) DSC and (**B**) first-order derivative curves of nonisothermal oxidation of (**a**) linoleic acid (LA), (**b**) LA + protocatechuic acid, (**c**) LA + protocatechuic methyl ester, (**d**) LA + protocatechuic ethyl ester, and (**e**) LA + protocatechuic propyl ester.

oxidation induction temperature (OIT) of the material after processing. Especially for polyolefins, OIT tests are well established for quality control purposes as a quick screening method to check the activity of the stabilization systems (*26*).

Therefore, the antioxidant activity of protocatechuic acid and its alkyl esters was also evaluated by DSC using pure linoleic acid (LA) as a lipid model system. Because of the presence of double bonds in LA, its oxidation starts at relatively low temperature in comparison with saturated fatty acids, and clear exothermic effects due to oxidation can be easily monitored (*27*). Studies reported in the literature showed that linoleic acid autoxidation processes should be assigned to initial exothermal effects recorded on DSC curves (*25*). Hence, extrapolated oxidation induction temperatures were obtained from the first exothermal peak of nonisothermal oxidation of LA.

Table 2. Oxidation Induction Temperature (OIT) and Peak Temperature of the Derivative Curve (PTDC) Obtained for Pure and Inhibited Linoleic Acid (LA)

compd	OIT (°C)	PTDC (°C)
pure LA	105.7	125.2
LA + protocatechuic acid	159.7	170.6
LA + protocatechuic methyl ester	152.4	166.6
LA + protocatechuic ethyl ester	164.9	175.6
LA + protocatechuic propyl ester	168.9	181.5
LA + gallic acid	156.7	182.0
LA + Trolox	139.5	151.1

DSC curves obtained for oxidation of LA inhibited by protocatechuic acid and its alkyl esters are presented in **Figure 4**. Their shapes are similar to the uninhibited LA oxidation curve, but oxidation kinetics appears slightly different (**Figure 4A**). By use of the described experimental conditions (see Materials and Methods), the oxidation induction temperature (OIT) obtained for pure LA was 105.7 °C. The addition of protocatechuic acid modifies the OIT of linoleic acid from 105.7 to 159.7 °C (**Table 2**). This phenolic acid acts as an antioxidant, slowing down significantly linoleic acid oxidation, this behavior being consistent with the data found in literature for related compounds (*28*). Nevertheless, LA oxidation pattern seems to be affected upon protocatechuic acid addition. In fact, the DSC curve presents, near the OIT for pure LA, a slight increase in the energy flow characteristic of the occurrence of an oxidation reaction. However, this oxidative process seems to be quickly counterbalanced, permitting only the main oxidation to occur at higher temperatures (**Figure 4A**, **Table 2**). The apparent inability of protocatechuic acid to initially block the oxidation of LA can be linked to the difficulty of its diffusion across the lipid matrix or to the occurrence of a rather different oxidation kinetics mechanism from that occurring for pure LA. The fact of this behavior being less evident for protocatechuic alkyl esters, compounds with higher lipophilicity (log *P*), could indicate that the diffusion assumption is more likely. Yet the data found for Trolox highlight another possibility. This subject will be clarified below.

To make the OIT readings from DSC thermograms simpler and faster, the data found were re-evaluated in terms of the thermogram first-order derivative (**Figure 4B**). These differential thermograms were found to provide sharper and better defined peaks, facilitating data reading and acquisition (**Table 2**).

The protocatechuic alkyl esters also exhibit antioxidant capacity toward LA oxidation, as shown by the increase observed in OIT (**Figure 4A**, **Table 2**). From the results it can be concluded that the alkyl esters display an antioxidant capacity comparable to that ascribed to the acid. However, an increasing antioxidant trend is observed with an increase of the number of methylenic groups on the alkyl ester chain. This same pattern is also described in the literature for some hindered phenol antioxidants (*29*).

The OIT for linoleic acid stabilized with the two reference compounds, gallic acid and Trolox, has also been measured and compared with the OIT results obtained for protocatechuic acid and its synthesized alkyl esters (**Figure 5**). An increase of the OIT to higher values, 139.5 and 156.7 °C for LA inhibition with gallic acid and Trolox, respectively, was observed indicating a clear delaying of lipid oxidation.

The DSC curve obtained for LA oxidation inhibited by Trolox presents, near the OIT for pure LA, a slight increase in the energy flow characteristic of the occurrence of an oxidation reaction. This same pattern had already been observed for protocatechuic acid inhibition of LA. Since this behavior was less evident for the

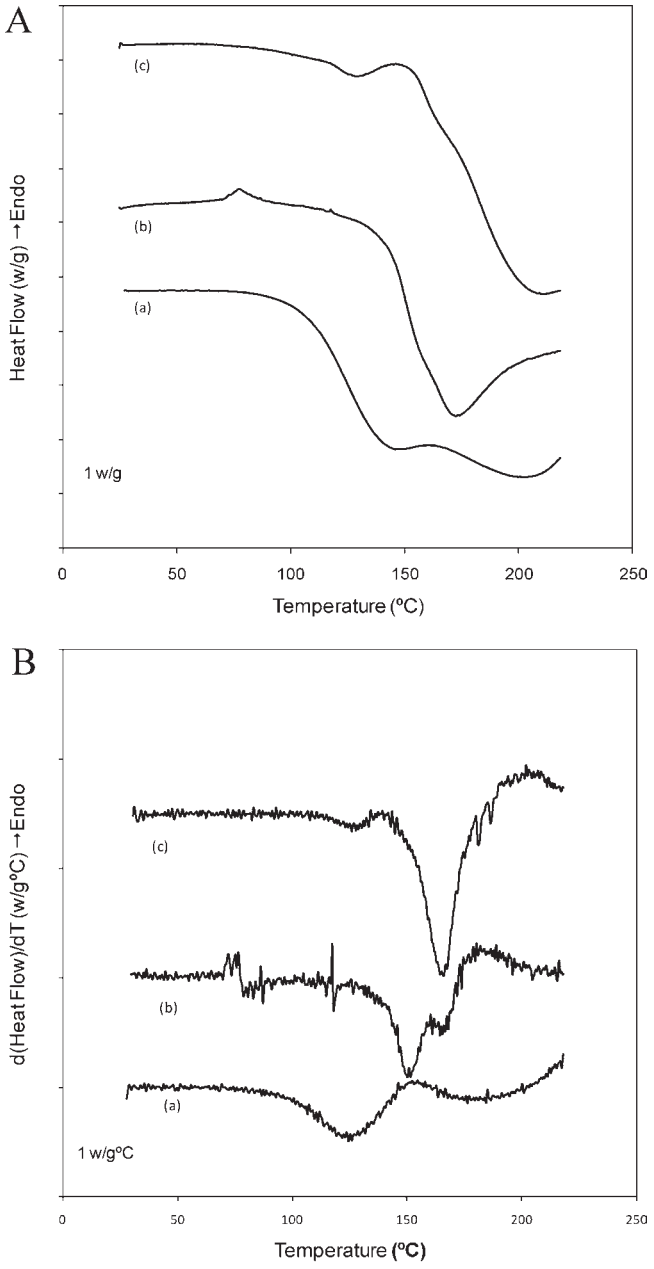

Figure 5. (**A**) DSC and (**B**) first-order derivative curves of nonisothermal oxidation of (**a**) linoleic acid (LA), (**b**) LA + gallic acid, and (**c**) LA + trolox.

alkyl esters derivatives, this conduct was ascribed to differences in the compound's lipophilicity. However, this assumption could not be valid with respect to Trolox, since it presents the highest log *P* of the molecules under study. Therefore, the occurrence of this pattern could be simultaneously related to a molecule's property other than lipophilicity. As was stressed during the electrochemical studies, besides its scavenging capabilities, phenolics with low reduction potentials could also exert prooxidant activity, mainly due to autoxidation. Protocatechuic acid, gallic acid, and Trolox present the lowest redox potentials of the compounds under study. Therefore, this could be the key to understanding the occurrence of a different oxidation pattern for inhibited LA. The occurrence of prooxidative effects could be rapidly counterbalanced by the antioxidative capacity of the compounds, resulting in the highest OIT values. Although gallic acid exhibits one of the smallest redox potential, it presents also the lowest log *P*, meaning that is the less lipophilic of the compounds under study. This redox potential−lipophilicity imbalance could therefore be the reason for the lesser propensity shown by gallic acid to inhibit LA oxidation. Further studies are currently in progress in order to clarify this subject.

Table 3. Radical-Scavenging Activity of Protocatechuic Acid, Its Alkyl Esters Derivatives, and Reference Compounds Using DPPH Assay

compd	EC_{50} (μM)	ARP	T_{EC50} (min)	AE ($\times 10^{-3}$)	AAI
protocatechuic acid (**1**)	15.0	0.067	300	0.22	22.19
protocatechuic methyl ester (**2**)	6.0	0.17	175	0.95	50.74
protocatechuic ethyl ester (**3**)	5.0	0.20	175	1.14	56.32
protocatechuic propyl ester (**4**)	4.0	0.25	175	1.43	65.71
gallic acid (**5**)	12.0	0.083	70	1.19	25.12
Trolox (**6**)	31.0	0.032	4	8.06	6.61

In conclusion, nonisothermal DSC data showed that protocatechuic acid and its alkyl ester derivatives are good antioxidants regarding the inhibition of lipid oxidation. Moreover, the gathered results allow the conclusion that the protocatechuic alkyl ester derivatives studied exhibit an antioxidant capacity higher than gallic acid and that closely matches that found for Trolox.

Radical-Scavenging Activity. The main mechanism of action of phenolic antioxidants is free radical scavenging. Since the DPPH assay is a simple method that enables measurement of the ability of antioxidants to trap free radicals, the reactivity of the protocatechuic acid and its alkyl esters toward this radical was evaluated. Two reference compounds, gallic acid and Trolox, were also included in these studies. The data obtained (**Table 3**) clearly show that protocatechuic acid has a lower DPPH radical-scavenging activity than its derivatives. All the different indexes considered for the potency expression of the antioxidant activity, namely, the antiradical power (ARP), the antiradical efficiency (AE), and the antioxidant activity index (AAI), revealed this trend. In fact, on the basis of AE values, protocatechuic propyl ester was 6 times more efficient than protocatechuic acid. Moreover, according to antiradical efficiency classification, protocatechuic propyl ester presents an AE similar to gallic acid but lower than Trolox. On the other hand, considering the AAI index, it is possible to conclude that all the compounds under study exert a strong antioxidant activity. Again, protocatechuic propyl ester emerges as the most effective of the synthesized compounds and even much more efficient than the gallic acid and Trolox standards.

This behavior is consistent with the antiradical activity described in the literature for related phenolic esters (*16*, *30*). It has been demonstrated that catechols bearing a strong electron-withdrawing substituent at C(1) exhibit high DPPH-radical-scavenging activity in alcoholic solvents, since electron-withdrawing substituents enhance the electrophilicity of the *o*-quinone intermediate (*15*). It was also suggested that the low reactivity of the protocatechuic acid compared to its methyl ester is due to the dissociation of the electron-withdrawing carboxylic acid function to the electron-donating carboxylate ion which decreases the electrophilicity of the *o*-quinone (*16*). Because the electron-transfer mechanism is the most important pathway in $DPPH^{\bullet}$ stabilization by hydroxycinnamic acid and its methyl esters, (*31*) it is expected that there is a relation between the increase of the length of the alkyl chain and the radical-scavenging capacity. In fact, it was observed that the increase in the length of the alkyl chain increases the radical-scavenging capacity. This effect is probably related to the electron-donating enhancement caused by the alkyl chain increase, from which a well-stabilized phenoxyl radical is achieved. The assumption is consistent with the data obtained during the electrochemical studies. The introduction of alkyl groups to the carboxylic group caused an increment of

the voltammetric signal, a feature that could be related to the increased ability of stabilization of protocatechuate radicals produced during the oxidative process.

Estimation of partition coefficients (log *P*). The failure of promising candidates as viable drugs can be traced to difficulties in their transport across membranes. Current approaches for predicting permeation through lipid barriers rely on highly generic physicochemical parameters such as the partition coefficients.

The activity of an antioxidant is mainly governed by lipophilicity, which determines partitioning of compounds in the lipid phase. In view of better understanding the overall properties of the compounds, the lipophilicity, expressed as the octanol−water partition coefficient and herein called log *P*, was calculated according to Crippen's fragmentation method (*21*). The examination of the data obtained allows the conclusion that esterification leads to a clear increase of the log *P* compared to the acid. Moreover, an increase of lipophilicity is also attained with the introduction of additional methylenic groups in the ester alkyl chain (**Table 1**).

The order of increasing lipophilicity on the basis of calculated partition coefficient values (log *P*) was gallic acid < protocatechuic acid < protocatechuic methyl ester < protocatechuic ethyl ester < protocatechuic propyl ester < Trolox (**Table 1**).

In conclusion, the alkyl protocatechuate compounds synthesized demonstrated a higher radical-scavenging activity than the natural antioxidant protocatechuic acid. The increase of the length of ester alkyl chain attached to the catecholic ring seems to influence the stabilization of the radicals formed in the oxidation process. Moreover, the introduction of alkyl groups in the carboxylic acid led to a significant increase of lipophilicity which seems to be positively influenced by the antioxidant activity of protocatechuic acid derivatives.

DSC measurements on the oxidation of linoleic acid with added protocatechuic acid and its alkyl esters enabled the assessment of the influence of the alkyl group on the catecholic ring and on the antioxidant activity of protocatechuic acid derivatives. The use of this technique, particularly of differential thermograms, was revealed to be a simple and to expedite evaluation and comparison of the efficiency of antioxidants.

Electrochemical measurements showed its ability for the study and elucidation of the oxidation process and its effectiveness for the understanding of structure−property−activity relations. The cooperative use of different analytical methods for the antioxidant activity determination has shown the interesting antioxidant potential of protocatechuic acid alkyl esters.

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