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# Solvent and pH Effects on the Antioxidant Activity of Caffeic and Other Phenolic Acids

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The antioxidant activity of several phenolic acids and esters has been investigated both in organic solutions and in large unilamellar phosphatidylcholine vesicles. In solution these compounds behaved as good antioxidants, with the exception of protocatechuic acid, due to the presence of the catechol moiety. Because their antioxidant activity followed an inverse dependence on the magnitude of their O−H bond dissociation enthalpies (BDE), the key mechanism of the chain-breaking action was attributed to hydrogen atom transfer (HAT) from the phenolic OH to peroxyl radicals. In unilamellar vesicles the antioxidant activity was strongly dependent on the pH of the buffer solution. In acid media (pH 4) all of the examined phenolic acids or esters behaved as weak inhibitors of peroxidation, whereas, with increasing pH, their antioxidant activity increased substantially, becoming comparable to or even better than that of Trolox. At pH 8 they also gave rise to lag phases 2−3 times longer than that of Trolox. The increased activity being observed in proximity of the p$K_a$ value corresponding to the ionization of one of the catecholic hydroxyl groups, this effect has been attributed to the high antioxidant activity of the phenolate anion.

**KEYWORDS: Antioxidants; catechol; liposomes; peroxyl radicals; pH effect; phenolic acids**

## INTRODUCTION

Polyphenols are found ubiquitously in both edible and nonedible plants and are important for normal plant growth and defense against infections and injury (*1*). There is currently an intense research interest regarding the phenolic constituents of the diet and their role in contributing to the maintenance of good health (*2*−*4*). It is generally assumed that their beneficial effects are due, at least partially, to their antioxidant activity, which may be brought about at three distinct levels: scavenging of reactive oxygen and nitrogen species; chelating of prooxidant transition metal ions such as copper and iron; and inhibition of prooxidant enzymes [lipoxygenase, myeloperoxidase, xanthine oxidase, NAD(P)H oxidase, cytochrome P-450 enzymes] (*3*, *5*).

Phenolic acids have been extensively investigated for their antioxidant activity in vitro and in vivo in humans and rats, and particularly as agents able to enhance the resistance to oxidation of low-density lipoproteins (*6*−*8*). Structure−activity relationship studies on cinnamic acids have pointed out the importance of the catechol group for the radical scavenging efficacy (*5*), although the role of the ethylenic side chain remains controversial. Some authors suggest that this structural feature is important because it may contribute to the resonance stabilization of the phenoxyl radical formed, whereas others claim that the conjugated double bond is not required for antioxidant efficacy (*9*−*13*). Despite many studies, important points regarding the structure−activity of cinnamic derivatives are still poorly clarified, especially those concerning the relative antioxidant activity in organic and aqueous environments (*5*) and the effect of pH on the antioxidant behavior in water systems (*14*). Actually, this information is of great importance in predicting many biological aspects of practical interest, given the large pH range experienced by food during its way through the digestive tract.

The present study was undertaken to obtain quantitative data on the peroxyl radical scavenging activity of caffeic acid and related derivatives and to better understand the mechanism of their chain-breaking activity in different experimental systems. These studies were carried out in a homogeneous nonpolar medium, such as the mixture styrene/chlorobenzene, and in a heterogeneous system consisting of large unilamellar vesicles of egg yolk lecithin, where interfacial interactions, molecular packing, and dynamics of the lipid phase can be envisaged to be similar to those of natural membranes.

## MATERIALS AND METHODS

**Chemicals.** Solvents were of the highest grade commercially available and were used as received. Egg yolk phosphatidylcholine (PC) was purchased as chloroform solution from Lipid Products (Redhill,

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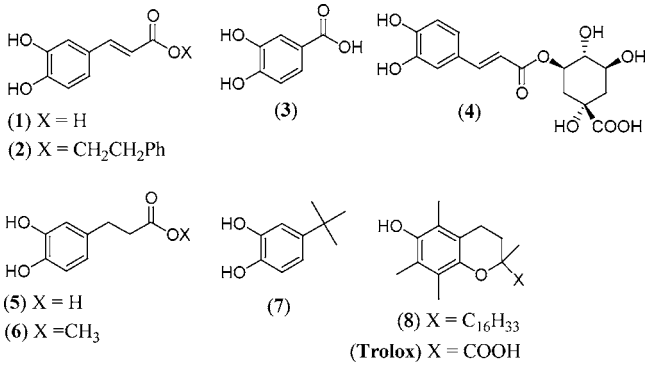


**Figure 1.** Structures of compounds **1**–**8** and Trolox.

U.K.), and styrene was was percolated over aluminum oxide to remove the stabilizer. The azo compounds 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) were from Wako Pure Chemicals Ind. Ltd. and were stored at −20 °C. Caffeic acid (**1**), caffeic acid phenethyl ester (**2**), protocatechuic acid (**3**), chlorogenic acid (**4**), dihydrocaffeic acid (**5**), *tert*-butylcatechol (**7**), 5-di-*tert*-butyl-4-hydroxycinnamic acid (**9**), α-tocopherol (**8**), and Trolox were commercially available (structures shown in **Figure 1**), whereas methyl dihydrocaffeate (**6**) was synthesized from dihydrocaffeic acid by Fischer esterification following the procedure described in the literature (*15*). All other compounds were commercially available and were used as received.

**Autoxidation Experiments in Homogeneous Solution.** Autoxidation experiments were performed in a two-channel oxygen uptake apparatus, based on a Validyne DP 15 differential pressure transducer, that has been described elsewhere (*16*). The entire apparatus was immersed in a thermostated bath ensuring a constant temperature within ±0.1 °C. In a typical experiment, a small amount of a methanol solution of the antioxidant was added to an air-saturated solution of styrene (final concentration of $5 \times 10^{-6}$ M) and equilibrated with the reference solution containing an excess of α-tocopherol ($1 \times 10^{-4}$ M) at 30 °C. This instrumental setting allowed us to have the $N_2$ production and the oxygen consumption derived from the azoinitiator decomposition already subtracted from the measured reaction rates. After equilibration, a concentrated chlorobenzene solution of AMVN (final concentration of $5 \times 10^{-3}$ M) was injected in both reference and sample flasks, and the oxygen consumption in the sample was measured from the differential pressure between the two channels recorded as function of time. The apparatus was calibrated before each series of experiments.

Induction period lengths ($\tau$) were determined by the intersection between the regression lines to the inhibited and the uninhibited traces. Initiation rates, $R_i$, were determined in preliminary experiments by the inhibitor method using α-tocopherol as reference antioxidant: $R_i = 2[\alpha\text{-TOH}]/\tau$.

**EPR and Bond Dissociation Enthalpy (BDE) Measurements.** The BDE of the phenolic O−H bond of 3,5-di-*tert*-butyl-4-hydroxycinnamic acid (**9**) was determined by using the radical EPR equilibration method described elsewhere (*17*, *18*). Briefly, a deoxygenated benzene solution containing a mixture of phenol **9** and 2,4,6-trimethylphenol in a ratio ranging between 1/20 and 1/50 and di-*tert*-butyl peroxide (10% v/v) were sealed under nitrogen in a Suprasil quartz EPR tube. The sample was inserted at room temperature in the cavity of an EPR spectrometer and photolyzed with the unfiltered light from a 500 W high-pressure mercury lamp. Relative radical concentrations, determined by computer simulation, afforded the equilibrium constant between the two phenol/phenoxyl couples, from which the ΔBDE(OH) for **9** was calculated with respect to the known BDE(O−H) value for 2,4,6-trimethylphenol (81.6 kcal/mol) (*17*, *19*) on the assumption that the entropic term can be neglected.

**Preparation of Large Unilamellar Vesicles.** The appropriate amount of a chloroform solution of PC was placed in a round-bottom tube, and the solvent was carefully removed with a gentle nitrogen stream. The thin film was vortex-stirred for 7 min with 0.6 mL of buffered isotonic solutions (phosphate-buffered saline for pH 5, 6, 7.2, and 8 or 0.1 M potassium hydrogen phthalate for pH 4) containing 1 $\times 10^{-3}$ M $Na_2EDTA$ to obtain a PC dispersion mainly constituted by multilamellar vesicles. The milky suspension was then transferred into LiposoFast (Avestin, Ottawa, Canada) and extruded 21 times back and forth through two polycarbonate filters with 100 nm pore size (Nucleopore Corp., Pleasanton, CA) to obtain large unilamellar vesicles (LUVET). The total volume was then adjusted to give a final concentration of $1.5 \times 10^{-2}$ M PC.

**Vesicle Peroxidation.** Autoxidation experiments in the presence and in the absence of antioxidants were carried out by monitoring the oxygen concentration with a miniaturized Clark-type electrode (Instech, Plymouth Meeting, PA) provided with an automatic data recorder (World Precision Instruments, Sarasota, FL). The measurement chamber (internal volume of 0.6 mL) was kept at constant temperature by circulating water and was protected from room light to avoid initiator photodecomposition. After thermal equilibration at 37 °C of the oxidizable substrate, the appropriate amount of AAPH (final concentration of $1.7 \times 10^{-2}$ M) was injected into the cell at the beginning of data collection. After a few minutes, an ethanol solution of the appropriate phenolic acid or ester was added to obtain a final concentration of $5 \times 10^{-6}$ M.

## RESULTS AND DISCUSSION

**Autoxidation Experiments in Homogeneous Solution.** The antioxidant activity of caffeic acid (**1**), caffeic acid phenethyl ester (**2**), dihydrocaffeic acid (**5**), methyl dihydrocaffeate (**6**), protocatechuic acid (**3**), and 4-*tert*-butylcatechol (**7**) was determined by following the oxygen consumption in a closed vessel during the autoxidation of an air-saturated chlorobenzene solution of styrene (4.3 M), in the absence and in the presence of one of the phenols or α-tocopherol (α-TOH), used as reference antioxidant, each at $5 \times 10^{-6}$ M concentration. All samples were added with a small amount (0.1 M) of methanol to improve the solubility of the inhibitors.

The reactions were initiated by thermal decomposition of AMVN at 30 °C and were followed by means of a gas absorption recording apparatus using a commercial differential pressure transducer as detector (*16*). Under these conditions, the slopes of the inhibited period of the oxygen consumption traces provide the rate constants for the reaction of the antioxidants with peroxyl radicals, $k_{inh}$, by means of eq 1:

$$-\frac{d[O_2]}{dt} = \frac{k_p[\text{styrene}]R_i}{nk_{inh}[\text{antioxidant}]} \quad (1)$$

The rate constant for the propagation reaction of styrene at 30 °C is known to be $k_p = 41\ M^{-1}\ s^{-1}$ (*20*); the rate of initiation, $R_i$, was measured in preliminary experiments as described under Materials and Methods, and the stoichiometric factor, $n$, that is, the number of peroxyl radicals trapped by each antioxidant molecule, was obtained from eq 2 and found to be equal to 2,

$$n = \frac{R_i\tau}{[\text{antioxidant}]} \quad (2)$$

except for compound **3**, for which the oxygen consumption trace does not show a definite induction period. This value is consistent with the mechanism of inhibition of catechols, which are known to react with two peroxyl radicals to give *o*-quinone derivatives (*21*).

In the case of protocatechuic acid, the rate constant of inhibition, $k_{inh}$, was instead determined by using eq 3 (*22*), which

$$\frac{R_{ox,0}}{R_{ox}} - \frac{R_{ox}}{R_{ox,0}} = \frac{nk_{inh}[\text{antioxidant}]}{\sqrt{2k_tR_i}} \quad (3)$$

holds when a distinct inhibited period is not observed. The terms

**Table 1.** Measured Rate Constants ($k_{inh}$) for the Reaction of Phenolic Antioxidants with Peroxyl Radicals at 30 °C in Chlorobenzene during the Thermally Initiated Autoxidation of Styrene and Estimated Bond Dissociation Enthalpies (BDE) for the O–H Bond

| antioxidant | $k_{inh}$[a] ($M^{-1}$ $s^{-1}$) | BDE[b] (kcal/mol) |
|---|---|---|
| **1** | $2.9 \times 10^5$ | 78.7 |
| **2** | $3.0 \times 10^5$ | 78.7 |
| **3** | $6.5 \times 10^4$ | 82.2 [c] |
| **4** | | 78.7 |
| **5** | $2.9 \times 10^5$ | 79.0 |
| **6** | $3.5 \times 10^5$ | 79.0 |
| **7** | $4.3 \times 10^5$ | 78.8 |
| **8** | $3.2 \times 10^6$ [d] | $77.1 \pm 0.1$[e] |

[a] In the presence of 0.1 M MeOH; mean of three measures, error within 10%. [b] BDE values calculated by using the additive rule with the revised BDE(PhO–H) = 86.5 kcal/mol (*19*) and the group contributions (in kcal/mol) reported in refs *17*, *18*, and *25*. [c] Value obtained with the COOH group in the para position. [d] From ref *26*. [e] Experimental value (*17*, *19*).

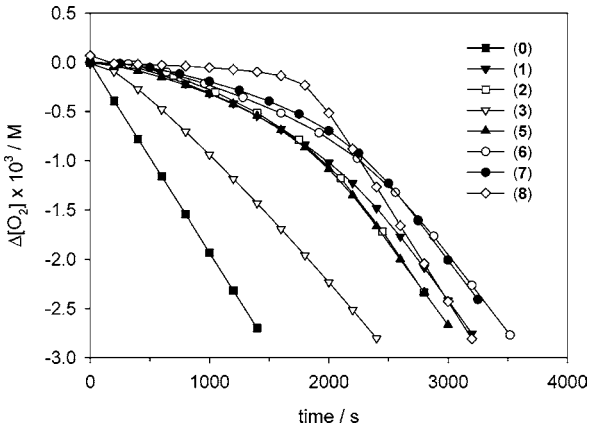


**Figure 2.** Oxygen consumption traces recorded during the autoxidation of styrene 4.3 M in PhCl at 30 °C initiated by AMVN ($5 \times 10^{-3}$ M) in the absence of any antioxidant (**0**) and in the presence of the antioxidants $5.0 \times 10^{-6}$ M.

$R_{ox,0}$ and $R_{ox}$ represent the initial oxygen consumption rates in the absence and in the presence of the inhibitor, respectively. The termination rate constant for styrene, $2k_t = 4.2 \times 10^7$ $M^{-1}$ $s^{-1}$ at 30 °C (*20*), was used, and $n$ was assumed to be equal to 2, similarly to the other phenolic acids.

The small amount of methanol present in the sample is expected to slightly reduce the $k_{inh}$ value because of the formation of a hydrogen bond complex between the alcohol and the phenolic OH. However, this effect should be nearly constant along the series of catechols, so that the relative reactivity should be the same (*23*).

**Table 1** reports the $k_{inh}$ values measured by analyzing the oxygen consumption traces of **Figure 2**. It can be seen that, in a homogeneous solution, the examined antioxidants possess a remarkable antioxidant activity with the only exception of **3**. This strong activity depends on the presence of the catechol moiety and is due to the formation, by hydrogen atom abstraction, of a protonated semiquinone radical stabilized by an intramolecular hydrogen bond stronger than that in the parent catechol (*24*). This determines a low value of the BDE of the catecholic O−H bond and a high rate of hydrogen atom transfer to the chain carrying peroxyl radicals. In most of the investigated compounds, the O−H BDE value of catechol undergoes a further reduction due to the presence of ring substituents capable of inducing an additional weakening of the O−H bond. The only exception is protocatechuic acid (**3**), for which the electron-withdrawing COOH group directly bonded to the aromatic ring has the opposite effect.

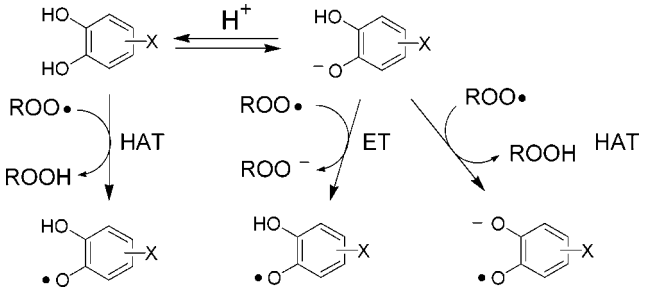


**Figure 3.** Mechanism for the reaction of catechols with peroxyl radicals.

Because it is well established that in phenolic antioxidants every substituent contributes to the overall BDE value and that this contribution is approximately constant and independent of the nature of the other substituents, except when they interact sterically with each other, the O−H bond strengths of the various derivatives can be easily estimated (*17*−*19*, *25*). The group contributions can be used to calculate the BDEs of **7** (78.8 kcal/mol), of **5** and **6** (79.0 kcal/mol, under the reasonable assumption that the alkyl chain has the same effect of a methyl group), and of **3** (82.2 kcal/mol). To calculate the O−H bond strength in caffeic acid and its esters, the unreported contribution of the acrylic substituent, CH═CH−COOH is needed. Thus, the O−H BDE value in 3,5-di-*tert*-butyl-4-hydroxycinnamic acid (**9**) was measured by means of the EPR radical equilibration technique as described under Materials and Methods. From the resulting value (79.7 kcal/mol), a contribution of −2.0 kcal/mol can be calculated for a para acrylic substituent, when it is considered that the additive term of two *tert*-butyl substituents ortho to the OH group is −4.8 kcal/mol (*18*). Thus, the BDE value for caffeic acid **1** and its esters **2** and **4** is estimated as 78.7 kcal/mol.

From **Table 1** it is seen that both BDE values and inhibition rate constants can be divided into three main groups: the first one contains only protocatechuic acid and is characterized by the highest O−H BDE value (82.2 kcal/mol) and a lower reactivity ($k_{inh} = 6.5 \times 10^4$ $M^{-1}$ $s^{-1}$); the second one includes all of the remaining catecholic compounds that show intermediate and similar reactivities ($2.9 \times 10^5$ $M^{-1}$ $s^{-1} \le k_{inh} \le 4.3 \times 10^5$ $M^{-1}$ $s^{-1}$) and bond strengths (78.7 ≤ BDE ≤ 79.0 kcal/mol); the third one contains the very reactive α-tocopherol ($k_{inh} = 3.2 \times 10^6$ $M^{-1}$ $s^{-1}$) (*26*), which shows also the lowest BDE value (77.1 kcal/mol). The fact that the antioxidant activity of phenolic acids and esters follows an inverse dependence on the magnitude of their O−H BDE values provides evidence that in weakly polar organic media, such as the mixture styrene/chlorobenzene used in the present work, the key mechanism of the chain-breaking action is the hydrogen atom transfer (HAT) from the phenolic OH to peroxyl radicals (**Figure 3**).

**Autoxidation Experiments in Large Unilamellar Vesicles.** The antioxidant activities of **1**, **2**, **3**, and **5**−**7** were also investigated in large unilamellar vesicles of PC, where interfacial interactions, molecular packing, and dynamics of the lipid phase can be envisaged to be similar to those of natural membranes, by using the water-soluble azo compound AAPH as initiator of radical peroxidation and Trolox, a hydrophilic analogue of vitamin E, as reference antioxidant. AAPH, owing to its hydrophilicity, gives rise to radical chain initiation at a constant rate in the aqueous phase and is commonly used for studying the effectiveness of hydrophilic and lipophilic peroxidation inhibitors against the attack of oxygen radicals to biomembranes

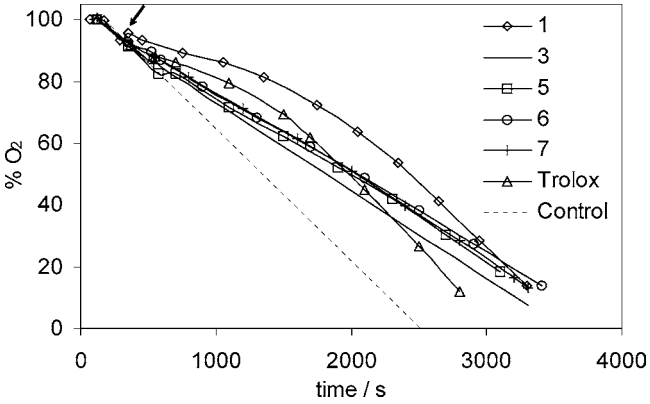


**Figure 4.** Oxygen uptake traces during AAPH ($1.7 \times 10^{-2}$ M) induced peroxidation of PC ($1.5 \times 10^{-2}$ M) unilamellar vesicles at 37 °C and pH 7.2 in the absence of inhibitor (control) and in the presence of one of the various phenolic antioxidants, each at the same concentration ($5 \times 10^{-6}$ M). The arrow shows antioxidant injection.

from the external water environment. Trolox is also largely partitioned in the water phase and in the polar interface of model membranes.

The oxygen uptake traces (**Figure 4**), obtained at 37 °C and pH 7.2, indicate that **1** behaves as a very effective antioxidant showing both a longer inhibition time and a higher rate constant for the reaction with peroxyl radicals than those measured with the same amount of Trolox. These results are similar to those reported by Laranjinha et al. (*7*) when assaying the antioxidant activity of **1** in low-density lipoproteins by monitoring the fluorescence decay of *cis*-parinaric acid previously incorporated into the particle. On the other hand, in the presence of equivalent amounts of **2**, **3**, and **5**−**7**, the rate of oxygen uptake was somewhat reduced with respect to control experiments, but no evident inhibition time occurred. These results are in striking contrast with what was observed in homogeneous solution, where caffeic acid is characterized by about the same antioxidant activity as the other phenolic acids.

We report, as a measure of the antioxidant activity, the ratio $nk_{inh(antioxidant)}/2k_{inh(Trolox)}$ (**Table 2**), where the two terms were obtained by means of eq 3 from the slopes of the oxygen consumption traces as described above. This was necessary because in aqueous media the stoichiometric coefficient, *n*, could not be determined for the majority of phenolic acids. Actually, at pH 7.2 only caffeic acid showed a clear induction period ranging between 4 and 5.

The data of **Table 2** show that the reactivity of caffeic acid toward peroxyl radicals is ∼5 times higher than that of the other phenolic acids and even larger than that of Trolox. Because the examined phenols contain two or three hydroxyl groups characterized by different p$K_a$ values, autoxidation experiments were carried out at various pH values of the buffered solution used to prepare PC vesicles, to test if there is any pH dependence of the antioxidant activity of these compounds.

The results of the measurements at pH 4 (**Figure 5A**) show that none of the tested phenolic acids, including caffeic acid, give any recognizable induction period. They only retard the autoxidation, with the exception of Trolox, which behaves as in neutral media. At pH 8 (**Figure 5B**), on the other hand, all tested phenolic acids behave as good inhibitors with an activity better than or comparable to that of Trolox. They give rise to lag phases, where oxygen consumption is strongly reduced, lasting for periods ∼2−3 times longer than that of Trolox. The less efficient antioxidant at pH 8 is **2**. On the basis of these results, it may be inferred that, under acidic conditions (for example, of gastric lumen), the tested phenolic compounds are not efficient radical scavengers. On the other hand, where the pH can reach 7−8, such as in the intestinal tract, in blood, in the extracellular fluid, and within cells, these diphenolic compounds are expected to behave as very good antioxidants.

To assess the antioxidant activity of phenolic acids at intermediate pH values, additional experiments were carried out

**Table 2.** Reactivities of Phenolic Antioxidants toward Peroxyl Radicals in PC Unilamellar Vesicles at 37 °C and pH 7.2 and p$K_a$ Values for the Hydroxyl Groups of Each Compound

| antioxidant | $nk_{inh(antioxidant)}/2k_{inh(Trolox)}$[a] | p$K_a$ |
|---|---|---|
| **1** | 3.40 | 4.4[b]<br>8.5[b]<br>11.2[b] |
| **2** | 0.70 | 8.4[c]<br>11.4[c] |
| **3** | 0.70 | 4.4[d]<br>8.9[d]<br>10.8[d] |
| **5** | 0.78 | 4.4[b]<br>9.2[b]<br>11.4[b] |
| **6** | 0.82 | 9.2[b]<br>11.1[b] |
| **7** | 0.72 | 9.4[e]<br>13.8[e] |
| Trolox | 1.00 | 4.4[f]<br>11.9[g] |

[a] Mean of three determinations, error within 20%. [b] From ref *13*. [c] Assumed to be identical to the p$K_a$ values of methyl caffeate (*13*). [d] From ref *27*. [e] From ref *28*. [f] Assumed to be identical to the p$K_a$ of other carboxylic acids (*13*). [g] From ref *29*.

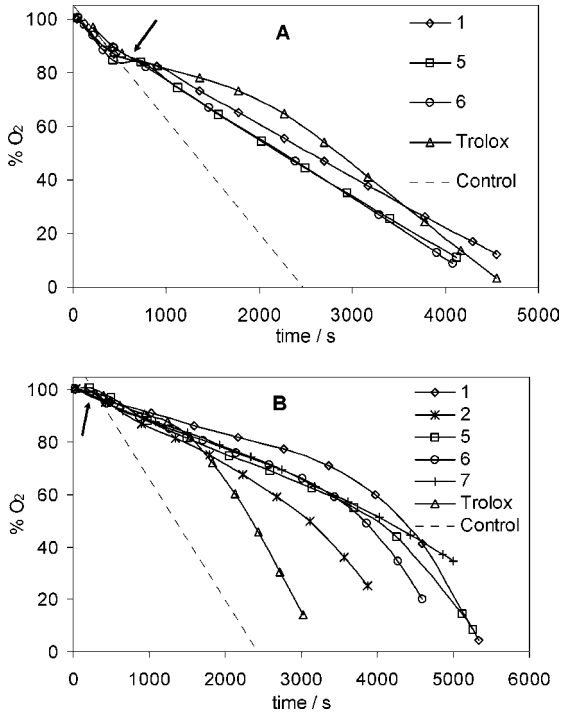


**Figure 5.** Oxygen uptake traces during AAPH ($1.7 \times 10^{-2}$ M) induced peroxidation of PC ($1.5 \times 10^{-2}$ M) unilamellar vesicles at 37 °C in the absence of inhibitor (control) and in the presence of one of the various phenolic antioxidants, each at the same concentration ($5 \times 10^{-6}$ M). The arrow shows antioxidant injection. Experiments were performed using (**A**) acid (pH 4.0) or (**B**) basic (pH 8.0) buffered isotonic solutions.

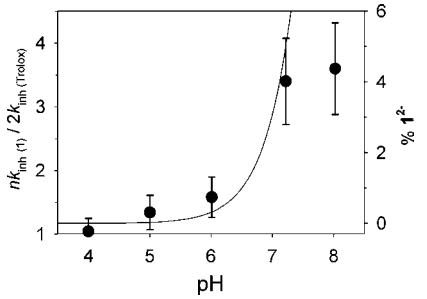


**Figure 6.** Antioxidant activity of caffeic acid with respect to Trolox measured at various pH values during the AAPH induced peroxidation of PC large unilamellar vesicles at 37 °C. Error bars: ± 20%. The line represents the percent molar fraction of the phenolate anion from caffeic acid ($\mathbf{1}^{2-}$) calculated from the p$K_a$ values given in **Table 2**.

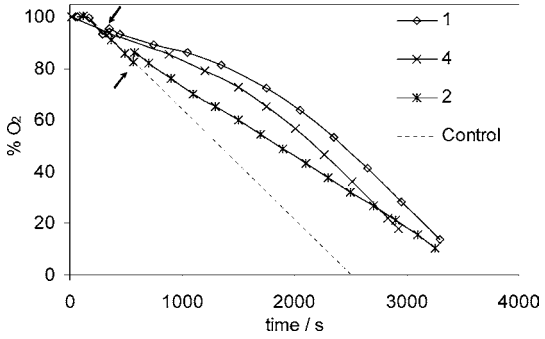


**Figure 7.** Oxygen uptake traces during AAPH ($1.7 \times 10^{-2}$ M) induced peroxidation of PC ($1.5 \times 10^{-2}$ M) unilamellar vesicles at 37 °C and pH 7.2 in the absence of inhibitor (control) and in the presence of caffeic acid (**1**), chlorogenic acid (**4**), and caffeic acid phenethyl ester (**2**), each at the same concentration ($5 \times 10^{-6}$ M). The arrow shows antioxidant injection.

with caffeic acid. The results are shown in **Figure 6**, reporting the ratio $nk_{inh(\mathbf{1})}/2k_{inh(Trolox)}$ as a function of pH.

The plot shows that the antioxidant activity of **1** undergoes a sudden increase at pH ~7, which is when the amount of the species $\mathbf{1}^{2-}$ ($\mathbf{1}^{2-}$ is the phenolate formed from **1**, i.e., X = CH═CHCOO$^-$ in **Figure 3**) is ~4%. We attribute the greater reactivity of caffeic acid and related compounds, observed at high pH, to the formation of the phenolate ion ($\mathbf{1}^{2-}$), the reaction of which with peroxyl radicals must be faster than that of the parent catecholic species. Consistent with this explanation is the fact that the other catechols characterized by higher p$K_a$ values and thus containing a lower percentage of the corresponding phenolate at pH 7.2 are less reactive than **1**, whereas under more basic conditions (pH 8.0) they become almost as reactive as caffeic acid (**Figure 5B**), independent on the nature of their side chain. Moreover, also caffeic acid behaves as a poor antioxidant at acidic pH, where the $\mathbf{1}^{2-}$ species is almost absent (**Figure 5A**).

These results imply that the anion $\mathbf{1}^{2-}$ behaves as a better antioxidant than the parent species $\mathbf{1}^-$. A similar effect has been observed by Mukai and co-workers (*14*) in the reaction between aroxyl radicals and several catechins at several pH values. The larger reactivity of the hydroxyl group in the ionized species was attributed to the greater electron-donating capacity of the O$^-$ group, which activates the adjacent OH.

An alternative explanation might be that in basic solution the inhibition process involves a rapid electron transfer (ET) to peroxyl radicals from the anion of the phenolic acid (**Figure 3**). The decrease of the reduction potential of phenols with deprotonation to give phenolates [for instance, for catechol $E_{pH7}$ = 0.53 and $E_{pH13.5}$ = 0.04 V vs NHE (*29*)] provides some support for this reaction path. It should be pointed out that the anomalous acceleration in alcoholic solvents of the reaction between substituted phenols and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), recently reported, has been explained on a similar basis (*30*, *31*), being attributed to partial ionization of the phenol to give the corresponding phenoxide anion followed by a very fast ET from the phenoxide to the DPPH radical.

A difficult aspect to be explained in terms of this model is the behavior of **2**, as shown in **Figure 4**. In fact, its p$K_a$ (8.4), corresponding to the first deprotonation of the catecholic function, is reported to be almost coincident with that of caffeic acid (8.5), so that acid and related ester are expected to show a similar reactivity. In contrast, **2** is considerably less reactive than **1**, both at pH 7.2 and at pH 8.0. A possible justification for the different antioxidant activities of acid and ester might be their different hydrophilicities, because the solubility of **2** in water is lower than that of **1** due to the substitution of the carboxylic OH with a $OCH_2CH_2Ph$ group. Different partition of the inhibitors between the water and the lipid phase of the PC vesicles is predictable, with **1** being highly partitioned in the water phase and **2** in the lipid region of model membranes.

To check this point we investigated the antioxidant behavior of chlorogenic acid (**4**), a derivative of **1** in which the carboxylic group is esterified with the highly hydrophilic D-quinic acid. These measurements, reported in **Figure 7**, show that **4** behaves as a much better inhibitor than **2**, although not so good as caffeic acid. Thus, it seems that the different partitionings between water and lipid phase is of considerable importance in determining the antioxidant activity of these compounds: the more efficient are those highly soluble in water such as caffeic acid and **4**.

Finally, the observation that caffeic acid at pH 7.2 and the other catecholic derivatives at basic pH give rise to inhibition periods 2−3 times longer than that of Trolox deserves some comments. Similar results have been previously reported by different authors when studying the antioxidant activity of polyphenols in micelles (*32*) or their oxidation in flow column electrolysis (*33*). We spent considerable effort to detect and identify the oxidation products of caffeic acid by analyzing the reaction mixtures with ESI-MS spectrometry without any appreciable success. Thus, the reason for these anomalous lag times could not be clarified. According to Roginsky (*32*) this behavior should be attributed to the formation of polymeric oxidation products having antioxidant activities comparable to those of the starting polyphenols.

In conclusion, the good antioxidant activity of caffeic acid and related derivatives is due to the presence in these compounds of the catechol moiety that gives rise, by hydrogen atom abstraction, to semiquinone radicals highly stabilized by an intramolecular hydrogen bond, thus determining a relatively small O−H BDE value. The low O−H bond strength is further reduced by the effect of ring substituents such as CH═CH−COOR and $CH_2$−$CH_2$R. The acrylic group is of some relevance in determining the good antioxidant properties of cinnamic acids.

The antioxidant activities measured in model membranes are different with respect to homogeneous solution and strongly dependent on the medium pH, this being attributed to the greater reactivity of phenolate anions toward peroxyl radicals.

The results here reported show the importance of thermodynamic and kinetic aspects in predicting the activity of chain-breaking antioxidants. Moreover, this study confirms that experimental measurements carried out in model membranes at different pH values are crucial in the understanding of all

factors responsible for the protective activity of antioxidants against peroxyl radical attack in biological systems.

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