An Experimental Approach to Structure–Activity Relationships of Caffeic and Dihydrocaffeic Acids and Related Monophenols

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ABSTRACT: The importance of a catechol moiety to the radical-scavenging activity of *p*-hydroxycinnamic derivatives is widely accepted, whereas the role of the carbon side chain remains controversial. Extension of the conjugation to this chain is a molecular feature that requires some attention. Differences in the activity of caffeic and dihydrocaffeic acids were examined using a series of experimental procedures: assays using free radicals [1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$)]; assays based on redox reactions; and accelerated tests in bulk oils and dispersed systems. To reinforce observations on the role of the side chain, a group of related monophenols, differing in one double bond and/or its position in the chain, was also examined. Extended conjugation was essential for the rapid scavenging of free radicals and for effectiveness in dispersed systems (both liposomes and emulsions). In the case of bulk oils, the contribution of conjugation remained unclear. Information based on redox reactions was not useful for the aim of our study. The experimental conditions may mask or enhance the effect of the chain characteristics on the activity, so the size and the order of activity may be influenced. Thus, structure–activity relationships should be drawn with caution.

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KEY WORDS: Bulk oils, caffeic acid, dihydrocaffeic acid, dihydroeugenol, dispersed systems, eugenol, isoeugenol, structure–activity relationships.

Consumption of foods containing phenolic antioxidants has been related to prevention of cardiovascular diseases and the reduction in the incidence of various types of cancer (1,2). Owing to the low pH in the human stomach, to enzymatic activity, and to the activity of intestinal microflora activity, these compounds may be transformed during digestion (hydrolyzed, methylated, demethylated) and thus not absorbed in the form of the intact molecules (3,4). As a result, the *in vivo* active forms may be the metabolites rather than the parent molecules. In the case of *p*-hydroxy derivatives, only low amounts of the parent molecules were found in blood plasma or urine. Compounds such as rosmarinic and chlorogenic acids are expected to be hydrolyzed to caffeic acid; the latter, owing to further transformations, may lead to the formation of the methylated derivative in the liver (ferulic acid) or to the hydrogenated one as a result of intestinal microflora activity (4). In this view it is quite challenging to examine differences in the activity of parent molecules and their metabolites for the purpose of a better appreciation of the sources for natural antioxidants.

The main mechanism of action of phenolic antioxidants (AH) is considered to be the scavenging of free radicals although other mechanisms may be involved (2). The radical-scavenging activity of phenolic compounds depends on structural characteristics that favor phenolic hydrogen donation and the stability of the resulting phenoxyl radicals (A$^{\bullet}$). Structure–activity relationship studies have pointed out the importance of a catechol moiety to the efficacy of *p*-hydroxycinnamic acid derivatives to scavenge free radicals (2,5). However, the contribution of the propenoic side chain with regard to radical-scavenging properties remains controversial (6,7). Only when at least a second phenol group is attached to this chain (e.g., rosmarinic or lithospermic acid) does its contribution becomes significant. Extension of the conjugation to the carbon chain is a molecular feature that requires some attention because it could participate by resonance to the stabilization of the phenoxyl radical (8). Moreover, it could also affect planarity of the molecule, thus causing steric hindrance toward free radical trapping (7,9).

In the present study differences in the activity of caffeic acid and its metabolite, dihydrocaffeic acid, were examined using a series of experimental procedures. To confirm observations made on the role of the side chain, a group of three monophenols, namely, isoeugenol, dihydroeugenol, and eugenol, differing in one double bond and/or its position in the chain, was also examined (Scheme 1). Assays frequently used in the literature for the appraisal of antioxidant activity were used although some of them have been criticized (10). The experimental approach involved free radical-scavenging assays, assays based on redox reactions, and accelerated tests in bulk oils (oven and Rancimat tests) and dispersed systems [liposomes and oil-in-water (o/w) emulsions].

MATERIALS AND METHODS

Standards, reagents, and solvents. Caffeic acid and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox,

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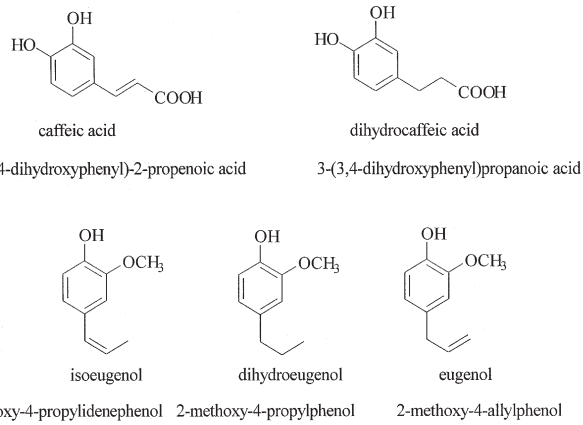

SCHEME 1

97%) were from Riedel de Haën (Seelze, Germany), and dihydrocaffeic acid (98%) was from Sigma Chemical Co. (St. Louis, MO). Isoeugenol, dihydroeugenol, and eugenol (99%) were from Aldrich Chemical Co. (Steinheim, Germany). DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] diammonium salt (approximately 98%) were from Sigma. Triolein, ~65%, used after purification, and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Fluka Chemie AG (Buchs, Switzerland). $FeCl_3 \cdot 6H_2O$ was from BDH (Dorset, United Kingdom). $FeSO_4 \cdot 7H_2O$, Na_2CO_3, NH_4SCN, and $FeCl_3$ were from Riedel de Haën. $BaCl_2 \cdot 2H_2O$, Folin–Ciocalteu reagent, and 1-octanol were from Panreac Quimica, S.A. (Barcelona, Spain). Tween 20 was from Merck Co. (Schuchardt, Germany). Lecithin [L-phosphatidylcholine (PC), ~40%, from soybean] and cupric acetate monohydrate were from Sigma. Absolute ethanol HPLC grade was from Riedel de Haën. HCl was from Fisons (Loughborough, United Kingdom).

Apparatus. A U-2000 Hitachi spectrophotometer (Tokyo, Japan) was used for the measurement of the reduction of DPPH$^{\bullet}$ absorbance at 516 nm and ABTS$^{\bullet+}$ at 734 nm. Induction periods of lipid substrates were measured using a Rancimat 617 apparatus (Metrohm AG, Herisau, Switzerland). For preparation of emulsion samples an UltraTurrax T25 (Janke & Kunkel, Berlin, Germany) homogenizer was used.

Triolein purification. Commercial triolein [TAG species expressed in equivalent carbon number (ECN, %) were: 50, 9.2%; 48, 65%; 46, 10%; 44, 7%; and 42, 8.6%] was purified in the laboratory on three chromatographic columns in series. The first two were packed with activated carbon/Kieselguhr (1:2, w/w), and the third one was packed with silicic acid. Eluates were checked for their tocopherol content with HPLC. Quality characteristics of the purified triolein were: PV = 0 meq O_2/kg; K_{232} = 1.2; K_{270} = 0.52; $Abs_{430,\ 460,\ 550,\ 630}$ = 0.011, 0, 0, 0; α-tocopherol = 0 mg/kg.

Radical-scavenging activity assays. (*i*) *The DPPH$^{\bullet}$ assay.* The radical-scavenging activity of phenols was determined using the free radical DPPH$^{\bullet}$ in ethanol (0.1 mM). Monitoring of [DPPH$^{\bullet}$] reduction was by absorbance measurement at 516 nm. The exact initial concentration in the reaction medium was calculated from a calibration curve, using Equation 1:

$$y = 2.223 \times \left[C_{DPPH^{\bullet}}\right] + 0.045 \quad (r = 0.999) \qquad [1]$$

For comparison, the radical-scavenging activity of Trolox was also tested. Different concentrations were used, expressed as moles of antioxidant [AH]/mole [DPPH$^{\bullet}$], and for each one the reaction kinetics was plotted. From these graphs the percentage of [DPPH$^{\bullet}$] remaining at the steady state was determined. The values were then transferred onto another graph showing the percentage of residual stable radical at the steady state as a function of moles [AH]/mole [DPPH$^{\bullet}$]. The latter was used to determine the efficient concentration (EC_{50}), that is, the amount of antioxidant necessary to decrease the initial [DPPH$^{\bullet}$] 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 = $1/EC_{50} \times T_{EC50}$, were also calculated (11). All tests were performed in triplicate. Statistical comparisons were by one-way ANOVA followed by the multiple Duncan test ($P < 0.05$).

(*ii*) *The ABTS$^{\bullet+}$ assay.* The ABTS$^{\bullet+}$ solution was prepared by reaction of 5 mL of a 7 mM aqueous ABTS solution and 88 μL of a 140 mM (2.45 mM final concentration) potassium persulfate ($K_2S_2O_8$) solution as proposed by Re *et al.* (12). After storage in the dark for 16 h, the radical cation solution was further diluted in ethanol until the initial absorbance value of 0.7 ± 0.05 at 734 nm was reached. Solutions of 0.5, 1.0, and 1.5 mM for each phenol under study were prepared (to achieve a 20–80% decrease in the initial absorbance of the reaction solution). An aliquot of the antioxidant solution (10 μL) was added to 1 mL of the radical solution, and the decrease in absorbance was recorded at 0 and after 6 min. Graphs of antioxidant concentration vs. % absorbance reduction were then constructed. The concentration of antioxidant giving the same percentage reduction of absorbance at 734 nm as the 1 mM Trolox solution was calculated from the three-point graphs. For each molecule and each concentration, measurements were made in triplicate. All tests were performed in triplicate. Statistical comparisons were by one-way ANOVA followed by the multiple Duncan test ($P < 0.05$).

Redox reaction assays. (i) The ferric reducing antioxidant power (FRAP) assay. Assay reagents included 10 mmol/L TPTZ in 40 mmol/L HCl, 20 mmol/L aqueous $FeCl_3 \cdot 6H_2O$, and 300 mmol/L acetate buffer (pH = 3.6). Working FRAP reagent was prepared by mixing acetate buffer, TPTZ solution, and $FeCl_3 \cdot 6H_2O$ in the proportions of 25:2.5:2.5, by vol. The assay was performed at 37°C according to the method of Benzie and Strain (13) and the absorbance recorded on a Shimadzu UV 160A spectrophotometer. Triplicate determinations were performed, and the FRAP reactivity determined by reference to the ferrous sulfate calibration line. The time to maximal activity was determined for each of the compounds of interest and then the linearity of response determined at the reaction time appropriate for the given compound. Student's *t*-test was used to determine the statistical significance of the experimental data.

(*ii*) *Assay based on the use of the Folin–Ciocalteu reagent.* The reducing antioxidant power of phenolic compounds was

measured as follows. Solutions of 0.5, 1.0, and 1.5 mM in methanol for each compound under study were prepared. An aliquot of the antioxidant solution (0.5 mL) was added to 5 mL of water and 0.5 mL of Folin–Ciocalteau reagent in a 10-mL volumetric flask. After 3 min, 1 mL of a saturated Na_2CO_3 solution was added, and the volume was brought to 10 mL with water. Absorbance at 725 nm was recorded 1 h after the addition of the sodium carbonate solution. Graphs of antioxidant concentration vs. % absorbance reduction were then constructed. The concentration of antioxidant giving the same percentage reduction of absorbance at 725 nm as the 1 mM Trolox solution was calculated from the three-point graphs. For each molecule and each concentration, measurements were made in triplicate with suitable blank solutions each time. Statistical comparisons were by one-way ANOVA followed by the multiple Duncan test ($P < 0.05$).

Lipid oxidation studies. (i) Oven test. Purified triolein samples containing the diphenols and Trolox at the level of 10 mg/kg and monophenols at the level of 30 mg/kg were held in an oven at 45°C. Aliquots of triolein (2.5 g) were distributed in a series of clear, open transparent glass vials of pharmacopeia quality (18 mm i.d.). The process of oxidation was followed by periodic measurement of PV in duplicate (CV% = 2.6, $n = 7$ for PV = 10).

(*ii) Rancimat test.* Samples of purified triolein (2.5 g) containing the antioxidants dissolved in ethanol at a concentration of 2.8 mM (~200 mg/kg) and of controls were prepared, and induction periods (IP) at 120°C were recorded automatically. IP is considered to be the time over which the oil is resistant to oxidation with or without the presence of an antioxidant. Protection factors (PF) were calculated as the ratio of IP in the presence of antioxidant/IP of control, i.e., PF = IP (time units)/$IP_{control}$ (time units). Each test was performed six times. Statistical comparisons were by one-way ANOVA followed by the multiple Duncan test ($P < 0.05$).

(*iii) Lecithin liposome test.* Lecithin was suspended in double-distilled water at a concentration of 8 mg/mL by stirring with a glass rod and by sonication in a bath-type sonicator for approximately 5 min. The solution was further sonicated with a sonicator rod (UP 200S; Dr. Hielscher GmbH, Berlin, Germany). Sonication time was approximately 2.5 min for 10-mL aliquots of the liposome sample. Ethanol solutions of phenolic antioxidants were added to screw-capped Erlenmeyer flasks at final concentrations of 5, 10, 15, 30, 45, 60, 80, 150, and 500 μM for caffeic and dihydrocaffeic acids and 5, 15, and 30 μM for Trolox and monophenols. Liposome samples were then weighed into the flasks and diluted with double-distilled water to a final lecithin concentration of 0.8% w/w and sonicated again for 1 min. Induced oxidation was achieved using cupric acetate (3 μM) and shaking (120 rpm) at 37°C in the dark. Liposome oxidation was monitored according to Yi *et al.* (14).

(*iv) O/w emulsion test.* O/w emulsion samples (10%, w/w) were prepared by homogenizing purified triolein (4.8 g), Tween 20 (0.49 g, 1%) and distilled water (44.5 g) at 13,500 rpm for 1 min. Samples were prepared to contain caffeic and dihydrocaffeic acids at concentrations of 150, 300, and 1000 μM (on oil weight basis) and Trolox or the monophenols at 15, 50, and 150 μM. Emulsions were placed in 100-mL Erlenmeyer flasks and subjected to oxidation at 37°C in an incubator (120 rpm). The course of oxidation was monitored by measuring PV using the ferric thiocyanate method (15). Results were expressed as meq O_2/kg oil.

Estimation of partition coefficient (P). A solution (0.1 mM) of each compound in 1-octanol was kept at 37°C for 30 min. A UV spectrum was then run, and the value of absorbance at λ_{max} was measured (A_o). Equal volumes of organic solution and water were vortexed (2500 rpm) for 1 min. The UV spectrum of the organic layer was obtained every 30 min until the absorbance value became constant (A_x). The partition coefficient P was calculated according to the relationship

$$P = A_x/(A_o - A_x) \quad [2]$$

A solution of 1-octanol saturated with water was used as the blank. All tests were performed in triplicate. Statistical comparison was by one-way ANOVA followed by the multiple Duncan test ($P < 0.05$).

RESULTS AND DISCUSSION

Finding differences in the activity of various antioxidants is a risky task, taking into account the limitations of each experimental procedure (10). In our study the following experimental procedures were applied to evaluate the performance of the compounds and to build up, if possible, structure–activity relationships.

Free radical-scavenging assays. Since the main mechanism of action of phenolic antioxidants is free radical scavenging, the reactivity of the *p*-hydroxycinnamic derivatives was tested against the stable radical DPPH$^\bullet$, which is frequently used (6–9). The results are presented in Table 1. In this table different expressions for the potency of the antioxidants are given. From EC_{50} or $1/EC_{50}$ (ARP) values, caffeic acid, having a propenoic side chain in the phenolic ring, was shown to have a lower scavenging activity toward DPPH$^\bullet$ than its saturated counterpart. The ratio of EC_{50} values for caffeic–dihydrocaffeic acids was 1.6, similar to that reported by Chen *et al.* (16) and almost half that found by Silva *et al.* (7). Our results were not in accord with those of Moon and Terao (6), who found no difference in the activity of the two compounds toward the same radical. It could be argued that the reduced activity of caffeic acid may be related to the electron-withdrawing character of the double bond in the side chain. On the basis of AE values, which are a function of the time required to attain the steady state, caffeic acid was found to be almost three times more efficient than dihydrocaffeic acid, as illustrated in Figure 1, where the kinetics of the reaction is presented. Kinetic studies were carried out to avoid possible discrepancies in antioxidant order using a rapid test (17). As suggested by Bondet *et al*. (18), some compounds react very quickly with DPPH$^\bullet$, reducing a number of molecules equal to the number of available hydroxyl groups of the

TABLE 1
Estimation of the Radical-Scavenging Activity of Phenols (AH) Using the DPPH• and ABTS•+ Assays[a]

	DPPH• assay					ABTS•+ assay
AH	EC_{50}	ARP	Mol DPPH•/ mol AH	AE	Time to reach steady state (min)	mmol AH equivalent to 1 mmol Trolox
Caffeic acid	0.18 ± 0.01[a]	5.5 ± 0.16[a]	2.90 ± 0.20[a]	1.10 ± 0.10[a]	4–15	0.96 ± 0.05[a]
Dihydrocaffeic acid	0.11 ± 0.01[b]	9.5 ± 0.45[b]	4.60 ± 0.30[b]	0.40 ± 0.04[b]	8–30	0.67 ± 0.02[b]
Isoeugenol	0.49 ± 0.03[c]	2.0 ± 0.11[c]	1.02 ± 0.06[c]	1.50 ± 0.23[c]	0.4–1.2	1.15 ± 0.03[c]
Dihydroeugenol	0.22 ± 0.01[d]	4.7 ± 0.28[d]	2.33 ± 0.11[d]	0.03 ± 0.00[d]	45–115	0.82 ± 0.08[d]
Eugenol	0.25 ± 0.01[d]	4.1 ± 0.18[d]	2.16 ± 0.20[d]	0.03 ± 0.01[d]	60–130	0.84 ± 0.05[d]
Trolox	0.21 ± 0.01[d]	4.75 ± 0.25[d]	2.40 ± 0.10[d]	1.77 ± 0.47[c]	0.9–3.2	1

[a]Values in the same column with different superscript letters are significantly different at $P < 0.05$; each value is the mean ± SD of three different experiments. Abbreviations: AH, phenolic antioxidant; EC_{50} = efficient concentration = amount of antioxidant necessary to decrease the initial [DPPH•] by 50%; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; AE = antiradical efficiency = $1/EC_{50} \times T_{EC50}$, where T_{EC50} is the time needed to reach steady state for EC_{50}; ARP = antiradical power = $1/EC_{50}$; ABTS•+, radical monocation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

antioxidant. Nevertheless, it seems that for the majority of antioxidants the reaction is slower and the mechanism more complex. Thus, caffeic acid scavenges fewer moles of DPPH• (2.9) than dihydrocaffeic acid (4.6), but the latter requires a longer period to attain steady state.

The complex mechanism for the reaction of *ortho* diphenols may be attributed to the ability of the diphenol to regenerate through the interaction of two phenoxyl radicals (19) or to polymerization reactions that may take place, leading to reproduction of hydroxyl moieties that enhance the radical-scavenging activity (20). The differences found between the two compounds were slight and sometimes disputable. This could be related to the presence of the powerful catechol group that may mask the influence of the other molecular

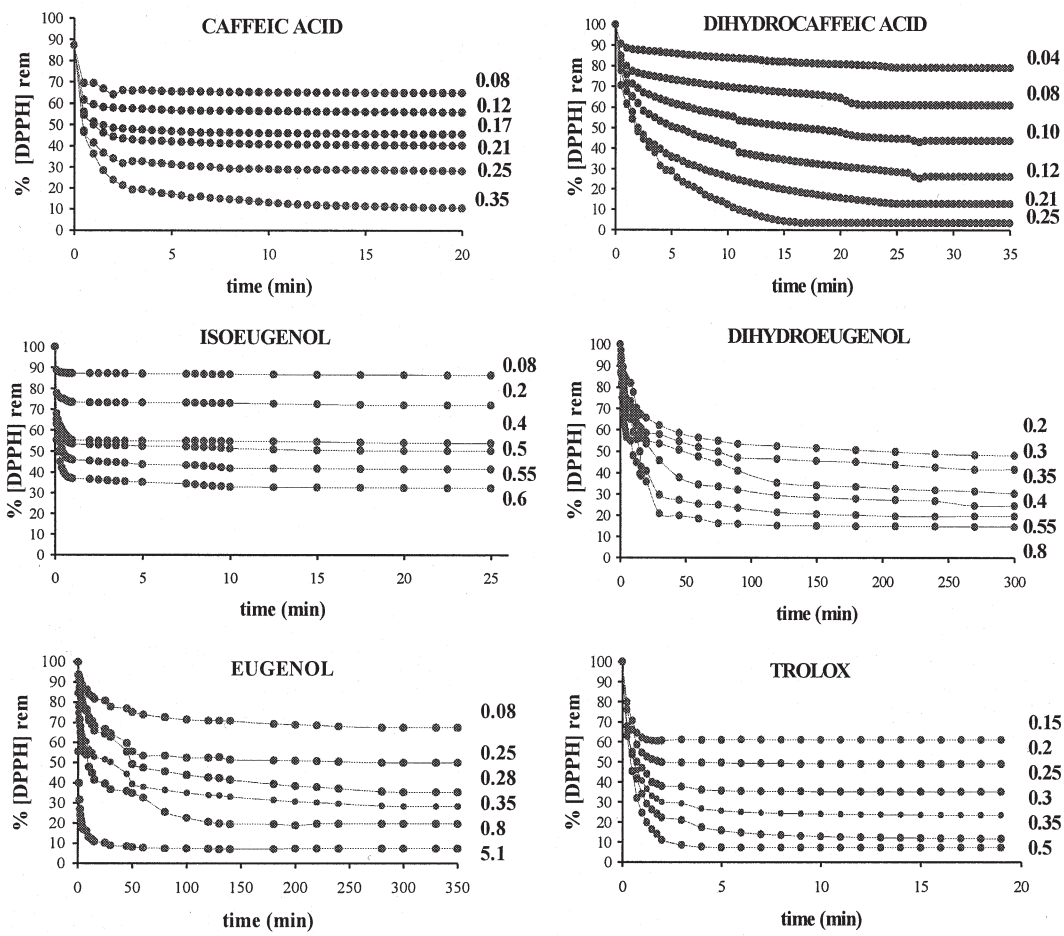

FIG. 1. Kinetic behavior of six phenols under study toward DPPH• in ethanol (concentrations are expressed as mol AH/mol DPPH•). Abbreviations: AH, phenolic antioxidant; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; rem, remaining.

characteristics and, to a lesser extent, to the presence of the remote carboxyl group (2).

Therefore, we chose to verify the effect of the presence and also of the position of a simple double bond in the side chain using three monophenol counterparts, namely, isoeugenol, eugenol, and dihydroeugenol. The data shown in Table 1 and Figure 1 were in accordance with the foregoing observations. However, the differences among the activities were more characteristic than those found for the catechol derivatives. The isolated double bond exerted the same effect as saturation. The overall kinetics indicated that isoeugenol reacted rapidly with the radical, whereas the AE values were 50 times lower for the other two compounds. The slow kinetics in the latter cases implied a more complex mechanism according to a scheme proposed for eugenol (19) that may also be considered for dihydroeugenol. Evaluation of the radical-scavenging activity was also examined using a modified $ABTS^{\bullet+}$ assay (12). Dihydro compounds were found to be significantly more active than the respective conjugated analogs (Table 1). The order of activity was in line with that based on the calculation of EC_{50} values but not with that based on AE values. Since no kinetic data could be obtained using the $ABTS^{\bullet+}$ assay (reaction is completed within 1 min), comparison with those obtained by the $DPPH^{\bullet}$ assay was not feasible. With regard to Trolox efficiency, both caffeic and dihydrocaffeic acids showed slower kinetics on the basis of AE values, whereas both compounds were slightly more active on the basis of $ABTS^{\bullet+}$ results. Trolox and isoeugenol had similar kinetic behavior, in contrast to the other two monophenols, which reacted too slowly. These discrepancies were less evident when the $ABTS^{\bullet+}$ test was performed.

Assays based on redox reactions. Although a considerable discussion exists on the usefulness of approaches that measure reducing capacity of potential antioxidants, the FRAP assay was applied to examine which counterpart was more readily oxidized. The data presented in Table 2, except for showing concentration-dependent activity of the compounds, offered some interesting information. The FRAP assay is an indirect procedure for the evaluation of antioxidant activity. The phenols that react with Fe^{3+} give rise to Fe^{2+}, which is more pro-oxidant than the former (21). If dihydrocaffeic and caffeic acids were present in a system containing Fe^{3+}, the order of their potential antioxidant activity should be the reverse of their reducing ability. Thus, actual values derived from the FRAP assay indicate the potential pro-oxidant activity. To highlight our remark, a direct redox reaction procedure, involving W^{6+} and Mo^{6+}, was also applied using the Folin–Ciocalteu reagent. This complementary study was supported by observations made in a previous work (22) on the differences in the reactivity of various phenolic compounds toward this oxidizing agent. The data, also shown in Table 2, were not very informative because of the very small differences observed within each group. This test may be useful to differentiate molecules with regard to the number of active hydroxyl groups but not to assign small differences as being due to the presence, for example, of a carbon side chain. In general, information based on redox reactions was not useful for our study.

Lipid oxidation studies. Moon and Terao (6) suggested that saturation of the side chain might affect the activity in bulk oil autoxidation. In bulk oils as well as in multiphase systems, the activity of the molecules, except for the number of active hydroxyl groups, seems to be defined by their polarity (23). When the compounds are of similar lipophilicity, structural characteristics determine the performance (24). Conjugation in the carbon side chain seems to be a structural feature that may affect polarity of the compounds since it influences the conformation of the molecule (9). Triolein oxidation was carried out using an oven test at 45°C. Estimation of the activity of caffeic–dihydrocaffeic pair showed that the latter was more effective (Fig. 2A). With the Rancimat test, dihydrocaffeic acid was found to be slightly better than caffeic acid (PF = 2.06 ± 0.06^{a} and 1.86 ± 0.06^{b}, respectively) and PF (Trolox) = 1.82 ± 0.06^{b}). (Values with different superscript letters are significantly different at $P < 0.05$.) With regard to the "polar paradox" concept, dihydrocaffeic acid seems to be more polar than caffeic acid, although its performance also may be related to the stability of the radicals formed or to side reactions (19). Owing to their volatility (dihydroeugenol, b.p. 124°C; isoeugenol, b.p. 141°C), monophenols were tested only at ambient conditions (Fig. 2B). According to the observations made for the two diphenols, isoeugenol was expected to be less active than

TABLE 2
Estimation of the Reducing Ability of Phenols (AH) Using the FRAP Assay and the Folin–Ciocalteu Reagent[a]

	FRAP assay		Folin–Ciocalteu assay
AH	Mean FRAP value (0.25 mM)	Mean FRAP value (1.0 mM)	Mmol AH equivalent to 1 mmol Trolox
Caffeic acid	0.60 ± 0.01^{a}	2.46 ± 0.05^{a}	0.30 ± 0.01^{a}
Dihydrocaffeic acid	1.09 ± 0.02^{b}	4.49 ± 0.07^{b}	0.34 ± 0.01^{b}
Isoeugenol	0.79 ± 0.03^{c}	2.62 ± 0.06^{c}	0.41 ± 0.02^{c}
Dihydroeugenol	1.04 ± 0.02^{d}	3.68 ± 0.02^{d}	0.39 ± 0.03^{c}
Eugenol	0.91 ± 0.03^{e}	3.45 ± 0.06^{e}	0.39 ± 0.01^{c}

[a]Values in the same column with different superscript letters are significantly different at $P < 0.05$; each value is the mean ± SD of three different experiments. FRAP, ferric reducing antioxidant power; for other abbreviations see Table 1.

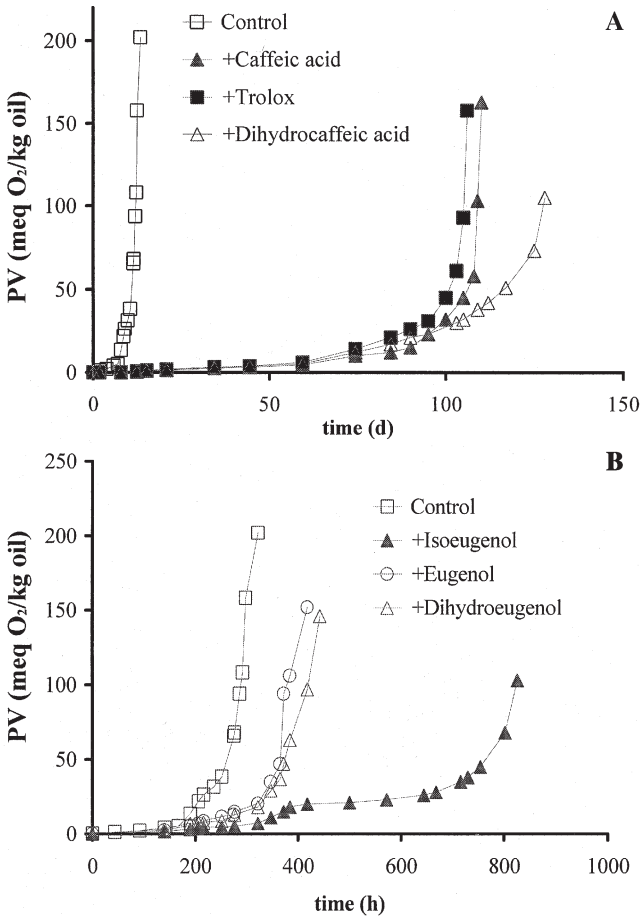

FIG. 2. Evaluation of antioxidant activity at 45°C in the dark of (A) diphenols at a level of 10 mg/kg oil; (B) monophenols at 30 mg/kg oil. PV are the mean of two measurements.

eugenol and dihydroeugenol. However, in this case extended conjugation seemed to enhance the activity and isoeugenol was the most effective compound, thus deviating from the polar paradox concept. Our finding supports an observation made by Brand-Williams *et al.* (19) concerning the activity of isoeugenol compared to that of eugenol that may be explained by other factors such as increased radical stability.

Differences in the activity of caffeic and dihydrocaffeic acids in multiphase models were considered next. Studies were carried out in lecithin liposomes and in an o/w emulsion system. Among the various factors affecting the ultimate performance of an antioxidant in such systems, phase partitioning is quite influential. Higher partition in the organic phase correlates well with the effectiveness of the tested phenols in liposomes and other dispersed systems (10). Partition coefficients (*P*) were therefore determined in an 1-octanol/water (1:1, vol/vol) mixture and the respective values are presented in Table 3. Diphenols partitioned to a lesser extent in the organic phase than monophenols. Trolox exhibited an intermediate behavior, which may explain its exceptional performance in both bulk (see also Fig. 2A) and multiphase systems (10,25). The nonconjugated derivatives were statistically more water-soluble than the conjugated counterparts. On the basis of the foregoing values all monophenols should be better antioxidants than the two diphenols (24,26). Still, differences in the antioxidant behavior within each group could hardly be expected due to tiny differences in *P* values.

TABLE 3
Partition Coefficient (*P*) of Phenols in a 1-Octanol–Water System (1:1, vol/vol) at 37°C[a]

AH	*P*	AH	*P*
Caffeic acid	0.18 ± 0.01[a]	Dihydroeugenol	0.84 ± 0.02[d]
Dihydrocaffeic acid	0.16 ± 0.01[b]	Eugenol	0.81 ± 0.01[d]
Isoeugenol	0.89 ± 0.01[c]	Trolox	0.46 ± 0.01[e]

[a]Values with different superscript letters are significantly different at a statistical level of $P < 0.05$; each value is the mean ± SD of three measurements.

A preliminary study showed that caffeic acid at a final concentration of 15 μM had no antioxidant activity, whereas its dihydro counterpart was a pro-oxidant. Owing to the low *P* values, diphenols are mainly expected in the aqueous phase. In such an environment and at low pH values (4.5–3.1), diphenols do not act as metal chelators (23,26) but as pro-oxidants, especially when present at low concentrations, as a result of an increase in their reducing capacity toward transition metals (27). Additional experiments in the range of 5–500 μM for the two diphenols suggested that the concentration of an AH is decisive in this test. Caffeic and dihydrocaffeic acids showed antioxidant activity for levels of addition higher than 30 and 60 μM, respectively. As illustrated in Figure 3A in the presence of

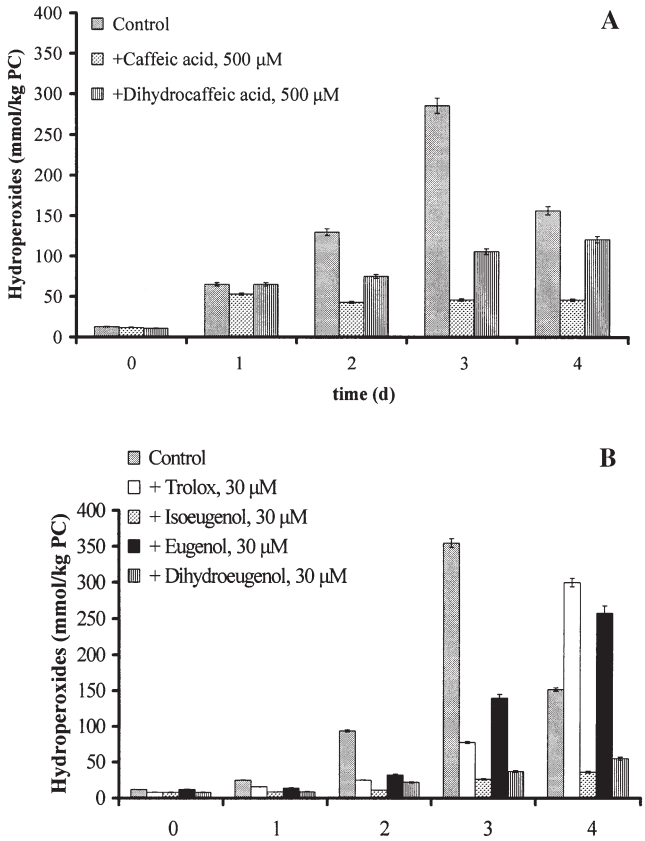

FIG. 3. Lecithin liposome oxidation course at 37°C in the presence of diphenols (A) and monophenols (B). Hydroperoxide values are means of three measurements ± SD.

caffeic acid at a level of 500 µM, hydroperoxides were rather stable during the experiment; dihydrocaffeic acid was less efficient. All monophenols were effective at lower levels of addition and, therefore, were tested at 5, 15, and 30 µM. Results are illustrated for the level of 30 µM in Figure 3B. Partitioning was a determining factor for the evaluation of the compounds in this system. Extension of conjugation seemed to be a positive characteristic for the enhancement of the activity. No deviation was observed for isoeugenol in this case.

On the basis of the previous findings and the literature survey, the diphenols were studied in an o/w emulsion (10%, w/w) at 150, 300, and 1000 µM, and the monophenols at 15, 50, and 150 µM level of addition. Results are given in Figure 4A for the diphenols and in Figure 4B for the monophenols. In this system conjugation in the side chain seemed to be a positive characteristic for the antioxidant activity. Looking for slight molecular differences that may affect the behavior of phenolic compounds in an emulsion is a difficult task. Thus, induced oxidation was avoided as was the use of buffers in order to avoid side reactions (28). Still, it can be said that conjugated phenolic antioxidants are more effective and that the presence of catechol group is not an advantage for the performance of the compounds. Monophenols were stronger antioxidants than the diphenols, probably because they could be oriented toward the water–oil interface, but differences related to structural characteristics were greater between diphenols and rather small among monophenols. Minor characteristicts like a double bond in the carbon side chain may differentiate the behavior of structurally related AH. The extended conjugation in the side chain seems to be essential for the rapid scavenging of free radicals.

Extended conjugation also is important for effectiveness in dispersed systems. In the case of bulk oils the contribution of conjugation remains unclear and the polar paradox concept has not been fully applied. Experimental conditions influenced the size of the activity difference and even defined the order of activity. The need for a different approach, which could highlight existing molecular characteristics that possibly are related to the behavior of the compounds under the various experimental conditions, is evident. DFT calculations of molecular descriptors that can support the experimental findings and offer mechanistic explanations for the antioxi-

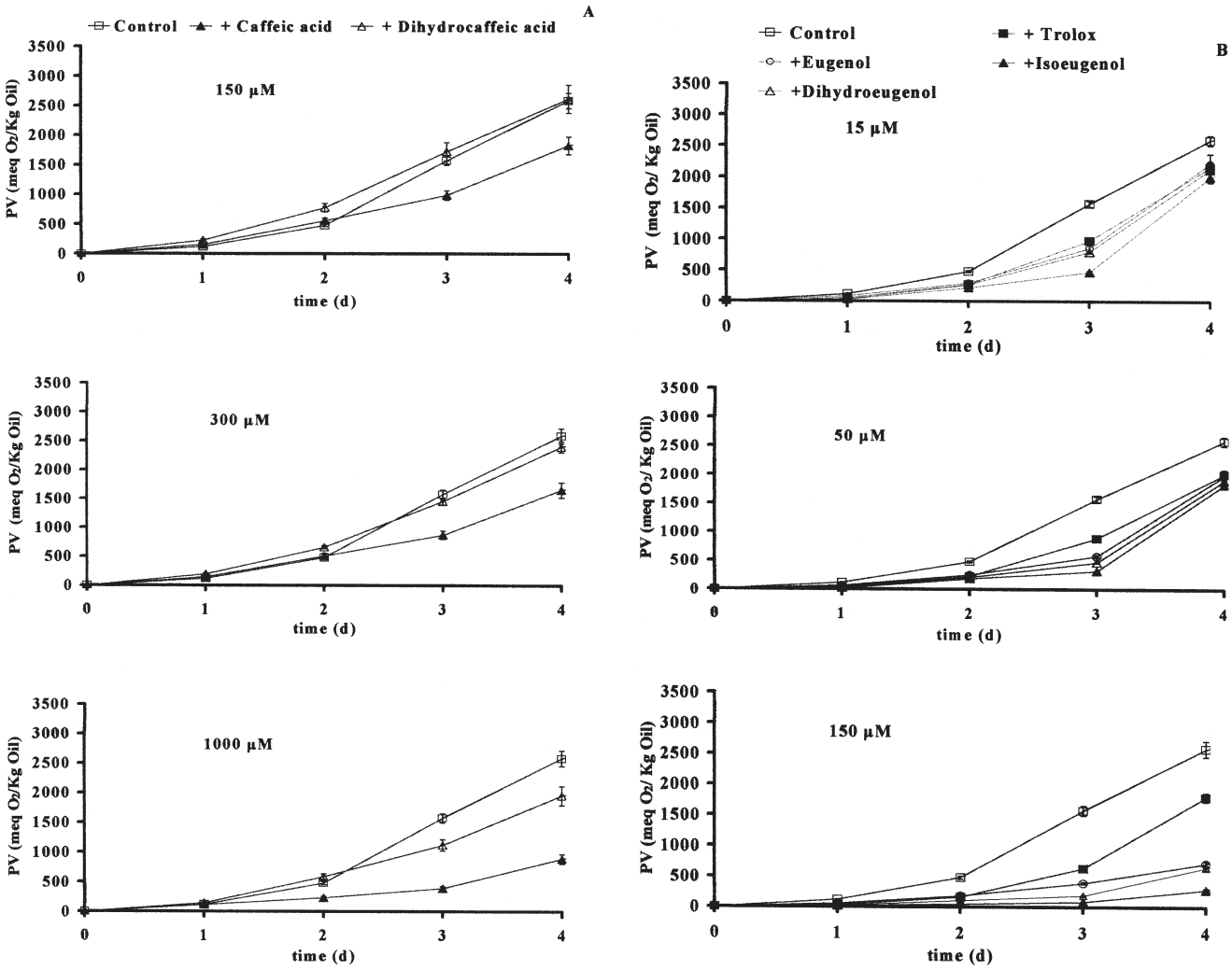

FIG. 4. Effect of diphenols (A) and monophenols (B) on oxidative stability of a 10% oil-in-water emulsion at 37°C. PV are means of three measurements ± SD.

dant activity of the compounds may be such an alternative approach (9).

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