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Structure−Antioxidant Activity Relationship of Ferulic Acid Derivatives: Effect of Carbon Side Chain Characteristic Groups

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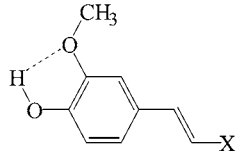
Differences in the antioxidant activity of some biosynthetically related ferulic acid derivatives induced by the presence of characteristic groups (−COOH, −CHO, $-CH_2OH$, $-CH_3$, and $-COOC_2H_5$) at the end of their carbon side chain were investigated using both experimental and computational methods. The relative order of the scavenging activity toward the DPPH radical derived from kinetic studies was isoeugenol ∼ coniferyl alcohol ≫ ferulic acid ∼ coniferyl aldehyde ∼ ethyl ferulate. In bulk oil autoxidation (45 °C) the same order of activity was obtained. In the o/w emulsion autoxidation, lipophilicity of the phenols was the determining factor because the least polar compounds bearing $-CH_3$ and $-COOC_2H_5$ were the most effective ones. The order of activity based on the O−H bond dissociation enthalpy (BDE) and ionization potential (IP) values, calculated by the density functional theory (DFT) method, was in accordance with the experimental radical scavenging order and with the electron-donating/withdrawing properties of the characteristic groups. Other molecular descriptors could not complement the experimental findings.

KEYWORDS: Ferulic acid derivatives; DFT; DPPH radical; bulk oil oxidation; emulsion oxidation; structure−antioxidant activity relationship

INTRODUCTION

Hydroxycinnamic acid derivatives are widely distributed in plants and are known antioxidants of various food products (*1*, *2*). Their antioxidant activity has been extensively studied in vitro. Structure−activity relationships have been mainly related to the type and number of characteristic groups on the aromatic ring (*2*, *3*). However, studies on the contribution of structural characteristics of the carbon side chain to the antioxidant performance of these precious compounds are rather limited (*4*−*6*). In the present study, a group of biosynthetically related ferulic acid derivatives (*7*) was selected to examine whether different characteristic groups at the end of the propenoic side chain would affect the antioxidant performance. These compounds, except for ferulic acid (4-hydroxy-3-methoxycinnamic acid, coniferic acid), were ethyl ferulate (4-hydroxy-3-methoxyethyl cinnamate), coniferyl aldehyde (4-hydroxy-3-methoxycinnamaldehyde), coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), and isoeugenol (2-methoxy-4-propylidenephenol) (**Chart 1**) and were examined using both experimental and theoretical methods. The experimental investigation involved studies using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, oven test in bulk oils, and tests in oil/water emulsions. A density functional theory (DFT) study was carried out to complement observations on the role of the characteristic groups in the antioxidant performance of the phenols under investigation.

Chart 1. Ferulic Acid and Derivatives under Study



X= -COOH (4-hydroxy-3-methoxy cinnamic acid, ferulic acid)
X= $-COOC_2H_5$ (4-hydroxy-3-methoxy ethyl cinnamate, ethyl ferulate)
X= -CHO (4-hydroxy-3-methoxy cinnamaldehyde, coniferyl aldehyde)
X= $-CH_2OH$ (4-hydroxy-3-methoxy cinnamyl alcohol, coniferyl alcohol)
X= $-CH_3$ (2-methoxy-4- propylidenephenol, isoeugenol)

MATERIALS AND METHODS

Standards, Reagents, and Solvents. Ferulic acid (99%), ethyl ferulate (98%), coniferyl aldehyde (98%), and isoeugenol (99%) were from Aldrich Chemical Co. (Steinheim, Germany). Coniferyl alcohol (97%) was from Fluka Chemie A.G. (Buchs, Switzerland). DPPH radical (98%) was from Sigma Chemical Co. (St. Louis, MO). Triolein (∼65%) was from Fluka. $FeSO_4 \cdot 7H_2O$, NH_4SCN, and $FeCl_3$ were from Riedel de Haën (Seelze, Germany). $BaCl_2 \cdot 2H_2O$ was from Panreac Quimica, S.A. (Barcelona, Spain). Tween 20 was from Merck Co.

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Table 1. Scavenging Activity of Phenols under Study toward DPPH•

phenol (AH)	EC_{50} (mol of AH/mol of DPPH•)	stoichiometry of reaction	$t_{EC_{50}}$ (min)	AE [(mol of AH/mol of DPPH•) × min]$^{-1}$	σ_p^{+} [a]
ferulic acid	0.47	1.06	48	0.04	0.45
ethyl ferulate	0.51	0.98	65	0.03	0.45
coniferyl aldehyde	0.48	1.04	70	0.03	0.42
coniferyl alcohol	0.43	1.16	2.7	0.86	0.00
isoeugenol	0.49	1.02	1.7	1.2	–0.17

[a] σ_p^{+} values according to ref *17*.

(Schuchardt, Germany). Absolute ethanol (HPLC grade), as well as acetic acid (analytical grade) and sodium acetate used for the preparation of buffer solutions, was from Riedel de Haën. HPLC grade chloroform, methanol, and acetic acid were from Panreac Quimica. TLC plates (silica gel 60, F_{254}) were from Merck Co. Silicic acid (100−200 mesh) was from Sigma Chemical Co.

Apparatus. A U-2000 Hitachi spectrophotometer (Tokyo, Japan) was used for the measurement of the reduction of DPPH radical absorbance at 516 nm. For preparation of emulsion samples an Ultra Turrax T25 (Janke and Kunkel, Berlin, Germany) homogenizer was used.

Triolein Purification. Commercial triolein [triacylglycerol 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.

Estimation of Radical Scavenging Activity. The activity of the phenols was determined using the free radical DPPH in ethanol (0.1 mM). Reduction in [DPPH•] was monitored by absorbance measurement at 516 nm. The exact initial concentration in the reaction medium was calculated from a calibration curve. Different concentrations expressed as number of moles of antioxidant [AH] per mole of [DPPH•] were used, and for each one the reaction kinetics was plotted. The repeatability of the procedure was checked for isoeugenol (CV % = 2.4, $n = 6$). From these graphs the percentage of [DPPH•] remaining at the steady state was determined. The values were transferred onto another graph showing the percentage of residual stable radical at the steady state as a function of the molar ratio of [AH]/[DPPH•]. The latter was used to determine the efficient concentration (EC_{50}) that is the amount of antioxidant necessary to decrease the initial [DPPH•] by 50%. Moreover, the stoichiometry of the reaction (moles of AH for 100% scavenging of the radical), the reaction time needed to reach the steady state for EC_{50} ($t_{EC_{50}}$), and antiradical efficiency, $AE = 1/EC_{50} \times t_{EC_{50}}$ were also calculated (*8*, *9*).

Lipid Oxidation Studies. *Bulk Oils.* Purified triolein samples containing the compounds under study at the level of 30 mg/kg were stored in an oven at 45 °C. Aliquots of triolein (2.5 g) were then distributed in a series of clear open transparent glass bottles of pharmacopeia quality (18 mm i.d.). The process of oxidation was followed by periodic measurement of peroxide value (PV) in duplicate (CV % = 2.6, $n = 7$ for PV = 10).

Oil in Water (o/w) Emulsions. Emulsions (10%, w/w) were prepared by homogenizing purified triolein (4.8 g), Tween 20 (0.49 g), and distilled water or buffer solution (44.5 g) at 13500 rpm for 1 min. Acetate buffers at pH 3.7 and 5.7 were used. Samples were prepared to contain the phenolic compounds at a concentration of 150 μM (on an oil weight basis). The samples were then placed in Erlenmeyer flasks (100 mL) and were incubated at 37 °C under continuous agitation (120 rpm). The course of oxidation was monitored by periodic measurements of PV by the ferric thiocyanate method (*10*). Results of triplicate measurements were expressed as milliequivalents of O_2 per kilogram of oil. Two series of experiments were carried out for each experimental condition.

DFT Study. For the antioxidants under investigation the phenolic O−H bond dissociation enthalpy (BDE) and the ionization potential (IP) values were calculated as follows: The geometry optimization and the determination of vibrational frequencies were performed using the semiempirical AM1 method (*11*). Then, single-point electronic energies were obtained by DFT using the B3LYP functional on the 6-311+G(2d,2p) level. Employing the electronic energies and the scaled thermal correction to energies (the scaled factor is 0.973) (*12*), BDE values for the phenolic O−H were obtained. The O−H BDE = $H_r + H_h - H_p$, where H_r is the enthalpy of phenoxyl radical generated after H-abstraction, H_h is the enthalpy of hydrogen atom (−0.49763 hartree), and H_p is the enthalpy of the parent phenol. For IP values, single-point calculations were performed on the 6-31G(d) level. IP = $H_c - H_p$, where H_c is the enthalpy of the cation radical generated after electron transfer and H_p is the enthalpy of the parent phenol. The enthalpy of the away conformation (H_p') for each parent phenol was also calculated at the 6-311+G (2d,2p) level, to estimate the hydrogen bond strength. All calculations were performed using GAUSSIAN 98 (*13*).

Estimation of Polarity. The polarity of the antioxidants was estimated on silica gel thin layer chromatographic plates according to ref *14*. The development system was chloroform/methanol/acetic acid (19:1:0.5, v/v/v).

Estimation of Partition Coefficient (Log *P*). Calculation of the Log *P* values, which express the partitioning of the phenols in an *n*-octanol/water system, was based on Broto's fragmentation method and was accomplished using the CS ChemDraw Pro (4.5 Ultra, Cambridge Soft Corp., 1985−1997) program (*15*).

RESULTS AND DISCUSSION

Experimental Investigation. The results of the experimental investigation are presented in **Table 1** and **Figures 1−3**.

Radical Scavenging Test. Because the main mechanism of action of phenolic antioxidants is considered to be the scavenging of free radicals (*16*), the reactivity of the ferulic acid derivatives was initially tested toward the stable radical DPPH. According to the stoichiometry of the reaction (**Table 1**), all of the tested compounds that possess one hydroxyl group in the aromatic ring seemed to have similar activity. However, when the overall kinetics of the reaction was taken into account (AE values in the same table), the effect of the different characteristic groups at the end of the carbon side chain was revealed. On the basis of AE values, the calculation of which involves the time required to attain the steady state, isoeugenol and coniferyl alcohol were almost 30 times more efficient than the rest of the compounds. The latter showed activity of the same magnitude. This very interesting observation is better illustrated in **Figure 1**, where the kinetics of the reaction is given. Because the structure of the five compounds presented an extended conjugation, based on the findings of a previous study (*3*), the molecules are expected to be fully planar in crystal or deviate only marginally from the plane in solution. Thus, any differences in activity among them should be rather ascribed to electronic phenomena than to steric hindrance effects. Electron-donating groups, (e.g., $-CH_3$ and $-OH$), when present in the aromatic ring, increase the ease of hydrogen atom abstraction. Groups with electron-withdrawing properties (e.g., $-COOH$, $-CHO$, and $-COOR$) have the opposite effect. The effect of such properties of substituents seems to be transmitted to the aromatic center through extended conjugation in the side chain of the

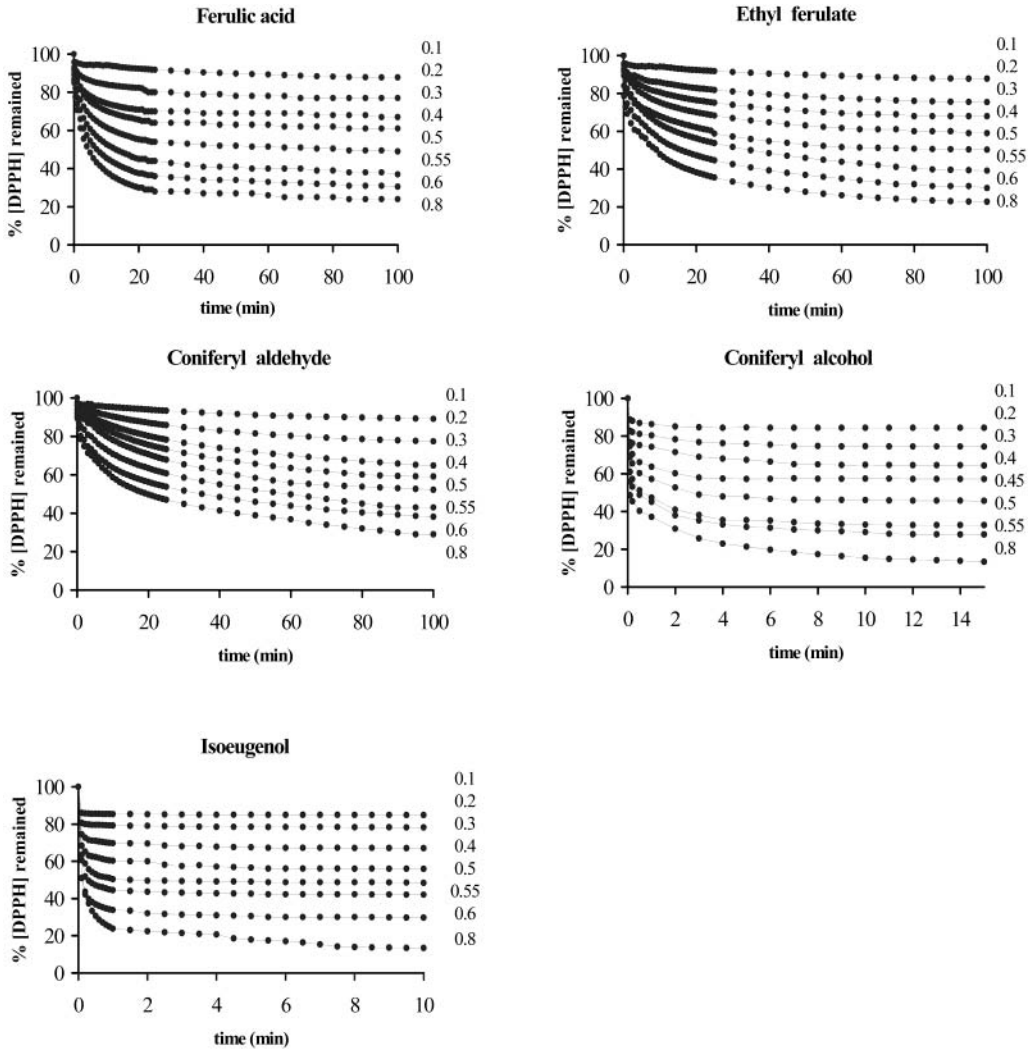

Figure 1. Kinetic behavior of phenols under study toward DPPH• in ethanol (concentration is expressed as moles of AH per mole of DPPH•).

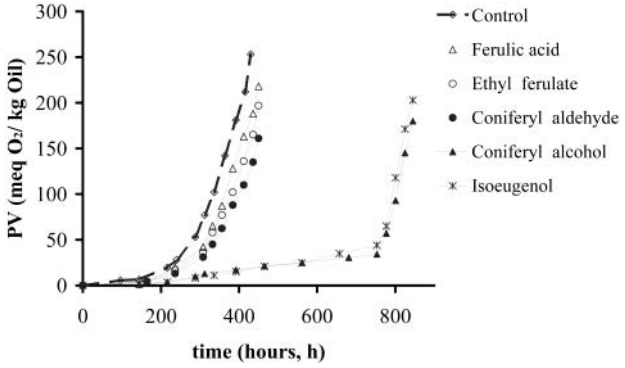

Figure 2. Evaluation of the antioxidant activity of phenols under study (30 mg/kg) in triolein stored at 45 °C in the dark. Each point represents a mean value of duplicate measurements.

examined compounds. The relative order of their activity is in line with that based on the values of the Brown parameter, σ_p^+, for the characteristic groups (*17*), also shown in **Table 1**.

Lipid Oxidation Studies. Except for the number of hydroxyl groups, differences in the activity of phenolic compounds in bulk oil autoxidation are usually related to their polarity (*14*). The polarity of the ferulic acid and its derivatives was estimated using the R_f values obtained by thin-layer chromatography. According to these values the relative order of decreasing polarity was coniferyl alcohol (R_f = 76 mm) < ferulic acid (R_f = 85 mm) < coniferyl aldehyde (R_f = 101 mm) < ethyl ferulate (R_f = 113 mm) < isoeugenol (R_f = 117 mm). On the basis of the polar paradox concept, it was expected that the most potent AH should be the most polar ones. Still, as can be seen in **Figure 2**, some deviations were experienced. The most polar compound, coniferyl alcohol, and the least polar one, isoeugenol, exhibited the same activity, which was much higher than those of the other examined compounds. The latter hardly protected the substrate from autoxidation at the level of addition used (30 ppm). A better performance of isoeugenol in comparison to that of ferulic acid in bulk oil autoxidation, although reported in the literature, did not gain the interest of authors as far as it concerns structure−activity relationships (*18*, *19*). The performance of the five compounds using the oven test at 45 °C revealed differences in the antioxidant performance that should be related to the electronic properties of the characteristic groups rather than to the polar paradox concept. The relative order of activity was the same with that obtained using the DPPH radical assay.

Food matrices are usually multicomponent systems, so studies were also carried out in o/w emulsions. Emulsions were prepared using buffers or distilled water to examine whether this parameter influenced the order of activity. Acetate buffers at pH 3.7, which is characteristic of acidic lipid food matrices such as salad dressings (*20*), and at pH 5.7 were used. The latter was chosen because it coincided with the initial pH value of

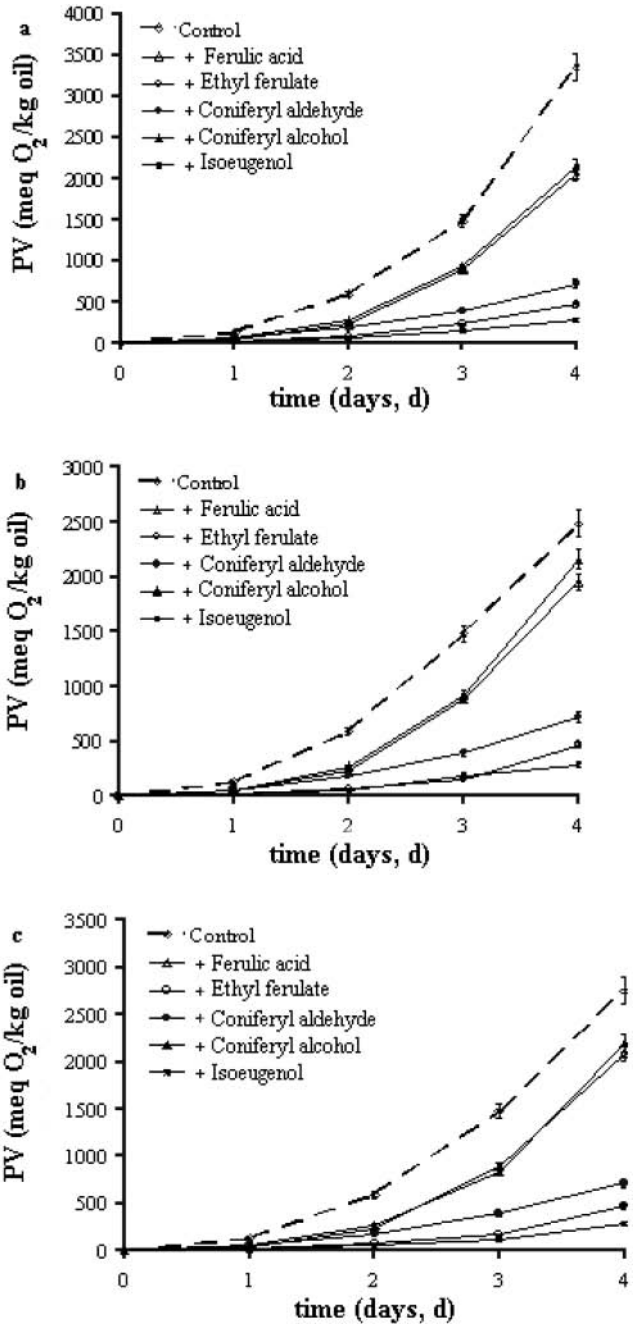

Figure 3. Effect of phenols under study (150 μM) on the stability of a 10% o/w emulsion prepared in (a) pH 3.7, (b) pH 5.7, and (c) distilled water incubated at 37 °C. Each point represents a mean value of triplicate measurements ± SD.

emulsion prepared with distilled water, which was found to decrease during the course of oxidation to a value of 4.3, possibly due to the formation of organic acids. For this reason experiments using emulsions prepared with buffers or distilled water were carried out to examine whether this parameter influences the final results.

Among the various factors affecting the ultimate performance of a phenolic antioxidant in a dispersed system is phase partitioning (*21*), which is a function of its polarity, and it is usually expressed in terms of partition coefficient values obtained experimentally (*22*). In this study, the Log *P* values of the tested compounds were calculated according to Broto's fragmentation method (*15*). The relative order of increasing partitioning in the organic phase was found to be coniferyl alcohol (1.33) < ferulic acid (1.42) < coniferyl aldehyde (1.82) < ethyl ferulate (2.02) < isoeugenol (2.49), in agreement with that determined for the polarity of the compounds. From the emulsion autoxidation study it was, thus, expected that among the five components the least polar ones, having higher partitioning in the lipid phase, should have a better performance. The results of the study, illustrated in **Figure 3**, showed a clear antioxidant behavior of all compounds at the three pH environments. The presence of the acetate buffers did not seem to influence the relative order or the size of activity of the AH. Acceleration of oxidation was observed at pH 3.7, possibly due to higher solubilization of metal traces (*16*). According to the obtained results, isoeugenol, ethyl ferulate, and coniferyl aldehyde were more potent than the two other compounds. In the emulsion test no deviant behavior was observed for isoeugenol. The better performance of the latter in comparison to that of ferulic acid in a dispersed system has also been reported (*19*). It can be suggested that when the affinity to lipid phase predominates and differences in the polarity within the group of components are important, the ultimate performance of the AH depends to a lesser extent on the electronic phenomena of the characteristic groups.

Table 2. O–H BDE and Intramolecular Hydrogen Bond Strength Values for the Phenols under Study Calculated by AM1/B3LYP/6-311+G (2d,2p), T = 298.15 K

phenol (AH)	H_p (hartree)	H_p' (hartree)	H_r (hartree)	BDE (kcal/mol)	H bond strength (kcal/mol)
ferulic acid	–687.99	–687.98	–687.36	84.32	3.91
ethyl ferulate	–766.37	–766.57	–765.94	83.90	4.55
coniferyl aldehyde	–612.72	–612.71	–612.0.9	84.38	4.01
coniferyl alcohol	–613.90	–613.89	–613.27	81.52	3.93
isoeugenol	–538.67	–538.49	–538.042	81.15	3.74

Table 3. IP Values for the Phenols under Study Calculated by AM1/B3LYP/6-31G(d), T = 298.15 K

phenol (AH)	H_p (hartree)	H_c (hartree)	IP (kcal/mol)
ferulic acid	–687.76	–687.49	167.49
ethyl ferulate	–766.32	–766.06	165.53
coniferyl aldehyde	–612.52	–612.25	169.78
coniferyl alcohol	–613.68	–613.44	155.10
isoeugenol	–538.49	–538.24	159.86

Theoretical Investigation. It was found experimentally that different characteristic groups at the end of the carbon side chain might differentiate the behavior of structurally related AH in different environments. To complement the above findings and the respective discussion, a theoretical study was also carried out for the same group of phenols. After full optimization of the geometry of the examined compounds, different molecular descriptors were calculated to characterize their antioxidant potency. The theoretical descriptors were O−H BDE, hydrogen bond strength, and IP, the use of which has been reasonably justified in the literature (**Tables 2** and **3**). As can be seen from the results, calculation of each molecular descriptor revealed differences among the five compounds. On the basis of the O−H BDE values that are commonly used to characterize the ease of hydrogen donation to free radicals (*3*, *12*, *23*−*29*) differentiation into two groups could be made. In particular, the O−H BDE values for isoeugenol and coniferyl alcohol were lower (∼2.4−3.1 kcal) than those of the other three compounds. BDE values are governed by the resonance effect (R^+), and electron-donating/withdrawing groups influence them (*27*, *28*). The latter finding was drawn from studying the effect of characteristic

groups directly attached to the aromatic ring (*27*). In the present research, it is interesting to observe that despite the distance of the side chain from the aromatic ring, the performance of the antioxidants was also considerably affected. The order of activity defined in this way was the same as that based on the DPPH radical assay when the kinetic parameter AE was calculated or with that in bulk oils where the free radical mechanism prevails.

It is known that phenolic compounds usually bearing a substituent in the ortho position to the hydroxyl group form an intramolecular hydrogen bond, which is energetically favorable (*25*, *29*). This bond may affect the ease of donation to free radicals. Therefore, a DFT calculation of the intramolecular hydrogen bond strength between the phenolic O−H and the methoxyl group may support the size of the BDE values obtained. Results presented in **Table 2** show that in the case of isoeugenol the strength of the intramolecular hydrogen bond was the lowest, which might enable the ease of the hydrogen donation. Coniferyl alcohol presented the same hydrogen bond strength with the coniferyl aldehyde and ferulic acid, whereas ethyl ferulate had the highest one. However, the hydrogen bond energy varies within 1 kcal/mol; thus, a small effect on the BDE is expected, and this molecular descriptor was not further examined.

Differences in the IP values among the compounds defined an order of activity similar to that defined by BDE values (**Table 3**). This parameter characterizes the ease of electron donation to free radicals (*12*, *28*, *30*). According to the calculated values, coniferyl alcohol and isoeugenol were the compounds that can be more easily oxidized through an electron transfer mechanism in comparison to the rest of the AH.

It seems that for the moment theoretical calculations are a useful complementary tool for the ranking of the antioxidants concerning their radical scavenging ability and the activity in bulk oil oxidation. However, the calculated molecular descriptors could not support the behavior of the compounds in emulsions.

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