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Free radical scavenging capacity of hydroxycinnamic acids and related compounds†

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ABSTRACT: The capacity of hydroxycinnamic acid derivatives to trap peroxyl radicals was evaluated by competitive kinetics and oxygen radical absorbance capacity (ORAC) indexes, using *c*-phycocyanin and pyranine as target molecules. The pattern of results is similar in all the systems, with the reactivity of the compound determined by the bond dissociation energy (BDE) of the hydrogen atom of the phenolic moiety. However, differences in the relative reactivity are observed depending upon the employed methodology (initial rate of consumption or ORAC-type methodology) and target molecule employed. These differences are explained in terms of the role played by secondary reactions of the initially formed phenoxyl radicals. This emphasizes the need for performing a complete kinetic analysis of the results in order to obtain meaningful evaluations of the relative reactivity of the tested compounds.

KEYWORDS: free radical scavenging; hydroxycinnamic acids; kinetic analysis; phenoxyl radicals

INTRODUCTION

Hydroxycinnamic acids and their derivatives are frequently considered to be among the most valuable antioxidants present in natural products and beverages.[1–4] The presence of phenolic moieties confers to these compounds their free radical scavenging capacities.[3,5–15] In particular, caffeic acid, due to the presence of two vicinal hydroxyl groups, is one of the most active compounds of the family,[16] and it is considered as the cause of the increase in the total antioxidant capacity of human plasma measured after drinking a cup of coffee.[17,18]

In spite of the large number of studies in which cinnamic acid derivatives have been reported to act as free radical scavengers, there are very few quantitative kinetic data on their reactivity towards reactive oxygen species (ROS) and their dependence upon the structure of the molecule.[11,14,19] Rice-Evans *et al.*[7] have established structure-antioxidant activity relationships by evaluating their capacity to bleach 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) derived radicals, but in this system the extent of the process is more related to stoichiometric factors than to reactivity parameters.[20,21] Natella *et al.*[16] have evaluated, by competitive kinetics employing crocin as the target molecule, the reactivity of different cinnamic acid derivatives towards peroxyl radicals. They reported the following pattern of reactivity:

$$\text{Coumaric} < \text{ferulic} < \text{caffeic} < \text{sinapic}$$

and that the reactivity of ferulic acid is very similar to that of the reference antioxidant Trolox. However, no molecular explanations were given to explain their relative reactivity. In fact, very limited attempts have been undertaken to relate their relative reactivity to molecular properties.[13] In the present work, we have evaluated the capacity of different cinnamic acids (Scheme 1) to protect *c*-phycocyanin and pyranine from their bleaching by peroxyl radicals derived from the thermal decomposition of 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH). These target molecules were chosen in order to ascertain the relevance of the chemical reactivity of the target molecule on the reactivity pattern observed for a family of closely related antioxidants. Cinnamic acid and phenyl propionic acid derivatives were employed in order to evaluate the importance of the conjugated double bond and the number and position of hydroxyl groups in the aromatic ring.

EXPERIMENTAL

Chemicals

2,2′-azo-bis(2-amidinopropane) dihydrochloride, (AAPH), was used as the peroxyl radical source. *c*-phycocyanin (*c*-Pc), pyranine (Py), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), all tested cinnamic

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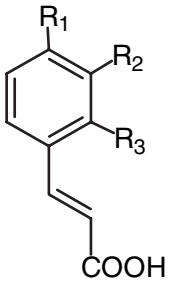
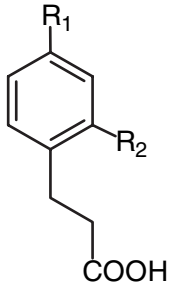


I (caffeic acid)	R_1 = OH, R_2 = OH, R_3 = H
II (ferulic acid)	R_1 = OH, R_2 = OMe, R_3 = H
III	R_1 = OMe, R_2 = OH, R_3 = H
IV (coumaric acid)	R_1 = OH, R_2 = H, R_3 = H
V	R_1 = H, R_2 = OH, R_3 = H
VI	R_1 = H, R_2 = H, R_3 = OH
VII	R_1 = OMe, R_2 = H, R_3 = H



VIII	R_1 = OH, R_2 = H
IX	R_1 = H, R_2 = OH

Scheme 1. Structures of the cinnamic and 3-phenylpropionic acid derivatives

acids (Scheme 1), and AAPH were purchased from Sigma-Aldrich (St. Louis, MO) and employed as received.

Competitive kinetics experiments

Solutions containing *c*-Pc or Py, with or without the tested cinnamic acid in phosphate buffer (10 mM) at pH 7.0, were incubated at 37 °C in a thermostated cuvette in a Fluorolog Spex 1681 spectrofluorimeter. The reaction was initiated by adding a small aliquot (50 μL) of AAPH (10 mM final concentration). The consumption of the target molecule (*c*-Pc or Py) was evaluated from the decrease in its fluorescence intensity. Fluorescence measurements were carried out using excitation wavelengths of 620 and 460 nm for *c*-Pc and Py, respectively. Fluorescence emission intensities were measured at 640 and 510 nm for *c*-Pc and Py, respectively. Cinnamic acid solutions in phosphate buffer were prepared daily by addition of small aliquots of a concentrated stock solution in ethanol.

Oxygen radical absorbance capacity (ORAC) determinations

The consumption of the probe molecule, *c*-Pc or Py, promoted by its incubation in the presence of AAPH, was estimated from fluorescence intensity (F) kinetic profiles. F/F_0 values, where F is the measured fluorescence and F_0 is the fluorescence measured prior to AAPH addition (corrected by dilution), were plotted as a function of the elapsed time. The area under the curve (AUC) was evaluated, up to a time such that (F/F_0) reached a value of 0.2, and employed to obtain ORAC values, defined by:

$$\mathrm{ORAC} = \frac{(\mathrm{AUC} - \mathrm{AUC}^0)}{(\mathrm{AUC}_{\mathrm{TROLOX}} - \mathrm{AUC}^0)} \left(\frac{[\mathrm{Trolox}]}{[\mathrm{Additive}]} \right) \quad (1)$$

where: AUC = area under the curve in presence of the tested cinnamic acid derivative; AUC^0 = area under the curve for the control without additive; $\mathrm{AUC}_{\mathrm{TROLOX}}$ = area under the curve obtained employing Trolox as reference antioxidant.

Bond dissociation energy (BDE) estimations

Bond dissociation energies of the tested compounds were calculated from their oxidation potentials measured by cyclic voltammetry. The cyclic voltammograms were collected with 100 μM of the tested compound in phosphate buffer (50 mM) with KCl 0.1 M at pH 7.0. The experiments were performed with a Wenking PO753 instrument at a scan rate of 100 mV/sec. A glassy carbon stationary electrode was used as working electrode, with a platinum wire as counter electrode. All potentials were measured against a saturated calomel electrode (SCE) under an atmosphere of pure (dry) nitrogen. BDE values were estimated according to Bordwell *et al.*[22]:

$$\mathrm{BDE} = 1.37\ \mathrm{p}K_a + 23.06\ E_{ox} + \mathrm{C} \quad (2)$$

where C is a constant that depends on the working pH and takes into account entropy changes associated with the electrochemical oxidation process.

RESULTS AND DISCUSSION

c-Phycocyanin bleaching promoted by peroxyl radicals: Protection by phenolic compounds

The decrease in *c*-Pc bleaching rate in the presence of an additive (XH) able to trap peroxyl radicals can be interpreted in terms of the following mechanism:

$$\mathrm{AAPH} \xrightarrow{O_2} 2\mathrm{ROO}^\bullet \quad (3)$$

$$\mathrm{ROO}^\bullet + c\text{-Pc} \rightarrow \text{bleaching} \quad (4)$$

$$\mathrm{ROO}^\bullet + \mathrm{XOH} \rightarrow \mathrm{ROOH} + \mathrm{XO}^\bullet \quad (5a)$$

and/or

$$\mathrm{ROO}^\bullet + \mathrm{XO}^- \rightarrow \mathrm{ROO}^- + \mathrm{XO}^\bullet \quad (5b)$$

If R^0 is the initial *c*-Pc bleaching rate, and R is the rate in the presence of the additive, this simplified mechanism predicts a monotonous increase in R^0/R values with the additive concentration.[23] If R^0/R values are plotted against the additive concentration, the initial slope of

the plot is related to the rate constant of process (5). The results obtained in the present work do not allow a distinction between hydrogen (5a) and electron (5b) transfer. The estimated rate constants must then be considered as the weighed sum of both processes. However, phenolic compounds are more prone to react through a hydrogen transfer mechanism,[11] particularly at pHs well below the pK_a of the target molecule, as under the present experimental conditions. Furthermore, it has to be considered that although AAPH-derived radicals, such as ROO, have a positive charge at the amidoyl moiety, these radicals are generally considered as a representative example of water-soluble peroxyl radicals, particularly in the evaluation of the relative reactivity of uncharged groups. However, the presence of net charge can be determinant when charged macromolecules are employed as targets, since in these systems adsorption promoted by electrostatic interactions can be particularly relevant.[24]

Profiles of *c*-Pc bleaching elicited by its exposure to peroxyl radicals in the presence of ferulic acid correspond to those typical of compounds with moderate free radical trapping capacities (Fig. 1). A plot of R^0/R values as a function of the additive concentration is shown in Fig. 2. This plot shows a noticeable downward curvature, incompatible with the simplified mechanism depicted by reactions (3) to (5). This behavior has been attributed to secondary reactions of the additive derived radicals, such as

$$XO^{\bullet} + c\text{-Pc} \rightarrow \text{bleaching} \qquad (6)$$

The maximum attainable R^0/R value can be considered as a rough indicator of the damaging capacity of the XO radical. This damaging capacity will be determined by the

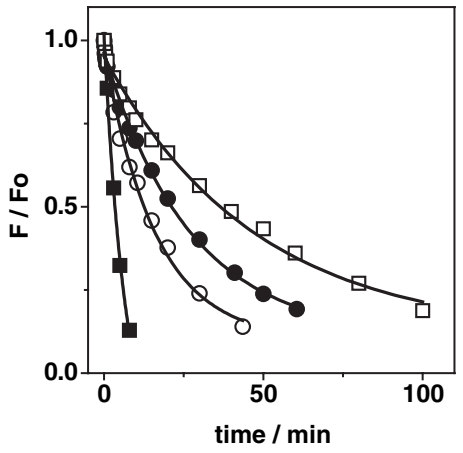


Figure 1. Bleaching of *c*-phycocyanin (0.01 mg/mL) elicited by AAPH (10 mM) derived peroxyl radicals in the presence of different ferulic acid concentrations. Control (■); ferulic acid: 10 μM (○); 50 μM (●) and 100 μM (□). The reaction was monitored through the decrease in *c*-Pc fluorescence intensity (excitation at 620 nm, emission at 640 nm) at 37 °C

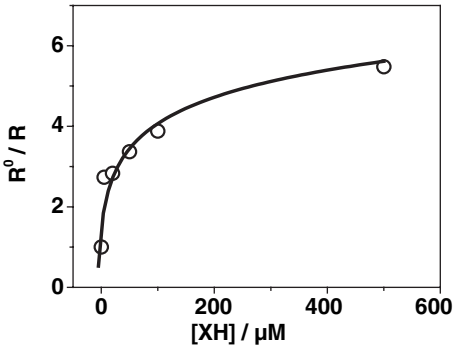


Figure 2. Protection of *c*-Pc elicited by compound III. The data are plotted as the ratio between the initial rate of *c*-Pc consumption in the absence (R^0) and presence (R) of the additive

reactivity of the radical (k_6) and its tendency to enter into radical–radical reactions, such as:

$$XO^{\bullet} + XO^{\bullet} \rightarrow \text{non radicals products} \qquad (7)$$

that must be the dominant termination process at high XOH concentrations. The data of Table 1 show that most radicals derived from cinnamic acid derivatives are able to bleach *c*-Pc. However, it is interesting to note that this secondary damage is considerably lesser for caffeic and ferulic acids, the most reactive compounds considered in this study. This can be explained in terms of a reduced rate of reaction (6) and, for caffeic acid, a faster rate of reaction (7) due to the favorable formation of the corresponding quinone.[25]

The curve of the R^0/R*versus* [XOH] plots makes a precise kinetic treatment difficult. To characterize the rate of the process, rather arbitrary indexes must be defined. In Table 1 we have tabulated two of these indexes:

(i) The value of R^0/R at an arbitrarily chosen additive concentration. This value was selected as 5 μM in order to minimize the contribution of reaction (6).
(ii) The ORAC index; calculated from the area under the curve of the F/F^0 *versus* time plot measured at a given XOH concentration (50 μM). This index, although

Table 1. *c*-Pc bleaching promoted by AAPH-derived radicals

Compound	Maximum (R^0/R)	[(R^0/R)−1] at 5 μM	ORAC at 50 μM
I (caffeic acid)	≈50	4.5	1.0
II (ferulic acid)	55	2.1	0.32
III	3.8	1.1	0.07
IV (coumaric acid)	2.7	1.3	0.11
V	3.9	0.8	0.09
VI	2.4	1.1	0.08
VII	2.6	0.8	0.05
VIII	4.8	0.8	0.08
IX	5.5	0.9	0.06
Trolox[a]	—	4.0	1.0

Protection by cinnamic acid derivatives.
[a] Taken from Ref. [23].

rather arbitrary, has the advantage that it can be calculated regardless of the bleaching profiles. However, it has the shortcoming that it can be influenced by the stoichiometry of the process and the secondary reactions of the additive derived products.

The data in Table 1 show a good correlation between R^0/R values (measured at a single additive concentration) and ORAC values. However, it must be noted that the range of values is larger for the ORAC index. This index could then magnify the differences in reactivity among the tested compounds.

The data shown in Table 1 imply that hydroxyl groups located at position four in cinnamic acids present the largest peroxyl radical trapping capacity (both as assessed by the R^0/R value and the ORAC index). In this family, the reactivity order is similar to that reported previously employing crocin as the target molecule.[16] However, the range of reactivities in the system under study (nearly a factor four in $[(R^0/R)-1]$ and a factor 10 in ORAC values) is considerably smaller than that reported in the crocin system (nearly one hundred). Furthermore, the data here presented imply that ferulic acid is significantly less efficient than Trolox, a result that contrasts with those obtained employing crocin as the target molecule.

An interesting feature of the data presented in Table 1 is the similar reactivity of cinnamic and propionic acid derivatives (compounds VIII and IX). Also, it is interesting to point out that even compound VII produces a small amount of protection, suggesting a moderate reactivity at the double bond in cinnamic acid derivatives.

Protection by phenolic compounds against pyranine bleaching promoted by peroxyl radicals

Pyranine (Py) is a target molecule whose consumption follows zero order kinetics over a wide range of concentrations.[26] If $XO^{\bullet}$ radicals do not react with Py, a simple reaction scheme predicts a Stern-Volmer's like relationship for the quotient between the rate of Py bleaching in the absence (R^0) and presence of additive (R)[23] such that:

$$\frac{R^0}{R} = 1 + \frac{k_{OH}}{k_{Py}}\left(\frac{[XOH]}{[Py]}\right) \qquad (8)$$

Typical data showing the decrease in Py bleaching rate elicited by XOH additives are shown in Fig. 3. Different profiles are observed for different compounds:

(i) Caffeic acid gives well-defined induction times (Fig. 3A).
(ii) Ferulic acid gives ill defined induction times (data not shown).
(iii) Compounds III to IX, reduce the rate of Py consumption in a concentration dependent way (Fig. 3B).

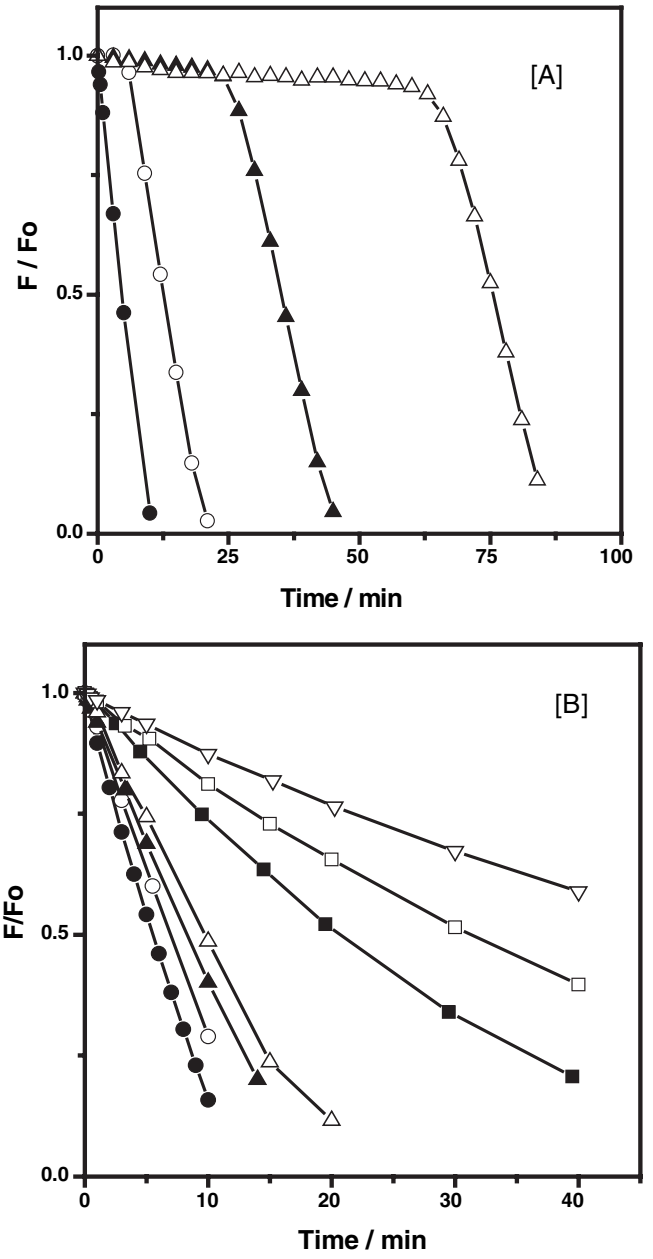


Figure 3. Bleaching of pyranine mediated by AAPH derived peroxyl radicals in the presence of cinnamic acid derivatives. **A**: Bleaching of pyranine (5 μM) elicited by AAPH (10 mM) in the presence of caffeic acid. Control (●); caffeic acid: 1 μM (○); 5 μM (▲) and 10 μM (△). **B**: Bleaching of pyranine (5 μM) elicited by AAPH (10 mM) in the presence of compound VIII. Control (●); compound VIII: 5 μM (○); 50 μM (▲); 100 μM (△); 500 μM (■); 1 mM (□); 2 mM (▽)

The behavior of caffeic acid is very close to that of an 'ideal' inhibitor that efficiently traps peroxyl radicals. In fact, induction times are proportional to the additive concentration and nearly independent of the target molecule (pyranine) concentration (data not shown). If it is considered that, under the present experimental conditions, the rate of radical production is 0.75 μM/min,[27] the induction time in Fig. 3A implies that *ca*. 4.8

radicals are trapped per caffeic acid molecule ($n = 4.8$). This value is similar to those obtained employing other *ortho*-diphenols, such as 3,4-dihydroxy-benzoic acid and 1,2-dihydroxybenzene (data not shown). These large values would imply that each hydroxyl group scavenges nearly two radicals. This suggests that, when pyranine is employed as the target molecule, a quinone is not the main product associated with *ortho*-diphenol oxidations (in this case, $n = 2$ is expected). This conclusion contrasts with that reached for caffeic acid when *c*-Pc is employed as the target molecule, implying that target-derived radicals can influence the secondary reactions of the additive.

The presence of an induction time in the protection of Py by caffeic acid is not compatible with the results obtained employing *c*-Pc as the target molecule. In fact, due to the lower reactivity of this molecule, better defined induction times would be expected. This suggests that the high protection afforded by caffeic acid to Py is due to a repair mechanism, where the target derived radical reacts with the additive. This type of process seems to be particularly relevant when Py is employed as the target molecule.[26]

Plots of R^0/R *versus* [XOH] for several of the compounds considered (ferulic acid, coumaric acid, V and IX) are nearly lineal over the whole concentration range considered. On the other hand, the data obtained with the other compounds show a moderate downward curvature (compound VIII, $(R^0/R)_{max} = 20$) or reach a plateau at relatively low R^0/R values (2.3 and 1.7 for compounds III and VI, respectively), indicating that in these systems damage of pyranine molecules by additive-derived radicals cannot be disregarded. However, taken together these data would suggest that pyranine is less prone to be degraded by additive derived radicals than *c*-Pc.

To obtain relative efficiencies of Py protection by cinnamic acid derivatives, we calculated the R^0/R and ORAC values. The results obtained are given in Table 2. These data show that, regarding both R^0/R and ORAC indexes, caffeic and ferulic acid are, as in the *c*-Pc system, the most efficient in the protection of pyranine. In particular, the ORAC values (Table 2) indicate that caffeic acid is nearly three times more efficient than Trolox, while ferulic acid presents a protection capacity very close to that of the reference compound. This result indicates that the catechol moiety is very efficient in the removal of peroxyl radicals.

Comparison of the data given in Table 2 with that obtained employing *c*-Pc as the target molecule (Table 1) allows the following conclusions:

(i) For most of the compounds considered, particularly the less reactive, R^0/R values are considerably lower when Py is employed as the target molecule. This is compatible with the fact that *c*-Pc consumption is at the first order limit, while Py consumption is a zero order process, indicating that Py is regulating the $ROO^{\bullet}$ steady state concentration. This would suggest that these compounds are competing for the initial radicals and not repairing an initial damage to the Py molecule (*vide infra*).

(ii) Caffeic acid is more efficient in the Py (ORAC = 3.2) than in the *c*-Pc system ORAC = 1.0). This is compatible with a role of a repairing mechanism in the protection afforded by caffeic acid to Py molecules.

(iii) Py, as the target molecule, amplifies the differences among compounds of different reactivity. In fact, the ratio of coumaric acid to caffeic acid reactivity observed is even higher than that previously reported employing crocin as the target molecule.[16] Similarly, differences in ORAC indexes are larger in the Py than in the *c*-Pc system. This could be related to the relevance of repair mechanisms in systems comprising Py and additives of high reactivity.

Bond-dissociation energies

The oxidation potentials, pK_a and BDE, estimated by Equation (2), are given in Table 3. These values show that the main features of the data obtained in this study (i.e., the high reactivity of caffeic acid, the small effect of the

Table 2. Pyranine bleaching promoted by AAPH-derived peroxyl radicals

Compound (XOH)	R^0/R (XOH = 5 μM)	R^0/R (XOH = 50 μM)	ORAC (XOH = 30 μM)
I (caffeic acid)	50	50	3.2
II (ferulic acid)	3.6	24	1.0
III	1.9	2.2	0.26
IV (coumaric acid)	1.0	1.3	0.01
V	1.0	1.2	0.01
VI	1.0	1.1	0.01
VII	1.0	<1.1	0.01
VIII	1.1	1.2	0.03
IX	1.0	<1.1	0.01

Protection by cinnamic acid derivatives.

Table 3. Oxidation potentials, pK_a and bond dissociation energies (BDE) of cinnamic and propionic acid derivatives[a]

Compound	E_{ox}/V	pK_a	BDE (kcal mol^{-1})
I (caffeic acid)	0.377	9.07	76.5
II (ferulic acid)	0.581	9.55	81.9
III	0.662	9.43	83.6
IV (coumaric acid)	0.694	9.45	84.3
V	0.839	9.75	88.1
VI	0.697	10.6	86.0
VIII	0.687	10.61	85.8
IX	0.702	10.47	85.9

[a] E_{ox} values referenced to standard hydrogen electrode.
pK_a values were taken from Ref. [28], compound VIII was evaluated in this study from changes in the UV spectrum with the pH. BDEs were evaluated according to Equation (2).

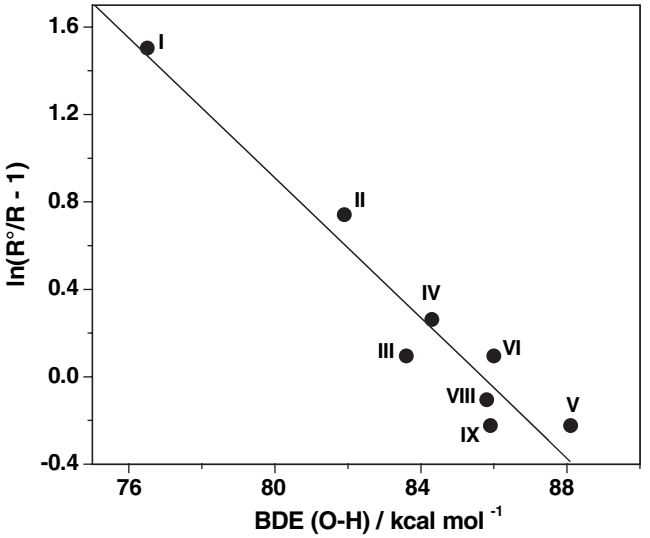


Figure 4. Plot of ln $[(R^0/R)-1]$ *versus* BDE. *R* values obtained employing *c*-Pc (0.01 mg/mL), AAPH (10 mM), and cinnamic acid derivatives (5 μM)

C—C double bond, and the higher reactivity of the phenolic groups located at position four) can be explained in terms of differences in the O—H bond dissociation energies. This is emphasized by plotting ln $[(R^0/R)-1]$ values obtained for the *c*-Pc system against the BDE of the compounds (Fig. 4). This figure shows a strong inverse correlation between the two sets of data ($r=-0.93$; $p=1\times10^{-4}$), but with a rather gentle slope (-0.16). This would indicate that each kcal decrease in BDE increases the rate of hydrogen abstraction from the phenolic moiety only by a factor 1.17. This rather small effect could imply that the transition state in the hydrogen transfer process lies close to that of the reactants. It is interesting to note that a similar treatment of the data obtained employing pyranine as the target molecule showed a weaker correlation with the BDE of the compounds ($r=-0.89$; $p=0.00255$), but a steeper negative slope (-0.61), in agreement with the greater selectivity observed in this system.

GENERAL CONCLUSIONS

Both sets of data, obtained employing *c*-Pc or Py as target molecules, show that the reactivity of the tested compounds towards peroxyl radicals is related to the BDE of the most labile hydroxyl group. However, there are significant differences between the two sets of data, related to differences in the secondary reactions of the phenol-derived radicals. This emphasizes the need to perform a complete kinetic analysis of the results to obtain meaningful evaluations of the relative reactivity of the tested compounds.

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