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Crocin Bleaching Assay (CBA) in Structure−Radical Scavenging Activity Studies of Selected Phenolic Compounds

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The applicability of the crocin bleaching assay (CBA) to structure−activity relationship (SAR) studies of a great number ($n = 39$) of selected phenolic compounds was thoroughly investigated. The focus was on the activity of hydroxybenzoic, hydroxyphenylacetic, hydroxyphenylpropanoic, and hydroxycinnamic acids. Other assays [oxygen radical absorbance capacity (ORAC), lipid oxidation] were applied when necessary. Hydroxybenzoic acids were less active than the respective simple phenols. The position of the −COOH group relative to hydroxyl substituents was critical. The number and position of the −OH groups governed the order and size of activity within the subgroup of these acids. Gallic acid was the most active, being 1.6- and 3.4-fold superior to protocatechuic and syringic acids, respectively. The effect of proximity of the −COOH group to the phenyl ring was more distinct for 3,4-guaiacol acids (ferulic ≫ dihydroferulic ≅ homovanillic > vanillic) than for 3,4-catechol ones (caffeic ≫ protocatechuic ≥ dihydrocaffeic ≅ homoprotocatechuic). Compounds such as vanillin, tyrosol, ferulic acid derivatives, rosmarinic acid, and quercetin were examined to reinforce discussion on the basis of physical organic chemistry principles. Taking into account the acidity of most compounds, the CBA-derived order of activity was meaningful.

KEYWORDS: Phenolic compounds; crocin bleaching assay; hydroxybenzoic; hydroxyphenylacetic; hydroxycinnamic acids; simple phenols; structure−radical scavenging activity relationship

INTRODUCTION

Various in vitro assays have been used so far to investigate the free radical scavenging properties of acid phenols (*1*−*7*), compounds closely related to biosynthesis and the in vivo degradation of flavonoids (*8*). Depending on the reaction mechanism underlying each assay, significant antioxidant activity is not always supported by structural characteristics of the test compounds (*9*, *10*). Because results from in vitro assays are often used to extrapolate the in vivo performance of acid phenols toward reactive oxygen species (ROS), it is important to clarify whether structure−activity relationships (SARs) are meaningful. Reliable SARs are used as a basis for designing potent model antioxidants and better evaluation of natural sources. That being the case, a considerable amount of research has been devoted over the past years to the development or reappraisal of analytical assays.

Among them, those based on competitive reactions (e.g., CBA, ORAC, etc.), seem to gain the interest of investigators due to the use of peroxyl radicals generated under conditions that imitate physiological ones (pH ∼7, $T = 37-40$ °C). Recently, we validated a CBA protocol and proposed an alternative way to evaluate antioxidant activity (*11*). As we reported therein, for the compounds studied by other investigators (*12*, *13*) and for those tested by us in the course of validation, CBA seems promising in SAR studies.

In the present work, a thorough investigation on the applicability of CBA to build SARs among acid phenols is presented. This group of antioxidants attracted our interest not only because of the significant antioxidant efficiency reported so far (*1*, *3*, *4*) but also due to the fact that their overall activity is debatable for various reasons (e.g., anion contribution, side-chain effect, decarboxylation). The activity of mono- and disubstituted phenols was used as a basis for discussion. The polarity index (log *P*), with regard to that of Trolox, was a supportive criterion for compound selection in line with previous suggestions (*11*). The contribution of structural features that are commonly viewed as essential for scavenging free radicals, for example, the number and position of hydroxyl and/or methoxyl substituents to the phenyl ring (*14*), was carefully explored. Literature data from different radical scavenging assays for the tested compounds (AHs) were used to support discussion. When necessary, the activity of AHs was tested using other assays to investigate whether performance under the CBA conditions should be accepted as meaningful.

MATERIALS AND METHODS

Samples and Standards. Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece). Refined olive oil was kindly donated by ELAIS S.A. (Piraeus, Greece). 2-Methoxy-4-(2-propenyl)-

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phenol (eugenol, 99%), 2-methoxy-4-propylphenol (dihydroeugenol, >99%), 2-methoxy-4-propenylphenol (isoeugenol, 98%), 2,4-dihydroxybenzoic acid (γ-resorcylic, 97%), 3,4-dihydroxyphenylacetic acid (homoprotocatechuic), 3-hydroxy-4-methoxycinnamic acid (isoferulic), 3-methoxy-4-hydroxycinnamic acid (ferulic), 3-(3,4-dihydroxyphenyl)-propanoic (dihydrocaffeic, 98%), 4-hydroxyphenethyl alcohol (tyrosol, >98%), 4-hydroxy-3-methoxycinnamaldehyde (coniferyl aldehyde, 98%), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Aldrich Chemie (Steinhem, Germany). 1,3-Benzenediol (resorcinol), 1,4-benzenediol (hydroquinone), 2-hydroxycinnamic acid (*o*-coumaric), 2-hydroxyphenylacetic, 2-methoxyphenol (guaiacol), 2,3-dihydroxybenzoic acid (*o*-pyrocatechuic), 2,5-dihydroxybenzoic acid (gentisic), 3-hydroxy-4-methoxybenzoic acid (isovanillic), 3-methoxy-4-hydroxybenzaldehyde (vanillin), 3-methoxy-4-hydroxybenzoic acid (vanillic), 3-methoxy-4-hydroxyphenylacetic acid (homovanillic), 3,4-dihydroxybenzoic acid (protocatechuic), 3,4,5-trihydroxybenzoic acid (gallic), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic), 4-hydroxybenzoic, 4-hydroxybenzyl alcohol, 4-hydroxycinnamic acid (*p*-coumaric), 4-hydroxyphenylacetic, ethyl-4-hydroxy-3-methoxycinnamate (98%), and 4-hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol, 98%) were purchased from Sigma Chemical Co. (St. Louis, MO). 3,4-Dihydroxycinnamic acid (caffeic) was a product of Riedel de Haën (Seelze, Germany). 1,2-Benzenediol (pyrocatechol >98%), 1,3,5-benzenetriol (phloroglucin, puriss), 2-hydroxybenzoic acid (salicylic, >99%), and DL-α-tocopherol (98% for HPLC) were from Fluka Chemie GmbH (Buchs, Switzerland). 1,2,3-Benzenetriol (pyrogallol, for synthesis) was obtained from Merck (Stuttgart, Germany). 3-(3-Methoxy-4-hydroxyphenyl)propanoic acid (dihydroferulic, 98%) was from Alfa Aesar (Heysam, Lancaster). Quercetin dihydrate (>98.5%) and rosmarinic acid (Rotichrom, ~CHR) were purchased from Carl Roth GmbH, (Karlsruhe, Germany).

Reagents and Solvents. 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH, >98%) was purchased from Fluka Chemie. Crocin stock and working solutions were prepared in our laboratory as recently described (*11*). NaCl, KH_2PO4, Na_2HPO4, and KCl used for the preparation of phosphate buffer saline (PBS) and acetic acid for the preparation of acetate buffer were from Panreac Quimica S.A. (Barcelona, Spain). Sodium acetate-3-hydrate was from Riedel de Haën. Silicic acid (mesh size = 100−200, Sigma, St. Louis, MO), Celite (Riedel de Haën), commercial sucrose, and activated carbon (<100 mesh, Aldrich, Dorset, U.K.) were used for column chromatography. HPLC grade methanol and iso-octane (spectranal) were obtained from Riedel de Haën. Acetonitrile (HPLC-isocratic preparative), diethyl ether stabilized with ethanol, *n*-hexane (95%), and 2-propanol (preparative) were purchased from Panreac Quimica S.A. Ultrahigh-purity water was produced in our laboratory using a Millipore Milli-Q System.

Apparatus. A Shimadzu UV-1601 spectrophotometer (Kyoto, Japan), accompanied with UVPC-1601 software, was used for all UV−visible absorbance measurements. For the CBA measurements, the system was thermostated at 39.5 ± 0.5 °C with the aid of an outer water-circulating bath. A Shimadzu RF 1501 spectrofluorometer equipped with a stirrer and a temperature-controlled cell holder at 37 °C was used for fluorescence measurements. Calculations were carried out by means of the RF 1501-PC software. Adjustment of pH was achieved using a Consort 5231 model portable pH-meter (Turnhout, Belgium).

Calculation of Partition Coefficient (Log *P*). Calculation of the log *P* values, simulating partitioning of tested compounds in an *n*-octanol/water (1:1, v/v) system, was based on Broto's fragmentation method and was accomplished using CS ChemDraw Ultra 5.0 software (*15*).

CBA Kinetic Study. The peroxyl radical scavenging activity of AHs was evaluated according to the protocol of ref *16* with the modifications reported in ref *11*. Briefly, crocin concentration was adjusted to ca. 10 μM, on the basisof the extinction coefficient reported in the literature $\epsilon_{433}^{MeOH} = 133000\ M^{-1}\ cm^{-1}$. A certain volume of crocin working solution was diluted with methanol to 5 mL (total volume) so that the A_{433} value was ~1.3. The same volume of crocin working solution was then transferred into a 5 mL volumetric flask, along with 100 μL of AH (from 0.5 mM solution in methanol). Stock AAPH solution (0.25 M) was daily prepared in 0.01 M PBS (0.08% w/v NaCl) and stored at 4 °C during the different sets of experiments. The reaction started with the addition of AAPH (250 μL) (t = 0 min). After dilution to 5 mL (total volume) with PBS and stirring for ca. 30 s, the test solution was transferred into a 3 mL quartz cell and reaction spectra recording started at exactly 50 s after the addition of initiator. Reaction spectra were recorded in the region of 200−600 nm within 10 min after AAPH addition. Within this time interval, absorbance reduction of crocin progressed at a linear rate (see Supporting Information).

Expression of Results. Loss in absorbance values within 10 min of reaction, in the absence (ΔA_0) or in the presence of AH, at an equimolar ratio to crocin (ΔA), was calculated and expressed in terms of percent inhibition of bleaching (% Inh) using the equation: $\%Inh = (\Delta A_0 - \Delta A/\Delta A_0) \times 100$. % Inh values were then divided by the respective value of Trolox, resulting in the index "Trolox equivalents" ($TEV_{\%Inh}$). Each measurement was made in triplicate. Due to the high number of the test compounds included in this study, different crocin working solutions were prepared within the time period of analysis. The performance of Trolox was tested many times in the course of the experimentation period. Intraday repeatability of ΔA_0 and ΔA_{Trolox} values (as % CV) at five random days of analysis varied from 2.5 to 6.1 and from 1.8 to 4.3, respectively.

Various levels of [AH] (other than 10 μM) were also tested so that linear regression curves of relative rates ($\Delta A_0/\Delta A$) against the [AH]/[C] ratios could be constructed. The range of [AH] levels was selected by trial and error, taking into account the linearity of regression curves ($r^2 > 0.96$) as well as the potency found for [AH]/[C] = 1. With the exception of the highly active compounds for which low levels of addition were used (≤10 μM), the concentrations of most AHs ranged within 10−90 μM. The five-point linear regression slopes representing relative rate constants ($k_{rel} = k_{AH}/k_C$) were then divided by the k_{rel} value of Trolox and the respective $TEV_{k_{rel}}$ were also calculated. Repeatability of k_{rel} measurements has been thoroughly examined for Trolox in our previous study (*11*). For each of the test compounds, k_{rel} calculation was based on one series of experiments and repeatability was checked randomly for some of them. Within-day repeatability (as % CV of the k_{rel} values, n = 3) was checked for eugenol (±2.87), dihydroeugenol (±5.20), isoeugenol (±3.77), gentisic acid (±8.63), and hydroquinone (±7.23).

ORAC Assay. Evaluation of peroxyl radical scavenging activity was based on the protocol described by Naguib (*17*) with the following modifications. Peroxyl radicals were generated at a controlled rate by thermal decomposition of AAPH. Working fluorescein solution (8.6 nM) was prepared from a stock solution (0.11 mM) in phosphate buffer (75 mM, pH 7) so that the initial fluorescence intensity was around 800 units (instrument range = 0−1000 units). The test solutions (5 mL) contained fluorescein (4 mL) along with AH solution in phosphate buffer (250 μL, 5 μM). The reaction started by the addition of AAPH (120 μL, 0.125 M) to the test solution. Decay of the fluorescence intensity (Exc, 490; Em, 515 nm) was monitored until zero fluorescence occurred. Trolox was used as the reference compound. The net area under curve (AUC) calculated in the presence (AUC_{AH}) and in the absence of the test compounds (AUC_{Blanc}) was used to express the antiradical activity of AHs relative to Trolox: $[(AUC_{AH} - AUC_{blank})/(AUC_{Trolox} - AUC_{blank}) \times$ (mol of Trolox/mol of AH). This value is known as the ORAC value. For each test compound, three series of such measurements were used for the calculation of ORAC values.

Oven Test. *Olive Oil Purification.* Refined olive oil was purified in the laboratory on three chromatographic columns in series, according to the procedure of Psomiadou and Tsimidou (*18*). Residual α-tocopherol content was measured by normal phase HPLC and fluorescence detection at 294 and 330 nm (*19*). Peroxide values (mequiv of O_2/kg) as well as k_{232} values [A_{232}/C (g/100 mL)] were also measured prior to the oxidative stability test (PV = 8 mequiv of O_2/kg, k_{232} = 1.6).

Bulk Oil Stability. Aliquots of the purified olive oil (2 g) were distributed in a series of clear open dark glass bottles of pharmacopeia quality (18 mm i.d.). Oil samples containing the compounds under study at the level of 50 mg/kg were stored at 45 and 62 °C in the dark. Oil solutions (0.15−0.30%, w/v) were prepared in iso-octane, and the UV spectrum was recorded between 190 and 350 nm. The process of oxidation was followed by periodic spectra recording in the region between 190 and 350 nm. All analyses were performed in duplicate.

K_{232} values of the control oil solution were also measured periodically. Repeatability of K_{232} values within 47 h of treatment under 45 ($n = 5$) or 62 °C ($n = 5$) was satisfactory: CV% = 3.7−7.8 and 4.2−12.6%, respectively.

Expression of Results. The first- and second-derivative spectra were calculated at optimized combinations of smoothing parameters (17 experimental points) and delta lambda values ($\delta\lambda = 2$ and 4 nm, for the first- and second-spectrum derivatives, respectively). Quantitative data were expressed as Δh values ($\Delta h = (h_{239} - h_{246})$/C), where Δh is the vertical distance between the two minima at ∼239 and ∼246 nm of the first-derivative spectrum divided by the concentration of the sample solution (%, w/v). This vertical distance has positive, zero, and negative values. High positive values are related to low levels of oxidation, whereas high negative values are expected for stored oils (*20*). Graphical plots of Δh values versus time were constructed to indicate the effect of each AH on the oil stability.

Statistical Analysis. Statistical comparisons of the mean values of each set of experiments were performed at a significance level of 95% by Student's test (*t* test) and one-way ANOVA using SPSS 11.5 software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Structural characteristics, calculated log *P* values, and data from application of CBA to a series of AHs are shown in **Table 1**. Chemically related simple phenols (**1a**−**1f**) were considered in this study as "parent molecules" of the respective acids (**2**−**5**). The latter are further subgrouped as hydroxy derivatives of benzoic (**2a**−**2g**), phenylacetic (**3a** and **3b**), phenylpropanoic (**4a** and **4b**), and cinnamic acids (**5a**−**5b′**). Phenol was not examined due to the very low activity toward free radicals it presents (*21*), which restricts its use as a reference compound in experimental SAR studies.

Compounds, included to reinforce discussion (vanillin, tyrosol, etc), were grouped together with those for which comparison of activity was examined and not necessarily according to structural characteristics. No codes were assigned in such cases. The alkyl−peroxyl radical scavenging activity of each AH is presented in terms of $TEV_{\%Inh}$ and $TEV_{k_{rel}}$. The former expression provides information about the competitive reaction of equal numbers of moles of crocins and AH with the AAPH-derived radicals. In this way, the order and magnitude of activity should be neither over- nor underestimated. TEV values based on k_{rel} calculation were also included in the study because most published CBA data so far are expressed in this way (*11*).

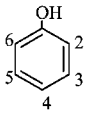
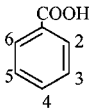
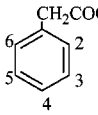
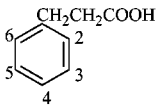
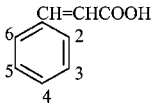
Simple Phenols. Considering the group of simple phenols of **Table 1** as a training set and taking into account the observations from application of CBA to the evaluation of antioxidant capacity of smoke phenols, published in the course of writing this paper (*22*), the number and position of −OH groups were found to govern the order and magnitude of activity. As shown in the table, the descending order of activity among the test phenols was **1f** > **1b** > **1d** > **1c** ≅ **1e** ≥ **1a**. Guaiacol (**1a**), lacking the *ortho*-dihydroxyl configuration, was 6-fold less efficient than catechol (**1b**). The latter was found to be somewhat more active than hydroquinone (**1d**), whereas both of them were 3−4 times more active than resorcinol (**1c**). Pyrogallol (**1f**), possessing three adjacent hydroxyl groups, was the most potent radical scavenger among test phenols, twice as active as **1b**. Introduction of a third hydroxyl group to the meta position, as in the case of phloroglucinol (**1e**), had only a marginal effect on the antiradical potential of **1c**.

The above trend was in agreement with so far accepted criteria for enhanced radical scavenging performance. As both −OH and −OMe substituents exert similar electronic effects, the feasibility of a H-atom abstraction and stabilization of the corresponding radical is related to the formation and strength of an intramolecular hydrogen bond to the parent and/or the radical, respectively (e.g., **1b** radical vs **1a** radical). The superior activity of **1f** is highly attributed to the stabilization of the respective phenoxyl radical by two hydrogen bonds. The stoichiometries of **1b** and **1d** toward peroxyl radicals should be quite similar, because stable *ortho*- and *para*-quinones are the end products of these reactions (*23*, *24*). Literature data for the activity of phenols using other radical scavenging assays or lipid oxidation tests were then collected for comparison with CBA ones and are presented in **Table 2**. Due to the various ways of activity expression the data were transformed as percentiles of catechol activity to ease discussion. The relative size of radical scavenging activity as estimated using CBA was comparable to that reported for the DPPH$^{\bullet}$ assay in the presence of methanol or an ethanol/buffer mixture (*26*, *27*). In such environments, resorcinol activity is enhanced, whereas in ethanol, its activity diminishes (*6*, *25*). This may also explain why resorcinol activity was low but measurable under the aqueous conditions of CBA. Significant was the difference in activity of **1f** and **1b**, as estimated by CBA. Such a difference was also observed by Hotta and co-workers (*27*), using the DPPH$^{\bullet}$ assay. The similar potency found for **1c** and **1e** was in line with DPPH$^{\bullet}$ data reported by Nenadis et al. (*6*) and is justified by theoretical calculations of BDE values (*23*). Data produced by CBA for **1c** and **1e** were in contrast to those reported using the ABTS$^{\bullet+}$ assay. The latter is considered to be inappropriate for SAR studies by some investigators (*6*, *9*). These first observations and justifications reinforced the idea of studying further the applicability of CBA to SAR studies of more acidic phenolic compounds.

Acid Phenols. Data for acid phenol activity are also shown in **Table 1**. All monohydroxy acid phenols examined (*o*- and *p*-hydroxybenzoic, *o*- and *p*-hydroxyphenylacetic, *o*- and *p*-hydroxycinnamic acids) were found to be inactive using the CBA, as expected. Consequently, differences in the activity of di- and trisubstituted acid phenols were examined with regard to those of simple phenols. These differences, expected to be influenced by the proximity of the carboxylic group to the phenyl ring and its relative position to existing substituents, are discussed, taking into account the analytical conditions (pH 7.4, 12% methanol in PBS) (*31*, *32*). Overall observations indicate that a carboxylic group, ionized or not, affected negatively the activity of acid phenols. The size of this effect is difficult to interpret, but it should correlate with the simultaneous presence of nondissociated and dissociated carboxylic groups. Differences in the activity within a certain subgroup (**2**) or among acids belonging to various subgroups (**2**−**5**) using the CBA are discussed in detail in the following paragraphs.

Importance of the Catechol Moiety. An *ortho*-dihydroxy configuration to the phenyl ring of the examined acids, that is, a catechol moiety, was shown to significantly enhance activity as expected. The effect depended on the relative position of the catechol moiety with regard to the carboxylic substituent. For example, 2,3- instead of 3,4-dihydroxy configuration accounted for the 3-fold lower activity of *o*-pyrocatechuic (**2b′**) compared to that of protocatechuic acid (**2b**). Reported BDE values [78.53 and 76.11 kcal/mol, respectively (*6*)] support the trend but not the size of the difference in activity. ORAC values, obtained under the conditions presented under Materials and Methods, suggested a different trend (ORAC values: **2b′**, 2.84 ± 0.28; **2b**, 1.51 ± 0.20, $n = 3$) that did not obey the classical criteria. Considering that steric phenomena or intramolecular H-bonding between the adjacent −OH and -COOH groups is

Table 1. Alkyl–Peroxyl Scavenging Activity of the Phenolic Compounds under Study

Code /no[a]	Test compounds[b]	Structure[c]	Log*P*[d]	Expression of results	
				$TEV_{\%Inh}$[e]	TEV_{krel}[f]
	Trolox		3.18	1.00	1.00
1	**Phenols**				
1a	guaiacol	2-OMe	1.61	0.21±0.07	0.14
1b	catechol	2-OH	1.09	1.34±0.04	0.53
1c	resorcinol	3-OH	1.09	0.32±0.08	0.09
1d	hydroquinone	4-OH	1.09	1.05±0.10	0.55
1e	phloroglucin	3,5-diOH	0.70	0.31±0.06	0.13
1f	pyrogallol	2,3-diOH	0.70	2.88±0.04	3.57
	p-benzyl alcohol	4-CH_2OH	0.73	0.04±0.07	0.08
	tyrosol	4-CH_2CH_2OH	1.18	0.07±0.14	0.05
2	**Benzoic acids**				
	o-OH benzoic	2-OH	0.83	0.10±0.03	0.08
	vanillin	-CHO	1.09	0.52±0.14	0.24
	p-OH benzoic	4-OH	0.83	0.06±0.11	0.06
2a	vanillic	3-OMe-4-OH	0.97	0.15±0.04	0.14
2a'	isovanillic	3-OH-4-OMe	0.97	-0.20±0.14	0.11
2b	protocatechuic	3,4-diOH	0.45	1.49±0.05	0.71
2b'	*o*-pyrocatechuic	2,3-diOH	0.45	0.55±0.04	0.18
2c	resorcylic	2,4-diOH	0.45	0.18±0.10	0.09
2d	gentisic	2,5-diOH	0.45	0.82±0.09	0.26
2f	gallic	3,4,5-triOH	0.06	2.34±0.04	2.58
2g	syringic	3,5-diOMe-4-OH	1.10	0.69±0.02	0.28
3	**Phenylacetic acids**				
	o-phenylacetic	2-OH	1.02	-0.12±0.06	0.04
	p-phenylacetic	4-OH	1.02	-0.05±0.09	0.05
3a	homovanillic	3-OMe-4-OH	1.15	0.31±0.04	0.10
3b	homoprotocatechuic	3,4-diOH	0.63	1.04±0.07	0.54
4	**Phenylpropanoic acids**				
4a	dihydroferulic	3-OMe-4-OH	1.61	0.26±0.09	0.23
4b	dihydrocaffeic	3,4-diOH	1.09	1.07±0.08	0.63
5	**Cinnamic acids**				
	o-coumaric	2-OH	1.57	0.03±0.11	0.08
	p-coumaric	4-OH	1.57	0.11±0.08	0.08
5a	ferulic	3-OMe-4-OH	1.70	1.02±0.02	0.84
5a'	isoferulic	3-OH-4-OMe	1.70	0.23±0.06	0.10
5b	caffeic	3,4-diOH	1.18	2.53±0.03	3.51
5b'	rosmarinic		1.90	2.79±0.06	4.53

[a] Arabic numbers correspond to different subgroups of compounds; small letters indicate the –OH and/or –OMe configuration of the aromatic ring (shown in the third column); within a subgroup, stressed letters stand for acid phenols with the same –OH substitution but different position and/or characteristics of the side chain. [b] Empirical names of the test compounds. [c] Structural formulas indicating the number and position of the ring substituents. [d] Calculated as described under Materials and Methods. [e] Mean value ± SD ($n = 3$). [f] Calculated from one set of experiments (see Materials and Methods).

Table 2. Relative Radical Scavenging Activity of Substituted Phenols, Expressed as Percentage of Catechol Activity, Using Other than CBA Assays

	phenols						
ref	1-OH, 2-OMe **1a**	1,2-diOH **1b**	1,3-diOH **1c**	1,4-diOH **1d**	1,3,5-triOH **1e**	1,2,3-triOH **1f**	reaction environment/expression of results
6	–[a]	100.0	4.2	74.7	5.7	–	DPPH assay, % RSA (absolute ethanol)
	–	100.0	117.5	70.1	168.0	–	TEAC assay, % Inhibition of ABTS reduction (PBS pH 7.4)
9	–	100.0	171.2	91.7	–	–	TEAC, % inhibition of ABTS reduction (PBS, pH 7.4)
	–	100.0	4.7	94.5	–	–	peroxynitrite scavenging, inhibition of DHR oxidation (IC_{50})
	–	100.0	1.5	100	–	–	superoxide scavenging, inhibition of NBT reduction (IC_{50})
21	–	100.0	0.6	6.4	–	–	% inhibition of microsomes peroxidation (Fe^{2+}, Tris-HCl–KCl buffer, pH 7.4)
22	15.5	100.0	-	40.9	–	–	DPPH assay, EC_{50}
	12.0	100.0		515.4	–	–	AE (methanol)
25	–	100.0	0.0	60.0	–	–	amount of two-electron reaction of 0.5 μmol of phenols with 2.5×10^{-3} μmol of DPPH, % (absolute and 95% ethanol)
26	–	100.0	0.7	100.7	–	–	DPPH assay, log *Z*
	–	100.0	19.7	73.7	–	–	EC_{50} (methanol)
	–	100.0	21.6	98.7	–	–	% inhibition of synaptosomal lipid peroxidation ($OH^{\bullet}$ and $O_2^{\bullet -}$ radicals)
27	–	100.0	23.7	59.5	–	220.0	DPPH assay, EC_{50} (1:1 ethanol/phosphate buffer, pH 7)
28		100.0				621.0	$O^{\bullet -}$ scavenging activity (IC_{25})
	–	100.0	0.64	6.41	–	–	inhibition of rat liver microsome peroxidation (Fe^{2+}, phosphate buffer, pH 7.4)
29	–	100.0	89.9	–	–	212.9	quenching reaction of singlet oxygen ($^1O_2$), second-order rate constants (ethanol)
30	22.2	100.0					DPPH assay, EC_{50}
	2.2	100.0					AE (absolute ethanol)
this study	15.7	100.0	23.9	78.4	23.1	214.0	CBA, $TEV_{\%inhib}$ (12% methanol/PBS, pH 7.4)

[a] No available data.

expected to hinder the abstraction of a H-atom from **2b′** (*33*), its weaker activity found under CBA conditions seems to be rational.

The 3,4-catechol moiety influenced positively the activity of all the examined acids in comparison to the respective guaiacol one (**2b** > **2a**, **3b** > **3a**, **4b** > **4a**, **5b** > **5a**). Such a trend was more apparent when the carboxylic group was next to the phenyl ring. These findings were in line with antiradical efficiency (AE) values produced in our recent work using the $DPPH^{\bullet}$ assay (*30*). It is interesting to stress that abstraction of a H-atom is readily feasible from either the 3- or 4-OH group of catechol acids, resulting in stable semiquinone radicals. This is not the case for guaiacol acids, for which the relative position of the −OH and −COOH groups to the phenyl ring plays a crucial role in the stability of the respective radicals. Such an effect was distinctly revealed under CBA conditions when the pairs vanillic (**2a**)−isovanillic (**2a′**) and ferulic (**5a**)−isoferulic (**5a′**) were examined. The 3-hydroxy-4-methoxy configuration was 3−4-fold less efficient than the 3-methoxy-4-hydroxy one, as a result of the resonance-stabilized radical formation.

Gallic acid (**2f**) was the most potent among hydroxybenzoic acids, 1.6- and 3.4-fold more than **2b** and syringic acid (**2g**), respectively. This finding implied the significance of the *ortho*-trihydroxy configuration compared to the respective dihydroxy and dimethoxyhydroxy ones for the antiradical performance of acid phenols. Such a relationship is in line with general SAR criteria concerning the effect of −OH and −OMe groups on the activity of compounds (*14*). It is worth noting that, among few available literature data for the relative size of activity of **2f**, **2b**, and **2g** under various experimental conditions (*2*, *34*), similar findings were reported only by Hotta et al. (*27*) using the $DPPH^{\bullet}$ assay, in terms of EC_{50} values.

Under the CBA conditions it was evidenced that the relative position of the −OH groups to the phenyl ring, such as the *meta*- or *para*-dihydroxy configuration, as well as their position relative to the carboxylic group affected significantly the activity of acid phenols. In particular, resorcylic acid (**2c**) was the least potent among dihydroxybenzoic acids, possessing only 12% of protocatechuic acid's activity. Moreover, *o*-pyrocatechuic acid (**2b′**) was less active than gentisic acid (**2d**), in line with the reported BDE values for these acids (*6*).

The advantage of CBA to distinguish small differences among acid phenols belonging to the same subgroup should not be overlooked.

Importance of the Proximity of the Carboxylic Substituent to the Phenyl Ring. The π-electron and field effect interactions of a substituent with the phenyl ring are expected to change when methylene groups are introduced between these moieties (*35*). Indeed, such structural differences were crucial for the activities of substituted phenols under the CBA conditions. For example, hydroquinone (**1d**) was far more potent than both 4-hydroxybenzyl alcohol and tyrosol, indicating the significant role of the quinoid structures to the H-atom abstraction as well as better stabilization of the corresponding radicals. The similar activities of the two latter phenols to that of 4-hydroxybenzoic acid showed that at high pH values the −COOH group is present as $-COO^-$ and *this* may be equivalent to a $-CH_2OH$ group, under CBA conditions. When conjugation of the carboxylic group was interrupted, for example, in the case of 4-hydroxyphenylacetic acid, the activity was further reduced. These findings are not in line with the trend suggested by the Brown σ^+ parameter values of the respective substituents [e.g., −COOH, 0.45; $-CH_2COOH$, −0.01; and $-CH_2OH$, 0.00 (*36*)]. The importance of the effect of carboxylic group proximity to the aromatic ring was then systematically examined for two series of 3,4-guaiacol and catechol acids.

(a) Direct Attachment to the Phenyl Ring. BDE values support a negative effect of the carboxylic substituent directly attached to the phenyl ring (*23*, *30*). Indeed, the clear reduction in activity of **2b** evidenced using the AE values in our aforementioned

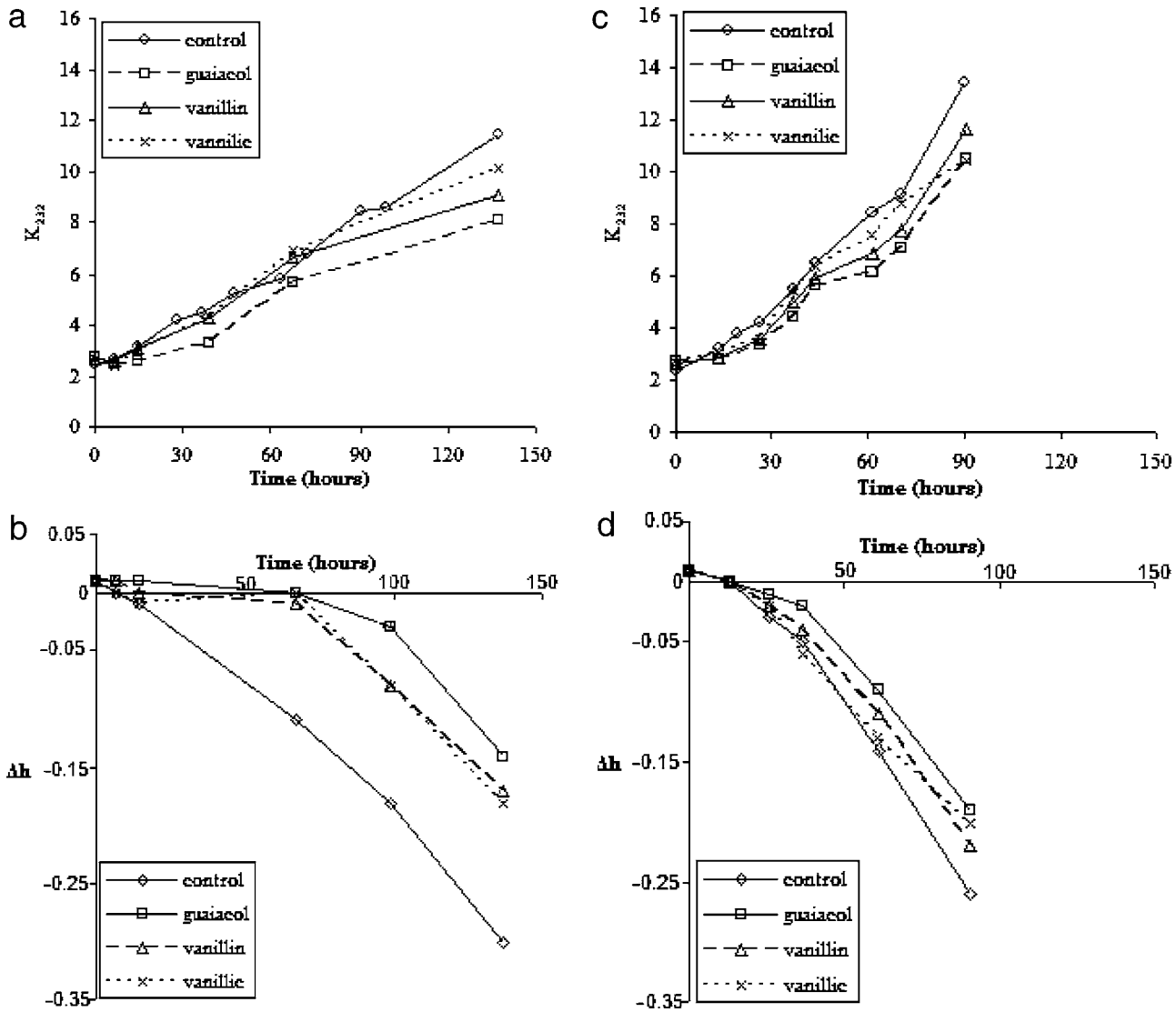


Figure 1. Evaluation of the antioxidant activity of guaiacol, vanillin, and vanillic acid (50 mg/kg) in refined olive oil stored at 45 °C (**a**, **b**) and 62 °C (**c**, **d**).

study was not observed using CBA. The slightly reversed trend indicated that a negative electronic effect might not be the determinant factor for the activity of acid phenols under the examined conditions. The poor activity of both **1a** and **2a** did not allow further discussion.

Under CBA conditions, the presence of dissociated carboxylic groups for both **2a** and **2b** is expected to affect the estimation of the overall activity, so that an electron-donating effect cannot be excluded. Information about the contribution of electronic phenomena to the activity of hydroxybenzoic acids was then sought by the comparative study of vanillin, an aldehyde for which a net electron-withdrawing effect was foreseen. The activity of vanillin was examined and compared to those of **1a** and **2a**. The aldehyde derivative was found to be far more active than the mentioned compounds. In view of the DPPH$^{\bullet}$ data for **1a** and **2a** (*30*), as well as for **1a** and vanillin (*22*), which support negligible activity for them, the high activity of the aldehyde toward the alkyl−peroxyl radicals used in the present study was difficult to interpret. Implications for a SET-based reaction of vanillin (*22*) or electrostatic interactions that may take place between the positively charged AAPH-derived radicals and the $-COO^-$ substituents of acid phenols (*37*) cannot be disregarded. Nevertheless, to be realistic, we carried out bulk oil oxidation experiments using the three compounds. Changes in the lipid substrate were expressed as K_{232} values corresponding to an increase in conjugated systems (**Figure 1a**,**c**). Analysis of first-derivative spectra in the course of oxidation and calculation of Δh values offered a more discriminatory means of monitoring the above changes (**Figure 1b**,**d**) as was explained in a previous work (*20*). Activity toward lipid−peroxyl radicals, at two temperatures (45 and 62 °C), showed that both the acid and the aldehyde were slightly less potent inhibitors than guaiacol. The slight differences could not be discussed in terms of log *P* values (see **Table 1)** and the "polar paradox" concept or easily assigned to electronic phenomena.

These data were supportive of the CBA finding for vanillic acid and guaiacol but could not support the performance of vanillin. Further investigation upon this subject is obviously needed.

(b) Linking through a Carbon Side Chain. A carbon side chain with a terminal carboxylic substituent is the main characteristic of phenolic compounds belonging to the classes of phenylacetic, phenylpropanoic, and cinnamic acids. The activities of guaiacol and catechol acids, belonging to the above subgroups (**3a**, **3b**, **4a**, **4b**, **5a**, and **5b**) were compared with those of the respective phenols (**1a** and **1b**) and of benzoic acids (**2a** and **2b**), so that differences within a series of acids reflect the effect of the side-chain characteristics (see **Table 1**).

Taking into account previous suggestions that the potency of the catechol moiety may mask small differences in the side chain (*38*), the order of activity among guaiacol acids was examined first. Under CBA conditions, the order found [**5a** ≫ **4a** ≅ **3a** > **1a** ≥ **2a**] indicated that ferulic acid (**5a**) could be distinctly characterized as the most potent one, almost 7-fold higher than **2a**. A slight prioritization of dihydroferulic acid (**4a**) and homovanillic acid (**3a**) compared to **2a**, in line with the BDE-based order (*30*), implied that a close proximity of the −COOH or $-COO^-$ substituents to the ring is not beneficial for the activity of acid phenols. Such a suggestion was more evident when kinetic data for the DPPH• reaction with **4a**, **3a**, and **2a** were viewed, indicating a high difference between the first two (*30*). Given that the acidity of the carboxylic group is affected by the electronic phenomena of the guaiacol ring as well as the side carbon chain characteristics, a different degree of dissociation could be brought about at pH 7.4 for each of the tested acids. Thus, fully dissociated molecules of ferulic acid (**5a**) are expected to be present in the assay solution at pH 7.4 (*6*). The electron-donating effect of the $-CH{=}CHCOO^-$ substituent is considered to significantly favor the stabilization of the radical formed after abstraction of a H-atom, offering an explanation for the superior activity found for **5a**, under CBA conditions. Besides, the extended conjugation in the side chain accounting for a planar structure of **5a** signifies the importance of electronic rather than steric phenomena to the activity of this compound.

In the presence of a catechol moiety, a similar behavior of the respective acids was evidenced except for the case of **2b.** The latter was found to be nearly 1.5-fold more active than homoprotocatechuic acid (**3b**) and dihydrocaffeic acid (**4b**) (**Table 1**) so that discussion about the effect of proximity of the carboxylic substituent to the catechol ring became quite perplexing. The findings obtained by CBA were similar to those evidenced on the basis of DPPH• data obtained when acetonitrile was used as a solvent (*30*). In acetonitrile, the conjugated carboxylic group of **2b** becomes highly acidic after the formation of electron-withdrawing quinone groups to the aromatic ring, accounting for the presence of a high number of dissociated **2b** molecules (*39*). Under the aqueous CBA conditions, dissociation is expected to be more intense. In fact, pK_a values reported in the literature for some of the examined acids (*4*, *31*) indicated that at pH 7.4, a considerable number of dissociated carboxylic groups are present in the assay solution. Apart from formation of $-COO^-$ groups, ionization of 4-OH ones is also favored, depending on the characteristics of the side chain. For example, dianionic forms of caffeic acid (**5b**) are more likely to be formed, compared to those of **4b**, as indicated by pK_a values of the 4-OH group of each acid (**5b**, pK_{a2} = 8.48; **4b**, pK_{a2} = 9.24) (*4*). Obviously, a similar effect may be observed for other acid phenols, as well, resulting in a different degree of formation of the highly active $-O^-$ goups, at pH 7.4, as also supported by the recent findings of Amorati et al. (*40*).

Additional evidence may be derived by some preliminary data produced in our laboratory for the CBA performance of certain compounds at pH 5.5. The behavior of Trolox, the reference antioxidant, indicated only slight differences in the calculated k_{rel} values (e.g., $k_{rel5.5} = 0.71 \pm 0.01$ and $k_{rel7.4} = 0.78 \pm 0.00$). However, such differences were far less significant than those observed in the case of catechol (**1b**) and caffeic acid (**5b**). For the latter compounds, 6- and 22-fold reductions in k_{rel} values were observed at pH 5.5 compared to those at pH 7.4, respectively (**Figure 2**). The profound pH effect on the activity of compounds should be at least partially attributed to dissociation.

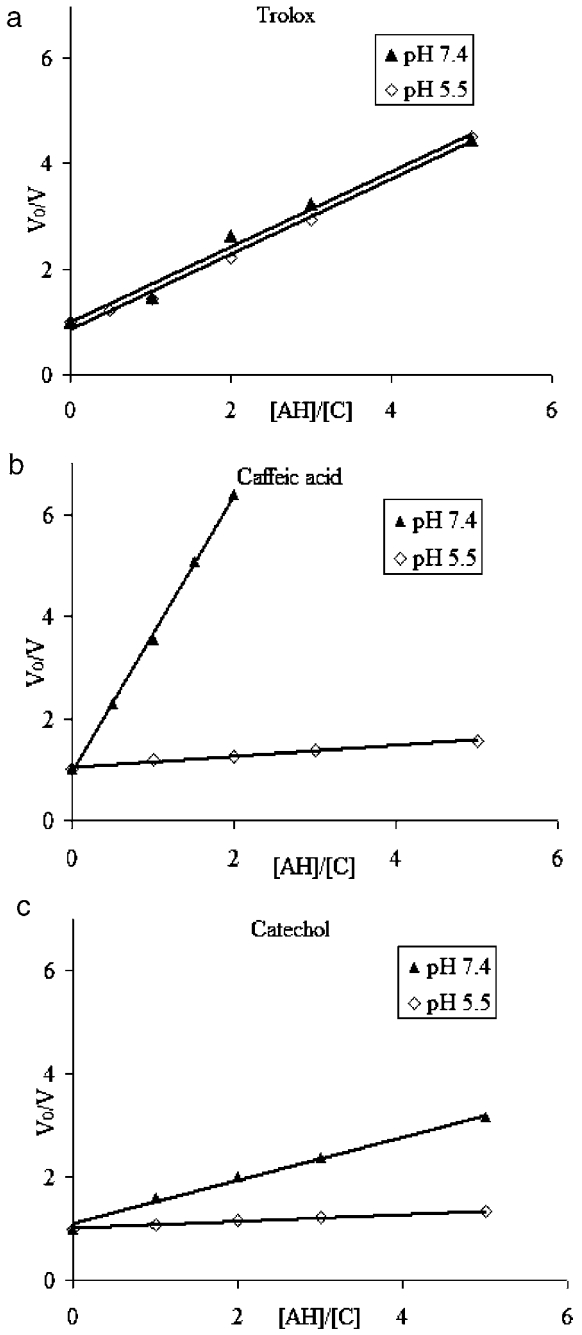
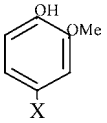


Figure 2. Effect of different pH values on the kinetic behavior (k_{rel}) of (**a**) Trolox, (**b**) caffeic acid, and (**c**) catechol.

Further Discussion on Acid Phenol Activity. Rosmarinic acid (**5b′**), a caffeoyl ester with proven medicinal properties and well-characterized physiological functions (*41*), has been examined in our laboratory in the past, along with caffeic and dihydrocaffeic acids, using DPPH• and $ABTS^{\bullet+}$ tests, as well as DFT calculations (*5*, *6*). Although **5b′** possesses a second catechol group, the reported BDE (72.63−73.95 kcal/mol) and IP (124.93−126.32 kcal/mol) values for these compounds suggested rather small differences in the antiradical activity of all three acids, regardless of the number of −OH groups or even the prevailing mechanism of activity (hydrogen or electron donation, respectively). Similar were the observations under CBA conditions as **5b′** was not found to be far more potent

Table 3. Alkyl–Peroxyl Scavenging Activity of Ferulic Acid Derivatives

test compound[a]	structure[b]	log *P*[c]	expression of results: $TEV_{\%Inh}$[d]	expression of results: $TEV_{k_{rel}}$[e]
Trolox		3.18	1.00	1.00
dihydroeugenol	$-CH_2CH_2CH_3$	2.94	0.33 ± 0.13	0.23
eugenol	$-CH_2CH{=}CH_2$	2.69	0.30 ± 0.06	0.17
isoeugenol	$-CH{=}CHCH_3$	2.49	0.90 ± 0.13	0.59
coniferyl alcohol	$-CH{=}CHCH_2OH$	1.33	1.00 ± 0.06	0.60
ferulic acid	$-CH{=}CHCOOH$	1.70	1.02 ± 0.02	0.84
ethyl cinnamate	$-CH{=}CHCOOEt$	2.46	1.27 ± 0.04	1.01
coniferyl aldehyde	$-CH{=}CHCHO$	1.82	1.32 ± 0.02	1.12

[a] Empirical names of the test compounds. [b] Structural formulas indicating the number and position of the ring substituents. [c] Calculated as described under Materials and Methods. [d] Mean value ± SD ($n = 3$), [e] Calculated from one set of experiments (see Materials and Methods).

than **5b** (**5b′**, $TEV_{\%Inh} = 2.79 \pm 0.06$, $TEV_{krel} = 4.53$; vs **5b**, $TEV_{\%Inh} = 2.53 \pm 0.03$, $TEV_{krel} = 3.51$).

The activities of the latter acids under CBA conditions were almost 7.1- and 5.5-fold superior to that of **4b**, respectively. Much lower differences have been evidenced using TEAC values, indicating that dihydrocaffeic was quite potent, compared to the mentioned acids. Such a behavior has been also observed in terms of AE values, toward the DPPH• in ethanol (e.g., AE_{4b}, 0.46; AE_{5b}, 1.12; $AE_{5b'}$, 0.91) (*5*, *6*). It is worth noting, though, that **4b** was found to be considerably less active than both **5b′** and **5b** (4.8- and 13.6-fold weaker, respectively) when AE values were calculated in acetonitrile. As discussed previously in the present paper, acid dissociation that is partially supported in the latter solvent is highly favored under CBA conditions, especially in the case of hydroxycinnamic acids. Thus, a higher number of **5b′** and **5b** anions or even dianions, compared to those of **4b**, could have played a role in the kinetic behavior of the both the caffeic and rosmarinic acids.

Differences in the antioxidant activity of some biosynthetically related ferulic acid derivatives ($-CHO$, CH_2OH, $-CH_3$, $-COOC_2H_5$) studied in the recent past using experimental and computational methods (*42*) were the basis for the discussion of the data obtained under the CBA conditions for the same compounds. These data are presented in **Table 3**.

In that study, the relative order of the DPPH• scavenging activity, derived by kinetic studies, revealed that isoeugenol and coniferyl alcohol were almost 30 times more efficient than the rest of the compounds. The relative order of activity was found to be in line with that based on the Brown parameter, σ^+, values, that is, the importance of the presence of electron-donating groups. Steric hindrance effects were not considered to influence the activity due to the planarity of the molecules (or slight deviation from the plane in solution). The same order of activity was evidenced in triolein oxidation system, in contrast to predictions based on the polar paradox concept, but in line with the trend indicated by the (rather small) differences in BDE values. Using the CBA, the order became quite different (coniferyl aldehyde ≅ ethyl cinnamate > ferulic acid ≅ coniferyl alcohol ≥ isoeugenol). Aldehyde became the most active compound, whereas isoeugenol was found to be the least potent, much less active than the respective alcohol. This trend, opposite to that discussed above, is indicative of the importance of inductive effects on the activity of ferulic acid (**5a**). The latter became equipotent to coniferyl alcohol, and this fact supports the hypothesis that the prevailed effect of the substituent was that of an electron donor. Brown parameter, σ^+, values provide an insight to this effect ($-CHO$, 0.42; $-COOEt$ and $-COOH$, 0.45; $-COO^-$, -0.02; $-CH_2OH$, 0.00; $-CH_3$, -0.17) (*36*).

Because electron-withdrawing substituents amplify the acidity of $-OH$ groups by stabilizing the O^- anions (*33*), which, in turn, favor the fast electron-transfer reactions (*43*), both hydrogen atom abstraction and electron-transfer reactions with the peroxyl radicals are taking place under CBA conditions.

Extending our search on the activity of isoeugenol, we found that even though its activity was rather limited under the experimental conditions of the present study, conjugation phenomena seemed to play a major role. This was indicated by the high differences observed among dihydroeugenol, eugenol, and isoeugenol (**Table 2**). The order of activity among these compounds was in line with that reported in oil-in-water emulsion tests or toward the DPPH• (*38*). It should be noted, though, that the exceptional activity observed for isoeugenol in the latter assay system (50-fold more active than its counterparts) was not evidenced in our study. The relative size of activity under CBA conditions showed that interruption of the conjugated system in the side chain of dihydroeugenol and eugenol accounted for a 3-fold lower activity compared to that of isoeugenol. Such an effect was even more adverse in the presence of the carboxylic substituent. For example, both cinnamic acids **5a** and **5b** were 4-fold more active than the respective dihydrocounterparts **4a** and **4b**. A limiting factor for the study of isoeugenol and the two other phenols could be the rather high partition coefficient values (2.49, 2.94, and 2.69, respectively) with regard to the upper limit value set for Trolox (log $P = 3.18$). Nevertheless, such a possibility was rejected because otherwise it was difficult to explain how the ester, with a similar log *P* value (2.46), was found to be more active than isoeugenol.

In our opinion thermodynamic parameters (BDE, IP) and kinetic ones (k_{rel}) using the CBA do not seem to complement each other, as has also been commented for AE values using the DPPH• scavenging assay (*30*, *44*). The comparative evaluation of activity of the aforementioned compounds illustrated that, using CBA, prioritization among compounds belonging to different classes of phenols is rather questionable. Nevertheless, within each class of compounds the information obtained is to a great extent in line with physical organic chemistry principles.

Because acid phenols are closely related to biosynthesis or in vivo degradation of flavonoids, differences in the size of activity between the most potent acid under CBA conditions, rosmarinic, and one of the most studied bioactive flavonoids, quercetin, were regarded to be worth examining. Quercetin was found to be slightly more active than rosmarinic acid (e.g., $TEV_{k_{rel}} = 5.03$ vs **5b′** $TEV_{k_{rel}} = 4.53$). This result indicated that a flavonol possessing the highest number of essential structural features (*45*) (a 3′,4′-catechol group to the B ring, a 2,3 double bond along with a C-3 hydroxyl group to the C ring) is nearly equipotent to a highly active acid phenol, possessing two catechol rings. The similar behavior of these compounds is rather supported by the respective BDE values (*6*), whereas experimental data indicate either a higher activity of **5b′** toward the DPPH• (*27*) or the reverse order, using the $ABTS^{\bullet+}$ and FRAP assays (*46*). ORAC values, calculated as described under Materials and Methods, indicated a similar trend for the two compounds (ORAC values: quercetin, 6.50 ± 0.62; **5b′**, 4.68 ± 0.49). Under all experimental conditions, the extent of activity seems to be comparable.

Conclusion. CBA-based order of activity for a large set of acid phenols was in line with the number of −OH and other molecular structural features. The acidity of these compounds, enhanced by the presence of the carboxylic substituent, which is highly affected by the experimental conditions, becomes a key factor for the estimation of activity. Our findings imply the suitability of CBA for exploring SARs among closely related compounds. SARs among compounds belonging to different classes of phenolics should be interpreted with caution.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); BDE, bond dissociation enthalpy; DFT, density functional theory; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant capacity; IP, ionization potential; ORAC, oxygen radical absorbance capacity; SET, single-electron transfer; TEAC, Trolox equivalent antioxidant capacity.

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Supporting Information Available: Kinetic curves obtained during reaction of crocin, in the absence or in the presence of different levels of Trolox concentration, with AAPH-derived radicals. This material is available free of charge via the Internet at http://pubs.acs.org.

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