JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Estimation of Scavenging Activity of Phenolic Compounds Using the ABTS$^{\bullet+}$ Assay

NIKOLAOS NENADIS,[†] LAN-FEN WANG,[‡] MARIA TSIMIDOU,*[,†] AND HONG-YU ZHANG*[,‡]

Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece, and Laboratory for Computational Biology, School of Life Sciences, Shandong University of Technology, Zibo 255049, People's Republic of China

Observations on the applicability of the ABTS$^{\bullet+}$ assay to define structure−activity relationships (SARs) among phenols (AH) were based on experimental data and theoretical calculations. All AH examined (hydroxycinnamic derivatives, simple polyphenols, polyhydroxybenzoates, and flavonoids) were found to be active toward ABTS$^{\bullet+}$. Moreover, known weak radical scavengers (i.e., coumaric and isoferulic acids) were found to be efficient or comparatively active to caffeic or rosmarinic acids in contradiction to the AH classification based on 1,1-diphenyl-2-picrylhydrazyl (DPPH) data or the bond dissociation enthalpy values. This behavior was observed both in ethanol and in buffered (pH 7.4) environment. Resorcinol and phloroglucin were found to be more active than catechol and hydroquinone, whereas, among polyhydroxybenzoates, 2,4-dihydroxybenzoic acid was the least active, in line with the DPPH and theoretical data. Therefore, it can be argued that the ABTS$^{\bullet+}$ assay may give an indication for the presence of antioxidants in a certain system but SARs cannot be readily inferred.

KEYWORDS: ABTS$^{\bullet+}$ assay; DPPH assay; DFT calculations; structure−activity relationship; phenolic antioxidants

INTRODUCTION

Due to the wide application of phenolic antioxidants (AH) in the food, pharmaceutical, and chemical industries, various methods have been developed to define structure−activity relationships (SARs) for AH. Among the methods that have been developed to estimate the radical-scavenging activity, assays based on the scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) are the most popular ones (*1*−*6*). The former has been widely used to measure the antioxidant activity of different phenolic compounds. The results obtained are, in most cases, in agreement with those derived by lipid peroxidation assays in bulk oils (*7*−*9*) and can be explained on the basis of the number and position of hydroxyl groups. The so-called ABTS$^{\bullet+}$ assay is a relatively recent one, which involves a more drastic radical, chemically produced, and is often used for screening complex AH mixtures such as plant extracts, beverages, and biological fluids. The excellent spectral characteristics, the solubility in both organic and aqueous media, and the stability in a wide pH range raised the interest in the use of ABTS$^{\bullet+}$ for the estimation of the antioxidant activity of pure compounds, both lipophilic and water-soluble (*5*, *6*). However, some limitations in the experimental procedures, which had to be overcome, confined the use of the assay. For this reason, the protocol for the production of the radical was gradually modified to ensure that AH reacted solely with the radical and not with other reactants (*6*). Currently, the radical is produced chemically (oxidation with $K_2S_2O_8$) and enzymatically, using horseradish peroxidase (HRP) or microperoxidase (MP8) (*4*, *6*, *10*). In this way problems related with the continuous production of radical during the reaction, the interaction of the AH with the system of radical production, or the formation of the dication radical are avoided. Moreover, although the method gave meaningful SAR data in some cases (*11*), in others this was not evident (*12*). The fact that an AH has been reported to scavenge ABTS$^{\bullet+}$ through hydrogen atom donation (*5*), as well as through electron transfer (*13*) or even with a combination of the two mechanisms (*10*), may explain difficulties in defining SARs. This may explain a current interest in the applicability of the ABTS$^{\bullet+}$ assay to define SAR of phenolic antioxidants (*12*). Toward this requirement, in the present study, the activities of a great number of phenolic compounds were evaluated. When necessary, the DPPH assay was also performed. To complement the experimental findings, theoretical calculations that seem to be very popular in SAR and QSAR studies (*9*, *10*, *14*, *15*) were also carried out.

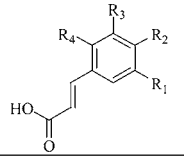
MATERIALS AND METHODS

Standards, Reagents, and Solvents. Caffeic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from

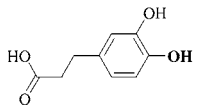
* Corresponding authors [(M.T.) telephone ++302310997796, fax ++302310997779, e-mail tsimidou@chem.auth.gr; (H.-Y.Z.) telephone ++86-533-2780271, fax ++86-533-2780271; e-mail zhanghy@sdut.edu.cn].
[†] Aristotle University of Thessaloniki.
[‡] Shandong University of Technology.

10.1021/jf0400056 CCC: $27.50
Published on Web 06/26/2004

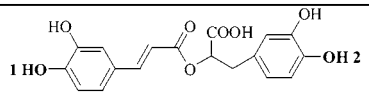
Chart 1. Cinnamic Acid Derivatives Examined



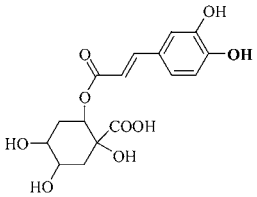
Hydroxycinnamates	R_1	R_2	R_3	R_4
trans-Cinnamic acid	H	H	H	H
p-Coumaric	H	**OH**	H	H
o-Coumaric	H	H	H	**OH**
m-Coumaric	H	H	**OH**	H
Ferulic acid	H	**OH**	OCH_3	H
Isoferulic acid	H	OCH_3	**OH**	H
Sinapic acid	OCH_3	**OH**	OCH_3	H
Caffeic acid	H	**OH**	OH	H



Dihydrocaffeic acid



Rosmarinic acid



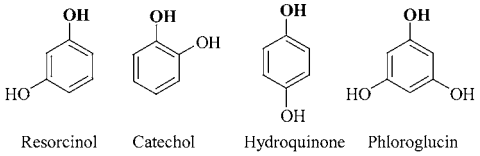
Chlorogenic acid

Riedel-de Haën (Seelze, Germany). Dihydrocaffeic acid, *o*-coumaric acid, *p*-coumaric acid, sinapic acid, chlorogenic acid, resorcinol, hydroquinone, 2,3- and 2,5-dihydroxybenzoic acids, gallic acid, quercetin, and morin were from Sigma Chemical Co. (St. Louis, MO). Ferulic acid, isoferulic acid, and 2,4-dihydroxybenzoic acid were from Aldrich Chemical Co. (Steinheim, Germany); *m*-coumaric acid and catechol were from Fluka Chemie (Buchs, Switzerland). Rosmarinic acid was from Röth (Karlsruhe, Germany). Phloroglucin was from Merck (Darmstadt, Germany). NaCl, KH_2PO_4, Na_2HPO_4, and KCl used for preparation of phosphate-buffered saline (PBS) were from Panreac Chimica S.A. (Barcelona, Spain). ABTS diammonium salt, DPPH, and potassium persulfate were from Sigma. Absolute ethanol of HPLC grade was from Riedel-de Haën.

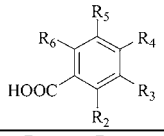
Apparatus. A U-2000 Hitachi spectrophotometer (Tokyo, Japan) was used for the measurement of the reduction of $ABTS^{\bullet+}$ absorbance at 734 nm and DPPH at 516 nm.

Estimation of Radical Scavenging Activity (RSA) Using the DPPH Assay. The %RSA activity of phenols was determined using

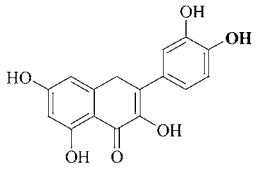
Chart 2. Polyphenols Examined



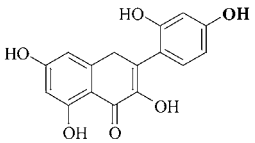
Resorcinol Catechol Hydroquinone Phloroglucin



Polyhydroxybenzoates	R_2	R_3	R_4	R_5	R_6
2, 3	OH	**OH**	H	H	H
2, 4	OH	H	**OH**	H	H
2, 5	OH	H	H	**OH**	H
3, 4	H	OH	**OH**	H	H
3, 4, 5	H	OH	**OH**	OH	H



Quercetin



Morin

the DPPH according to ref *16*. The decrease of the absorption at 516 nm of the DPPH solution after addition of the antioxidant (AH) was measured in a cuvette; 2960 μL of 0.1 mM ethanolic DPPH solution was mixed with 40 μL of a 1.85 mM AH solution so that the relative concentration of AH to the stable radical (moles of AH per mole of DPPH) in the cuvette was 0.25. The absorption was monitored at the start and after 20 min. The results are expressed as $\%RSA = [Abs_{516\ nm}(t = 0) - Abs_{516\ nm}(t = t')] \times 100/Abs_{516\ nm}(t = 0)$. Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions.

Estimation of TEAC Activity Using the $ABTS^{\bullet+}$ Assay. The $ABTS^{\bullet+}$ solution was prepared by reaction of 5 mL of a 7 mM aqueous ABTS solution and 88 μL of a 140 mM (2.45 mM final concentration) potassium persulfate ($K_2S_2O_8$) solution as proposed in ref *6*. After storage in the dark for 16 h, the radical cation solution was further diluted in ethanol until the initial absorbance value of 0.7 ± 0.05 at 734 nm was reached. Solutions of each phenol under study were prepared in ethanol so that their final concentration after the addition of 10 μL to the radical solution (2 mL) was 0−15 μM and a 20−80% decrease in the initial absorbance of the reaction solution was achieved. The decrease in absorbance was recorded at 0 and after 6 min. Graphs

Table 1. TEAC Values for Cinnamic Acid Derivatives in Different Media and Theoretical Parameters Calculated Using the B3LYP Functional at the 6-31G (d) Level

	exptl investigation		theor investigation		
AH	$TEAC_{eth}$[a]	$TEAC_{buf}$[a]	O–H BDE_{eth}[b] (kcal/mol)	IP_{p-eth}[b] (kcal/mol)	IP_{a-buf}[c] (kcal/mol)
dihydrocaffeic acid	1.52 ± 0.04aA	1.48 ± 0.06aA	72.63	120.63	124.93
rosmarinic acid	2.13 ± 0.09bA	2.18 ± 0.08bA	72.70[d]	120.98	126.32
			75.62[e]		
caffeic acid	1.01 ± 0.05cA	1.15 ± 0.09cA	73.95	125.87	126.33
chlorogenic acid	0.95 ± 0.10cA	1.03 ± 0.05dA	72.69	122.67	133.07
sinapic acid	1.27 ± 0.07dA	2.09 ± 0.11bB	70.49	121.24	127.99
ferulic acid	1.32 ± 0.07dA	1.97 ± 0.02eB	75.08	124.46	128.12
isoferulic acid	0.82 ± 0.01cA	1.18 ± 0.03cB	76.88	125.29	128.07
o-coumaric acid	1.05 ± 0.02cA	0.97 ± 0.05dA	78.08	133.98	133.19
m-coumaric acid	0.87 ± 0.03cA	0.90 ± 0.03dA	82.39	136.03	128.39
p-coumaric acid	2.00 ± 0.12bA	2.39 ± 0.09fB	79.86	130.43	132.45
trans-cinnamic acid	0	0	102.73	144.34	128.61

[a] Mean values of three different experiments ± SD. Different lower case letters indicate significantly different values within each column at $p < 0.05$. Different upper case letters within each row are significantly different at $p < 0.05$. [b] Data for parent molecule in ethanol. [c] Data for anions in buffer. [d] Data for 1-OH. [e] Data for 2-OH (see **Chart 1**).

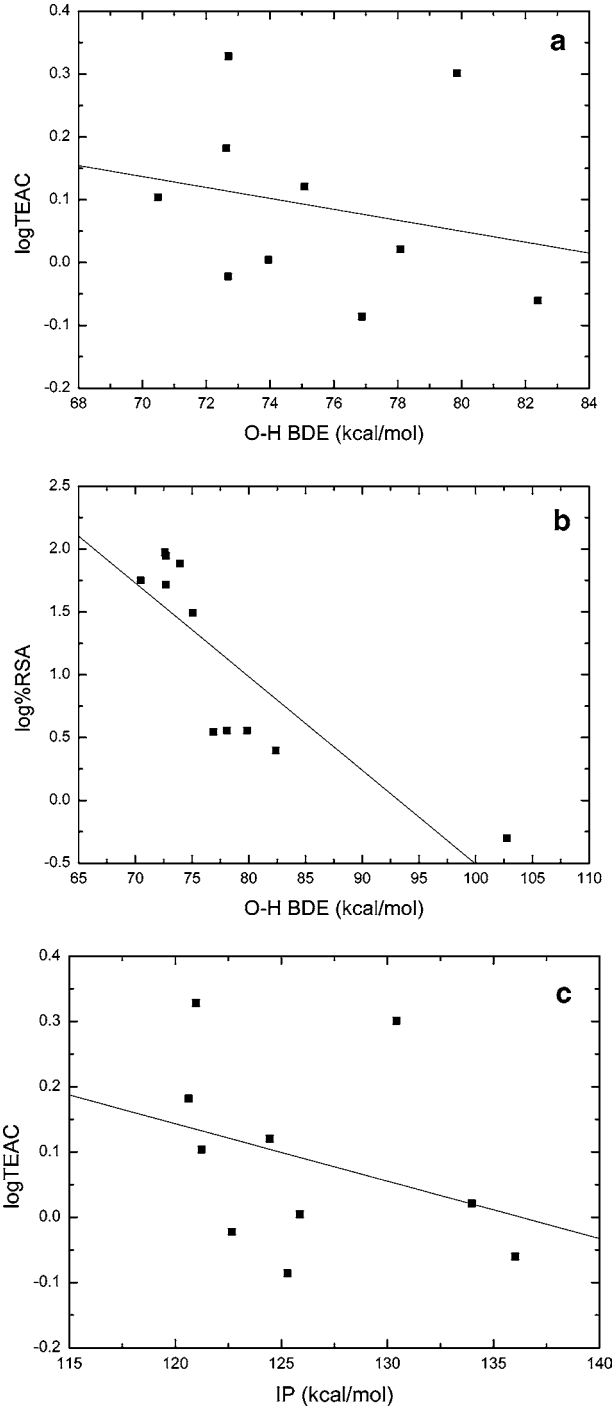


Figure 1. Correlation between (**a**) log TEAC and O–H BDE ($r = -0.23$, $P = 0.53$), (**b**) log %RSA and O–H BDE ($r = -0.84$, $P < 0.002$), and (**c**) log TEAC and IP in ethanol for cinnamic acid derivatives ($r = -0.34$, $P = 0.34$).

of antioxidant concentration versus percent absorbance reduction were then constructed. The concentration of Trolox giving the same percentage reduction of absorbance at 734 nm as the 1 mM antioxidant solution was calculated from the three point graphs. The results were thus expressed as Trolox equivalent antioxidant capacity (TEAC) values. For each molecule and each concentration, measurements were made in triplicate. All tests were performed in triplicate. For hydroxycinnamic acid derivatives experiments were also carried out using a buffer solution (PBS) with a pH value of 7.4 to dilute the free radical and prepare stock solutions of the acids. For each molecule and each concentration, measurements were made in triplicate. All tests were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions.

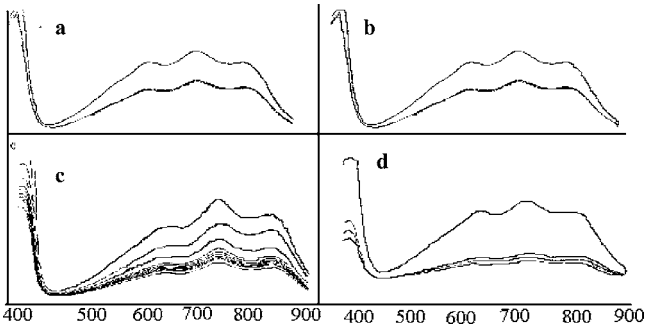


Figure 2. $ABTS^{\bullet+}$ spectra recorded at 0 and 10 s and 1–6 min after the addition of AH: (**a**) caffeic acid in ethanol; (**b**) caffeic acid at pH 7.4; (**c**) *p*-coumaric acid in ethanol; (**d**) *p*-coumaric acid at pH 7.4. The final concentration of the AH was 10 μM.

DFT Study. For the antioxidants under investigation the O−H bond dissociation enthalpies (BDEs) 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 (*17*). Then, single-point electronic energies were obtained by density functional theory (DFT) using the B3LYP functional on the 6-31G(d) level. Employing the total electronic energies (TEs) and the zero-point vibrational energies (ZPVEs, scaled by a factor of 0.973) (*18*), IP values were calculated using the equation $IP = (TE_c + ZPVE_c \times 0.973) - (TE_p + ZPVE_p \times 0.973)$. The first term in this equation stands for the cation radical generated after electron transfer, whereas the second corresponds to the parent molecule. Employing the molecular enthalpy in the gas phase at 298 K [which is the sum of B3LYP/6-31G(d) calculated TE, AM1 calculated ZPVE, and vibrational contribution to energy, scaled by a factor of 0.973, translational, rotational, and PV-work terms], the O−H BDE values were calculated using the equation $BDE = H_r + H_h - H_p$. In the equation H_r is the enthalpy for the radical generated after the H-abstraction reaction, H_h is the enthalpy for the hydrogen atom (−0.49792 hartree), and H_p is the enthalpy for the parent molecule. The solvent effects on O−H BDE and IP were taken into account by employing the self-consistent reaction field (SCRF) method with polarized continuum model (PCM). After the most stable conformation had been determined, it was possible to decide which OH would be the H-atom-abstraction target, on the basis of the respective O−H BDE values. For phenolics containing a catechol moiety, the intramolecular hydrogen bond (IHB) was considered to give the most stable isomer, as IHB efficiently stabilizes the conformation. The active centers are shown in bold in **Charts 1** and **2**. In the case of rosmarinic acid two reactive centers were assigned (1 OH, 2 OH). All calculations were performed using GAUSSIAN 98 (*19*).

Statistical Analysis. Statistical comparisons of the mean values for each experiment were performed by one-way ANOVA, followed by Duncan's multiple-range test ($p < 0.05$ confidence level) using SPSS 7.5 software.

RESULTS AND DISCUSSION

The compounds examined in this study were selected on the basis of structural characteristics such as the number and position of the hydroxyl groups. The activity of the compounds toward the $ABTS^{\bullet+}$ radical was tested in ethanol, an environment that does not favor deprotonation of the tested compounds and in certain cases in PBS at pH 7.4. The ability of compounds to scavenge the DPPH was also estimated when necessary. The possible interference of reaction products to the TEAC of the compounds under investigation was monitored through spectra recording in the region of 400−900 nm. Furthermore, molecular descriptors such as the BDE and IP values that are used to estimate the H-atom- and electron-donating ability of an AH, respectively (*9*, *14*, *15*), were also calculated theoretically to complement the experimental findings.

Table 2. %RSA and TEAC Values for Selected Polyphenols and Theoretical Parameters Calculated Using the B3LYP Functional at the 6-31G (d) Level

	exptl investigation		theor investigation	
AH	%RSA[a]	TEAC[b]	O–H BDE_{eth}[c] (kcal/mol)	IP_{p-eth}[c] (kcal/mol)
simple polyphenols				
resorcinol	2.7 ± 0.8a	1.14 ± 0.04a	81.07	127.42
catechol	65.1 ± 0.8b	0.97 ± 0.02b	73.91	127.35
hydroquinone	48.6 ± 0.4c	0.68 ± 0.03c	73.92	120.19
phloroglucin	3.7 ± 0.6a	1.63 ± 0.03d	81.84	126.80
polyhydroxybenzoates				
2,3	75.2 ± 2.9d	1.94 ± 0.07e	78.53	130.80
2,4	2.9 ± 0.7a	0.63 ± 0.09c	87.00	136.86
2,5	82.1 ± 1.0e	1.81 ± 0.02f	77.66	126.28
3,4	68.7 ± 0.8f	0.84 ± 0.07g	76.11	131.38
3,4,5	96.3 ± 0.4g	2.18 ± 0.04h	73.74	128.34
flavonoids				
quercetin	68.2 ± 1.0f	1.85 ± 0.08f	71.96	120.96
morin	43.2 ± 0.8h	1.20 ± 0.03a	81.46	121.97

[a] Mean values of three measurements ± SD. [b] Mean values of three different experiments ± SD. Different lower case letters indicate significantly different values within each column at $p < 0.05$. [c] Data for parent molecule in ethanol.

Cinnamic Acid Derivatives. This group was composed of AH known for their excellent antioxidant activity as well as compounds known to present mediocre or even no activity (*20*). The examined compounds were rosmarinic, caffeic, dihydrocaffeic, chlorogenic, sinapic, ferulic, isoferulic, *o*-, *m*-, and *p*-coumaric, and cinnamic acids (**Chart 1**). Their activity has been found to be in line with the number and position of hydroxyl groups when the DPPH assay was used (*16*). Thus, the relative order of activity on the basis of %RSA values was dihydrocaffeic (93.9), rosmarinic (88.4), caffeic (76.6), sinapic (56.1), chlorogenic (52.0), ferulic (30.9), isoferulic (3.5), *o*-coumaric (3.6), *p*-coumaric (3.6), *m*-coumaric (2.5), and cinnamic (0.5). However, using the $ABTS^{\bullet+}$ assay, serious discrepancies from the above order of activity were evidenced. The estimated TEAC values are given in **Table 1**.

A close inspection of the TEAC values shows that all of the compounds possessing at least one hydroxyl group in the aromatic ring were considerably active toward $ABTS^{\bullet+}$. Moreover, the relative activity differences among the compounds, in comparison to those observed in the DPPH assay, were rather suppressed. In particular, the most active AH (rosmarinic acid) toward $ABTS^{\bullet+}$ was only 2.6 times more efficient than the least active one (isoferulic acid), but in the case of the DPPH assay, the respective ratio was almost 38 (dihydrocaffeic acid/*m*-coumaric acid). An increase in the number of hydroxyl groups in the aromatic ring did not necessarily lead to an increase of the TEAC values. For instance, AH such as the coumaric acids and isoferulic acid, found to be inactive toward the DPPH, were significantly active toward $ABTS^{\bullet+}$ and even more active than certain diphenolic counterparts. Specifically, *p*-coumaric acid was almost equally active to rosmarinic acid, and *o*- and *m*-coumaric and isoferulic acids were almost equal in activity to caffeic acid. This finding, which was in line with those reported in ref *6* for ferulic, caffeic, and *p*-coumaric acids, is confusing because monophenols are known to be less active as scavengers than polyphenols. This may be attributed to the mechanism of reaction of AH with the $ABTS^{\bullet+}$, which is rather unclear.

Because the BDE is a molecular descriptor related to the hydrogen atom donating ability and the IP to their electron-donating ability, their values were calculated in an effort to highlight the behavior of hydroxycinnamates (**Table 1**). Prior to calculation of the molecular descriptors, the molecular structure of each AH was optimized. The optimization was carried out using the AM1 model. The selection was based on previous findings that the resulting geometry with AM1 was in agreement with that derived from a B3LYP/6-31G(d,p) optimization with an error of 0.01 (*21*, *22*). Thus, the combined AM1/DFT calculation methodology was employed as accurate and less time-consuming and suitable for the objective of this study.

As shown in **Figure 1a**, no obvious linear relationship between BDE and TEAC values was found, although the BDE values correlate well with %RSA (**Figure 1b**). In addition, the IP values could not correlate with the TEAC values (**Figure 1c**). For instance, despite the fact that the IP value for rosmarinic acid was among the lowest, consistent with its highest activity, the rather high IP value for *p*-coumaric acid could not justify its high TEAC value. Likewise, sinapic acid, although less active than *p*-coumaric acid, had a lower IP value than the latter.

Effect of Reaction Environment. Taking into account experimental pK_a values reported for phenolic acids in methanol (i.e., benzoic acid, 3-hydroxybenzoic acid, and 4-hydroxybenzoic acid with $pK_a = 9.3$, 9.58, and 9.99, respectively) (*23*), it is expected that the phenolic acids should remain undissociated also in ethanol. In this way any influence on the TEAC values of hydroxycinnamates due to formation of anions is not expected to occur under the present experimental conditions. Anions, in general, are reported to influence the radical scavenging reaction kinetics (*24*, *25*). Because cinnamic acids are expected to be fully deprotonated at pH 7.4, the activity of the compounds was also tested under such conditions. In the buffered aqueous environment, the electron-donating effect of $-CH{=}CH{-}COO^-$, instead of the electron-withdrawing effect of the nondissociated form, could influence the relative activity of the acids to a certain extent. The respective TEAC values given in **Table 1** differed slightly in the two media, and a poorer correlation was observed between the logarithm of TEAC and IP in buffer ($r = -0.21$, $P = 0.56$).

Effect of Reactivity of Reaction Products. Recently, the unexpected activity of phenolic compounds toward $ABTS^{\bullet+}$ has been related to the formation of side products that contribute to the overall TEAC values (*12*). This view was supported on the grounds of examination of the spectra of the $ABTS^{\bullet+}$ solution during the reaction with AH. None of the hydroxycinnamic acid derivatives seemed to obey such a behavior as is illustrated for caffeic and *p*-coumaric acids (**Figure 2**). Similar observations for the two compounds were also made when tested

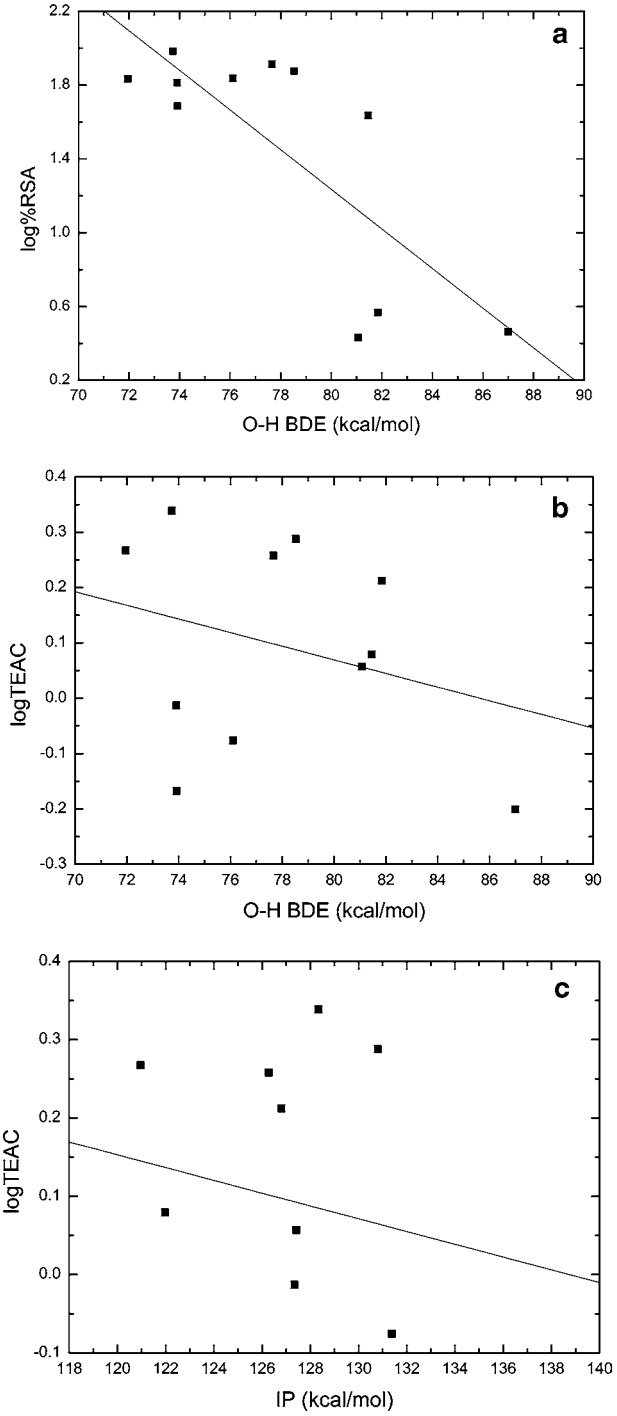


Figure 3. Correlation between (**a**) log %RSA and O–H BDE ($r = -0.78$, $P < 0.01$), (**b**) log TEAC and O–H BDE ($r = -0.29$, $P = 0.38$), and (**c**) log TEAC and IP for polyphenols in ethanol ($r = -0.21$, $P = 0.54$).

in an aqueous environment (pH 7.4). However, spectrum recording revealed that the reaction with the radical continued over the period of 6 min for all of the AH with the unique exception of caffeic acid. The latter seems to react almost instantly.

Polyphenols. As stated previously Arts and co-workers (*12*) reported alterations in the spectra of $ABTS^{\bullet+}$ mainly after reaction with resorcinol and to a lesser extent with catechol or hydroquinone. Because in the group of hydroxycinnamates no alterations in the $ABTS^{\bullet+}$ spectra were evidenced, we decided to study compounds, namely, phloroglucin, polyhydroxybenzoates, and flavonoids (**Chart 2**), that had structural characteristics similar to those of the three diphenols mentioned above. Thus, the focus was on the relative position of the hydroxyl groups in the aromatic ring.

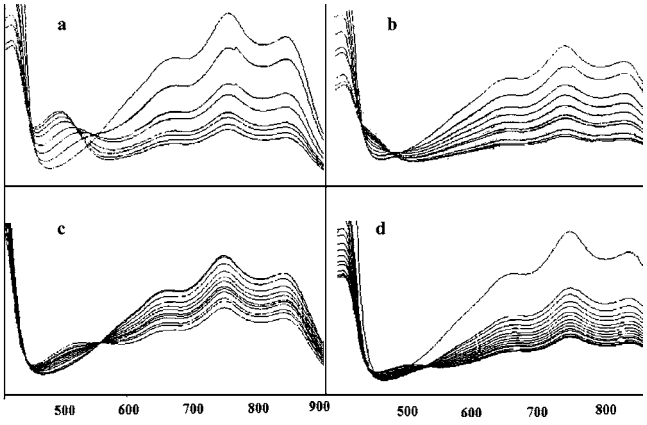


Figure 4. $ABTS^{\bullet+}$ spectra recorded at 0 and 10 s and 1–6 min after the addition of AH: (**a**) resorcinol; (**b**) phloroglucin; (**c**) 2,4-dihydroxybenzoic acid; (**d**) morin. The final concentration of the AH was 10 μM, and the solvent was ethanol.

Effect of the Relative Position of the Hydroxyl Groups in the Aromatic Ring. To determine whether the selected compounds followed the commonly accepted SAR principles, their reactivity was initially tested toward the DPPH. The %RSA values presented in **Table 2** indicated that the order of activity was the expected one and that compounds possessing hydroxyl groups having only electron-withdrawing properties were almost inactive. When the TEAC values were determined, all of the AH were found to be active. Discrepancies in the order of activity were evidenced in the case of the simple polyphenols, as resorcinol and phloroglucin were more active than catechol and hydroquinone. This observation was not verified in the case of polyhydroxybenzoates, where the relative order of scavenging activity was the same as that based on %RSA values. The electron-withdrawing *meta*-hydroxyl group was not found to affect positively the activity of morin in comparison to that of quercetin.

Once more, the calculated BDE values in ethanol were in line with the relative order of radical-scavenging ability (%RSA) (**Figure 3a**). However, the BDE and IP values in the same solvent could not support the activity of the AH as estimated using the $ABTS^{\bullet+}$ assay (**Figure 3b**,**c**).

Effect of Reactivity of Reaction Products. Spectrum monitoring during the reaction with the $ABTS^{\bullet+}$ indicated that alterations in the spectra occurred only for the compounds that had electron-withdrawing hydroxyl groups, that is, resorcinol, phloroglucin, 2,4-dihydroxybenzoic acid, and morin (**Figure 4**). Notable alterations in the spectra were observed only for resorcinol but not for phloroglucin, which bears more electron-withdrawing groups than the former. Moreover, the reaction of all polyphenols with the radical continued within the 6 min monitoring period with no exception as was observed for caffeic acid.

In conclusion, AH, expected to be inactive due to commonly accepted SAR, were proven to be as efficient as or even more potent than well-known antioxidants. It can be argued that the $ABTS^{\bullet+}$ assay may give an indication for the presence of antioxidants in an unknown mixture, but a SAR cannot be readily inferred. Moreover, considering that the $ABTS^{\bullet+}$ needs to be produced in the laboratory and is not very stable (fresh working solution is prepared almost every 2 h), its application seems to have more drawbacks. Still, the simplicity and rapidity,

as well as the applicability, of the method to both lipophilic and polar AH seem to attract the interest of some investigators.

ABBREVIATIONS USED

AH, phenolic antioxidants; AM1, Austin model 1; BDE, bond dissociation enthalpy; B3LYP, Becke's 3 Lee Yang Parr correlation functional; DFT, density functional theory; IP, ionization potential; %RSA, percent radical scavenging activity; SAR, structure−activity relationship; SCRF, self-consistent reaction field; PCM, polarized continuum model; TEAC, Trolox equivalent antioxidant capacity; TE, total energy; ZPVE, zero-point vibrational energy.

LITERATURE CITED

(1) Blois, M. S. Antioxidant determination by the use of a free radical. *Nature* **1958**, *26*, 1199−1200.

(2) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **1995**, *28*, 25−30.

(3) Arnao, M. B.; Cano, A.; Acosta, M. Methods to measure the antioxidant activity in plant material. A comparative discussion. *Free Radical Res.* **1999**, *31*, 89−96.

(4) Arnao, M. B. Some methodological problems in the determination of antioxidant activity using chromogen radicals: A practical case. *Trends Food Sci. Technol.* **2000**, *11*, 419−421.

(5) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *7*, 933−956.

(6) Re, R.; Pellegrini, A.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(7) Bakalbassis, E. G.; Chatzopoulou, A.; Melissas, V. S.; Tsimidou, M.; Tsolaki, M.; Vafiadis, A. An *ab initio* and a DFT study for the explanation of the antioxidant activity of certain phenolic acids. *Lipids* **2001**, *36*, 181−190.

(8) Nenadis, N.; Boyle, S.; Bakalbassis, E. G.; Tsimidou, M. An experimental approach to structure−activity relationships of caffeic and dihydrocaffeic acids and related monophenols. *J. Am. Oil Chem. Soc.* **2003**, *80*, 451−458.

(9) Nenadis, N.; Zhang, H. Y.; Tsimidou, M. Z. Structure-antioxidant activity relationship of ferulic acid derivatives: effect of carbon side chain characteristic groups. *J. Agric. Food Chem.* **2003**, *51*, 1874−1879.

(10) Tyrakowska, B.; Soffers, A. E. M. F.; Szymusiak, H.; Boeren, S.; Boersma, M.; Lemanska, K.; Vervoort, J.; Rietjens, I. M. C. M. TEAC antioxidant activity of 4-hydroxybenzoates. *Free Radical Biol. Med.* **1999**, *27*, 1427−1436.

(11) Lemanska, K.; Szymusiak, H.; Tyrakowska, B.; Zielinski, R.; Soffers, A. E. M. F.; Rietjens, I. M. C. M. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Radical Biol. Med.* **2001**, *31*, 869−881.

(12) Arts, M. J. T. J.; Dallinga, J. S.; Voss, H. P.; Haenen, G. R. M. N.; Bast, A. A critical appraisal of the use of the antioxidant capacity (TEAC) assay in defining optimal antioxidant structures. *Food Chem.* **2003**, *80*, 409−414.

(13) Pannala, A. S.; Chan, T. S.; O'Brien, P. J.; Rice-Evans, C. A. Flavonoid B-ring chemistry and antioxidant activity: Fast reaction kinetics. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 1161−1168.

(14) Zhang, H. Y.; Sun, Y. M.; Chen, D. Z. O−H bond dissociation energies of phenolic compounds are determined by field/inductive effect or resonance effect? A DFT study and its implication. *Quant. Struct.-Act. Relat.* **2001**, *20*, 148−152.

(15) Zhang, H. Y.; Sun, Y. M.; Wang, X. L. Substituent effects on O−H bond dissociation enthalpies and ionization potentials of catechols: A DFT study and its implications in rational design of structure−activity relationships for flavonoid antioxidants. *Chem.-Eur. J.* **2003**, *9*, 502−508.

(16) Nenadis, N.; Tsimidou, M. Observations on the estimation of scavenging activity of phenolic compounds using rapid 1,1-diphenyl-2-picrylhydrazyl (DPPH) tests. *J. Am. Oil Chem. Soc.* **2002**, *12*, 1191−1195.

(17) Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. P. AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **1985**, *107*, 3902−3909.

(18) Wright, J. S.; Johnson, E. R.; DiLabio, G. A. Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J. Am. Chem. Soc.* **2001**, *123*, 1173−1183.

(19) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*, revision A.11; Gaussian, Inc.: Pittsburgh, PA, 2001.

(20) Yanishlieva, N. Y. Inhibiting oxidation. In *Antioxidants in Food*; Pokorny, J., Yanishlieva, N., Gordon, M., Eds,; Woodhead: Cambridge, U.K., 2001; pp 22−70.

(21) Wright, J. S.; Carpenter, D. J.; McKay, D. J.; Ingold, K. U. Theoretical calculation of substituent effects on the O−H bond strength of phenolic antioxidants related to vitamin E. *J. Am. Chem. Soc.* **1997**, *119*, 4245−4252.

(22) Sun, Y. M.; Chen D.; Liu, C. Evaluation of the effectiveness of AM1 geometry used in calculating O−H bond dissociation enthalpy. *J. Mol. Struct.* (*THEOCHEM*) **2002**, *618*, 181−189.

(23) Rived, F.; Roses, M.; Bosch, E. Dissociation constants of neutral and charged acids in methyl alcohol. The acid strength resolution. *Anal. Chim. Acta* **1998**, *374*, 309−324.

(24) Zhang, H. Y.; Ge, N.; Zhang, Z. Y. Theoretical elucidation of activity differences of five phenolic antioxidants. *Acta Pharmacol. Sin.* **1999**, *20*, 363−366.

(25) Litwinienko, G.; Ingold, K. U. Abnormal solvent effects on hydrogen atom abstractions. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (DPPH) in alcohols. *J. Org. Chem.* **2003**, *68*, 3433−3438.

Received for review January 7, 2004. Revised manuscript received April 27, 2004. Accepted April 27, 2004.

JF0400056