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MODIFIED TEAC TEST FOR DETERMINATION OF THE ANTIOXIDANT PROPERTIES OF DIETARY POLYPHENOLIC COMPOUNDS OVER A WIDE pH RANGE

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In this study the effect of additional hydroxyl or metoxyl substituent, at *ortho* position to OH group, on the radical scavenging antioxidant activity and on pKa as well as on electron and hydrogen donating abilities of structurally related 4-hydroxybenzoic acid, 4-hydroxycynnamic acid, 4',3,5,7-tetrahydroxyflavone, and 4',3,5,7-tetrahydroxyanthocyanidins was investigated experimentally and also based on computer calculations. Antioxidant activity of the polyphenolic compounds over a wide pH range (4.5-9.5) was quantified using the modified TEAC assay. The results show significant pH-dependence on the radical scavenging antioxidant activity of polyphenolic compounds studied and reveal that important factors influencing TEAC antioxidant activity of polyphenolic antioxidants are not only the number and position of hydroxyl moieties in the molecule, but also their protonation state, influenced by the pKa of hydroxyl moieties, the pH of the surrounding medium, the effect of intramolecular hydrogen bridges, and *O*-methylation of hydroxyl groups. The results obtained demonstrate the applicability of the modified TEAC assay for studying the pH-dependent effects on the radical scavenging antioxidant activity of (poly)phenolic antioxidants. Moreover, this assay allows the determination of the TEAC value for the neutral form as well as for deprotonated form of (poly)phenolic compound, which is a proper basis for studying the mechanism of antioxidant action by comparison to calculated electronic parameters.

INTRODUCTION

Phenolic antioxidants such as hydroxybenzoates, hydroxycinnamates and flavonoids are important classes of natural antioxidants [Rice-Evans *et al.,* 1996; Miller & Rice-Evans, 1997a; Jovanovic *et al.*, 1994, 1996, 1997; Cao *et al.,* 1997]. They are an integral part of human diet as they occur ubiquitously in food of plant origin. There is an evidence that a polyphenolic-rich diet inversely correlates with the risk of coronary heart disease or carcinogenesis and that the antioxidant properties of polyphenols may contribute to this chemoprotective effect [Middleton & Kandaswami, 1994; Hollman & Katan, 1997; Prior & Cao, 2000]. The antioxidant efficiency of the phenolic acids and flavonoids has been related to the number of hydroxyl groups in the molecule and also to their hydrogen donating abilities [Rice-Evans *et al.*, 1996; Miller & Rice-Evans, 1997b; Cao *et al.*, 1997; Couvelier *et al.*, 1992].

Several assays have been introduced for the measurements of the antioxidant activity of a single compound and/or complex mixtures [Tyrakowska *et al*., 1999; Miller *et al*., 1993; Rice-Evans & Miller, 1994]. However, chemically different methods for measuring antioxidant activity produce different hierarchies of antioxidants, which is difficult to compare and interpret.

The intrinsic antioxidant capacity of polyphenolic compounds is often quantified by the so-called TEAC value, which is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\bullet+}$ (2,2'-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water soluble vitamin E analogue, Trolox® [Miller *et al.*, 1993; Rice-Evans & Miller, 1994]. This method was introduced and accepted in the field a few years ago for measuring antioxidant activities of many phytochemicals, synthetic compounds as well as food components, food extract and beverages [Rice-Evans *et al.*, 1995; Miller *et al.*, 1995, 1996; Strube *et al.*, 1997; Cao & Prior, 1998; van den Berg *et al.*, 1999; Proteggente *et al.*, 2002]. Furthermore, the method has been shown to result in TEAC values that, especially for a series of carotenoid model compounds, correlate with outcomes of other chemical assays for the antioxidant characterization, such as for example the degradation of the antioxidant by various types of radicals (peroxy, Fenton, and phenoxyl radicals) as well as with the potential to prevent the formation of example thiobarbituric acid reactive substances in multilamellar liposomes [Soffers *et al.*, 1999]. The TEAC values are generally measured at pH values of 7.4. This implies that the value reflects the intrinsic antioxidant capacity of the antioxidants at a physiological pH range. From a mechanistic point of view, however, this implies that TEAC values as presented in the literature may not reflect the intrinsic antioxidant activity of the compound in its neutral state. This holds especially for the phenolic antioxidants where introduction of additional hydroxyl moieties may affect the pKa of the hydroxyl moieties, resulting in deprotonation at physiological pH. In spite of this, the possible influence of OH group on pKa values and OH group deprotonation on the antioxidant potential is generally not taken into account. In recent studies we demonstrated that

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the antioxidant activity of the other hydroxybenzoates and hydroxyflavones [Tyrakowska *et al.*,1999; Lemańska *et al.*, 2001] is dependent on pKa of OH group deprotonation.

The objective of the presented study was to investigate the effect of additional hydroxyl or methoxyl substituent, at *ortho* position, on the radical scavenging antioxidant activity and pKa as well as electron and hydrogen donating abilities of hydroxybenzoic and hydroxycinnamic acids as well as hydroxyflavones and anthocyanidins, representing active antioxidants from the series of phenolic acids and flavonoids, respectively. The approach chosen was a combination of experimental studies and quantum mechanical calculations, where experimental data for OH group deprotonation and the TEAC antioxidant activities for the neutral form of phenolic compounds were obtained and compared to calculated parameters for hydrogen atom and electron donation. Radical scavenging antioxidant activity of selected polyphenolic compounds was quantified using the modified TEAC assay [Tyrakowska *et al.*, 1999]. In this modified TEAC assay, microperoxidase-8 (MP8) instead of methmioglobin is used to generate the $ABTS^{\bullet+}$. The major advantage of this assay is that it enables studies of radical scavenging antioxidant activity over a wide pH range (4.5-9.5) in contrast to many other commonly used assays such as ORAC, FRAP, TRAP, TEAC/mioglobin [Arnao *et al.*, 1999]. Using the modified TEAC assay, the effect of pH on the radical scavenging antioxidant activity was investigated for several relevant hydroxyphenolic acids and flavonoids (shown in Figure 1) to obtain better insight in the factors affecting TEAC antioxidant activity and mechanism of action of polyphenolic antioxidants.

MATERIALS AND METHODS

Materials. All hydroxybenzoic and hydroxycinnamic acids (p-hydroxybenzoic, protocatechuic, vanillic, p-coumaric, caffeic and ferulic) were purchased from Sigma (St. Louis, MO, USA). Quercetin, Trolox® and microperoxidase-8 (MP-8) were obtained from Aldrich (Steinheim, Germany). Isorhamnetin was obtained from Indofine Chemical (Somerville, NJ, USA). Anthocyanidins (pelargonidin, cyanidin and malvidin) were purchased from Extrasynthese (Genay, France). Kaempferol and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) were purchased from Fluka (Buchs, Switzerland). Hydrogen peroxide (30%), ethanol, methanol (HPLC grade) and buffer substances (analytical reagent grade) were obtained from Merck (Darmstad, Germany).

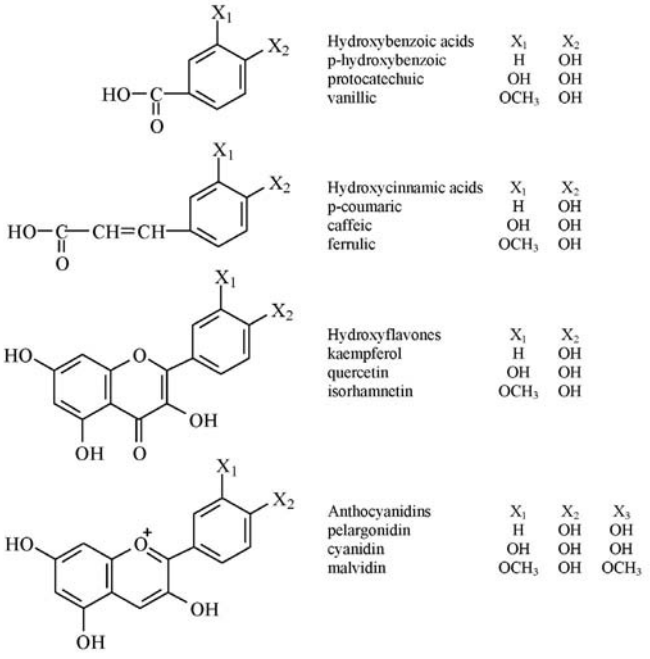

FIGURE 1. Chemical formulas and names of the model compounds studied.

TEAC assay. The antioxidant activity of the compounds studied was measured by TEAC assay performed essentially as described by Miller *et al.* [1993] and Rice-Evans & Miller [1994] with some modifications introduced by Tyrakowska *et al.* [1999]. Determination of the TEAC value is based on the ability of the antioxidant to scavenge the $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue – Trolox®.

Microperoxidase-8 (MP8) instead of metmyoglobin, was used to generate the $ABTS^{\bullet+}$ in PBS (potassium phosphate buffered saline) pH 7.4.

The $ABTS^{\bullet+}$ radical cation was made by the MP8 (f. conc. 0.2 µmol/L)/H_2O_2 (f. conc. 0.1 mmol/L) system by incubating MP8/ H_2O_2 with ABTS (f. conc. 3.0 mmol/L) in PBS, pH=7.4 for a few min (30°C) resulting in a solution in which no further reaction occurred. In such H_2O_2 driven MP8 reactions the MP8 catalyst has previously been shown to be completely inactivated upon 1-min incubation [Spee *et al.*, 1996; van Haandel *et al.*, 2000]. After generation of the $ABTS^{\bullet+}$ radical cation and inactivation of MP8, the solution was diluted to give the absorbance of about 0.6 at 734 nm. This dilution was carried out using 0.2 mol/L sodium acetate or potassium phosphate of various pH values to give $ABTS^{\bullet+}$ solutions at pH values varying between 4.5-9.5. This solution, which has a stable absorbance at 734 nm for over 2 h, was used for the TEAC assay measurements in which the antioxidants were added as 1% (v/v) of a 100 times concentrated stock solution in methanol or ethanol to give the final concentration required. The decrease in absorbance caused by the antioxidant compound, measured at 6 min, reflects the ABTS radical scavenging capacity and was plotted against the concentration of the antioxidant. The linear correlation obtained for this plot allows the assumption that the decrease in absorbance reflects especially the reaction between the $ABTS^{\bullet+}$ radical cation and the antioxidant and it is not significantly affected by possible side reactions. The TEAC value represents the ratio between the slope of this plot for scavenging of $ABTS^{\bullet+}$ by the antioxidant under investigation, compared to the slope of plot for $ABTS^{\bullet+}$ scavenging by Trolox®, used as an antioxidant standard.

Quantum-chemical calculations. All geometries were optimized with the B3LYP method a hybrid functional of density functional theory (DFT) by using a 6-31G (d) basis set as implemented in the Gaussian 98 computational package. Single-point energies were then evaluated by using a higher 6-311G (d,p) basis set. The calculated deprotonation energies (DE), ionization potentials (IP) and the O-H bond dissociation

TABLE 1. TEAC values for the series of 4-hydroxybenzoates, 4-hydroxycinnamates, and flavonoid-type phenolic antioxidants and their pKa values.

Compound/substitution	TEAC pH 7.4	TEAC monoanion/neutral*	pKa values
4-HYDROXYBENZOIC ACIDS			
4-OH	0.08 ± 0.01	0.00	9.3 [Mac Faul *et al*., 1996]
3,4-*di*OH	1.50 ± 0.03	1.12 ± 0.05	8.8 [Eppink *et al*., 1997]
3-OCH_3-4-OH	1.20 ± 0.05	0.00	9.2 [Eppink *et al*., 1997]
4-HYDROXYCINNAMIC ACIDS			
4-OH	1.05 ± 0.08	0.10 ± 0.02	8.0**
3,4-*di*OH	1.53 ± 0.01	1.23 ± 0.10	7.6 [Jovanovic *et al*., 1994]
3-OCH_3-4-OH	1.50 ± 0.02	1.42 ± 0.15	8.1 [Sauerwald *et al*., 1998]
POLYHYDROXYFLAVONES			
4',3,5,7-*tetra*OH	1.40 ± 0.08	1.14 ± 0.04	8.2 [Jovanovic *et al*., 1994]
3',4',3,5,7-*penta*OH	4.20 ± 0.10	3.32 ± 0.14	7.03 [Sauerwald *et al*., 1998]
3'-OCH_3-4',3,5,7-*tetra*-OH	1.49 ± 0.30	1.06 ± 0.04	8.06***
ANTHOCYANIDINS			
4',3,5,7-*tetra*OH	1.39 ± 0.10		
3',4',3,5,7-*penta*OH	4.01 ± 0.12		
3',5-*di*-OCH_3-4',3,7-*tri*-OH	2.07 ± 0.14		

* values for monoanionic form for phenolic acids and the neutral form-for flavonoids were determined from the pH-dependent TEAC profiles taking the TEAC values at a pH two units below the pKa value; ** predicted based on the equation from Tyrakowska *et al*. [1999]; *** predicted based on the equation from Lemańska *et al*. [2001].

energies (BDE) for the O-H bond homolytic cleavage were not corrected for zero-point-energy assuming a negligible error and thus sparing computer time.

DE was calculated as the energy of the parent molecule minus the energy of the deprotonated anion. BDE for homolytic O-H bond cleavage was calculated as the energy of the neutral molecule minus the energy of the radical resulting from hydrogen atom abstraction. IP was calculated as the energy of neutral molecule minus the energy of cation radical resulting from electron donation.

RESULTS AND DISCUSSION

The antioxidant potency of naturally occurring phenolic acids as well as flavonoids is known to be strongly related to the structure, in particular to electron delocalization in the aromatic system [Cuvelier *et al*., 1992; Rice-Evans *et al*., 1996]. It can be expected that these compounds upon formation of a phenoxyl radical during oxidation are stabilized by the resonance effect of the aromatic ring when H atoms are substituted by any electron donating groups. In the present study derivatives of 4-hydroxybenzoic acid, of 4-hydroxycinnamic acid, 4'-hydroxyflavone and 4'-anthocyanidin in which H-atom at *ortho* position was substituted by hydroxyl or methoxyl group were chosen as a model compounds. Table 1 lists the various phenolic acid and flavonoid model compounds of the presented study, their TEAC values, as well as the pKa values of these phenolic antioxidants obtained form literature and/or predicted based on the previous study [Tyrakowska *et al*., 1999; Lemańska *et al*., 2001]. The results presented in Table 1 indicate that hydroxyflavones and anthocyanidins are generally more potent antioxidants than phenolic acids studied, whereas hydroxycinnamic acids are better antioxidant than corresponding hydroxybenzoic acids. This is because flavonoids are completely conjugated, which gives more stable phenoxyl radical due to a higher degree of electron delocalization [Bors & Saran, 1987; Rice-Evans *et al.,* 1996]. Moreover, the data indicate that additional hydroxyl group at *ortho* position increases TEAC antioxidant activity, more than methoxyl group. Finally these data reveal that, especially for catechol containing derivatives, pKa values are such that partial deprotonation of hydroxyl moieties of the antioxidant molecule can be expected to occur at physiologically relevant pH.

On the basis of quantum chemically calculated deprotonation energies of the OH group (Table 2), it was found that hydroxyflavones in the neutral form are more acidic than monoanions of cinnamic acids, which are more acidic than corresponding benzoates. As a result of computer-calculated deprotonation energy (DE) it was predicted that the hydroxyl groups in hydroxyflavones are able to deprotonate at lower pH range than hydroxyl groups in phenolic acids, which is in agreement with experimentally observed pKa values (Table 1).

In subsequent experiments the effect of pH on radical scavenging activity was investigated. This was done to obtain better insight in the factors determining the antioxidant activity of phenolic compounds at physiological pH. Our previous studies revealed a significant influence of pH on the antioxidant activity of some polyphenolics [Tyrakowska *et al*., 1999; Lemańska *et al*., 2001]. The presented study demonstrates that derivatives of benzoic acids, cinnamic acids, hydroxyflavones as well as anthocyanidins studied behave similarly showing an increase in the TEAC value with increasing pH of surrounding medium.

Figure 2 presents the effect of the pH on the TEAC antioxidant activity of model compounds containing catechol moiety: protocatechuic acid, caffeic acid, quercetin and

TABLE 2. Calculated deprotonation energy (DE) of OH group, bond dissociation energy (BDE) of OH group and ionization potentials (IP) for studied phenolic compounds in their neutral (N) COOH/OH or OH/OH, monoanionic (M) COO^-/OH, anionic (A) O-/OH and dianionic (D) COO^-/O^- forms. All values are given in kcal/mol. The number between brackets refers to the position of the OH group.

Compound/substitution	DE	BDE(N)	BDE(M)	IP(M)	IP(D)
4-HYDROXYBENZOIC ACIDS					
4-OH	431.5(4)	92.1(4)	78.6(4)	69.6	37.8
3,4-*di*OH	422.4(4)	82.6(4)	71.1(4)	71.3	36.2
3-OCH_3-4-OH	430.9(4)	90.1(4)	79.8(4)	69.9	36.0
4-HYDROXYCINNAMIC ACIDS					
4-OH	410.7(4)	87.9(4)	74.4(4)	67.1	21.2
3,4-*di*OH	401.5(4)	78.8(4)	67.5(4)	67.6	18.8
3-OCH_3-4-OH	411.0(4)	87.1(4)	75.5(4)	66.7	20.4
	DE	BDE(N)	BDE(A)	IP(N)	IP(A)
POLYHYDROXYFLAVONES					
4',3,5,7-*tetra*OH	338.7(7)	86.8(3)	74.1(4')	163.3	63.0
3',4',3,5,7-*penta*OH	331.6(4')	78.6(4)	73.7(7)	162.1	62.0
3'-OCH_3-4',3,5,7-*tetra*-OH	338.7(7)	86.9(3)	77.1(4')	159.8	70.1
ANTHOCYANIDINS					
4',3,5,7-*tetra*OH					
AH^+		87.4(3)	-	-	-
B		74.0(3)	65.4(7)	151.1	49.6
A_1		73.2(3)	65.3(3)	153.9	69.4
A_2		74.7(3)	67.1(3)	151.6	69.9
A_3		75.6(3)	67.1(3)	153.1	69.9
Z-Chalcone		80.0(4)		159.4	68.0
4',3',3,5,7-*penta*OH					
AH^+		85.3(3)	-	-	-
B		73.7(3)	65.2(7)	150.6	50.7
A_1		72.5(3)	66.1(3)	154.2	69.0
A_2		73.5(3)	67.4(3)	151.5	71.1
A_3		75.1(3)	67.4(3)	149.0	71.1
Z-Chalcone		79.9(4)		158.6	65.9
3',5-*di*-OCH_3-4',3,7-*tri*OH					
AH^+		84.3(3)	-	-	-
B		74.5(3)	64.3(7)	150.0	51.0
A_1		72.1(3)	65.6(3)	150.0	65.7
A_2		73.2(3)	67.5(3)	148.2	66.2
A_3		72.0(3)	67.3(3)	140.1	66.2
Z-Chalcone		80.2(4')		156.2	61.9

cyanidin the most potent antioxidant of the presented study. The antioxidant action of Trolox was previously shown to be unaffected over the whole pH range tested [Tyrakowska *et al*., 1999].

It can be seen that quercetin and cyanidin are much stronger antioxidants than protocatechuic- and caffeic acid over the whole pH range. The reason for the relatively high TEAC value of quercetin and cyanidin may be related to the fact that in these flavonoids two antioxidant active structural elements are present. Both the 3'4'-catechol moiety and also the chromane ring substituted by several OH groups contribute to the antioxidant activity. Exceptionally strong antioxidant activity of these flavonoids results from high stabilization of 4'$O^\bullet$ phenoxyl radical formed due to electron delocalization which is extended to the conjugated chromane system. Results presented in Figure 2 also show that the antioxidant activity of protocatechuic and caffeic acids is very similar in the whole pH range studied. It seems that in this case more important contribution to the observed antioxidant activity results from the presence of catechol moiety than from the presence of ethylenic group as in the case of cinnamic acids.

Figure 3 illustrates the effect of additional hydroxyl or methoxyl substituent, at *ortho* position on the pH-dependent TEAC antioxidant activity of 4-hydroxybenzoic and 4-hydroxycinnamic acid, 4',3,5,7-tetra-hydroxyflavone and 4',3,5,7-tetra-anthocyanidin. It can be seen that the additional hydroxyl group at *ortho* position strongly increases antioxidant activity while methoxyl group generally to a lesser degree increases ABTS radical scavenging activity of compounds studied when compared to reference compounds. It seems that *o*-methylation of the OH group in the catechol-containing phenolic derivatives results in a decrease in the

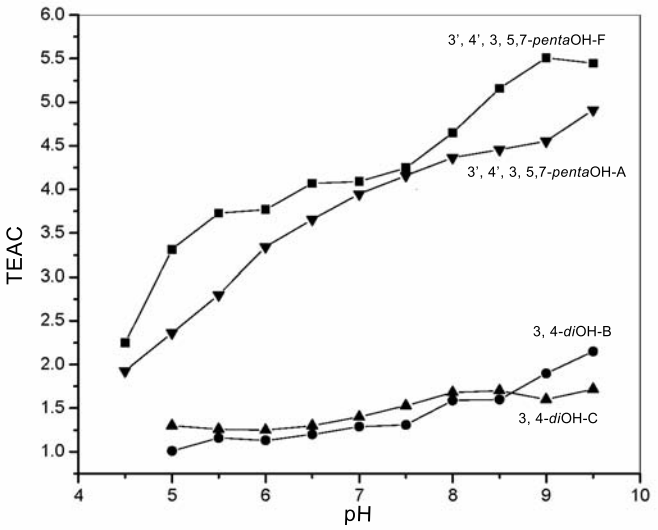

FIGURE 2. Effect of pH on the TEAC values of catechol containing derivatives of hydroxybenzoic acids (3,4-*di*OH-B), hydroxycinnamic acid (3,4-*di*OH-C), hydroxyflavones (3',4',3,5,7-*penta*OH-F) and anthocyanidins (3',4',3,5,7-*penta*OH-A).

TEAC antioxidant activity, because the important structure active element, *i.e*. catechol moiety, is disturbed (Figure 4). Phenoxyl radical resulting from hydrogen atom abstraction from *O*-methyl derivative cannot be stabilised by hydrogen bonding, thus it is less stable.

The observed pH dependence of the TEAC value of hydroxybenzoic, hydroxycinnamic acids and hydroxyflavones studied might be attributed to an effect of the pH on the deprotonation of their OH moiety, as it was shown in our previous studies [Tyrakowska *et al*., 1999; Lemańska *et al*., 2001]. On the basis of a comparison of the pKa values to the pH-dependent TEAC antioxidant profiles, it is concluded that a significant increase in the TEAC antioxidant activity of model polyphenols studied is related to deprotonation of their most acidic hydroxyl moiety (C4-OH - for phenolic acids and C4'-OH or C7-OH for flavonoids). Upon deprotonation phenolic compounds become better antioxidants.

From the pH-dependent TEAC profiles the TEAC values of monoanionic form (COO^-/OH) for phenolic acids and of neutral form for flavonoids have been determined. These TEAC values of the (poly)phenolic compounds in well defined protonation states form a better basis for mechanistic studies and comparison to calculated electronic characteris-

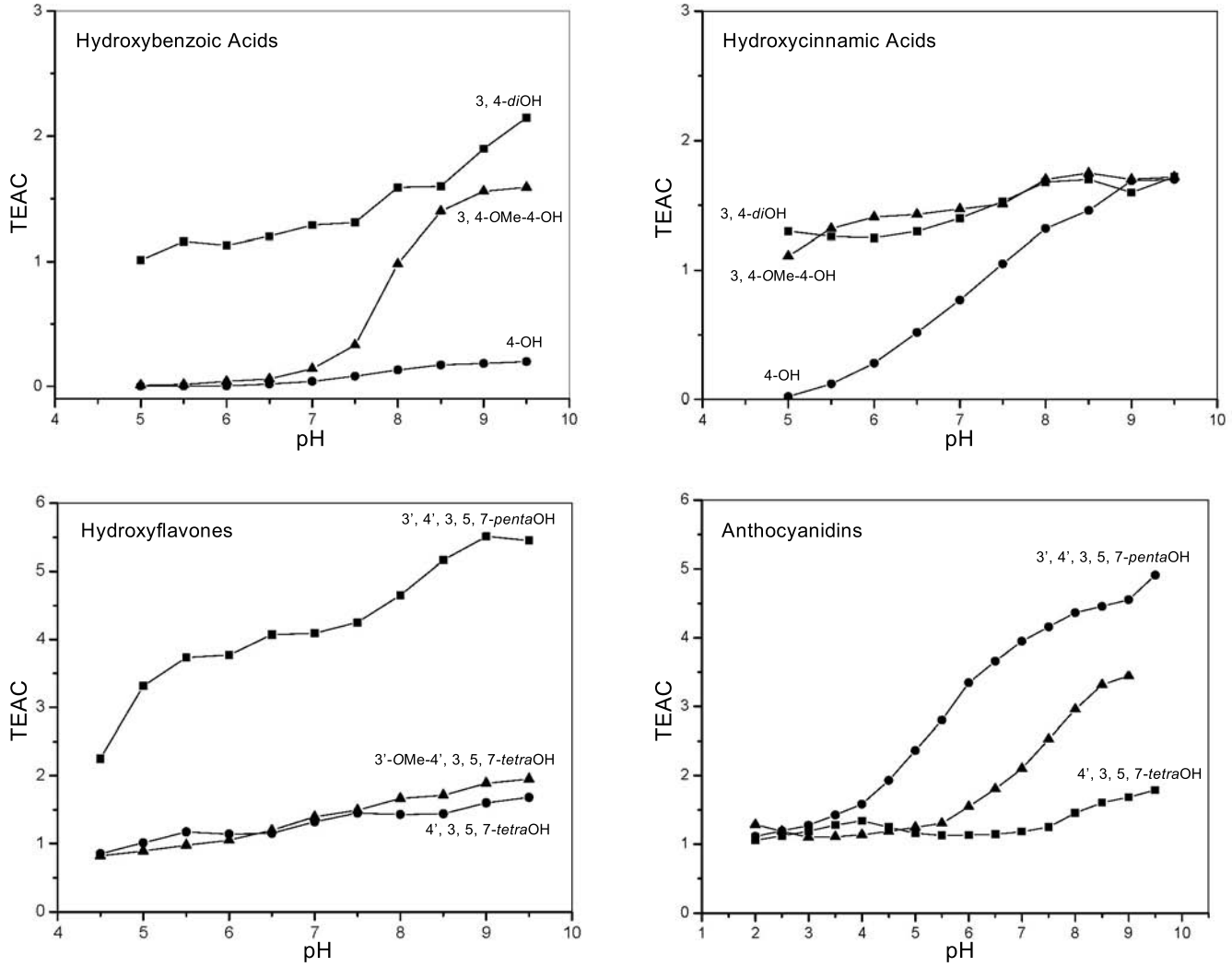

FIGURE 3. The effect of additional hydroxyl or methoxyl substituent at *ortho* position on the pH-dependent TEAC profiles for 4-hydroxybenzoic acid, 4-hydroxycinnamic acid, 4',3,5,7-*tetra*-hydroxyflavone and 4',3,5,7-*tetra*-hydroxyanthocyanidin.

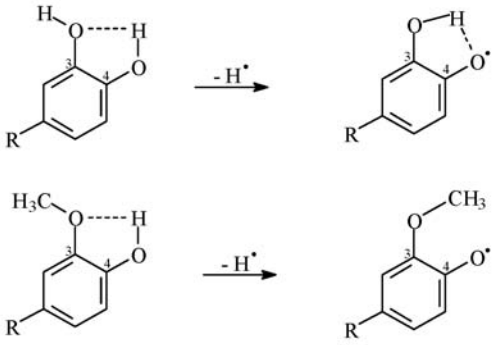

FIGURE 4. Schematic presentation of phenoxyl radicals stabilization in: catechol group containing derivative and 3-O-methyl derivative of polyphenolic compounds. Illustration of extra-stabilization of phenoxyl radical in case of catechol group due to hydrogen bonding.

tics than the TEAC values presented in literature which are generally measured at pH=7.4 [Rice-Evans *et al*., 1996], and often reflect the antioxidant behaviour of mixtures of various (de)protonation states.

To obtain more insight in the effect of additional hydroxyl or methoxyl substituents at *ortho* position to OH group on the radical scavenging antioxidant activity and in the effect of OH group deprotonation on the TEAC activity of polyphenolic compounds studied, the TEAC values, derived in the present study, were compared to theoretically calculated electronic parameters. Table 2 presents these parameters for the neutral and anionic form of the compounds under investigation, including OH bond dissociation energy (BDE) representing the ease of hydrogen atom donation and ionization potential (IP) representing the ease of electron donation. The results indicate that the BDE value, for the monoanionic form of phenolic acids and for the neutral form of hydroxyflavones and anthocyanins, is the best parameter that explains the relative antioxidant activity quantified by the TEAC values of the present study. Indeed, an additional hydroxyl group at *ortho* position to C4'OH moiety decreases BDE, which is reflected in relatively higher TEAC value, while substitution of one of the OH groups in the catechol moiety with methoxyl group increases BDE, reflected in its lowering effect on the TEAC value in comparison to catechol containing derivative. For anthocyanidins, the situation is much more complicated. Anthocyanidins exist in an aqueous phase in a mixture of essentially four molecular species: flavylium cation (AH^+) at pH 1-3, carbinol pseudo-base (B), which is formed at pH 4-5, quinoidal form-3 isomers (A_1, A_2, A_3) which are in a dynamic equilibrium with parent flavylium cation (AH^+). Another con-

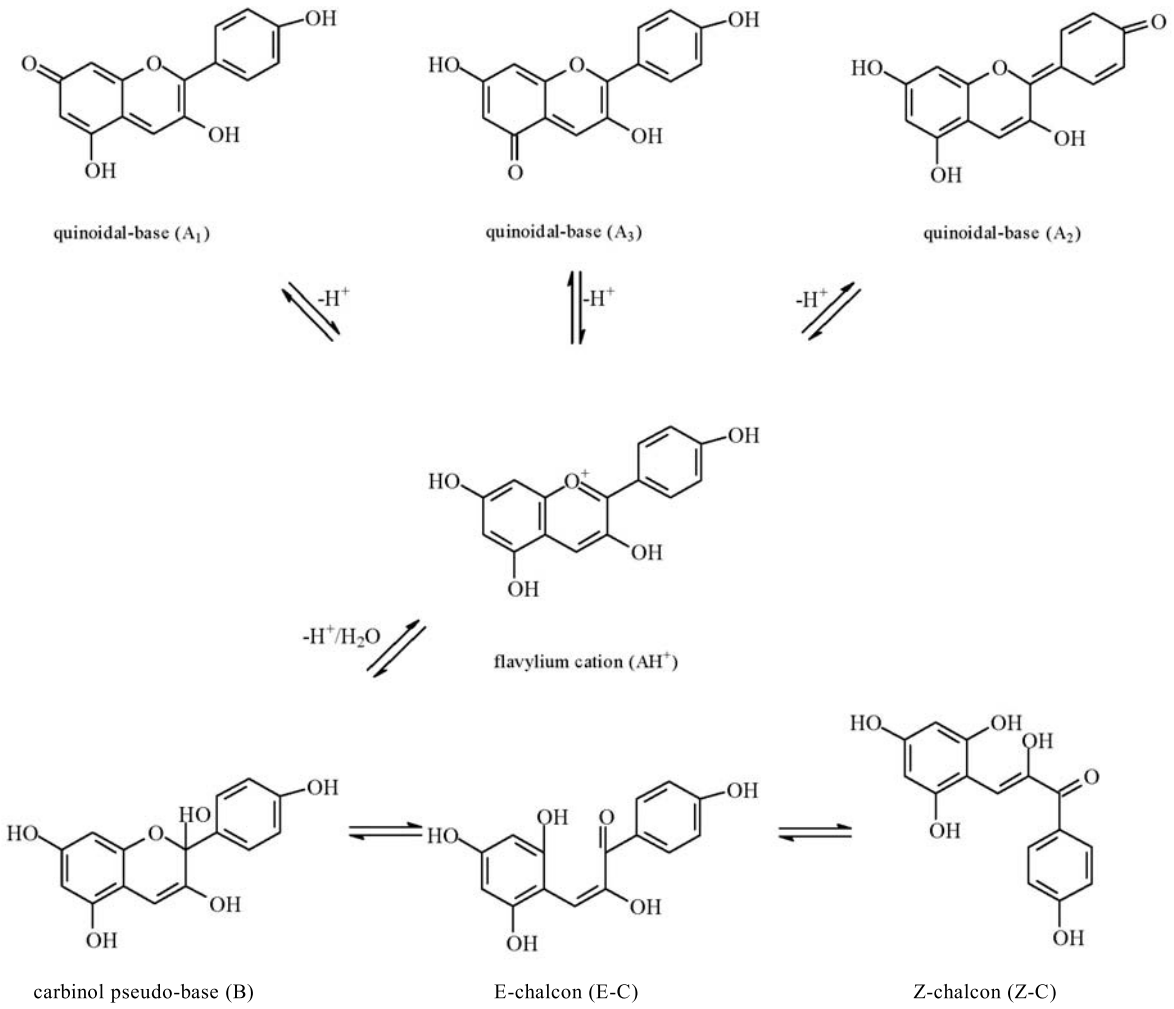

FIGURE 5. Schematic presentation of different forms of anthocyanidins in aqueous solution.

stituents are chalcones (E-C and Z-C), which could be formed from carbinol pseudobase (B) at pH 7-8 [Lapidot *et al*., 1999]. The main forms of anthocyanidins are presented in Figure 5. These forms exist in aqueous solution in different molar ratio which again is pH-dependent. On the basis of BDE values presented in Table 2 for different forms of anthocyanidins, it can be concluded that, irrespective of the percentage composition of fractions present in the solution, all forms of anthocyanidins are good hydrogen atom donors except flavylium cation and Z-chalcone.

Furthermore, the analysis of calculated electronic parameters and the antioxidant potential of the deprotonated forms of the phenolic derivatives was performed, providing BDE(M) and BDE (A) as well as IP(A) and IP (D) values for the anionic forms of phenolic acids and hydroxyflavones, respectively.

This was done to obtain more insight in the mechanism underlying the increase in TEAC value with increasing pH. The actual mechanism for the antioxidant action of these deprotonated forms can still be either hydrogen atom or electron donation or both in different rates. Therefore, Table 2 lists, not only the BDE values as a measure for the ease of hydrogen atom donation from the weakest remaining OH moieties in the anion (BDE(A)), but also the ionization potential of the deprotonated anionic molecules (IP(A)).

A comparison of the BDE values of the deprotonated forms of polyphenolic compounds to those already presented in Table 2 for their neutral forms reveals that there is a decrease in the BDE values upon polyphenolic antioxidants deprotonation. In contrast, the parameter reflecting the ease of electron donation in the form of calculated ionization potential (IP) is dramatically influenced by the deprotonation step; the ionisation potentials become significantly lower. This result is in agreement with previous findings for other hydroxyflavones [Lemańska *et al*., 2001] and supports the conclusion that upon deprotonation the TEAC value of polyphenolic compounds increases due to an increase in their electron donating ability.

Many authors have attempted to elucidate structure-activity relationships for phenolic compound-mediated radical scavenging antioxidant activities and have related the antioxidant efficiency of phenolic acids and flavonoids to the number of hydroxyl groups and also to their hydrogen donating abilities [Cao *et al*., 1997; Couvelier *et al*., 1992; Miller & Rice-Evans, 1997b; Rice-Evans *et al*., 1996]. The results of this study illustrate that the important factors influencing TEAC antioxidant activity of polyphenolic antioxidants are not only the number and position of hydroxyl moieties in the molecule, but also their protonation state, influenced by the pKa of hydroxyl moieties, the pH of the surrounding medium, the effect of intramolecular hydrogen bridges and *O*-methylation of hydroxyl group. Moreover, the results obtained demonstrate the applicability of the modified TEAC assay for studying the pH-dependent effects on the radical scavenging antioxidant activity of (poly)phenolic antioxidants. It was also shown that modified TEAC assay enables the determination of the TEAC value for the neutral form, as well as for the deprotonated form of (poly)phenolic compound, which is a proper basis for studying the mechanism of antioxidant action by comparison to calculated electronic parameters.

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