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EXPERIMENTAL AND THEORETICAL STUDY ON ANTIOXIDANT ACTIVITY OF STRUCTURALLY RELATED 4-HYDROXYCINNAMIC AND 4-HYDROXYBENZOIC ACIDS

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The effect of substituent on the pKa value and antioxidant activity reflected by the TEAC (Trolox equivalent antioxidant capacity) value of series of 4-hydroxybenzoic acids and 4-hydroxycinnamic acids was investigated. It was shown that the TEAC value of both 4-hydroxybenzoic and 4-hydroxycinnamic acids increases significantly with increasing pH of the surrounding environment. Comparison of the experimental data with quantum chemically computed parameters indicates that the antioxidant behavior of the monoanionic forms of 4-hydroxybenzoic acids and 4-hydroxycinnamic acids is not determined by the tendency of the molecule to donate an electron but rather by its ability to donate a hydrogen atom. Results of our experiments and computations explain qualitatively the effect of substituent on the antioxidant behavior of both 4-hydroxybenzoic and 4-hydroxycinnamic acids. Our results confirm that in the wide pH range (5–10) 4-hydroxycinnamic acids are more efficient antioxidants than structurally related 4-hydroxybenzoic acids. It is stressed that 4-hydroxycinnamic acids are potent antioxidants already in weakly acidic media (pH ≡ 6).

INTRODUCTION

Hydroxybenzoic acids (HBAs) and hydroxycinnamic acids (HCAs) constitute a major part of phenolic compounds widely represented in fruits (Miller & Rice-Evans, 1997). Their distribution may vary strongly with species, cultivator and other factors. In most cases HBAs and HCAs are not present in a free state but rather as chemically modified forms either soluble and accumulated in the vacuole or insoluble and covalently linked to cell-wall components (Macheix & Fleuriet, 1999). A general picture of HBAs and HCAs distribution in fruits and their importance as food constituents has been already described in several papers (Macheix, Fleuriet & Billot, 1990; Herrmann, 1989). Phenolic acids are directly involved in the response of fruits to different kinds of stress (Harborne, 1995), mechanical wounding, chemical treatment and microbiological infection. HBAs and HCAs are involved in fruit resistance by contributing to the healing of wounds by lignification of cell walls around wounded zones (Nicholson & Hammerschmidt, 1992) or through the antimicrobial properties (Harborne, 1995). Free phenolic acids are the best inhibitors of growth of the fungi that appear during storage and their structure activity relationships have been studied *in vitro* (Latanzio, De Cicco, Di Venere, Lima & Salerno, 1994). It is interesting that the additional methoxyl group caused an increase in biological activity of HBA and HCA derivatives.

The most important biological activity of HBAs and HCAs is their observed inhibitory effects on mutagenesis and carcinogenesis in direct relation with their antioxidative activity (Macheix & Fleuriet, 1999). Attention is now focused on natural antioxidants, since the synthetic antioxidants are suspected to act as cancer promoters (Ho, 1992). Various fruits (e.g., grape, citrus, black pepper, olive) are a good source of phenolic compounds constituting an important part of daily diet. It is already known that among numerous phenolic acids, caffeic acid, gallic acid and their derivatives show strong antioxidative properties acting as free radical scavengers (Cuvelier, Richard & Berset, 1992; Huang & Ferraro, 1992; Rice-Evans, Miller & Pananga, 1996). They belong to the most abun-

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dant phenolic compounds in plant tissues and are usually found at higher concentrations than flavonoids. HCAs occur naturally as various conjugated forms resulting from enzymatic hydroxylation, O-methylation, O-glycosylation or esterification principally as quinic or carbohydrate esters. In relation to HCAs their antimicrobial, antialergic and antiflamatory activity as well as antimutagenic properties were reported (Pannala, Razaq, Halliwell, Singh & Rice-Evans, 1998; Rice-Evans *et al.*, 1996). It was already noticed (Rice-Evans *et al.*, 1996) that an insertion of the ethylenic group between a phenyl ring carrying hydroxyl groups and the carboxylic group enhances antioxidant activity of these compounds in comparison to HBAs.

The aim of this study was to investigate both theoretically and experimentally the effect of the ethylenic group as well as the presence of additional hydroxyl or methoxyl substituents at C3 position on the pK_a value and the radical scavenging antioxidant activity of HCAs in comparison with HBAs. Experimental data for deprotonation and antioxidant activities were determined and compared with theoretically computed parameters for OH group deprotonation, electron donation and hydrogen atom donation. The antioxidant activity of a series of structurally related HBAs and HCAs was quantified by the TEAC (Trolox equivalent antioxidant capacity) value (Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993; Rice-Evans & Miller, 1994). The TEAC values were determined using a system consisting of microperoxidase-8 (MP8), hydrogen peroxide and ABTS in PBS (phosphate-buffered saline) in pH values varying between 5 and 10 (Tyrakowska, Soffers, Szymusiak, Boeren, Boersma, Lemańska, Vervoort & Rietjens, 1999). The pH dependent TEAC profiles were determined for several relevant 4-hydroxybenzoic and 4-coumaric acid derivatives shown in Fig. 1.

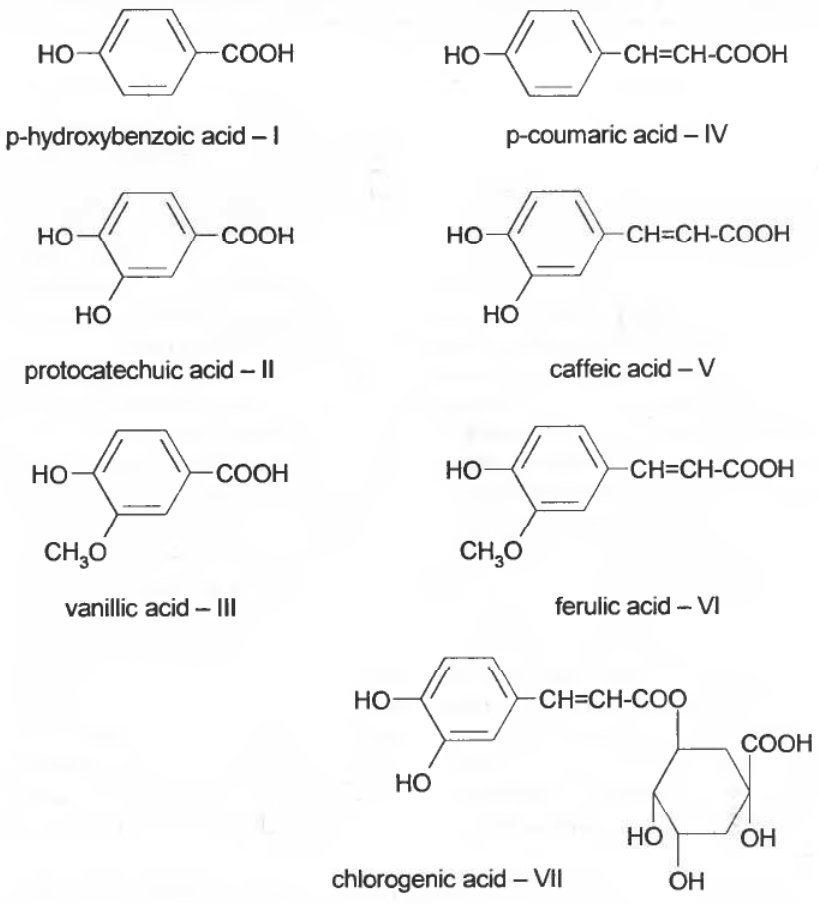

Fig. 1. Chemical formulas and symbols of the compounds studied

MATERIALS AND METHODS

Microperoxidase-8 (MP8), a heme octapeptide was obtained by enzymatic hydrolysis of horse heart cytochrome c (Aron, Baldwin, Marques, Pratt & Adams, 1986). Commercial samples of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (Aldrich), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (Aldrich), *p*-hydroxybenzoic acid (Sigma), protocatechuic acid (ICN Bioch), vanillic acid (ACROS Chemicals), 4-coumaric acid (ICN Bioch), caffeic acid (Aldrich), ferulic acid (Aldrich) and chlorogenic acid (Fluka) were used as supplied without further purification.

Antioxidant capacity of a series of phenolic acids (HBAs and HCAs) was determined using the method based on so-called TEAC assay developed by Miller *et al.*, (1993) and Rice-Evans & Miller (1994) with some modifications (Tyrakowska *et al.*, 1999). The TEAC value is defined as the concentration of Trolox (in mM) having an antioxidant capacity equivalent to 1 mM of the tested compound. A modified assay based on H_2O_2-driven peroxidase activity of microperoxidase-8 (MP8) instead of metmyoglobin was recently applied to 4-hydroxybenzoic acid derivatives by Tyrakowska *et al.* (1999). In this method determination of the TEAC value can be carried out in mixed solvents consisting of varying fraction of organic solvents in aqueous solutions giving a possibility to study antioxidants weakly soluble in water.

To determine the TEAC value for antioxidants (HBAs or HCAs) we have used a system consisting of microperoxidase-8 (MP8), hydrogen peroxide and ABTS in PBS (phosphate buffered saline). $ABTS^{\cdot+}$ cation radical was generated by addition of MP8 (0.24 μM, final concentration) and H_2O_2 (0.133 μM, final concentration) to ABTS solution (1.5 mM, final concentration) in PBS. In the assay MP8 and ABTS were mixed and the reaction was initiated by the addition of hydrogen peroxide. The $ABTS^{\cdot+}$ solution thus obtained was diluted 1:1 (v/v) using 0.2 M aqueous solution of potassium phosphate of pH varying between 5.0 and 9.5 or potassium phosphate and 0.2 M KOH for pH = 10. The antioxidant (dissolved in ethanol) was added, when formation of $ABTS^{\cdot+}$ cation radical was stable. At a fixed time we have measured the decrease in absorption (ΔA) of the solution (at 734 nm) due to the presence of an antioxidant. The decrease in absorption reflects $ABTS^{\cdot+}$ cation radical scavenging capacity of the compound being tested. From the slope of linear portion of the plot of ΔA against antioxidant concentration the values of TEAC were determined.

For ferulic acid the value of pK_a was determined spectrophotometrically for the solutions obtained by addition of 1% (v/v) ethanol stock solutions of model compounds to 0.2 M potassium phosphate buffer (or 0.2 M KOH) solution of pH varying from 5 to 11. In order to define (de)protonation state in which the compound acts as antioxidant in solution we used the pK_a value of the acid. The value of pK_a was calculated according to the method described by Albert and Serjeant (1971). The TEAC values of well defined (monoanionic) form of HBAs or HCAs were obtained from the pH-dependent TEAC profile at $pH = pK_a - 2.0$.

Computational Details

All quantum chemical computations were performed using Gaussian 98 program package. First, semiempirical method AM1, was used to obtain optimized geometries for neutral, anionic, dianionic and semiquinone forms. More accurate geometry parameters were then obtained using density functional theory (DFT) as B3LYP hybride functional including 6-31G* split-valence basis (adding polarization functions on the heavy atoms). In the next step the electronic energies of each species were calculated on the B3LYP/6-311G** level of DFT. Calculated values quoted in this study are bond dissociation energies (BDE), dissociation energies (DE) and ionization potentials (IP) referred to 0.0 K. The computed thermochemical data (BDE, DE and IP) were not corrected for zero-point energies (ZPE) nor for any other thermal corrections.

RESULTS AND DISCUSSION

The antioxidant potency of naturally occurring phenolic acids as well as their esters is known to be strongly related to the structure, in particular to electron delocalization of the aromatic nucleus (Cuvelier *et al.*, 1992; Rice-Evans *et al.*, 1996). It can be expected that these compounds upon formation of a free radical during autoxidation are stabilized by the resonance effect of the aromatic ring when H atoms are substituted by any electron donating groups. In our model studies we have chosen such derivatives of 4-hydroxybenzoic acid and those of 4-hydroxycinnamic acid where H-atom in *ortho* position was substituted by hydroxyl or methoxyl group. Results of our experimental studies together with some data found in literature are listed in Table 1.

The first finding from rough analysis of the data on antioxidant activity obtained by using different methods (Table 1) is that HCAs are generally stronger than corresponding HBAs. This conclusion can be drawn from comparison of the published data obtained by different methods like TEAC assay (Rice-Evans *et al.*, 1996), ORAC assay (Guo, Cao, Sofic & Prior, 1992) and methyl linoleate autoxidation assay (Cuvelier *et al.*, 1992). Data obtained by quite different assays shows that unsubstituted 4-hydroxybenzoic acid and 4-coumaric acid are the least active compounds but results from the three assays are different for substitution effect of hydroxyl or methoxyl group. Results reported by Cuvelier *et al.* (1992) show that hydroxyl group has stronger effect on enhancing antioxidant activity than methoxyl group, e.g. in this assay protocatechuic acid is much more active than vanillic acid and caffeic acid is slightly more active than ferulic acid. It was also found that esterification slightly decreases autoxidant activity. Results from ORAC assay (Guo *et al.*, 1997) are very similar to those obtained by Cuvelier *et al.* (1992). Also results from

Table 1. pK_a values, antioxidant activity (TEAC and ORAC values) and efficient antioxidant quantity (EQ) values for various derivatives of 4-hydroxybenzoic acid and 4-hydroxycinnamic acid

Structure	pK_a	TEAC pH = 7.4	TEAC monoanion	TEAC[e] pH = 7.4	ORAC[f] pH = 7.0	EQ[g] [ppm]
4-hydroxybenzoic acids						
I	9.3[a]	0.08	0.00	0.08	0.17	> 300
II	8.67[b]	1.50	1.18	1.19	2.06	11.5
III	9.2[c]	0.20	0.00	1.43	1.11	> 300
4-hydroxycinnamic acids						
IV	8.0*	1.05	0.10	2.22	1.09	126
V	7.6[b]	1.53	1.23	1.26	2.23	8
VI	8.1	1.50	1.42	1.90	1.33	72
VII	8.5[d]	1.04	0.95	1.30		28

Symbols: [a]Hussain, Entsch, Ballou, massey & Chapman, 1980; [b] Jovanovic, Steenken, Tosic, Marjanovic & Simic, 1994; [c]Eppink, Boeren, Vervort & Van Berkel, 1997; [d]Sauerwald, Schwenk, Polster & Bengsch, 1998; [e]Rice-Evans *et al.*, 1996; [f]Guo *et al.*, 1997; [g]Cuvelier *et al.*, 1992

*pK_a value estimated from regression line given in Fig. 2

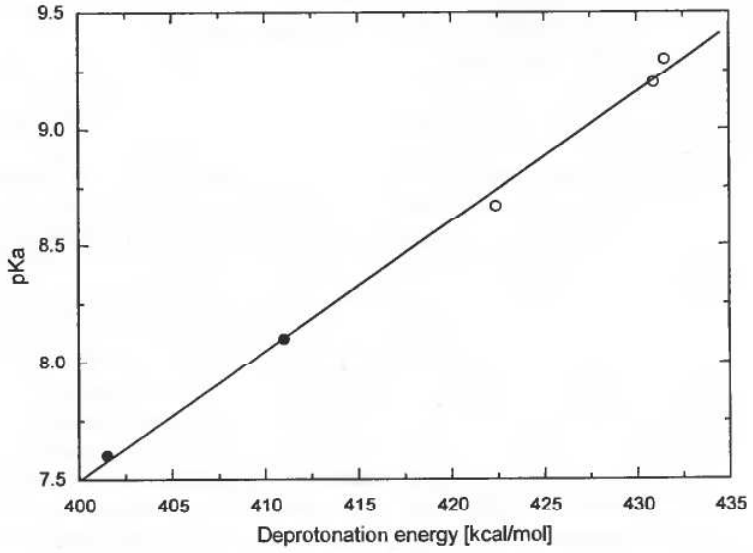

Fig. 2. Correlation between experimental pK_a values and calculated deprotonation energy (DE) of phenolic OH group for several 4-hydroxybenzoic acids (open symbols) and 4-hydroxycinnamic acids (filled symbols). Correlation equation: $pK_a = 0.05554$ DE [kcal/mol] – 14.721; r = 0.9980; N = 5

our study presented in Table 1 for pH = 7.4 are in qualitative agreement with those obtained from other assays clearly showing the importance of substitution with hydroxyl group on antioxidant activity. It should be noted, however, that in contrast, data reported in literature from TEAC assay (Rice-Evans *et al.*, 1996) indicate that methoxyl group has a stronger effect on antioxidant efficiency than hydroxyl group, e.g. in this case vanillic acid was found to be more active than protocatechuic acid and ferulic acid was much more active than caffeic acid.

We have found that there is a satisfactionary linear correlation between experimental pK_a values and calculated deprotonation energy (DE) for 4-hydroxybenzoic and 4-hydroxycinnamic acids as shown in Fig. 2. It should be noted that data for both series follows the same line. Existence of this correlation can be applied for estimation of pK_a values based on the equation of regression and the values dissociation energy calculated for phenolic OH group.

On the basis of quantum chemically calculated deprotonation energies of the OH group it was found that monoanions or esters of HCAs are more acidic than the corresponding HBAs. As a result of our computations we have predicted that the hydroxyl groups in HCAs are able to deprotonate at lower pH range being in agreement with experimental observations. We have found that the calculated deprotonation energies (DE) of phenolic OH group are consistent with experimental pK_a values reported so far (Table 1). These data can be used for estimation of unknown pK_a values for other derivatives (calculating DE for the compound and interpolating its pK_a from known pK_a *vs.* DE linear relationship).

Figures 3–8 present the pH dependence of TEAC values for structurally related benzoic and cinnamic acid derivatives. It can be seen (Fig. 3) that p-coumaric acid is a strong antioxidant in the whole pH range studied while p-hydroxybenzoic acid reveals hardly detectable TEAC antioxidant activity even in basic solutions.

It is interesting to note that the antioxidant activity of protocatechuic acid and caffeic acid (Fig. 4) was found to be very similar in the whole pH range studied (from pH 5 to pH 10). It seems that in this case more important contribution to the observed activity results from the presence of the catecholic moiety than from the presence of the ethylenic group. It should be noted that antioxidant efficiency of protocatechuic acid and caffeic acid determined by different methods (as shown in Table 1) are very similar. In Fig. 5 the pH-dependent TEAC profiles of vanillic acid and ferulic acid are compared showing negligible antioxidant activity of vanillic acid at pH values lower than 7 and a comparable high activity of both acids at pH values above *ca.* 8.5. Fig. 6 illustrates the effect of substitution at C3 position in 4-hydroxybenzoic acid molecule clearly showing that protocatechuic with the catecholic moiety has strong antioxidative potency over the whole pH range, whereas vanillic acid is active only in basic media but it is not active in acidic media. Fig. 7 shows analogous relations for 4-hydroxycinnamic acid series. It is interesting to note that at high pH the TEAC values for all derivatives are nearly the same; in acidic media only 4-hydroxycinnamic acids substituted at C3

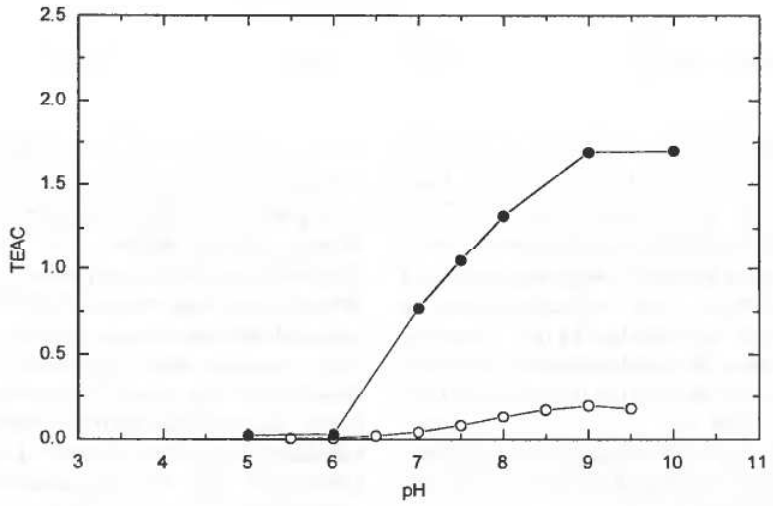

Fig. 3. Effect of pH on the TEAC value for p-hydroxybenzoic acid (I, open symbols) and p-coumaric acid (IV, filled symbols)

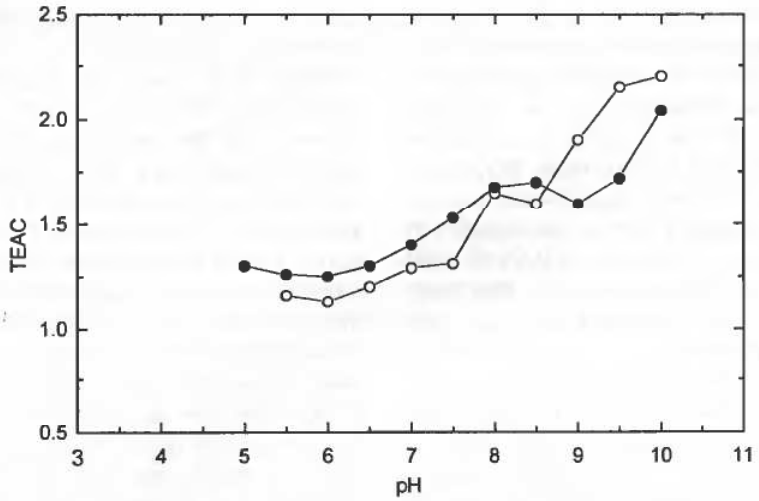

Fig. 4. Effect of pH on the TEAC value for protocatechuic acid (II, open symbols) and caffeic acid (V, filled symbols)

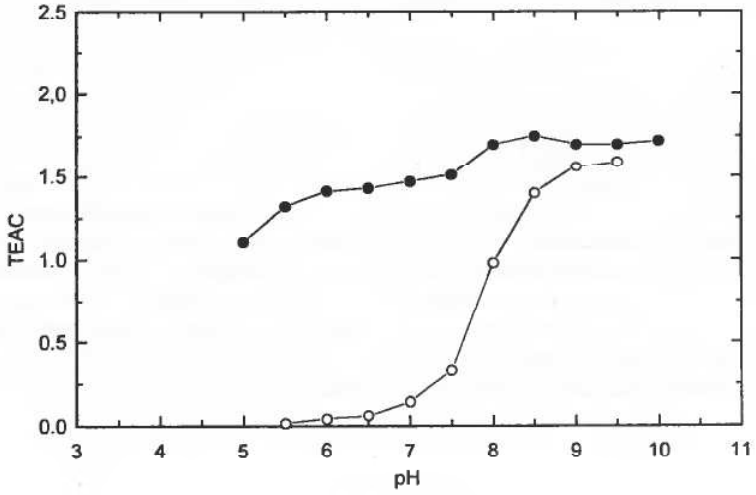

Fig. 5. Effect of pH on the TEAC value for vanillic acid (III, open symbols) and ferulic acid (VI, filled symbols)

position were found to be active, whereas p-coumaric acid is active only in neutral and basic solutions. In Fig. 8 the effect of esterification of caffeic acid is shown. Interestingly esterification of this cinnamic acid derivative tends to decrease its antioxidative potency in acidic media. From pH-dependent TEAC profiles the TEAC values for various derivatives of 4-hydroxybenzoic acid and 4-hydroxycinnamic acid in the monoanionic form were derived (Table 1).

In Table 2 quantum chemically calculated thermochemical data are shown for both HBA and HCA derivatives studied.

The calculated bond dissociation energies (BDE) and ionization potentials (IP) could be compared to the values characterizing antioxidant activity listed in Table 1. In general, quantum chemically calculated data usually refer to well defined form of the species. Therefore, we have to point out that also in analysis of antioxidant activity a proper definition of the form of the acid (in aqueous solution) plays a very important role. It seems that the value of BDE regardless whether calculated for neutral or monoanionic form of HBA or HCA is the best parameter that fits to relative antioxidant activity obtained from our TEAC values. Indeed, the substitution of 4-hyd-

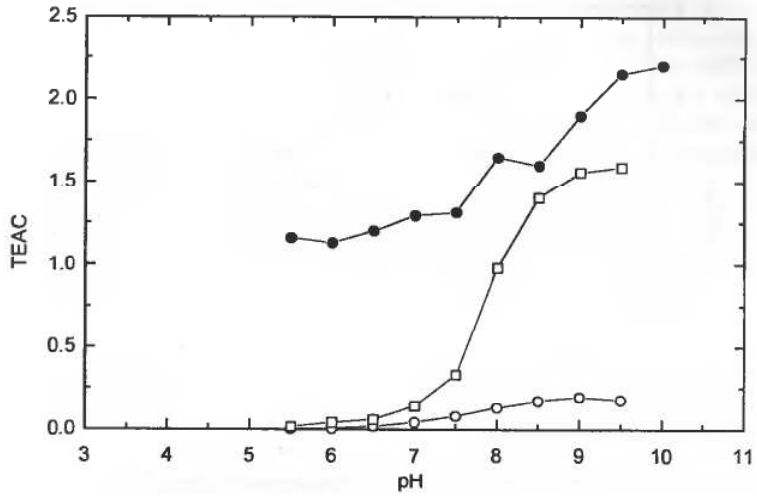

Fig. 6. Effect of C-3 substituent in 4-hydroxybenzoic acids on pH dependence of the TEAC value for several derivatives. Symbols: p-hydroxybenzoic acid (I, 3-H, open circles), protocatechuic acid (II, 3-OH, filled circles), vanillic acid (III, 3-OCH_3, squares)

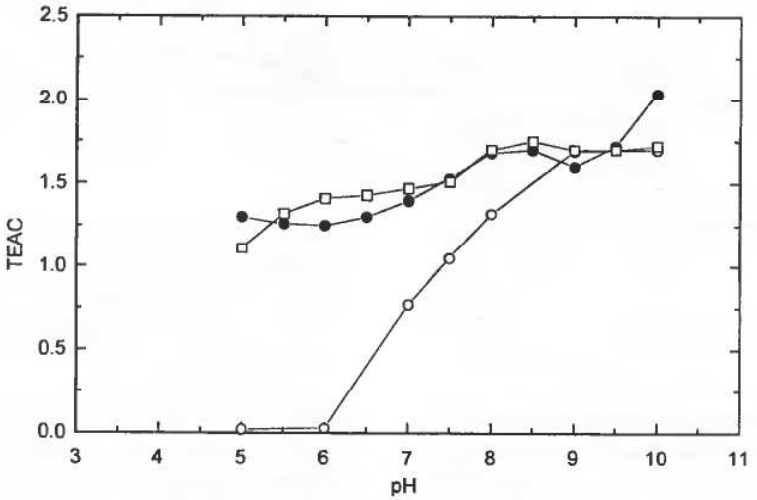

Fig. 7. Effect of C-3 substituent in 4-hydroxycinnamic acids on pH dependence of the TEAC value for several derivatives. Symbols: p-coumaric acid (IV, 3-H, open circles), caffeic acid (V, 3-OH, filled circles), ferulic acid (VI, 3-OCH_3, squares)

roxybenzoic acid or 4-hydroxycinnamic acid with OH group lowers BDE by 7–10 kcal/mol independent of whether neutral form (in lipophilic phase) or monoanionic form of the acid (in aqueous solution) exists. Ionization potentials (IP) calculated for dianionic forms may be compared with experimental data obtained at very high pH (i.e. $pH > pK_a + 2$). Because of complexity of the real solutions where phenolic acids practically appear as a mixture of monoanions and dianions it is very difficult to compare directly any experimental data and any computed parameters. It should be pointed out that the superiority of 3-OH substitutions both in HBAs and HCAs is nicely reflected in the results from both experimental and theoretical study pointing at the importance of the presence of additional ethylenic group on the increase in the radical scavenging TEAC antioxidant activity observed in HCAs over HBAs.

We have also found that the hydroxyl groups in HCAs are much better hydrogen donors than those in the corresponding HBAs. In the case of esters

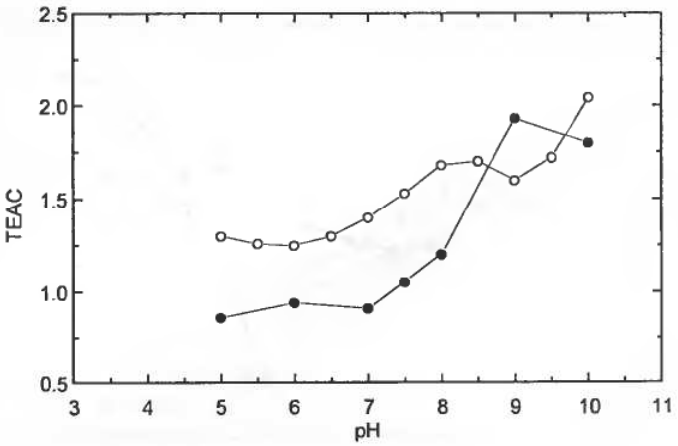

Fig. 8. Effect of esterification on pH dependence of the TEAC value for 3,4-dihy-droxycinnamic acids. Symbols: caffeic acid (V, free acid, open symbols), chlorogenic acid (VII, ester, filled circles)

Table 2. Calculated deprotonation energy (DE) of OH group, bond dissociation energy (BDE) of OH group and ionization potential (IP) for various derivatives 4-hydroxybenzoic acids and 4-hydroxycinnamic acids in their neutral (N) COOH/OH, monoanionic (M) COO^-/OH and dianionic (D) COO^-/O^- forms. All values are given in kcal/mol

Structure	DE	$BDE^{(N)}$	$BDE^{(M)}$	$IP^{(N)}$	$IP^{(D)}$
4-hydroxybenzoic acids					
I	431.5	92.1	78.6	195.9	37.8
II	422.4	82.6	71.1	186.6	36.2
III	430.9	90.1	79.8	181.6	36.0
4-hydroxycinnamic acids					
IV	410.7	87.9	74.4	181.9	21.2
V	401.5	78.8	67.5	176.4	18.8
VI	411.0	87.1	75.5	173.0	20.4
VII		78.4		173.9	

and monoanions (carboxylates) the predicted decrease in the value of bond dissociation energy (BDE) of an OH group is *ca.* 3–4 kcal/mol. It is presumably due to the presence of the additional electron donating ethylenic group. According to the commonly accepted mechanism (Jovanovic, Steenken, Tosic, Marjanovic & Simic, 1994) the activity of phenolic antioxidants depends mainly on the ability of electron or hydrogen atom donation. In view of that mechanism our results confirm that HCAs and their esters are better antioxidants than the corresponding HBAs.

In conclusion we can state that influence of the additional ethylenic group on the acidity of the 4-hydroxyl group of HCAs compared with HBAs can be predicted by quantum chemical calculations. 4-hydroxyl groups in HCAs are able to deprotonate in lower pH range than those in structurally related HBAs. The pK_a value of 4-hydroxyl moiety in HBAs and HCAs appears to dominate the pH-dependent TEAC behavior of a radical scavenger reflected by observed increase in its TEAC value upon deprotonation of 4-OH group. It was shown that the effect of additional ethylenic group on a increase in the radical scavenging antioxidant activity of HCAs compared with HBAs can also be predicted by quantum chemical calculations. Results of both our theoretical (BDE) and experimental data (TEAC) indicate that caffeic acid in its monoanionic form is the most efficient antioxidant among the compounds studied. Comparison of pH-dependent TEAC antioxidant behavior of HCAs and HBAs leads to the conclusion that in contrast to HBAs the HCAs are also active in slightly acidic media that can be met in digestive or urine bladder tract.

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