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EFFECT OF SUBSTITUTION PATTERN ON TEAC ANTIOXIDANT ACTIVITY OF MONO- AND DIHYDROXYFLAVONES

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The effect of position of the hydroxyl substituent on the antioxidant activity reflected by the so-called TEAC value of a series of mono- and dihydroxyflavones was investigated in pH ranging from 5 to 9.5. The TEAC value of flavones substituted by hydroxyl group at C3, C5 or C6 position significantly increases with increasing pH of the environment. Flavones with hydroxyl groups at C4' and C7 positions are essentially not active over the whole pH range studied. Surprisingly some combinations of double OH substitutions in the flavone system like 7,3'-diOH or 7,4'-diOH were found to be also not active. Comparison of the experimental data to calculated ionization potentials indicates that the hydroxyl moiety at the C3 or C5 position in the flavone system (in the vicinity of the C4=O group) is an important structural element causing an increase in the antioxidant activity (TEAC value) of the mono- and dihydroxyflavones. The presence of catechol moiety at 3'4' position as well as at 6,7 position and 7,8 position at the A ring was found to be necessary for any antioxidant action of dihydroxyflavones if hydroxyl group at the C3 or C5 position is absent.

INTRODUCTION

It is well known that diets rich in fruits and vegetables are protective against cardiovascular disease and certain forms of cancer. These protective effects have been attributed to the antioxidants present in food including vitamin C and carotenoids and plant phenolics such as the flavonoids (Rice-Evans, Miller & Papanga, 1996). Flavonoids are polyphenolic compounds that occur ubiquitously in food of plant origin. The basic structure of flavonoids allows a multitude of substitution patterns in the benzene rings A and B like hydroxyl groups, O-glycosides, methoxyl groups and/or sulfates. Changes in the heterocyclic ring C give rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. Over 4000 different naturally occurring flavonoids have been described (Cao, Sofic & Prior, 1997; Cotelle, Bernier, Catteau, Pommery, Wallet & Gaydo, 1996). As many other polyphenolic compounds, flavonoids have the ability to act as antioxidants by scavenging reactive radical species with the formation of less reactive flavonoid phenoxyl radicals (Cook & Samman, 1996; Cao *et al.*, 1997; Cotelle *et al.*, 1996). The mechanism of this activity of the flavonoids is a subject of endless debate. The antioxidant efficiency of the phenolic acids and flavonoids have been related to the number of hydroxyl groups in the molecule and also to their hydrogen radical donating abilities (Rice-Evans *et al.* 1996; Cao *et al.*, 1997). While the role of flavonoids polysubstituted with hydroxyl groups (i.e. quercetin, kaempferol or fisetin) as possible dietary antioxidants has been studied extensively (Hodnick, Milosavljevic, Nelson & Pardini, 1988; Hanasaki, Ogawa & Fukui, 1994; Cotelle *et al.*, 1996, Jovanovic, Steeken, Hara & Simic, 1996; Van Acker, De Groot, Van den Berg, Tromp, Den Kelder, Van der Vijgh & Bast, 1996), considerably little attention was focused on the antioxidant potential of simple model compounds such as monohydroxyflavones and dihydroxyflavones.

The aim of the present study was to investigate the influence of the position of the hydroxyl substituent on the pK_a as well as on the radical scavenging antioxidant activity of several mono- and dihydroxyflavones. The pH-dependent antioxidant activity was quantified by the TEAC value (Rice-Evans & Miller, 1994; Tyrakowska, Soffers, Szymusiak, Boeren, Boersma, Lemańska, Vervoort & Rietjens, 1999) and compared to calculated theoretical parameters for OH group deprotonation, electron donation and hydrogen atom donation to obtain better insight in the factors determining the

antioxidant activity of monohydroxyflavones and dihydroxyflavones studied.

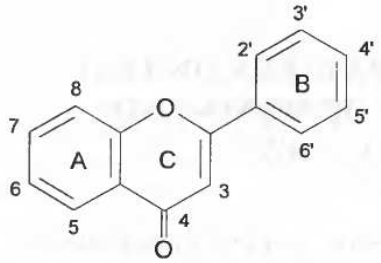

Fig. 1. Chemical structure and atom numbering system of flavone

MATERIALS AND METHODS

Monohydroxyflavones: 3-, 5-, 6-, 4'-, and 7-hydroxyflavone (Indofine Chemicals); dihydroxyflavones: 4',7-, 3',7- , 4',5-, 3',3-, 3',4'-, 6,7- and 7,8-hydroxyflavone (Indofine Chemicals); 5,7-, 3,7- and 3,6-dihydroxyflavone (Aldrich); 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulphuric acid) diammonium salt (abbreviated as ABTS) (Aldrich), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (abbreviated as Trolox) (Aldrich), hydrogen peroxide (30%) (Merck) were used as supplied without further purification.

TEAC assay

The antioxidant activity of the compounds studied was measured by so-called TEAC assay, performed essentially as described previously (Rice-Evans *et al.*, 1994) with some modifications (Tyrakowska *et al.*, 1999). Determination of the TEAC value is based on the ability of the antioxidant to scavenge the $ABTS^{\cdot+}$ radical cation. Microperoxidase-8 (MP8) was used instead of metmyoglobin, to generate the $ABTS^{\cdot+}$ in potassium phosphate-buffered saline. The reaction for generation of the $ABTS^{\cdot+}$ radical by MP8 was initiated by the addition of hydrogen peroxide. The $ABTS^{\cdot+}$ solution thus obtained was diluted 1:1 (v/v) using 0.2 M potassium phosphate of various pH values between 4.5 and 9.5, to give an absorption of *ca.* 0.7 at 734 nm. Hydroxyflavones were added as 1% (v/v) of stock solutions in methanol. The decrease in absorption caused by the antioxidant compound, measured at 6 min, is reflecting the $ABTS^{\cdot+}$ radical scavenging capacity and was plotted against the concentration of antioxidant. The TEAC value represents the ratio between the slope of the plot for scavenging of $ABTS^{\cdot+}$ by the antioxidant under investigation, compared to the slope of this plot for $ABTS^{\cdot+}$ scavenging by Trolox (a water-soluble analog of vitamin E) used as the antioxidant standard.

Quantum-chemical calculations

All geometries were optimized with the unrestricted B3LYP a hybrid 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 with the unrestricted B3LYP a hybrid density functional theory by using a higher 6-311G(d,p) basis set. The calculated deprotonation energies (DE), ionization potentials (IP) and bond dissociation energies (BDE) were not corrected for zero-point-energy assuming a negligible error and thus sparing computer time.

RESULTS AND DISCUSSION

Tables 1 and 2 present monohydroxyflavones and dihydroxyflavones used in the present study as well as experimentally determined pK_a values of their hydroxyl moieties published so far in literature (Thompson, Williams & Elliot, 1976; Wolfbeis, Leiner, Hochmuth & Geiger, 1984) as well as DFT calculated deprotonation energies (DE) of the hydroxyl group of the series of monohydroxy- and dihydroxyflavones. Some discrepancies between literature data for 3-OH and 7-OH indicate that the pK_a data for these compounds should be remeasured. Experimental pK_a values of the individual OH moieties were compared to deprotonation energies and this resulted in the quantitative structure activity relationship (QSAR) as shown in Fig. 2.

A good correlation obtained validates these pK_a values for hydroxyl group deprotonation as well as the DFT method used for calculations on the hydroxyflavones studied. Moreover, this QSAR provides the possibility to predict the pK_a values of OH moieties in other dihydroxyflavones. Comparison of the pK_a values (both experimental and theoretically predicted) leads to the conclusion that position of hydroxyl substituent(s) affect the pK_a of the hydroxyl groups in the monohydroxy- and dihyhydroxyflavones. Furthermore, hydroxyl moiety situated at C-4' and C-7 position deprotonates preferentially (Table 1). Much higher pK_a values for derivatives substituted with hydroxyl group at C-3 and C-5 position results from strong H-bondings to C4=O carbonyl group.

Table 1. Experimental pK_a and TEAC values for neutral form of monohydroxyflavones and calculated by (U)B3LYP/6-311G**//(U)B3LYP/6-31G* method deprotonation energies (DE), ionization potentials (IP) and the bond dissociation energies (BDE) for monohydroxyflavones. For comparison pK_a values predicted from regression line (Fig. 2) are included

Compound	pK_a exp.	pK_a pred.	TEAC neutral	DE [kcal/mol]	IP [eV]	BDE [kcal/mol]
3-OH	10.34[a]	9.8	1.25	350.5	7.48 (7.36)[c]	89.9
	9.60[b]					
5-OH	11.44[a]	11.2	0.93	361.1	7.82 (7.50)[c]	105.7
	11.56[b]					
6-OH	9.09[b]	9.6	0.35	349.2	7.84	90.2
7-OH	8.48[a]	8.6	0.00	341.5	8.02	92.1
	7.39[b,d]					
8-OH	7.82[b,d]	9.1	–	344.9	7.96	87.6
2'-OH	8.85[b]	8.4	–	339.6	7.93	89.9
3'-OH	9.75[b]	9.5	–	348.2	7.91	91.6
4'-OH	8.28[b]	8.2	0.00	338.0	7.72	89.2

Experimental pK_a data: a – Thompson *et al.* (1976), b – Wolfbeis *et al.* (1984). c – IP values given in parenthesis are calculated for the isomeric structure of the formed radical cation stabilized by H atom shift from C3-OH or C5-OH to C4=O, d – Because of large difference between the two sets of data these pK_a values should be revised

Table 2. Experimental pK_a and TEAC values for neutral form and calculated by (U)B3LYP/6-311G**// (U)B3LYP/6-31G* method deprotonation energies (DE), ionization potentials (IP) and bond dissociation energies (BDE) for several dihydroxyflavones. For comparison pK_a values predicted from regression line (Fig. 2) are included

Compound	pK_a exp.	pK_a pred.	TEAC neutral	DE [kcal/mol]	IP [eV]	BDE [kcal/mol]
3,6-diOH	–	9.3 (6)[a]	1.70	346.7 (6)	7.42 (7.33)[c]	89.9 (6)[b]
						89.6 (3)
3,7-diOH	–	8.0 (7)	1.10	337.1 (7)	7.39 (7.19)	92.1 (7)
						89.8 (3)
3,3'-diOH	–	9.5 (3')	0.80	348.4 (3')	7.46 (7.36)	89.5 (3')
	–					90.7 (3)
5,7-diOH	8.37 (7)	–	0.03	341.7 (7)	7.74 (7.48)	94.9 (7)
5,4'-diOH	–	7.7 (4')	0.02	334.6 (4')	7.62 (7.35)	90.0 (4')
6,7-diOH	–	7,5 (7)	1.66	333.1 (7)	7.76	83.3 (7)
				337.8 (6)		81.8 (6)
7,8-diOH	–	7.8 (7)	1.09	335.5 (7)	7.73	81.9 (7)
	–					78.5 (8)
7,3'-diOH	–	8.5 (7)	0.01	340.2 (7)	7.87	91.9 (7)
						91.3 (3')
7,4'-diOH	–	8.2 (4')	0.01	338.1 (4')	7.66	89.0 (4')
						91.6 (7)
3',4'-diOH	–	7.1 (4')	0.66	330.1 (4')	7.54	80.1 (4')
						81.8 (3')

Symbols: a – Thompson *et al.* (1976), b – position of OH moiety given in parenthesis, c – IP for the tautomeric structure of the formed radical cation by H atom shift C3-OH or C5-OH to C4=O

Using this extended set of pK_a values (Tables 1 and 2) the pH-dependent TEAC antioxidant activity of the monohydroxy- and dihydroxyflavones was investigated to test whether the pK_a of the hydroxyl group may be a factor affecting the antioxidant activity. This would provide an information on the (de)protonation state in which the compound acts the most effectively as an antioxidant in the solution. The TEAC values of a series of monohydroxy- and dihydroxyflavones were measured as a function of pH.

Fig. 3–7 present the pH-dependent TEAC profiles of a series of monohydroxy- and dihydroxyflavones. The antioxidant activity of Trolox was

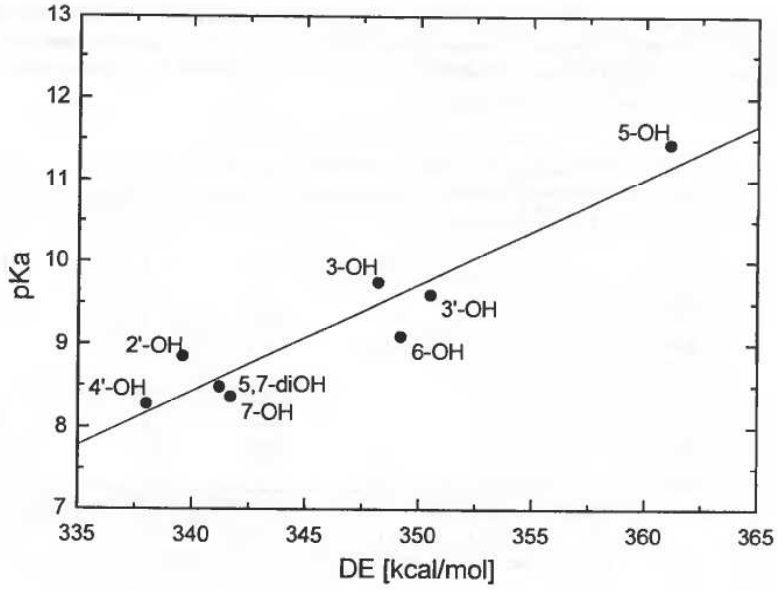

Fig. 2. Plot of experimental pK_a value of monohydroxyflavones and 5,7-dihydroxyflavone against the calculated deprotonation energy of their hydroxyl moiety. Deprotonation energies (DE) were calculated using DFT method. Solid line corresponds to the following regression equation: $pK_a = 0.12955 \times DE\ [kcal/mol] - 35.617$, $r = 0.9473$, $N = 8$

previously shown to be unaffected over the whole pH range (Tyrakowska *et al.*, 1999).

From the results obtained it can be derived that substitution pattern has significant influence on the pH-dependent antioxidant behavior of both monohydroxy- and dihydroxyflavones. We have found that 7-hydroxyflavone and 4'-hydroxyflavone are essentially not active over the whole pH range tested. In contrast, the TEAC value of 3-hydroxy-, 5-hydroxy- and 6-hydroxyflavones significantly increases with increasing pH of the surrounding environment over the whole pH range tested (Fig. 3).

The position-specific antioxidant behavior of the various hydroxyl moieties is observed also in various dihydroxyflavones. The antioxidant activity of dihydroxyflavones substituted with hydroxyl group at the C-7 and C-4' position (7,3'-dihydroxy- and 7,4'-dihydroxyflavones) are almost not active in the monoanionic form, at pH higher than 8.0 (Fig. 4). Substitution with hydroxyl moiety at the C-3 or C-5 position causes significant increase in the TEAC antioxidant activity of the dihydroxyflavones studied (Fig. 5 and 6). Comparison of pH-dependent TEAC profiles of dihydroxyflavones substituted with hydroxyl moiety at the C3 position (Fig. 6) or at the C5 position (Fig. 5) indicate clear difference in the antioxidant behavior of the compounds, namely the C3-OH derivatives start to be active already at slightly acidic medium (pH ≅ 6.0) while the C5-OH derivatives are active only in the basic medium, at pH higher than *ca.* 8.0.

Noteworthy is remarkable influence of the *ortho* substitution with hydroxyl group at the A or B ring on an increase of the antioxidant potential of dihydroxyflavones. From the plots presented in Fig. 7 it follows that the TEAC values increase significantly with increasing pH not only for the 3',4'-dihydroxyflavone but also for the 6,7-dihydroxy- and 7,8-dihydroxyflavone. This effect of substitution with the catechol moiety on an increase of the antioxidant activity of flavonoids was previously reported in several studies on qualitative structure-activity relationships only for flavonoids substituted with catechol moiety at 3',4' position at the B ring (Bors & Saran, 1987; Bors, Heller, Michael & Saran, 1990; Rice-Evans *et al.*, 1996; van Acker *et al.*, 1996). The effect of substitution with the catechol moiety in the A ring was never observed nor discussed.

The pH dependence of the TEAC value found for monohydroxy- and dihydroxyflavones might be attributed to an effect of the pH on the deprotonation of their OH moiety and also to the solute solubility. The problem of the effect of solubility on the pH dependence for dihydroxyflavones is currently investigated in our laboratory. Comparison of he pH profiles to the pK_a values of the hy-

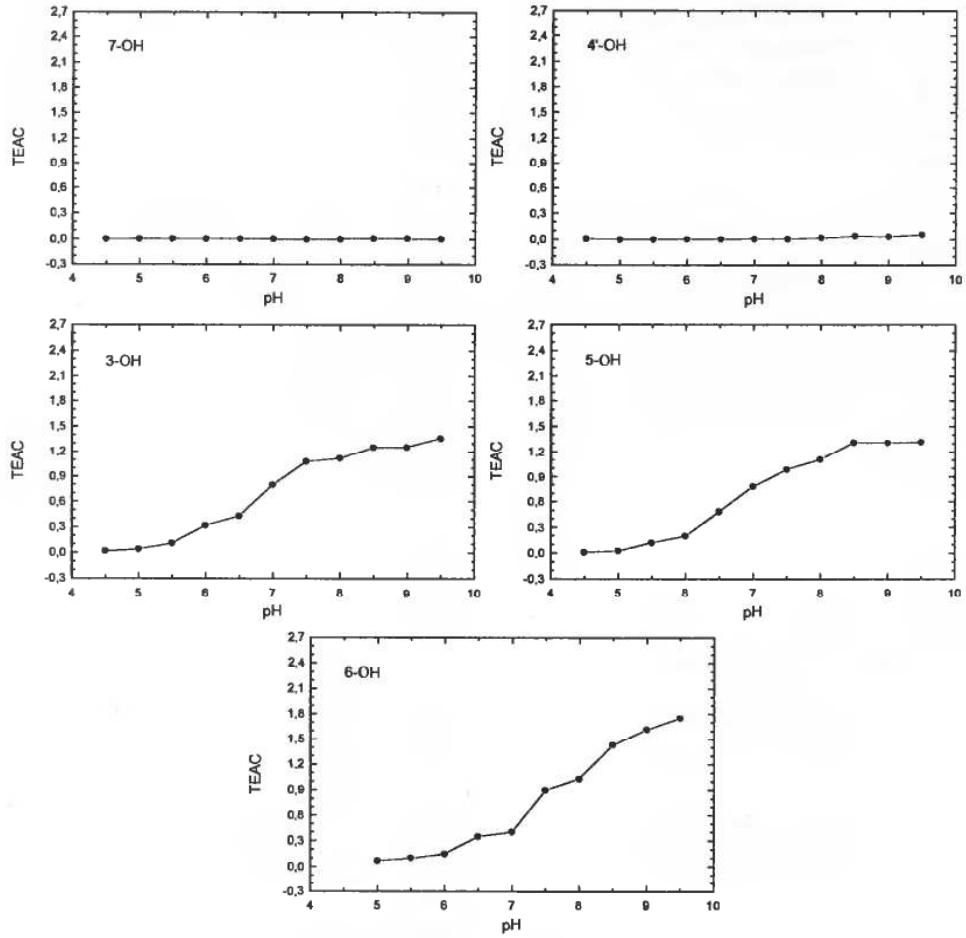

Fig. 3. Effect of pH on the TEAC value of various monohydroxyflavones

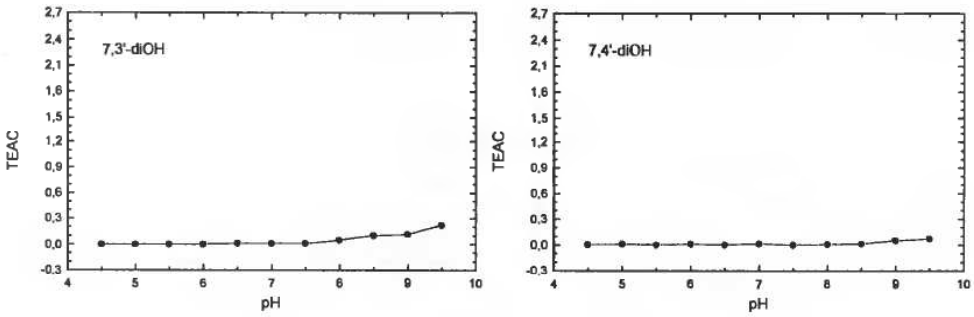

Fig. 4. Effect of pH on the TEAC value of dihydroxyflavones substituted with 7-OH group

droxyl moieties indicates that for hydroxyflavones the antioxidant behavior is generally not influenced by the hydroxyl moiety deprotonation. It should be noted, however, that only in the case of 5,7-dihydroxyflavone, in which the hydroxyl moiety which preferentially deprotonates is positioned in the same ring as the hydroxyl moiety dominating the antioxidant behavior, the pH dependent

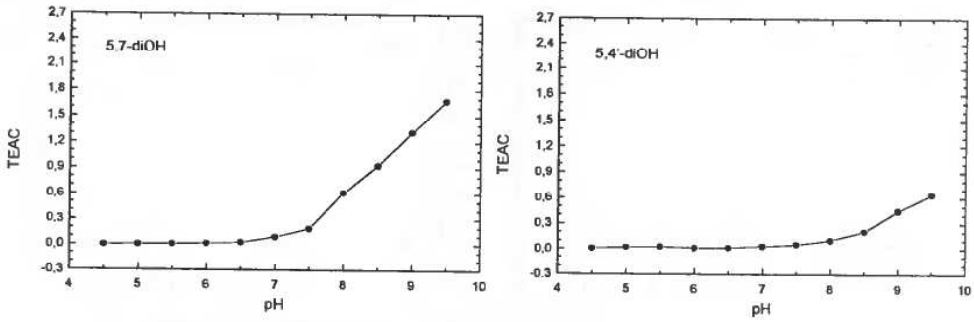

Fig. 5. Effect of pH on the TEAC value of dihydroxyflavones substituted with 5-OH group

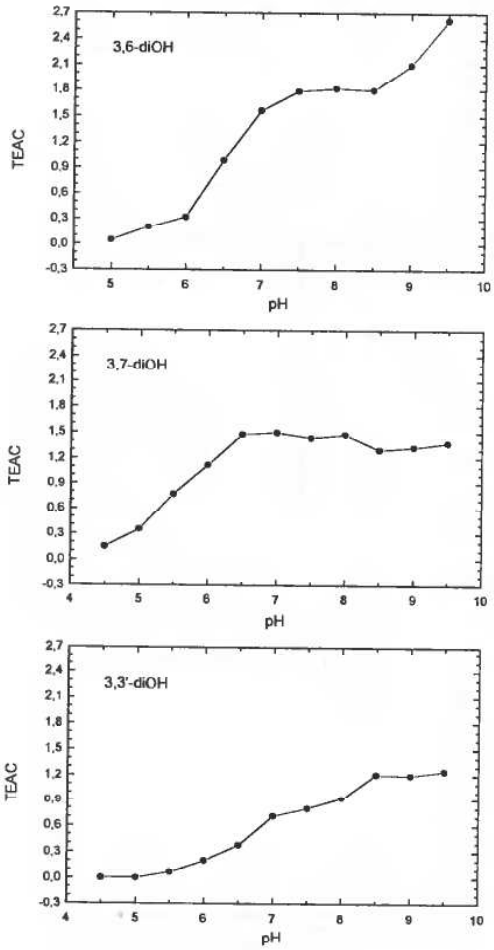

Fig. 6. Effect of pH on the TEAC value of dihydroxyflavones substituted with 3-OH group

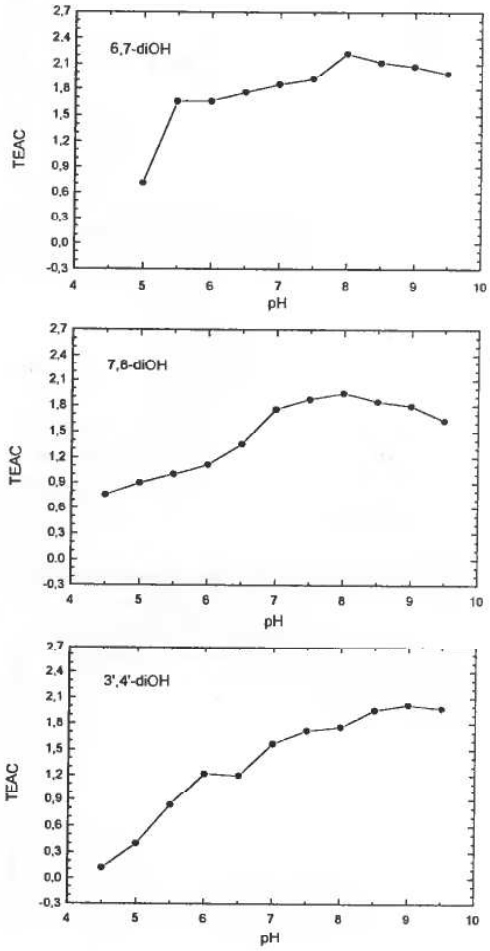

Fig. 7. Effect of pH on the TEAC value of dihydroxyflavones substituted with a catechol moiety

effect on the TEAC value seems to coincide with the pK_a for hydroxyl moiety deprotonation (Fig. 5). From the pH dependent TEAC profiles the TEAC values of neutral forms of the (poly)hydroxyflavones could be derived. These TEAC values of the hydroxyflavones in well defined protonation states form a better basis for mechanistic studies and comparison to calculated electronic

characteristics than the TEAC values presented in literature, which are generally measured at pH = 7.4, and often reflect the antioxidant behavior of mixtures of various (de)protonation states.

Tables 1 and 2 present the TEAC values for various hydroxyflavones in their neutral form, derived from pH-dependent TEAC profiles (Fig. 3–7). Because measurements at high pH values (> 9.5) become hampered by: i) rapid autoxidation of the flavonoids, ii) instability of the $ABTS^{\cdot+}$ radical cation and iii) difficulties in determination of the second pK_a value, determination of the TEAC values of mono- and/or dianionic forms of the monohydroxy- and dihydroxyflavones was not possible.

The TEAC values as derived in the present study for the neutral form of the monohydroxy- and dihydroxyflavones were compared to computer-calculated electronic characteristics (Tables 1 and 2). From the comparison between our experimental TEAC values for the neutral forms and quantum-chemically computed characteristics (Tables 1 and 2) it can be derived that the TEAC values of the investigated compounds do not correlate with the calculated OH bond dissociation energies. The only molecular parameter that can reasonably explain the observed antioxidant behavior of the neutral forms of the monohydroxy- and dihydroxyflavones is their calculated ionization potential, reflecting the electron donating ability of the molecule (Table 1). The high activity of 3-hydroxyflavone and 5-hydroxyflavone in scavenging the $ABTS^{\cdot+}$ radical cation may result from the proximity of their OH moiety to the C4=O carbonyl

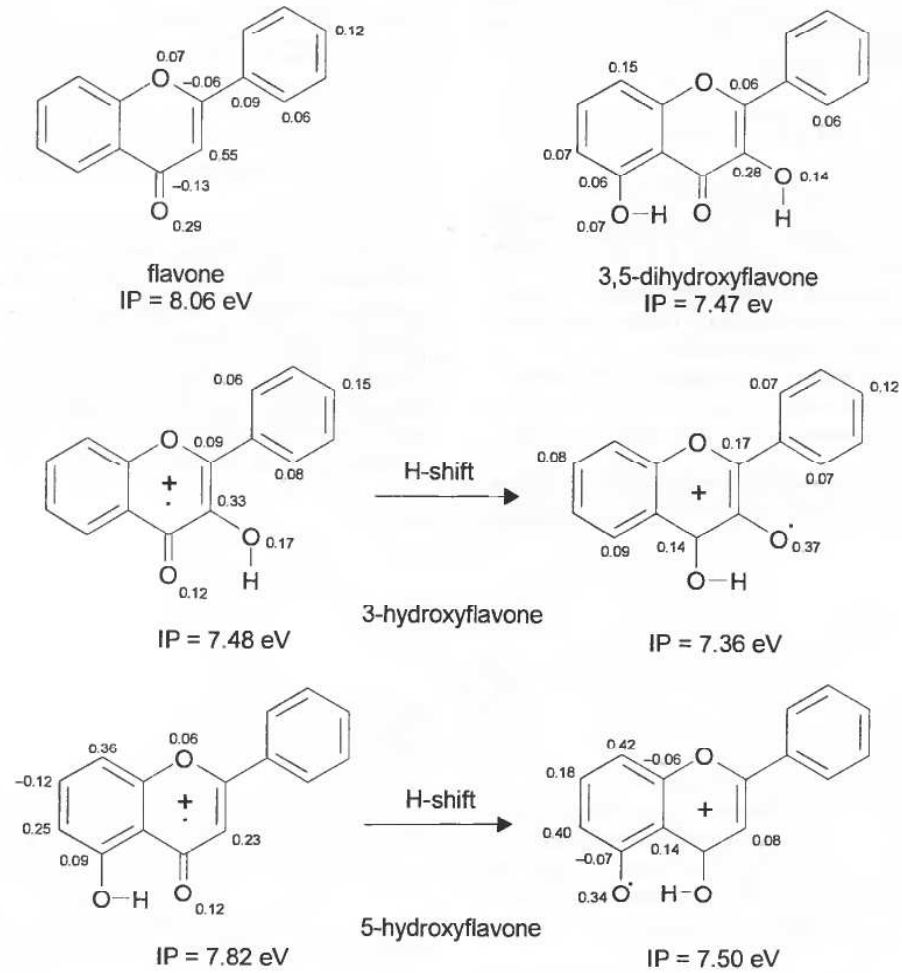

Fig. 8. Spin distributions and ionization potentials (IP, [eV]) of 3-hydroxyflavone and 5-hydroxyflavone and their isomeric forms in comparison to flavone and 3,5-dihydroxyflavone

group, enabling stabilization of the transient radical cation by isomerisation, thus lowering their ionization potential as shown in Fig. 8.

From results obtained in our study it may be concluded that C-3 and C-5 substitutions cause an increase of the TEAC value due to a decrease in redox potential of the flavone system. Due to strong hydrogen bonding 5-hydroxyflavone and 3-hydroxyflavone can not be a good hydrogen atom donor but can be a good electron donor due to the proton shift from C5-OH or C3-OH to to C4=O carbonyl group (Fig. 8). Thus, the C4=O carbonyl group was found to be an important structural element in stabilization of transient flavonoid phenoxyl radicals.

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