Reduction potentials of flavonoid and model phenoxyl radicals. Which ring in flavonoids is responsible for antioxidant activity?

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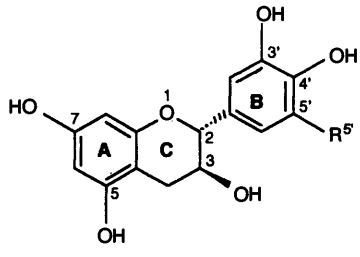
Model phenoxyl and more complex flavonoid radicals were generated by azide radical induced one-electron oxidation in aqueous solutions. Spectral, acid–base and redox properties of the radicals were investigated by the pulse radiolysis technique. The physicochemical characteristics of the flavonoid radicals closely match those of the ring with the lower reduction potential. In flavonoids which have a 3,5-dihydroxyanisole (catechins), or a 2,4-dihydroxyacetophenone (hesperidin, rutin, quercetin)-like A ring and a catechol- or 2-methoxyphenol-like B ring, the antioxidant active moiety is clearly the B ring [reduction potential difference between the model phenoxyls is ΔE(A–B ring models) > 0.1 V]. In galangin, where the B ring is unsubstituted phenyl, the antioxidant active moiety is the A ring. Even though the A ring is not a good electron donor, E_7 > 0.8/NHE V, it can still scavenge alkyl peroxyl radicals, E_7 = 1.06 V, and the superoxide radical, E_7 > 1.06 V. Quercetin is the best electron donor of all investigated flavonoids (measured $E_{10.8}$ = 0.09 V, and calculated E_7 = 0.33 V). The favourable electron-donating properties originate from the electron donating O-3 hydroxy group in the C ring, which is conjugated to the catechol (B ring) radical through the 2,3-double bond. The conjugation of the A and B rings is apparently minimal, amounting to less than 2.5% of the substituent effect in either direction. Thus, neglecting the acid–base equilibria of the A ring, and using those of the B ring and the measured values of the reduction potentials at pH 3, 7 and 13.5, the pH dependence of the reduction potentials of the flavonoid radicals can be calculated. In neutral and slightly alkaline media (pH 7–9), all investigated flavonoids are inferior electron donors to ascorbate. Quercetin, E_7 = 0.33 V, and gallocatechins, E_7 = 0.43 V, can reduce vitamin E radicals (assuming the same reduction potential as Trolox C radicals, E_7 = 0.48 V). Since all investigated flavonoid radicals have reduction potentials lower than E_7 = 1.06 V of alkyl peroxyl radicals, the parent flavonoids qualify as chain-breaking antioxidants in any oxidation process mediated by these radicals.

Introduction

Flavonoids (representative structures are shown below) are secondary metabolites of higher plants.[1,2] These polyphenols are present in substantial amounts (0.5–1.5%) in epidermal plant cells,[3] and in plant derived foods and beverages (catechins constitute approximately 40% of dry weight of tea leaves[4,5]). The pharmacological significance of flavonoids was recognized very early.[6–8] Flavonoids are known to have beneficial action in cardiovascular disorders.[9] This can be attributed in part to their ability to inactivate enzymes and proteins. It is noteworthy that the phenol groups of the A ring and O-1 and the OH group at C-3 of the C ring are important positioning points in the enzyme inactivation. If these groups are blocked through binding to a 'receptor site', is any antioxidant action of a flavonoid still possible?

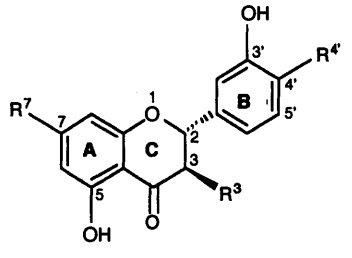
The ability of flavonoids to act as antioxidants *in vivo* and *in vitro* has been extensively studied.[5,10–24] Various structure–activity relationships were derived, some of them are rather contradictory.[13,17,18] The consensus appears to be, however, that these polyphenols act as electron donors in aqueous media and H-atom donors in nonpolar systems. One-electron reduction potentials of phenoxyl radicals provide the quantitative measure of the ability of parent flavonoids to act as electron donors. Numerous studies offer indirect[15,19,23–25] and some direct[17,18] evidence that the catechol-type (B) ring in the catechins and selected flavones and flavanones is the antioxidant active moiety. In view of the importance of the reduction potentials of



Flavan-3-ols

$R^{5'}$= H, (±)-Catechin

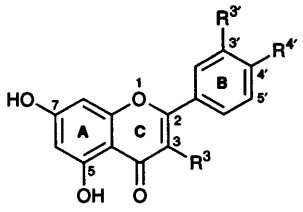
$R^{5'}$= OH, (–)-Epigallocatechin



Flavanones

R^3=R^7=$R^{4'}$=OH, Dihydroquercetin (Taxifolin)

R^7=O-Rutinose; R^3=H; $R^{4'}$=OCH_3, Hesperidin



Flavones
$R^{3'} = R^{4'} = H$, $R^3 = OH$, Galangin
$R^{3'} = R^{4'} = OH$, R^3 = O-Rutinose, Rutin
$R^3 = R^{3'} = R^{4'} = OH$, Quercetin

flavonoid radicals for the assessment of their antioxidant potential, and the identification of the antioxidant active moiety in these complex polyphenols for their pharmacological action, we investigated the redox properties of the model phenoxyl and selected flavonoid radicals in the pH range 3–14. Our previous findings [17,18] that the ring with lower reduction potential of radicals is the antioxidant active site in the catechins are fully confirmed and expanded to other flavonoids.

Materials and methods

(−)-Epicatechin and (−)-epigallocatechin were extracted from green tea and purified by Mitsui Norin Co., as detailed elsewhere.[4] Trolox C, rutin, galangin, 2,4-dihydroxyacetophenone, 4-methylcatechol and 2-methoxy-4-methylphenol (Aldrich), taxifolin and promethazine hydrochloride (Sigma), quercetin and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine hydrochloride (Fluka), 4-methoxyphenol, sodium azide, potassium bromide, phosphate and borate buffers, NaOH, $HClO_4$ and potassium thiocyanate (Merck), were used as received. Water was purified through a Millipore Milli-Q system. All solutions were prepared freshly before each experiment. The solutions were purged with high purity (>99.99%) N_2O to enable conversion of e^-_{aq} to the hydroxyl radical.[26]

The 3 MeV van de Graaff pulse radiolysis equipment with optical detection at the Max-Planck-Institut für Strahlenchemie[27] was used for the pulse radiolysis studies. A 2 cm Suprasil quartz cell with temperature variation through a thermostatically controlled liquid jacket was used for sample irradiation. The spectra of the radicals and the dissociation constants were measured at 3–5 Gy/pulse, whereas the rate constants were determined at lower 1–2 Gy/pulse to minimize interference from radical–radical reactions. Thiocyanate dosimetry was used in dose determinations, assuming $G\varepsilon_{480}[(SCN)_2^{\bullet -}] = 4.56 \times 10^{-4}$ J^{-1} m^2 in N_2O-saturated 10 mmol dm^{-3} KSCN aqueous solutions.

The rate constants of the azide radical induced one-electron oxidations of the phenols were determined by monitoring the build up of the absorption of the phenoxyl radical. The concentration of phenols was varied from 0.03 to 0.5 mmol dm^{-3}, which ensured the pseudo-first order of the $N_3^{\bullet}$ + phenol reaction. The second-order rate constant was then derived from the dependence of the pseudo-first-order rate on the concentration of the phenol.

The flavonoid and radicals of redox standards for the measurements of the reduction potentials were generated in N_2O-saturated aqueous solutions of 0.1 mol dm^{-3} NaN_3, containing 1 mmol dm^{-3} phosphate buffer for the measurements at pH 7 or 0.32 mol dm^{-3} KOH for pH 13.5 at 20 °C. Promethazine radical cation was generated by the bromide radical induced oxidation of promethazine hydrochloride at pH 3.0 in 0.1 mol dm^{-3} KBr aqueous solutions at 20 °C, because of favourable solubility of promethazine·HCl. Low dose rates from 0.6 to 1.3 Gy/pulse, trace averaging (10–50 traces per measurement), and high 0.1–20 mmol dm^{-3} solute concentrations were used in these measurements to obtain accurate pseudo-first-order rates. The flavonoid radical anions decay at $2k$ *ca.* 10^7 dm^3 mol^{-1} s^{-1},[28] whereas the decay rates of the radicals of redox standards (except for the 4-methoxyphenoxyl radical, $2k$ *ca.* 10^9 dm^3 mol^{-1} s^{-1}) are generally lower, $k < 10^6$ dm^3 mol^{-1} s^{-1}. In the presence of the flavonoid radicals the radicals from redox standards decayed more quickly than in their absence, however, the concentrations of the solutes were always adjusted to enable the accurate determination of the equilibrium absorbances. In all studied reactions the equilibrium was verified from the absorbance measurements at several ratios of solute concentrations (see Table 1), and more weight has been given to these values. Fig. 1 is given as a typical example of this procedure.

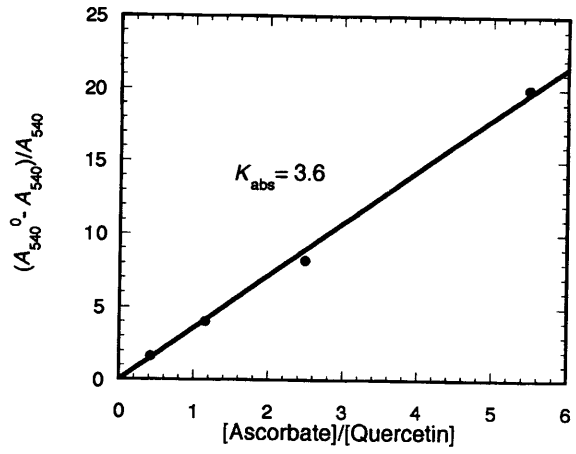


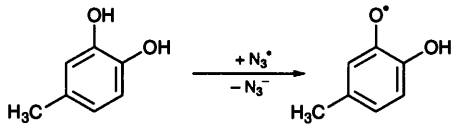
Fig. 1 Equilibrium absorbance of the quercetin radical anion as a function of the concentrations of quercetin and ascorbate, measured by pulse radiolysis in 0.1 mol dm^{-3} NaN_3 at pH 10.80, 20 °C, 0.08 Gy/pulse. The concentrations of quercetin and ascorbate were 2 mmol dm^{-3} and from 0.84 to 11 mmol dm^{-3}, respectively.

Results and discussion

Reduction potentials of model phenoxyls

The models for the A and B rings have been chosen to enable the comparison with various classes of flavonoid radicals. As may be seen from Table 2, the physicochemical properties of the A and B rings of the investigated flavonoids closely resemble those of 3,5-dihydroxyanisole, 2,4-dihydroxyacetophenone, 1,2-dihydroxy-4-methylbenzene (4-methylcatechol), 2-methoxy-4-methylphenol and methyl gallate. We have reported previously the dissociation constants and reduction potentials of 3,5-dihydroxyanisole and methyl gallate radicals,[17] so in this study we investigated the properties of 2,4-dihydroxyacetophenone, 4-methylcatechol and 2-methoxy-4-methylphenol radicals.

The phenoxyl radicals were generated by the azide radical induced one-electron oxidation of model phenols, *e.g.* the reaction with 4-methylcatechol. The rate constant of one-



electron oxidations are close to diffusion controlled, $k = (5.0 \pm 0.5) \times 10^9$ dm^3 mol^{-1} s^{-1} for 2-methoxy-4-methylphenol, $k = (2.7 \pm 0.3) \times 10^9$ dm^3 mol^{-1} s^{-1} for 4-methylcatechol and $(1.1 \pm 0.1) \times 10^9$ dm^3 mol^{-1} s^{-1} for 2,4-dihydroxyacetophenone. In spite of the rapid decay of the azide radical, $2k = 9 \times 10^9$ dm^3 mol^{-1} s^{-1},[13] at concentrations of any substituted phenol higher than 0.1 mmol dm^{-3} and at dose rates of 1–2 Gy/pulse (corresponding to 0.6–1.2 μmol dm^{-3} radicals), more than 99.9% of the initially generated $^{\bullet}$OH (*i.e.* azide) radicals are converted to phenoxyl radicals.

The 2-methoxy-4-methylphenoxyl radicals are neutral and do not have any pK_a values in the accessible pH range (3–14). The dissociation constant of the 4-methylcatechol radical,

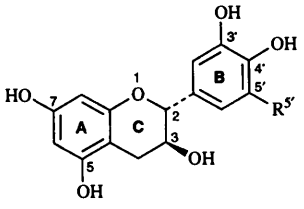
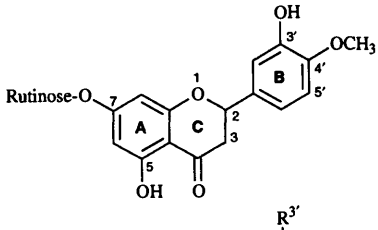
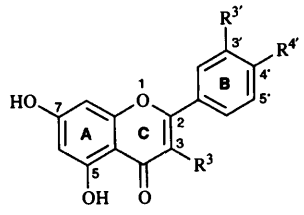
Table 1 One-electron transfer reactions of flavonoids

$$A^{\bullet} + D \underset{k_r}{\overset{k_f}{\rightleftharpoons}} A + D^{\bullet}$$

Radicals, A$^{\bullet}$, from	Donor, D	pH	k_f[a]/dm^3 mol^{-1} s^{-1}	k_r[a]/dm^3 mol^{-1} s^{-1}	K_{kin}	K_{abs}[b]	ΔE[c]/V
Hesperidin	Promethazine	3.0	3.0×10^9	—	—	69	0.11
Promethazine	Epigallocatechin	3.0	1.4×10^7	8×10^5	18	19	0.08
Catechin	Promethazine	3.0	1.5×10^8	—	—	6	0.05
TMPD	Rutin	13.5	1.3×10^9	—	—	41	0.1
Hesperidin	TMPD	13.5	1.4×10^9	—	—	460	0.16
Galangin	TMPD	13.5	1.6×10^9	—	—	55	0.1
Quercetin	Ascorbate	10.8	2.4×10^5	7×10^4	3	4	0.032

[a] Estimated to be accurate to ±10% for the rate constants in the forward direction, ±20% for the others. [b] Equilibrium constant derived from the absorbances of the radicals at equilibrium. The emphasis has been given to these values, because they are more reliable. [c] The reduction potential difference calculated from the equilibrium constant using the Nernst equation, $\Delta E = 0.059 \log K$.

Table 2 Structures of selected flavonoids and their models

Flavonoid	Structure	A ring	B ring
Catechin ($R^{5'} = H$) Epigallocatechin ($R^{5'} = OH$)		3,5-Dihydroxyanisole	Catechol
Hesperidin			2-Methoxy-4-methylphenol
Galangin ($R^{3'} = R^{4'} = H$; $R^3 = OH$) Rutin ($R^{3'} = R^{4'} = OH$; R^3 = O-Rutinose) Quercetin ($R^3 = R^{3'} = R^{4'} = OH$)		2,4-Dihydroxyacetophenone	4-Methylcatechol, 1-Catechol-2-hydroxy-ethylene, or Catechol

$pK_a = 5.1 \pm 0.1$, was derived from the pH-dependent changes in the spectra [Fig. 2(*a*)]. It is only slightly higher than $pK_a = 5.0$[29,30] of the catechol radical. The substituent effect of the electron-donating methyl group, which is apparently in the *meta* position to the radical site, is indeed very small [*e.g.* $\sigma^+_m(CH_3) = -0.07$[31]]. Similarly [Fig. 1(*b*)], $pK_r = 5.9 \pm 0.1$ for the 2,4-dihydroxyacetophenone radical. This value is lower than $pK_r = 6.7$ of 3,5-dihydroxyanisole,[17] because of the electron-withdrawing effect of the acetyl group, $C(=O)CH_3$.

The reduction potentials of 4-methylcatechol, 2-methoxy-4-methylphenol and 2,4-dihydroxyacetophenone radicals were measured at pH 7 against 4-methoxyphenol ($E_7 = 0.73$ V)[32,33] and Trolox C ($E_7 = 0.48$ V)[22] as standards. These redox standards are chosen because of the reliability of the values of the reduction potentials and the favourable spectral properties of their radicals. The *ortho*-substituted phenoxyl radicals of 2,4-dihydroxyacetophenone, 2-methoxy-4-methylphenol and 4-methylcatechol do not absorb appreciably at 420 and 425 nm which are the absorption maxima of 4-methoxyphenoxyl and Trolox C chromanol radical, respectively.

The method for measuring the reduction potential difference between the radicals of unknown reduction potential and those of the redox standard has been thoroughly described elsewhere,[34] and its virtues and faults repeatedly evaluated.[32,34-37]

Following the usual procedure, the phenoxyl radicals were generated by the azide radical induced one-electron oxidation of parent phenols. One-electron transfer equilibria between 4-methoxyphenol and 2-methoxy-4-methylphenol, and between 2,4-dihydroxyacetophenone and 4-methoxyphenol radicals were followed at 420 nm, whereas the equilibrium between the 4-methylcatechol and Trolox C radicals was monitored at 425 nm. Both the kinetics of the reaction, and the absorbances of the radicals depended on the ratio of the concentrations of the parent phenols, which is taken to indicate the equilibrium conditions, as presented in Scheme 1. The reduction potentials of the model phenoxyls studied are summarized in Table 3, together with the previously measured dissociation constants and reduction potentials of the other model phenoxyl radicals.

Obviously, the A ring models, 2,4-dihydroxyacetophenone and 3,5-dihydroxyanisole phenol radicals, have the highest reduction potentials, $E_7 = 0.89$ and $E_7 = 0.84$ V,[18] respectively (Table 3). The reduction potentials of the B ring models, 4-methylcatechol, $E_7 = 0.52$ V, and methylgallate, $E_7 = 0.56$ V,[18] radicals are considerably lower. This trend agrees well with the previously published reduction potentials of the unsubstituted resorcinol ($E_7 = 0.81$ V),[22] catechol ($E_7 = 0.53$ V)[22] and pyrogallol ($E_7 = 0.575$ V)[22] phenoxyls.

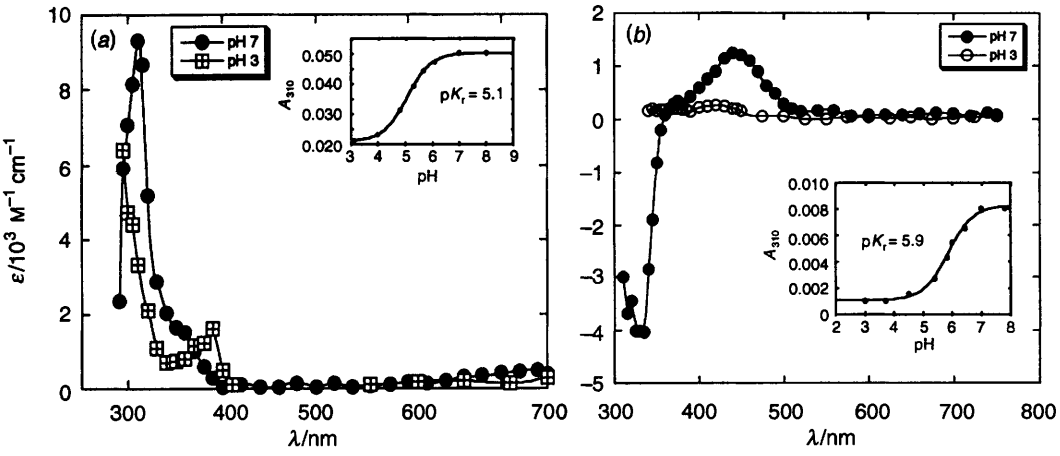
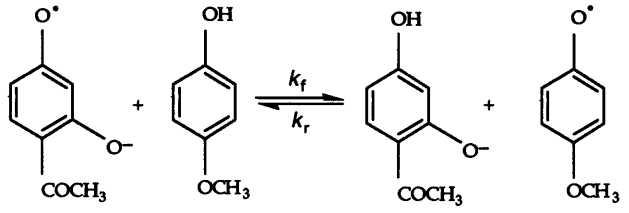


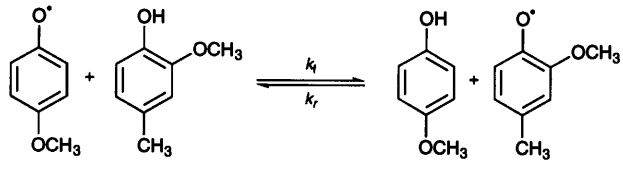
Fig. 2 Transient absorption spectra of phenoxyl radicals at pH 3 and 7 obtained upon $N_3^{\cdot}$ oxidation of (*a*) 4-methylcatechol in N_2O-saturated aqueous solution of 0.37 mmol dm^{-3} 4-methylcatechol, 2 mmol dm^{-3} phosphate buffer and 30 mmol dm^{-3} NaN_3, at dose/pulse *ca.* 2 Gy, 20 °C and (*b*) 2,4-dihydroxyacetophenone in N_2O saturated aqueous solution of 0.94 mmol dm^{-3} 4-methylcatechol, 2 mmol dm^{-3} phosphate buffer and 40 mmol dm^{-3} NaN_3, at dose/pulse *ca.* 3 Gy, 20 °C. The pH-dependent changes of the absorption at 310 nm are presented as an inset.



$k_f = 6.5\times10^7$ dm^3 mol^{-1} s^{-1}; $k_r = 4\times10^5$ dm^3 mol^{-1} s^{-1}

$K_{abs} = 210$

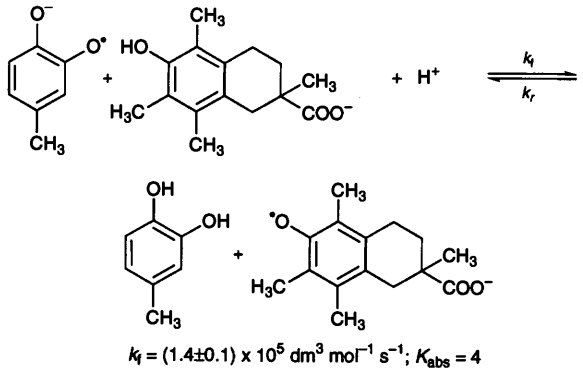
$\Delta E = 0.14$ V, E_7[2,4-(OH)O$^{\cdot}$C$_6$H$_3$COCH$_3$] = 0.89 V



$k_f = (5.7\pm0.6)\times10^5$ dm^3 mol^{-1} s^{-1}; $k_r = (7\pm1)\times10^4$ dm^3 mol^{-1} s^{-1}

$K_{kin} = 8$ $K_{abs} = 8$

$\Delta E = 0.05$ V, E_7(2-OCH$_3$-4-CH$_3$C$_6$H$_3$O$^{\cdot}$) = 0.68 V



$k_f = (1.4\pm0.1)\times10^5$ dm^3 mol^{-1} s^{-1}; $K_{abs} = 4$

$\Delta E = 0.04$ V, E(2-O$^{\cdot}$-4-CH$_3$C$_6$H$_3$O$^{\cdot}$) = 0.52 V

Scheme 1

Table 3 Reduction potentials of model phenoxyl radicals

Phenoxyl	E_7/V, NHE	pK_r[a]
3,5-Dihydroxyanisole	0.84[b]	6.7[b]
2,4-Dihydroxyacetophenone	0.89	5.9
2-Methoxy-4-methylphenol	0.68	<3
Catechol	0.53[c]	5.0[c]
4-Methylcatechol	0.52	5.1
Methyl gallate	0.56[b]	4.4; 9.2[b]

[a] Refers to the equilibrium between neutral and semiquinone radical anion. [b] From ref. 18. [c] From ref. 29.

Table 4 Reduction potentials of flavonoid radicals

Radicals from	E_3/V	E_7/V	$E_{13.5}$/V
Hesperidin	1.09	0.72[a]	0.43
Galangin	n/a	n/a	0.37
Rutin	1.02[b]	0.6[a]	0.17
Catechin	1.04	0.57[a]	0.08[c]
Epigallocatechin	0.9	0.43[d]	n/a
Quercetin	n/a	0.33[b]	−0.037[c]
			$E_{10.8}$/V = 0.090

[a] From ref. 17. [b] Calculated from the pH dependence (see text). [c] From ref. 22. [d] From ref. 18.

Reduction potentials of flavonoid radicals

It is conceivable that the electron-donating moiety in any flavonoid will be the ring whose daughter radical has the lowest reduction potential. For example, the reduction potential difference between the A and B ring phenoxyls in the catechin derivatives is $\Delta E_7 > 0.1$ V (see Table 3), which corresponds to $K > 100$. This means that even if the A ring radical is somehow generated, it will practically irreversibly oxidize the B ring. Such reactions indeed occur intra- and inter-molecularly as previously reported[18] for the azide radical induced oxidation of the catechin derivatives. The question remains, however, as to the extent of coupling of the A and B rings, especially in the flavone derivatives through canonical structures involving the 2,3-double bond, O-1 and C-4 carbonyl group of the C ring. To address this question quantitatively, and to enable the evaluation of the pH dependence of the reduction potentials of various flavonoid radicals, we investigated one-electron transfer equilibria of catechin, epigallocatechin and hesperidin with promethazine at pH 3 ($E_3 = 0.98$ V),[32] of rutin, hesperidin and galangin with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine at pH 13.5 ($E = 0.27$ V),[22] and of quercetin with ascorbate ($E_{10.8} = 0.06$ V)[22] at pH 10.80. The results are summarized in Tables 1 and 4.

Clearly, the reduction potentials of the phenoxyl radicals derived from flavan-3-ols (catechins), hesperidin (flavanone) and rutin (flavone) are similar to those of the model phenoxyls for the B ring (see Tables 3 and 4). This is in full agreement with the spectral and acid–base properties of the flavonoid phenoxyl radicals, which resemble in every detail those of the B ring models (for detailed spectra see ref. 18). It is interesting to note that, as already pointed out in our earlier paper,[17] the influence of the fully saturated C ring on the flavonoid radical is minimal, amounting to a Brown $\sigma^+ \approx 0$. The spectra of rutin radicals resemble those of the anthocyanidins,[25] which indicates some coupling of the B ring radical with the C ring. However, the

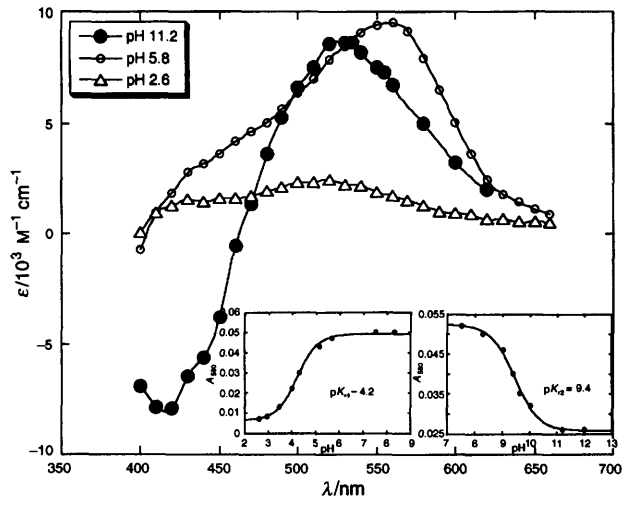


Fig. 3 Transient absorption spectra of quercetin radicals at different pH obtained upon $N_3^{\bullet}$ oxidation of quercetin in N_2O-saturated aqueous solution of 40 μmol dm^{-3} quercetin, 0.1 mmol dm^{-3} phosphate buffer and 10 mmol dm^{-3} NaN_3, at dose/pulse *ca.* 2 Gy, 20 °C. The changes in the spectra with pH monitored at 580 nm are given as insets.

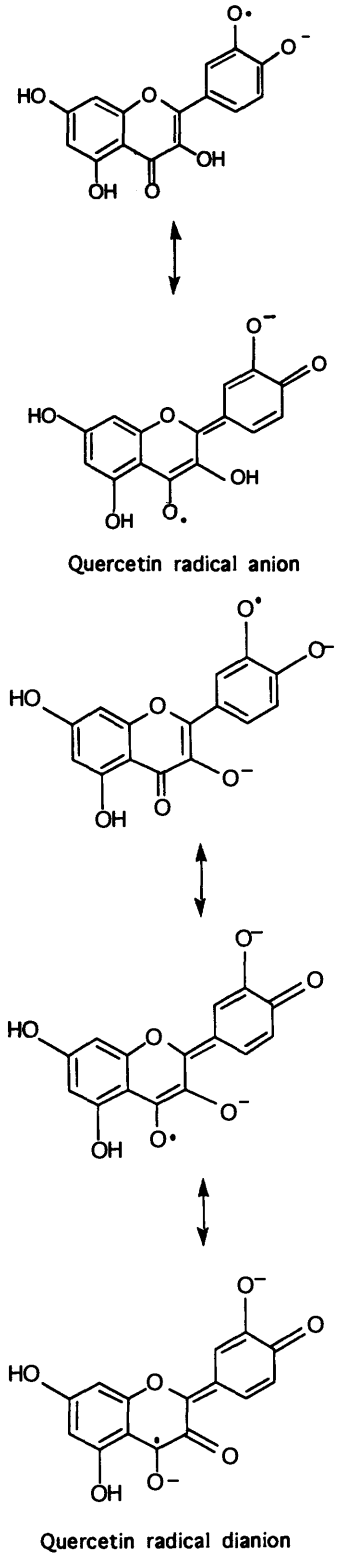
2,3-double bond in the rutin radical has an electron-withdrawing effect, and its reduction potential is higher at any pH than that of the catechin radical, which lacks the 2,3-double bond.

The reduction potential of the epigallocatechin radical is lower than those of hesperidin, rutin and catechin in the pH range 3–7, because of the electron rich gallate moiety. It should be emphasized that this water soluble constituent of green tea should be considerably more stable in acidic (pH 3) than in neutral media, similarly to other phenolic antioxidants (*e.g.* ascorbate) because of less favourable electron-donating properties under acidic conditions.

Quercetin radicals have the lowest reduction potential of all investigated flavone radicals. At pH 13.5, $E_{13.5} = -0.037$ V[22] is even lower than $E_{13.5} = 0.015$ V[22] of the 'electron-sink' ascorbate. In comparison with similar rutin radicals, which have $E_{13.5} = 0.17$ V, such favourable electron-donating properties of quercetin indicate efficient coupling between the B ring radical with the OH (or O^-) group in the C ring through the 2,3-double bond. However, to establish the electron-donating properties of this flavonoid in more biologically significant neutral media, we first had to thoroughly investigate the spectral and acid–base properties of the daughter radicals. The representative spectra of various forms of the quercetin radicals and the pK_r curves derived from the spectral changes are presented in Fig. 3.

The existence of the two pK_r values for the quercetin radicals is at first sight surprising, because the catechol radical (B ring) has only one ionizable hydroxy group. On the basis of the similarity with the 3,4-dihydroxycinnamate radical,[17] the $pK_{r1} = 4.2$ is easily assigned to the OH group at C-4′ (catechol), thus $pK_{r2} = 9.4$ may only originate from the ionization of the OH group at C-3 in the radical. Some canonical structures of the quercetin radical anion and dianion are presented in Scheme 2, to highlight the coupling between B and C rings.

How good an antioxidant is the A ring in flavones? The electron-donating properties of galangin, which has no hydroxy substituents in the B ring, may only reflect those of the A ring. However, $E_{13.5} = 0.37$ V is lower than the value calculated for the model 3,5-dihydroxyanisole radical, $E_{13.5} = 0.46$ V, which probably indicates the influence of the C ring hydroxy group. In acidic and neutral media (pH 3–9), galangin radicals undergo a first-order reaction, transforming from the 540 nm spectra to the 440 nm spectrum (Fig. 4). This transformation, although interesting, precludes accurate measurements of redox and acid–base properties of the galangin radicals below pH 9.



Scheme 2

pH Dependence of reduction potentials of flavonoid phenoxyls

The lack of influence of the ring with the higher reduction potential of daughter phenoxyl on the ring with the lower reduction potential simplifies the pH dependence of the reduction potentials of the flavonoids containing these rings. For example, with an error of *ca.* 2.5% (calculated on the basis of *ca.* 40% attenuation of the substituent effect per saturated bond), we can assume that the dissociation constants of the A ring in catechin, epigallocatechin, and rutin can be neglected and only use the dissociation constants of the B ring in the evaluation of the pH dependence of the reduction potentials of

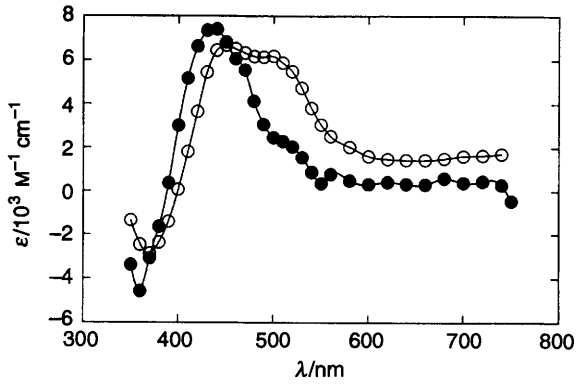


Fig. 4 The spectra of galangin radicals obtained by pulse radiolysis in an N_2O-saturated aqueous solution of 0.016 mmol dm^{-3} galangin, 0.2 mmol dm^{-3} phosphate buffer, 7 mmol dm^{-3} NaN_3, at pH 7.2, 20 °C. —○— Upon completion of the one-electron oxidation of galangin at 140 μs after the pulse, and —●— upon the first-order transformation in the radical spectra at 6 ms after the pulse.

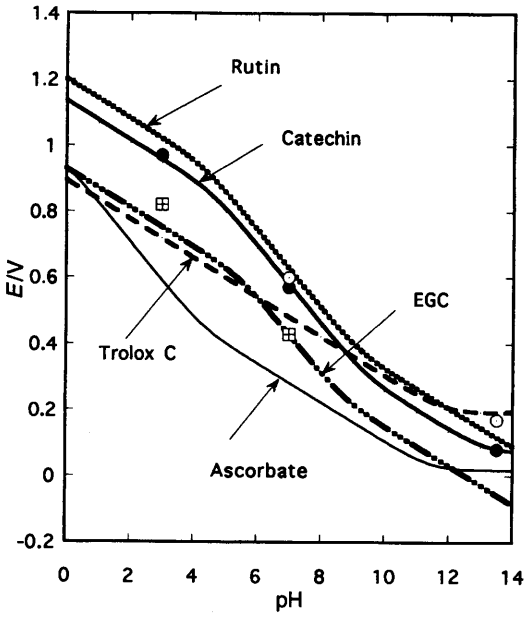


Fig. 5 pH Dependence of the reduction potentials of the flavonoid and simple phenol radicals. The points are experimental measurements, and the curves are calculated from the formulae in the text. The dependences for Trolox C and ascorbate radicals[22] are given for comparison.

corresponding radicals. Consequently, the 'half-electrode reaction' is, ^-O–Fl–$O^\bullet + e^- + 2H^+ \longrightarrow$ HO–Fl–OH, which leads to the formula, eqn. (1) where pK_{a1} and pK_{a2} are the dissociation

$$E_{pH} = E^{0\prime} + 0.059 \log \frac{(10^{-pK_{a1}}10^{-pK_{a2}} + 10^{-pK_{a1}}10^{-pH} + 10^{-2pH})}{(10^{-pK_r} + 10^{-pH})} \quad (1)$$

constants of the B ring in the flavonoid,[17,18] and pK_r is the dissociation constant of the corresponding radical. $E^{0\prime}$ is the reduction potential at pH 0, which is not necessarily equal to the standard potential (the difference amounts to the deviation of the activity coefficients of reduced and oxidized forms of flavonoids from 1). The pH dependence of the reduction potentials of catechin and rutin radicals is presented in Fig. 5 together with those of Trolox C and ascorbate radicals.

Clearly, there is good agreement between the calculated pH dependence and the measured values of the reduction potentials of the flavonoids. We would like to emphasize the well-known rigidity of the *E vs.* pH function. The calculated curve leaves little room for speculation about the accuracy of

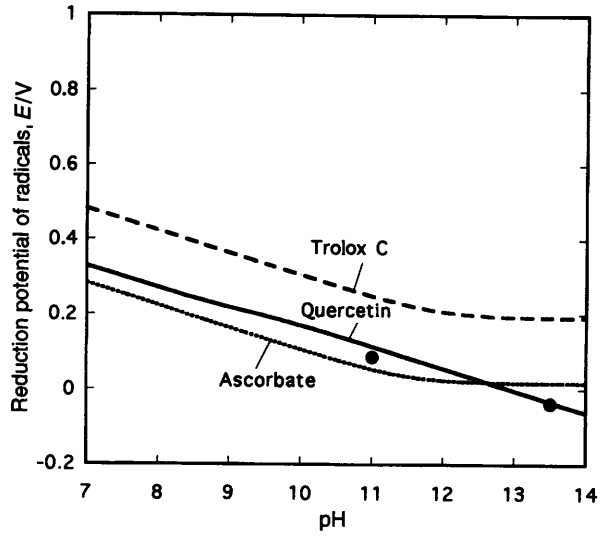


Fig. 6 pH Dependence of the reduction potentials of quercetin radicals. The points are experimental measurements and the curve is calculated from the formulae in the text. The dependences for Trolox C and ascorbate radicals[22] are given for comparison.

the measurements, because the agreement of the independently determined experimental values at different pH is better than ±0.03 V in the pH range 7–14. At pH 3, the calculated values are about 0.1 V lower than the measured values. This might be either due to the inaccuracy in the reduction potential of the promethazine radical (*e.g.* another study[36] gives a 0.08 V lower value, $E = 0.91$ V) or to the existence of another pK_a of quercetin in the low pH range.

The notable exception from the simple pH dependence is the quercetin radical. First, the radical has two pK_r values indicating the coupling of the B and C rings, and second, the value measured at pH 10.80, $E = 0.09$ V, is lower than $E = 0.22$ V derived from the simple pH dependence. A reasonable fit through both measured values at pH 10.80 and pH 13.5 may only be obtained if the following 'half-electrode reaction' is assumed, ^-O–Fl(O^-)–$O^\bullet + e^- + 2H^+ \longrightarrow$ HO–Fl(O^-)–OH which leads to the formula, eqn. (2).

$$E_{pH} = E^{0\prime} + 0.059 \log \frac{(10^{-pH})(10^{-9.02} + 10^{-pH})}{(10^{-4.2-9.4} + 10^{-4.2-pH} + 10^{-2pH})} \quad (2)$$

The first term in the denominator accounts for the fact that the second pK_{a2} of the B ring in quercetin (probably above pH 13) is not known with sufficient accuracy. The pH dependence of the reduction potential of the quercetin radical is presented in Fig. 6 together with ascorbate.

From the above 'half-electrode' reaction, the quercetin radical has apparently one pK_r without a corresponding pK_a in the parent molecule. However, provided that this 'hidden' pK_a of quercetin lies below pH 7 (*e.g.* keto–enol tautomerism of the C-4 carbonyl), it cannot influence the pH dependence of the reduction potential of the radical in the pH range 7–13.5. Unfortunately, the low solubility of quercetin in water precludes meaningful measurements of the reduction potential of the radicals at lower pH values, to extend the pH dependence below pH 7 (see Table 3).

We would like to comment on the recent paper[13] where the reduction potentials of some flavonoid radicals were determined using ascorbate as a redox standard at pH 8.5. The value of the reduction potential of quercetin estimated in that study, $E_{8.5} = 0.398$,[13] is higher than $E_{8.5} = 0.245$ V from our pH dependence, whereas the value of rutin, $E_{8.5} = 0.275$ V,[13] is considerably lower than our $E_{8.5} = 0.46$ V. Apparently, not only the values of the reduction potentials, but also the trend in substituent effects published in that study are inconsistent

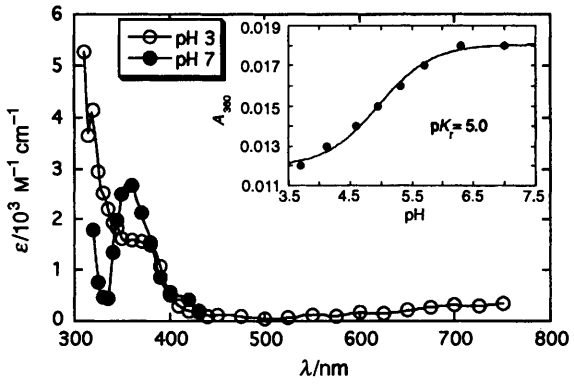


Fig. 7 The spectra of dihydroquercetin radicals obtained upon $N_3^•$ oxidation of dihydroquercetin in N_2O-saturated aqueous solution of 0.04 mmol dm^{-3} dihydroquercetin, 0.4 mmol dm^{-3} phosphate buffer and 10 mmol dm^{-3} NaN_3, at dose/pulse *ca.* 2 Gy, 20 °C. The pH-dependent changes of the absorption at 360 nm are presented as an inset.

with our observations. In an attempt to rationalize these inconsistencies, we investigated the reduction of the rutin radicals by ascorbate at pH 8.5, which was claimed [13] to lead to one-electron transfer equilibrium with $K = 24$. In an N_2O-saturated aqueous solution of 64 mmol dm^{-3} NaN_3, 1.2 mmol dm^{-3} rutin, 1 mmol dm^{-3} phosphate buffer, containing 0.18–1.84 mmol dm^{-3} ascorbate, at dose/pulse = 1 Gy (corresponding to 0.6 μmol dm^{-3} radicals), the reduction of the rutin radical by ascorbate monitored at 460 nm is found to be slow, $k = (8.0 \pm 0.8) \times 10^5$ dm^3 mol^{-1} s^{-1}. However, the reaction proceeded to completion for every concentration of ascorbate, which means that an electron-transfer equilibrium is not established in this system, and K is in fact higher than 100, in support of our value for rutin radicals from which $\Delta E = 0.26$ V ($K = 2.55 \times 10^4$). Furthermore, the value of the rate constant $k = 8 \times 10^5$ dm^3 mol^{-1} s^{-1} is 50% lower than $k = 1.25 \times 10^6$ dm^3 mol^{-1} s^{-1},[13] which is well outside the margin of error in the measurements. The reason for the higher rate constant and the erroneous assignment of equilibrium conditions in that study [13] is very probably very high dose (20 Gy, corresponding to 11.6 μmol dm^{-3} of radicals, and solute concentrations in the μmol dm^{-3} range), which must affect the accuracy of the determination of any pseudo-first-order rate because of large interference from the second-order radical–radical and, possibly, radical–solute processes. Emphasis was also given to the computer analysis of the kinetic data,[13] however, unless the establishment of one electron-transfer equilibrium is verified through the absorbance measurements, as repeatedly pointed out,[32,34,36] erroneous evaluation of the data is inevitable.

We have also tried to repeat the measurement of the reduction potential of dihydroquercetin, which is claimed [13] to be a better electron donor than ascorbate. However, because of nearly complete overlap between the spectra of dihydroquercetin and ascorbate radicals (consistent with the published spectra [13]), the electron transfer in either direction could not be measured.

The spectral properties of the dihydroquercetin radical are found to be similar to those of the 4-methylcatechol radical. The pH dependent changes in the spectra of the dihydroquercetin radical are presented in Fig. 7. The $pK_r = 5.0$ is similar to $pK_r = 5.2$ of the 4-methylcatechol radical. The difference of 0.1 pH unit corresponds to less than 0.03 V difference in the reduction potentials of corresponding radicals, which means that the reduction potential of the dihydroquercetin radical should be between 0.52 V (4-methylcatechol, $pK_r = 5.1$) and 0.50 V (catechol $pK_r = 5.0$). This estimated higher reduction potential of the dihydroquercetin radical, $E_7 = 0.5 \pm 0.04$ V, more like the catechin than quercetin radicals, is consistent with the cyclic voltammetric measurements.[38] Although the electrochemical determinations [38] give consistently lower reduction potentials of the flavonoid radicals than our measurements under equilibrium conditions, the trend is similar to ours, reflecting well-established electronic substituent effects in the electron-transfer reactions of phenoxyl radicals.[33,35]

Finally, we would like to comment on the feasibility of the 'sparing' effect flavonoids may have on vitamin C.[13] Based on our results, even quercetin, which has the best electron-donating properties of the investigated flavonoids, cannot repair the vitamin C radicals in a physiologically meaningful pH range. Let alone that hesperidin, with the highest reduction potential of all investigated flavonoid radicals, which was the main constituent of Szent-Györgyi's vitamin 'P',[6–8] can 'spare' vitamin C in one-electron transfer reactions in aqueous media. If the hesperidin radicals are generated in the presence of ascorbate, hesperidin will be restored at the expense of vitamin C. However, there is little doubt that flavonoids can and do act as antioxidants, because of their high concentration in biological systems and the ability to scavenge various peroxyl radicals.[2,5,10–12,14–20,23,24]

Conclusions

The conjugation between the A and B rings in the flavonoid radicals is very inefficient. The substituent effects in either direction amount to less than 2.5%.

The ring whose radical has lower reduction potential is the antioxidant active moiety in any flavonoid. In the catechins, hesperidin, rutin, dihydroquercetin and quercetin the catechol B ring dominates their antioxidant action, whereas in galangin, where the 2,4-dihydroxyacetophenone A ring radical has the lower reduction potential, the A rings takes over.

The investigated flavonoids are inferior electron donors to ascorbate in a physiologically significant pH range. The gallocatechins and quercetin may restitute vitamin E (provided the reduction potential of vitamin E radicals is similar to that of Trolox C radicals) under physiological conditions. The favourable reduction potentials of the investigated flavonoids, which are lower than the reduction potentials of biologically damaging peroxyl radicals [*e.g.* alkyl peroxyl $E_7 = 1.06$ V and $E_7(O_2^{•-}/HO_2^-) > 1.06$ V [37]], and their abundance in foods and vegetables make them an important class of nutritional antioxidants.

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