One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media

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Quercetin (3,3′,4′,5,7-pentahydroxyflavone) and quercetin derivatives (3-methylquercetin, rutin) are strong flavonoid antioxidants abundant in plants and in human diet. Their oxidation by DPPH, CAN or dioxygen (autoxidation) is studied in protic and non protic solvents. From kinetic investigations by UV–visible spectroscopy, oxidation rate constants are estimated. Fast disproportionation of flavonoid radicals is shown to give quinones which can be identified by their adducts with methanol (quercetin quinone) or sodium benzenesulfinate (rutin quinone). In strongly alkaline non aqueous conditions, the quercetin quinone can also be evidenced by strong charge transfer absorption bands in the range 700–800 nm.

The consequences of these observations for the antioxidant properties of quercetin and quercetin derivatives are discussed.

Introduction

Flavonoids are the most important class of polyphenolic secondary metabolites in plants.[1] They generally display a chromophore of the 2-phenyl-1-benzopyran type diversely substituted by OH and OMe groups. Additional structural diversity comes from the structure of the central ring (presence of a keto group at C-4, of a C-2–C-3 double bond...) which differentiates the flavonoid sub-families (flavanones, flavones, flavonols...) and further modifications of the chromophore (*O*- and *C*-glycosidation, *C*-isoprenylation, *O*-sulfation...).

Besides their important biological roles in plant pigmentation, nitrogen fixation and chemical defense, flavonoids possess anti-cancer, anti-viral and anti-inflammatory properties[2] which are the consequence of their affinity for proteins (including enzymes) and their antioxidant properties.

Antioxidants[3] are compounds which, at low concentrations, can protect biomolecules (proteins, nucleic acids, polyunsaturated lipids, sugars) from oxidative degradation, in particular by reactive oxygen species (*e.g.*, hydroxyl, alkoxyl and alkylperoxyl radicals, singlet dioxygen...) arising from incomplete reduction of dioxygen in the electron transporting chain or from the catalytic cycle of redox enzymes involved in purine and lipid metabolism or in antibacterial defense.[4] Flavonoid antioxidants may act in a variety of ways including direct quenching of the reactive oxygen species, inhibition of enzymes involved in the production of the reactive oxygen species, chelation of low valent metal ions (Fe^{2+}, Cu^{+}) able to promote radical formation through Fenton type reactions, regeneration of membrane-bound antioxidants such as α-tocopherol (vitamin E).[5] Consequently, flavonoids may be promising compounds to combat pathologies in which oxidative damage is involved such as cancers, cardiovascular and neurodegenerative diseases. In addition, epidemiological studies suggest that flavonoids which are relatively abundant in food could be key compounds in the relationship between health and diet.[6] Owing to the wide variety of flavonoid structures ranging from hydrophilic to relatively hydrophobic compounds, flavonoids could exert their antioxidant activity in biological sites of varying polarity (membranes, plasma...).

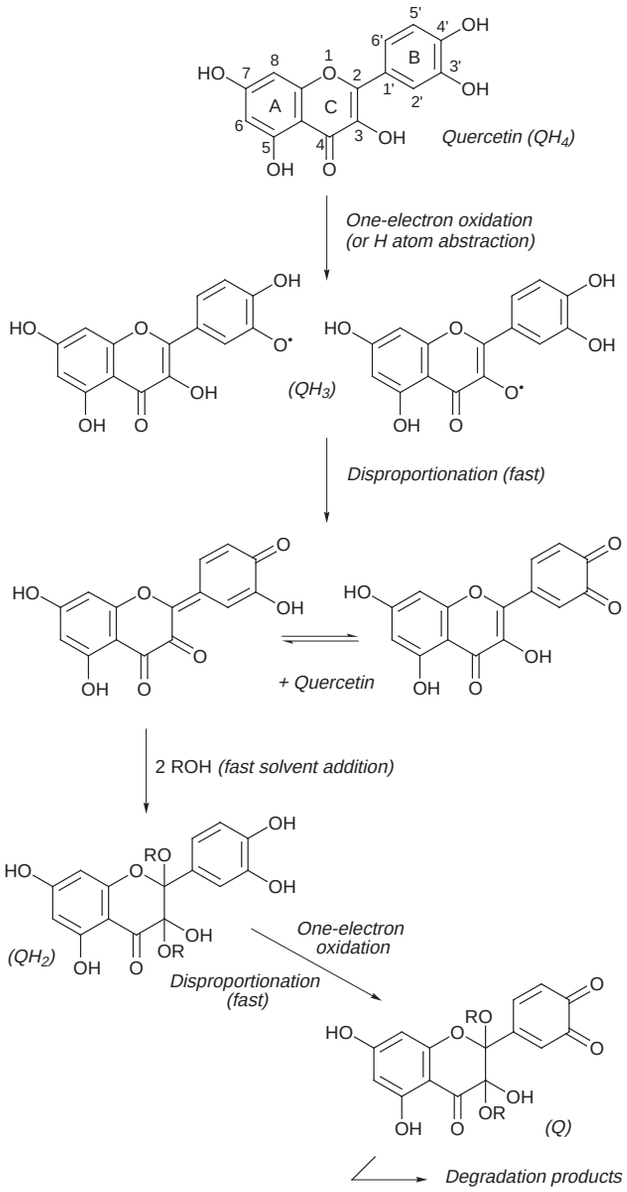
Until recently, there was a lack of information on the molecular mechanisms by which flavonoids may exert their antioxidant properties. In the last ten years however, investigations of flavonoid oxidation using the fast pulse radiolysis technique have allowed determination of the spectral properties, reduction potentials and pK_a values of flavonoid radicals as well as the kinetic parameters for the reactions of flavonoids with reactive oxygen species.[7] The structural requirements for strong antioxidant activity could thus be firmly established. These investigations also showed that the flavonoid radicals quickly decay through second-order kinetics ($2k$ in the range 10^6–10^7 dm^3 mol^{-1} s^{-1}). However, little information is so far available on the structure and reactivity of secondary oxidized products, a point which may have biological significance. For instance, the possible formation of quinones and/or quinonoid compounds upon radical disproportionation may have deleterious consequences since such compounds have well-documented oxidizing and electrophilic properties which may eventually cause biopolymer modifications through oxidation and/or covalent coupling.[8]

In this work, one-electron oxidation of quercetin and quercetin derivatives (flavonols) is quantitatively investigated by UV–visible spectroscopy in protic and non protic media (DMF, DMSO, methanol, aqueous buffers). Formation of quinone intermediates (through radical disproportionation) is investigated as well as the reactivity of the quinones in the different media under consideration. The consequences of these observations for the antioxidant properties of quercetin and its derivatives are discussed.

Results and discussion

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, Scheme 1) has been selected because it is abundant in plants and food and displays the structural requirements (a C-2–C-3 double bond, an *o*-dihydroxy substitution on ring B, a free OH group at C-3) favourable to strong antioxidant activity.[5,7]

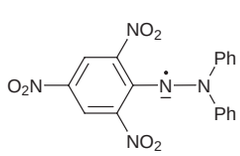
Rutin (3-(α-L-rhamnopyranosyl-1,6-β-D-glucopyranosyl)-quercetin), a very abundant quercetin glycoside, and 3-methylquercetin are also considered in order to outline the influence of common substituents on flavonoid oxidation.



Scheme 1

Oxidation by DPPH and CAN

Oxidation by DPPH.† DPPH (diphenylpicrylhydrazyl, Scheme 2) is a highly coloured commercially available radical



Scheme 2

widely used for a rough estimation of the ability of antioxidants to trap potentially damaging one-electron oxidants (H atom abstracting agents).[5i,9] In particular, antioxidants can be characterized by their stoichiometry *n i.e.* the number of DPPH molecules reduced by one molecule of antioxidant. Hence, the antioxidant can be regarded as a source of *n* H atoms that will convert DPPH into the corresponding hydrazine.

† *Oxidation* is here used as a general term. In the case of DPPH, direct abstraction of H atoms from the flavonoid OH groups is more likely than a true electron transfer.

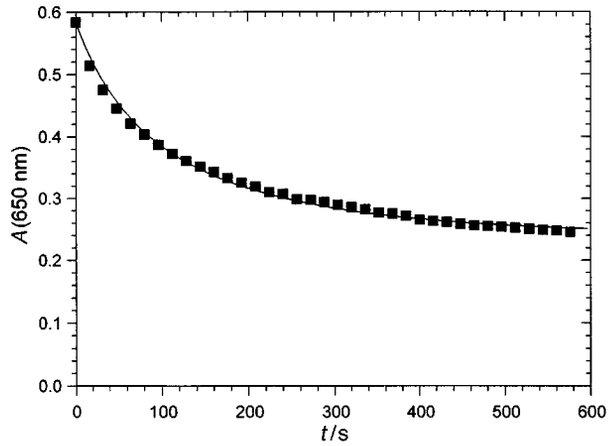


Fig. 1 Decay of the visible absorption of DPPH after addition of quercetin in DMF at 25 °C. DPPH:quercetin molar ratio = 4. The solid line is the result of the curve-fitting procedure for the determination of the antioxidant stoichiometry and of the overall rate constant for abstraction of H atoms from the antioxidant (for details, *see text*).

The antioxidant stoichiometry is determined by the structure of the antioxidant and its mechanism of oxidation. For instance, a monophenol ArOH can react with DPPH to give an aryloxyl radical $ArO^{\bullet}$ that will dimerize into $(ArO)_2$. If the dimers are not reactive toward DPPH, the stoichiometry will be 1. If the dimers are further oxidized by DPPH, the stoichiometry will be higher than 1. Similarly, *o*- and *p*-diphenols may be oxidized twice by DPPH into the corresponding *o*- and *p*-quinones. If the quinones are not reactive toward DPPH, the stoichiometry will be 2. If the quinones are further oxidized by DPPH, the stoichiometry will be higher than 2.

In order to achieve a more quantitative description, the following simple model was used. An antioxidant of stoichiometry *n* is regarded as *n* independent sub-units AH which all transfer an H atom to DPPH with the same second-order rate constant *k*. Hence, eqn. (1) and (2) can be used in the curve-

$$A = A_0\,[\mathrm{DPPH}]/c_0 \qquad (1)$$

$$-\mathrm{d[AH]}/\mathrm{d}t = -\mathrm{d[DPPH]}/\mathrm{d}t = k[\mathrm{AH}][\mathrm{DPPH}] \qquad (2)$$

fitting of the kinetic traces featuring the decay of DPPH. *A*: visible absorbance at time *t*, A_0: initial absorbance, c_0: initial DPPH concentration. The initial condition on [AH] is: [AH] = *nc* (*c*: initial antioxidant concentration).

In the kinetic runs, the initial DPPH:flavonol ratio (typically, 4–8 in our experiments) must be higher than *n* for the model to apply. In DMF, the reaction was monitored at 650 nm instead of 520 nm (DPPH absorption maximum in DMF) in order to avoid interference with the oxidized forms of the flavonoids which absorb at the latter wavelength (*see below*). This procedure gave satisfying curve-fittings ($r > 0.995$) for quercetin and rutin (Fig. 1) and consistent values for *n* and *k* at different DPPH:flavonoid ratios (Table 1). The antioxidant stoichiometry is 2 for rutin and slightly higher for quercetin, a result which suggests the possible involvement of OH-3 in trapping DPPH.

Close values for rate constant *k* were found for quercetin and rutin. Not only the parent flavonoid but also its oxidation and degradation products can transfer H atoms to DPPH (especially in protic media, *see below*). Hence, the *k* value provides a quantitative estimation of the overall reactivity toward DPPH of the antioxidant and the products it forms in the course of the reaction. This overall reactivity is roughly the same for quercetin and rutin.

Oxidation by DPPH is faster by a factor of *ca.* 10 in methanol than in DMF (Table 1). Similar solvent effects were reported in the oxidation of α-tocopherol and phenol by

Table 1 Oxidation of quercetin and rutin by DPPH at 25 °C. Stoichiometries (n) and rate constants (k) (for definition, *see text*) are deduced from curve-fittings of the kinetic traces at λ_{max}(DPPH). Typical time interval: 10 min (DMF), 1 min (protic solvents)

Flavonol	Quercetin	Quercetin	Rutin	Rutin
DPPH/equiv.	4	8	4	8
k/dm^3 mol^{-1} s^{-1} in DMF [a]	57.4 (±0.8)	51.5 (±0.9)	61.9 (±1.1)	66.2 (±0.8)
n (DMF) [a]	2.35 (±0.01)	2.37 (±0.01)	1.94 (±0.09)	2.00 (±0.01)
k/dm^3 mol^{-1} s^{-1} in MeOH	723 (±15)	583 (±13)	669 (±9)	718 (±7)
n (MeOH)	3.19 (±0.02)	3.61 (±0.02) 6.8 [c]	2.12 (±0.01)	2.33 (±0.01)
k/dm^3 mol^{-1} s^{-1} in MeOH–H_2O (4:1)	[b]	695 (±19)	1139 (±16)	1385 (±28)
n (MeOH–H_2O (4:1))	[b]	4.53 (±0.04) 6.5 [c]	2.30 (±0.01)	2.57 (±0.01)

[a] Determined at 650 nm. [b] Model not valid (DPPH:flavonol ratio lower than n). [c] Determined over 10 min using $n = c_0(1 - A_f/A_0)/c$ (A_f: visible absorbance at the end of the kinetic run, A_0: initial absorbance, c: initial antioxidant concentration, c_0: initial DPPH concentration).

DPPH.[9a] They were attributed to hydrogen bonding between an alcohol molecule (donor) and the diphenylamino group of DPPH (acceptor) that would reduce electron delocalization in DPPH and thus enhance its reactivity. A slight acceleration is also observed when going from methanol to methanol–water (4:1). More interesting is the very significant increase in quercetin stoichiometry when going from non protic to protic solvents.

^{1}H-NMR monitoring of quercetin oxidation by DPPH (1–2 equiv.) in CD_3CN only resulted in uninterpretable spectra. TLC analysis on silica gel showed the formation of complex mixtures of compounds more polar than quercetin. After chromatography on silica gel (eluent = ethyl acetate–MeOH 95:5), small amounts of a quercetin dimer ($m/z = 603$ (MH^+), $m/z = 625$ (MNa^+) in electrospray MS, positive mode) were isolated from the less polar fractions.

When the experiment is repeated in CD_3OD, a simple NMR spectrum is obtained which is identical to that of the product of quercetin oxidation by sodium periodate in CD_3OD (compound QH_2 in Scheme 1).[10] Such a compound is formed upon oxidation of quercetin into the corresponding quinone and subsequent addition of two methanol molecules on C-2 and C-3. Similar structures were reported in the literature for compounds resulting from the oxidation of quercetin and other flavonols by periodate or Cu(II) in alcohols.[11]

In order to form quinones, aryloxyl radicals may further react with the oxidant or with themselves (disproportionation). The latter process is more probable since fast second-order decay of polyphenolic aryloxyl radicals in water ($2k$ in the range 10^6–10^7 dm^3 mol^{-1} s^{-1}) has already been reported in the case of flavonoids from pulse radiolysis experiments[7] and in the case of caffeic acid from electrochemical investigations.[12] Hence, the quercetin quinone is proposed to form through the following mechanism (Scheme 1): one-electron oxidation of quercetin (QH_4) giving aryloxyl radicals (QH_3); disproportionation of the aryloxyl radicals into quercetin and its quinone.

From semi-empirical quantum mechanics calculations, the two most stable tautomers have been selected to depict the quercetin radical, *i.e.* the tautomers resulting from H abstraction on OH-3 and OH-3′, the former being *ca.* 7.5 kJ mol^{-1} more stable. H abstraction on OH-4′, OH-5 and OH-7 leads to less stable radicals (+13.4, +59.4 and +46.0 kJ mol^{-1}, respectively) in agreement with pulse radiolysis experiments which clearly showed that radical formation essentially involves the B and C rings.[7f] Similarly, the quercetin quinone may be depicted as a mixture of two tautomeric *o*-quinone and *p*-quinonoid forms, the former being *ca.* 7.5 kJ mol^{-1} more stable.

In the case of rutin, the presence of a glycosyl group on O-3 cancels the *o*-quinone–*p*-quinonoid tautomerism and leaves the *o*-quinone as the sole possible structure. Such a compound does not react as quickly with solvent molecules and can be characterized by ^{1}H-NMR in CD_3OD if the spectrum is recorded immediately after DPPH addition (2 equiv.). As expected, the

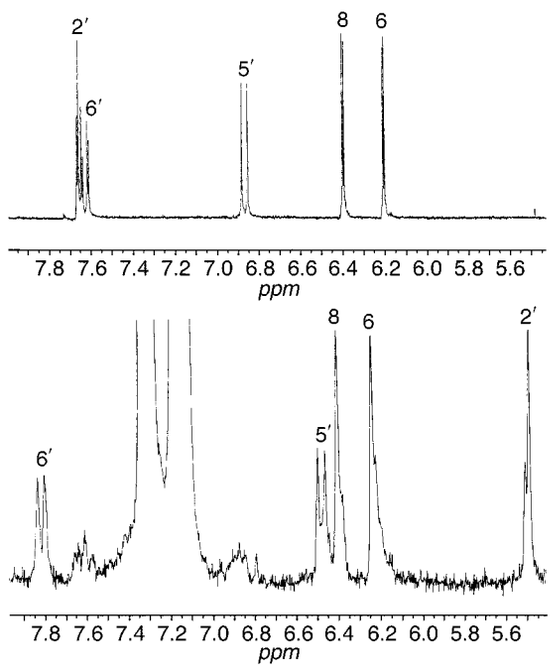


Fig. 2 *Upper part*: ^{1}H-NMR spectrum (300 MHz, 27 °C) of rutin (2×10^{-2} mol dm^{-3}) in CD_3OD. *Lower part*: spectrum recorded just after addition of DPPH (2 equiv.). The intense signals in the range 7.0–7.5 ppm are due to diphenylpicrylhydrazine.

H-6′ signal is deshielded (+0.19 ppm) and the H-2′ and H-5′ signals are strongly shielded (respectively, −2.18 and −0.39 ppm) with respect to the corresponding signals in rutin whereas the H-8 and H-6 signals are essentially unaffected (Fig. 2). After a few minutes, the quinone signals were no longer detectable. However, no signals typical of a quinone–methanol adduct could be observed, a result indicating that processes faster than solvent addition (probably, oligomerization) were taking place. When the oxidation of rutin in methanol is conducted in the presence of sodium benzenesulfinate, the quinone can be easily trapped as a stable biaryl sulfone resulting from regioselective Michael addition of the sulfinate anion on C-6′.

Formation of quinones upon oxidation of quercetin and rutin is consistent with stoichiometries close to 2 in a non nucleophilic solvent such as DMF. The significant increase in the quercetin stoichiometry in nucleophilic solvents (methanol and methanol–water (4:1)) is also consistent with solvent addition on ring C of the quinone nucleus, thus regenerating a catechol group on ring B (Scheme 1) which is susceptible to being further oxidized.

Final products in the oxidation of quercetin have been reported to be 2,4,6-trihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid.[13] Such compounds may also take part in the antioxidant activity. Indeed, when the oxidation of quercetin in

methanol and methanol–water (4:1) is prolonged over 10 min (final equilibrium state), stoichiometries higher than 6 were estimated from the total amplitude of the kinetic traces (Table 1).

A refined kinetic model can be used which takes into account the different steps in quercetin oxidation (Scheme 1): one-electron oxidation of quercetin (QH_4) into aryloxyl radicals QH_3 (rate constant k_1); fast disproportionation of QH_3 into quercetin and its quinone and subsequent fast solvent addition on the quinone to give adduct QH_2; one-electron oxidation of QH_2 into aryloxyl radicals QH (rate constant k_2); fast disproportionation of QH into QH_2 and the corresponding quinone Q. Finally, further oxidative degradation of quinone Q (after eventual solvent addition) may occur (overall rate constant k_3).

The pulse radiolysis experiments reported in the literature[7f] in the pH range 2–11 gave UV–visible absorption maxima between 520 and 560 nm for quercetin radicals in their neutral, monoanionic and dianionic forms. In our kinetic experiments, the absence of significant absorption in this range means that quercetin radicals do not accumulate in the course of the kinetic runs. Assuming a quasi-stationary state for the radicals, eqn. (3)–(6) were derived.

$$-d[QH_4]/dt = k_1[QH_4][DPPH]/2 \quad (3)$$

$$d[QH_2]/dt = k_1[QH_4][DPPH]/2 - k_2[QH_2][DPPH]/2 \quad (4)$$

$$d[Q]/dt = k_2[QH_2][DPPH]/2 - k_3[Q][DPPH] \quad (5)$$

$$-d[DPPH]/dt = k_1[QH_4][DPPH] + k_2[QH_2][DPPH] + k_3[Q][DPPH] \quad (6)$$

Curve-fitting of the kinetic traces featuring the oxidation of quercetin by DPPH (8 equiv.) in methanol–water (4:1) gave the following values for the rate constants: $k_1 = 39.3\ (\pm 1.9) \times 10^2$, $k_2 = 13.1\ (\pm 1.0) \times 10^2$, $k_3 = 3.9\ (\pm 0.7) \times 10^2$ dm^3 mol^{-1} s^{-1}. With a DPPH : quercetin molar ratio of 4, the last step in the kinetic model could be neglected ($k_3 = 0$). Consistent values for k_1 and k_2 were obtained: $k_1 = 41.9\ (\pm 1.4) \times 10^2$, $k_2 = 13.0\ (\pm 0.4) \times 10^2$ dm^3 mol^{-1} s^{-1}. As expected from its restricted electron delocalization, the quinone–solvent adduct reduces DPPH *ca.* 3 times as slowly as quercetin. However, its significant ability to trap radicals must prolong the overall antioxidant activity of quercetin beyond that of rutin.

For comparison, values of the absolute rate constant for abstraction by DPPH of the phenolic H atom from α-tocopherol and phenol in Bu^{t}OH at 25 °C were estimated to be 5.7×10^2 and 2.9×10^{-3} dm^3 mol^{-1} s^{-1}, respectively.[9a] Hence, in protic media, DPPH is trapped by quercetin *ca.* 7 times as quickly as by the potent antioxidant α-tocopherol.

Oxidation by CAN. Since quinones have typical UV–visible absorption bands in the range 300–500 nm[14] where DPPH strongly absorbs, oxidation by ceric ammonium nitrate (CAN) should be a better way to get UV–visible spectroscopic evidence for the formation of quinones. Addition of one equivalent of CAN to a solution of quercetin in DMF is followed by a fast decrease in the quercetin absorption band ($\lambda_{max} = 376$ nm) and a simultaneous building-up of a shoulder (λ_{max} in the range 425–430 nm) which is ascribed to the quinone. When oxidation is carried out in quercetin solutions added with 1, 2 or 3 equivalent(s) of Bu^{t}OK, the quinone can be characterized by a well-defined absorption band (Fig. 3) whose intensity gradually increases with the base concentration (control experiments showed no significant reaction between CAN and Bu^{t}OK and no overlapping between the absorption bands of the quinone and those of CAN in its oxidized or reduced form). Simultaneously, the λ_{max} value of the quinone absorption band shifts from 447 (1 equiv. Bu^{t}OK) to 463 nm (3 equiv. Bu^{t}OK). In

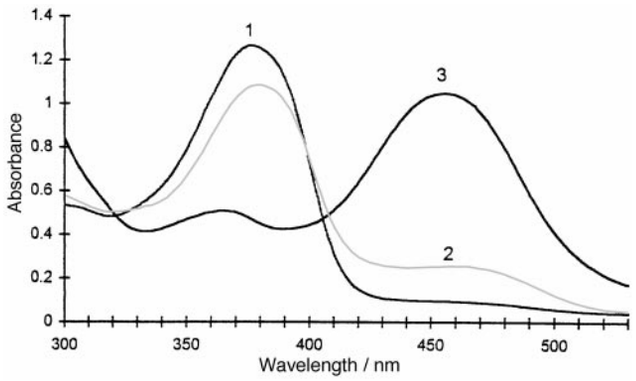


Fig. 3 UV–visible monitoring of quercetin oxidation by CAN (1 equiv.) in DMF at 25 °C. Spectrum 1: quercetin. Spectrum 2: quercetin + Bu^{t}OK (2 equiv.). Spectrum 3: oxidation completed (*ca.* 30 s after CAN addition). Initial quercetin concentration: 5×10^{-5} mol dm^{-3}.

addition, a very broad weak absorption band significantly builds up in the range 550–800 nm when oxidation is carried out after addition of 2 or 3 equivalents of Bu^{t}OK. It may be attributed to quercetin–quinone charge transfer complexes (*see below*).[14]

The kinetic runs can be quantitatively interpreted from the one-electron oxidation–radical disproportionation mechanism according to the kinetic model shown in eqn. (7)–(9). A:

$$d[QH_2]/dt = -d[QH_4]/dt = k_1[QH_4][CAN]/2 \quad (7)$$

$$-d[CAN]/dt = k_1[QH_4][CAN] \quad (8)$$

$$A = A_0[QH_4]/c + \varepsilon_1[QH_2] \quad (9)$$

absorbance at time t, A_0: initial absorbance, c: total quercetin concentration, ε_1: molar absorption coefficient of quinone QH_2.

Curve-fitting of the kinetic traces at the wavelengths of quercetin and quinone absorption maxima gave consistent values for k_1 and good correlation coefficients ($r > 0.99$) (Table 2). Although the quinone can be easily detected in dilute solutions in DMF, it starts slowly decaying soon after having reached its plateau concentration. The decay of the quinone absorbance is much faster in the presence of 2–3 equivalents of Bu^{t}OK and can be quantitatively interpreted by assuming either a reaction between quercetin and its quinone that would give biflavonoids[15] or a dimerization of the quinone.

Oxidation of quercetin by CAN (1–2 equiv.) in methanol did not afford the spectral characteristics typical of quinones: the quick decay of the quercetin absorption bands ($\lambda_{max} = 371$, 256 nm) was followed by the simultaneous raising of a band with an absorption maximum at 293 nm. The same spectral changes were observed upon oxidation of quercetin by sodium periodate in methanol.[10] They are consistent with a two-step mechanism of quinone formation and subsequent addition of methanol on the quinone (Scheme 1).

Autoxidation

Autoxidation of flavonoids in strongly alkaline aqueous[5i] and organic[16] (DMSO) solutions has already been used for the EPR detection of flavonoid radical anions. In strongly alkaline aqueous solutions of quercetin,[5i] not only quercetin radical anions but also radical anions having an additional OH at C-2′ (subsequently referred to as *secondary* radicals) could be observed.

Autoxidation of quercetin has also been investigated in physiological conditions (pH 7.5 aqueous buffers)[17] and shown to generate reactive oxygen species such as superoxide and hydrogen peroxide. Such prooxidant effects are of interest in the context of tumor cell cytotoxicity.

Table 2 Oxidation of quercetin (5×10^{-5} mol dm^{-3}) by CAN (1 equiv.) in DMF at 25 °C

Bu^{t}OK/equiv.	0	1	2	3
k_1/dm^3 mol^{-1} s^{-1} [a]	37.3 (±0.7) × 10^2	40.6 (±0.7) × 10^2	38.6 (±0.5) × 10^2	42.9 (±1.3) × 10^2
$\lambda_{max}(QH_2)$/nm	425–430 [b]	447	456	463
$\varepsilon_1(QH_2)$ at λ_{max}/ dm^3 mol^{-1} cm^{-1}	17780 (±50)	25700 (±70)	36700 (±50)	41760 (±260)

[a] Determined at $\lambda_{max}(QH_2)$. [b] Shoulder.

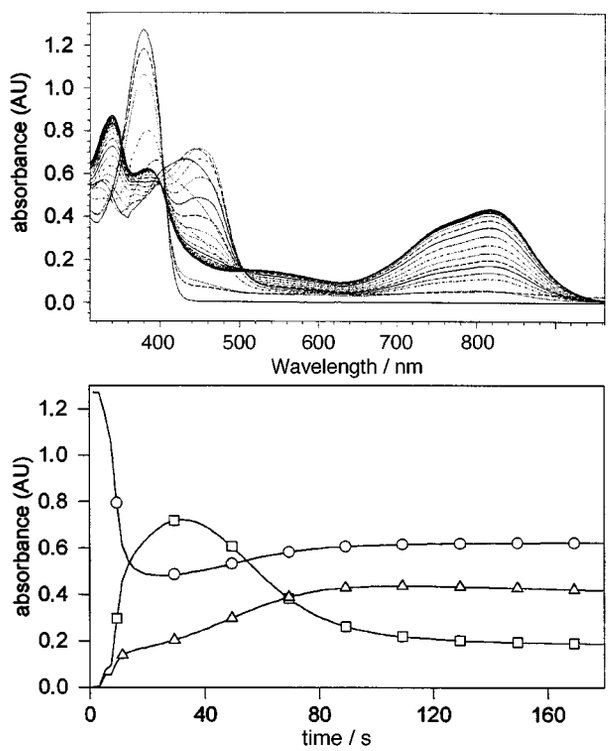


Fig. 4 *Upper part*: UV–visible monitoring of quercetin autoxidation in DMSO containing Bu^{t}OK (18 equiv.) at 25 °C. Initial flavonoid concentration: 5×10^{-5} mol dm^{-3}. *Lower part*: kinetic traces at 816 (△), 442 (□) and 380 nm (○).

Autoxidation in DMSO. When a large excess of Bu^{t}OK (18 equiv.) is added to a solution of quercetin in DMSO, a dark colour quickly appears. When followed by UV–visible spectroscopy (Fig. 4), the whole process can be deconvoluted into three steps: (i) A fast step of quercetin deprotonation (λ_{max} = 380 nm) with apparent first-order kinetics ($k = 0.171$ (±0.007) s^{-1}). Deprotonated quercetin (λ_{max} = 442 nm) may be a mixture of tautomeric anions. Since proton transfer reactions between oxygen atoms are usually diffusion-controlled, the k value may seem surprisingly weak. However, the final steps in the overall deprotonation of quercetin (*e.g.*, deprotonation of tetraanions into the pentaanion) could be dramatically slowed down by strong electrostatic repulsions between the *t*-butoxide anion and the quercetin polyanions. (ii) A first-order decrease in the absorption band of quercetin anions accompanied by the raising of a broad band with a maximum at 816 nm. (iii) A very slow first-order decay of the band at 816 nm. This step is much faster when oxidation is carried out in the presence of small amounts of water (2–5%).

From the results of pulse radiolysis investigations in the literature,[7] it seems unlikely that the UV–visible absorption band at 816 nm refers to primary quercetin radicals. Indeed, quercetin radicals display typical UV–visible absorption bands below 600 nm even in fairly alkaline conditions (pH 11.2) and probably disproportionate too quickly for their steady-state concentration to reach the threshold of detection in the dilute solutions (5×10^{-5} mol dm^{-3}) used in UV–visible spectroscopy. Hence, we assume that the absorption band at 816 nm (ΔE *ca.* 1.5 eV) is evidence of the intermediate formation of quercetin–quinone charge transfer complexes. Charge transfer must occur primarily from the HOMO of the donor (quercetin) to the LUMO of the acceptor (quinone). The calculated energy difference ($\Delta E = 1.35$ eV) between the HOMO of the quercetin trianion deprotonated at O-3, O-4′ and O-7 and the LUMO of the *o*-quinone dianion deprotonated at O-3 and O-7 which is identical to the *p*-quinonoid deprotonated at O-3′ and O-7 (most stable tautomers selected for both donor and acceptor) actually falls in the correct range to account for a charge transfer process from the flavonoid to its parent quinone. Quinone charge transfer bands were also detected upon oxidation of free or albumin-bound quercetin by periodate in slightly alkaline aqueous buffers.[10]

The following mechanism can be proposed for quercetin autoxidation: one-electron oxidation of quercetin to primary radicals which rapidly disproportionate into a mixture of quercetin and quinone in charge transfer interaction; addition of water and/or the hydroxide anion on the quinone. Water addition on *o*- or *p*-quinone intermediates has already been postulated to account for the formation of secondary radicals of phenols upon autoxidation [18] or enzymatic oxidation.[19]

The theoretical treatment (eqn. (10) and (11)) of the kinetic

$$-\mathrm{d}[QH_4]/\mathrm{d}t = k'_1[QH_4]/2 \quad (10)$$

$$\mathrm{d}[QH_2]/\mathrm{d}t = k'_1[QH_4]/2 - k'_2[QH_2] \quad (11)$$

data is directly adapted from that used in the oxidation by CAN, the oxidation rate constant k'_1 being now an apparent first-order rate constant ($k'_1 = k_1[O_2]$). In addition, allowance is made for the first-order decay of the quinone (rate constant k'_2). Table 3 gives the values for the rate constants k'_1 and k'_2 as well as the spectral characteristics of the quinone.

Water addition can occur at C-2 and C-3 to give compounds similar to those resulting from oxidation by periodate or Cu(II) in alcohols.[10,11] However, in aqueous alkaline solutions, solvent addition is followed by the opening of the pyran ring and subsequent cleavage of the carbon chain to give a distribution of degradation products.[13,20] New absorption bands at 340 and 384 nm could thus be assigned to water adducts and/or degradation products. In DMSO–water (97.5 : 2.5), the former band is shifted to 326 nm and the latter band appears as a shoulder around 380 nm.

Michael addition at C-2′, C-5′ or C-6′ would give quercetin derivatives with an additional OH group on these positions. These routes cannot be discarded since they account for the observation of secondary radical anions in strongly alkaline conditions (hydroxylation at C-2′).[5i] However, such highly hydroxylated compounds must be even more oxidizable than quercetin and thus rapidly degraded. In the case of the 3-methylquercetin *o*-quinone, addition of water and/or the hydroxide ion can only occur at C-2′, C-5′ or C-6′, thus regenerating a flavonoid nucleus. Consistently, the decay of the *o*-quinone absorption band is accompanied by the appearance of a strong absorption band at 447 nm (Fig. 5), typical of flavonoid anions.

Autoxidation does not necessarily require complete one-electron transfer from the substrate to dioxygen. An alternative

Table 3 Autoxidation of flavonol anions in DMSO–Bu′OK systems at 25 °C. Flavonoid concentration = 5×10^{-5} mol dm^{-3}. Bu′OK : flavonoid molar ratio = 18

Flavonol	Quercetin	Quercetin[a]	3-Methylquercetin
$\lambda_{max}(QH_2)$/nm	816 (CT band)	790 (CT band)	520 (shoulder)
k'_1/s^{-1}	62 (±3) × 10^{-3}	0.74 (±0.02)[b]	66 (±1) × 10^{-3}
k'_2/s^{-1}	15.0 (±0.1) × 10^{-5}	39.0 (±0.8) × 10^{-4}	37.4 (±0.7) × 10^{-3}
$\varepsilon_1(QH_2)$ at λ_{max}/ dm^3 mol^{-1} cm^{-1}	9790 (±150)	10090 (±50)	12930 (±120)

[a] In DMSO–H_2O 97.5 : 2.5. [b] Apparent rate constant because of probable interference with the deprotonation kinetics.

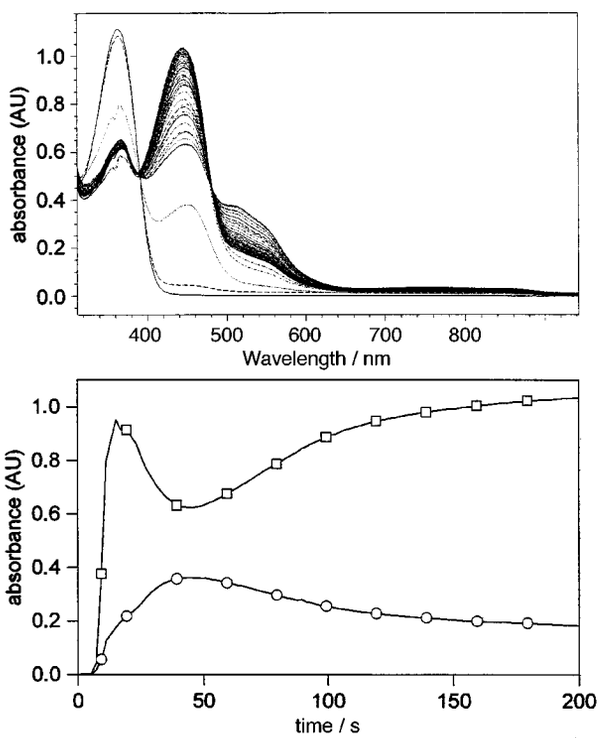


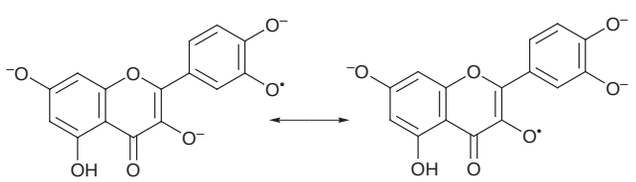
Fig. 5 *Upper part*: UV–visible monitoring of 3-methylquercetin autoxidation in DMSO containing Bu′OK (18 equiv.) at 25 °C. Initial flavonoid concentration: 5×10^{-5} mol dm^{-3}. *Lower part*: kinetic traces at 447 (□) and 520 nm (○).

mechanism has been proposed which involves the formation of substrate–dioxygen charge transfer complexes.[21] However, from calculated values of ionization potentials,‡ complete electron transfer and subsequent radical formation seems a thermodynamically favourable process with highly oxidizable substrates such as dianions of catechol and hydroquinone.[21]

^{1}H-NMR experiments show that autoxidation proceeds quickly in DMSO for Bu′OK : quercetin ratios higher than 3. Aliquots of 0.5 equivalent of Bu′OK were successively added to a 10^{-2} mol dm^{-3} solution of quercetin in DMSO-d_6. Up to 2 equivalents, adding Bu′OK only resulted in a shielding of the aromatic protons which points to the deprotonation of phenolic OH groups. The magnitudes of the δ shifts (in ppm) after addition of 2 equiv. of Bu′OK are in the following order: H-8 (0.71), H-6 (0.63), H-5′ (0.25), H-2′ (0.20), H-6′ (0.12). They are consistent with deprotonation occurring on OH-7 and OH-4′ in agreement with the literature.[22] Above 2 equivalents, adding Bu′OK brought about a broadening of all aromatic proton peaks which ultimately vanished when the Bu′OK : quercetin ratio reached approximately 4. Such observations are consistent with the formation of an increasing concentration of paramagnetic quercetin radicals in fast equilibrium with diamagnetic quercetin anions.

OH-3 is probably the third most acidic proton of quercetin. Indeed, OH-5 (δ = 12.5 ppm in DMSO-d_6) is strongly stabilized through hydrogen bonding with the 4-keto group. Moreover, in semi-empirical quantum mechanics calculations, the trianion formed upon deprotonation of OH-3, OH-4′ and OH-7 ($A_{3,4',7}$) was found to be *ca.* 40 kJ mol^{-1} more stable than the tautomer formed upon deprotonation of OH-3′, OH-4′ and OH-7 ($A_{3',4',7}$).

When going from neutral to highly alkaline solutions, trianion $A_{3,4',7}$ is probably the first structure whose electron density is high enough to transfer one electron to the empty π* orbitals of dioxygen, thus giving $R_{3,4',7}$ which is actually the most stable quercetin radical dianion (9.6 and 24.3 kJ mol^{-1} more stable than $R_{3',4',7}$ and $R_{3,3',7}$, respectively). Deprotonation of $R_{3,4',7}$ and $R_{3',4',7}$ gives $R_{3,3',4',7}$ which is the most stable radical trianion (17.6 and 24.3 kJ mol^{-1} more stable than $R_{3,4',5,7}$ and $R_{3',4',5,7}$, respectively) as expected from its highly delocalized structure (Scheme 3).



Scheme 3

Consistent with the picture of an autoxidation process triggered by deprotonation of OH-3, glycosidation of OH-3 dramatically reduces the oxidizability of the flavonol nucleus in strongly alkaline aerated solutions. Thus, adding Bu′OK in large excess to a solution of rutin in DMSO or DMF only resulted in deprotonation. Methylation of OH-3 still allows autoxidation at large Bu′OK : flavonol ratios. However, large differences were noticed when autoxidations of quercetin and 3-methylquercetin were run under the same conditions (DMSO, 18 equiv. Bu′OK). Autoxidation of 3-methylquercetin led to the formation of the free quinone characterized by a broad shoulder in the range 500–600 nm (Fig. 5). No significant charge transfer band could be detected in the range 600–800 nm. In DMSO, the quercetin quinone is *ca.* 250 times as stable as the 3-methylquercetin quinone as evidenced by the respective decays of their absorption bands (Table 3).

Unlike the 3-methylquercetin quinone which only displays an *o*-quinone structure, the quercetin quinone has two mesomeric *o*-quinone and *p*-quinonoid forms in these strongly alkaline conditions (Scheme 4). The strong contribution of the latter form could be the origin of the high stability of the quercetin quinone and its ability to act as an acceptor in charge transfer complexation.

It must be emphasized that autoxidation is markedly affected by the presence of water. For instance, quinone formation in DMSO containing 2.5% of water is *ca.* 12 times as fast as in DMSO (Table 3). Small amounts of water may promote a specific stabilization by hydrogen bonding of the charge density

‡ Whereas the electron affinity of dioxygen is 0.43 eV, the ionization potentials of hydroquinone in its neutral, monoanionic and dianionic forms are 8.03, 1.39 and −3.58 eV, respectively.

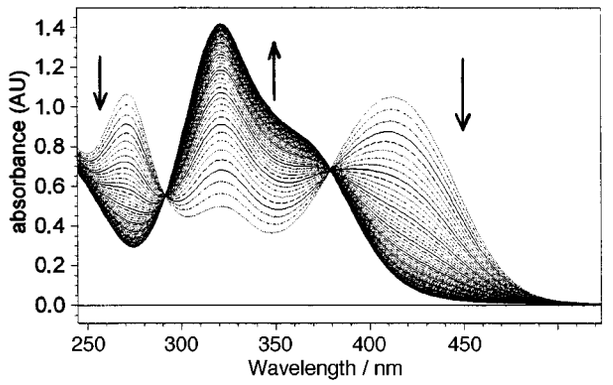
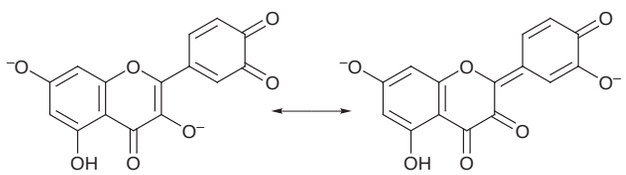


Fig. 6 UV–visible monitoring of quercetin autoxidation in a pH 9.4 carbonate buffer (initial quercetin concentration = 5×10^{-5} mol dm^{-3}, 25 °C, ionic strength = 0.2 mol dm^{-3}). Time interval between two consecutive spectra: 10 s.



Scheme 4

transferred in the π* orbitals of O_2 in the transition state of the first step (radical formation) in agreement with theoretical investigations.[21] Since the first step is rate-limiting, this must result in a rate enhancement.

In DMSO–water (97.5:2.5), the decay of the quinone–quercetin charge transfer band (λ_{max} shifted from 817 to 790 nm by the presence of water) was found to be *ca.* 26 times as fast as in DMSO (Table 3). This strong destabilization of the quinone is consistent with the higher concentration of nucleophilic hydroxide ions in the solution.

Autoxidation in aqueous solution. When monitored by UV–visible spectroscopy, autoxidation of quercetin in aqueous carbonate buffers (pH 8.6–11.2) is manifested by the apparent first-order decay of the quercetin absorption band (λ_{max} = 399–428 nm depending on the pH) and the simultaneous raising of absorption bands at shorter wavelengths typical of water adducts (324–314 nm) (Fig. 6). These spectral changes are entirely analogous to those occurring upon oxidation of quercetin by CAN in methanol. No evidence for the free quinone or its charge transfer complex with quercetin could be detected because of a fast addition of water on the quinone under such conditions. Similar observations were made when the quinone was generated upon oxidation of quercetin by sodium periodate in neutral to weakly alkaline aqueous buffers.[10] The apparent first-order kinetics of autoxidation can be accounted for by a rate-determining one-electron oxidation of quercetin anions followed by two fast steps of radical disproportionation and water addition on the resulting quinones.

As expected, the apparent first-order rate constant ($k_{app} = k'_1/2$) is strongly pH-dependent and increases by a factor of *ca.* 30 from pH 8.6 to pH 11. The apparent rate constant *vs.* pH plot (Fig. 7) could be fitted assuming a single proton transfer with pK_a = 10.09 (±0.04) at 25 °C and 0.2 M ionic strength. This value is close to that reported in the literature for the fourth deprotonation of quercetin.[23] Hence, although already detectable at the stage of the trianion, autoxidation would be faster by a factor of *ca.* 30 at the stage of the tetraanion.

Quercetin autoxidation is significantly faster (by *ca.* a factor of 2 at pH 11) in a carbonate buffer than in a glycine buffer of the same concentration and pH. Much more spectacular is the ionic strength effect. At pH 9.6 (glycine buffer), the autoxidation rate constant *vs.* NaCl concentration is linear in the concentration range 0–2.5 mol dm^{-3} and is increased by a factor of *ca.* 17 between these two boundaries. In the autoxidation process, the small superoxide anion that is formed is expected to interact much more strongly with the atmosphere of sodium ions than the large quercetin anions with their highly delocalized negative charge. Hence, it is proposed that specific stabilization by the atmosphere of the sodium ions of the superoxide being formed in the transition state of the autoxidation process is mainly responsible for the large ionic strength effect observed.

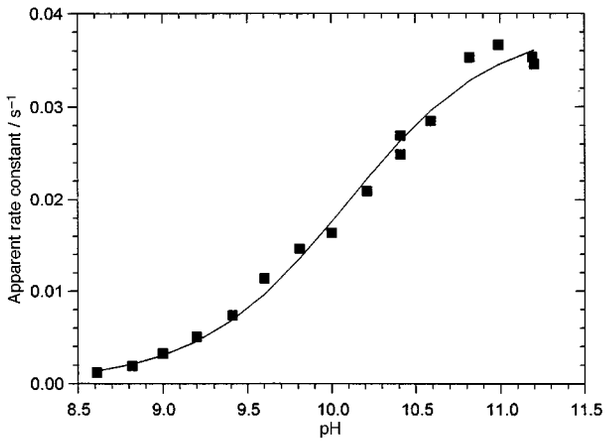


Fig. 7 pH-dependence of the first-order apparent rate constant of quercetin autoxidation in carbonate buffers (initial quercetin concentration = 5×10^{-5} mol dm^{-3}, 25 °C, ionic strength = 0.2 mol dm^{-3}). The solid line is the result of the curve-fitting procedure assuming a single proton transfer (pK_a = 10.09 (±0.04)).

Autoxidation of rutin is quite slow in the pH range 8.6–11.0. In 0.2 mol dm^{-3} NaOH, the apparent first-order decay (k_{app} = 6.3 (±0.2) × 10^{-4} s^{-1}) of the absorption band typical of rutin (λ_{max} = 405 nm) was found to be *ca.* 34 times as slow as that of quercetin in the same conditions. The strong contribution of OH-3 (in its deprotonated form) to the stability of quercetin radical anions in alkaline conditions is probably mainly responsible for such differences in reactivity.

Conclusion

Antioxidants able to directly react with reactive oxygen species must be converted into relatively innocuous species upon quenching. For instance, α-tocopherol (vitamin E) is known to break the chain mechanism of lipid peroxidation upon reducing alkylperoxyl radicals into the corresponding hydroperoxides.[24] Simultaneously, α-tocopherol is converted into the long-lived α-tocopheryl radical which is not reactive enough to efficiently abstract a labile H atom from a polyunsaturated fatty acid molecule and thereby initiate another chain. The α-tocopheryl radical itself can undergo one-electron oxidation into an unreactive *p*-quinone. Thus, the reactivity of the oxidized forms of a given antioxidant can be a factor controlling the overall antioxidant activity and the eventual toxicity of the antioxidant.

The eventual toxicity of flavonoids may arise from the following mechanisms:

Formation of superoxide radical anion during autoxidation (prooxidant activity).[17] Superoxide disproportionation can produce hydrogen peroxide which is a source of highly damaging hydroxyl radicals through the Fenton reaction.

Formation of reactive aryloxyl radicals (one-electron oxidation) and quinones (two-electron oxidation) that may covalently modify biopolymers.

The present work shows that quercetin radicals quickly disproportionate to regenerate quercetin and produce a quinone which adds water molecules and is then degraded. Oligomerization might also be a minor route, especially in media of low

water content. On the other hand, it may be pointed out that oxidation of the quercetin–serum albumin complex did not result in flavonol–protein covalent coupling but simply retarded water addition on the quercetin quinone.[10] Thus, one possible mechanism for the antioxidant activity of flavonoids could be their easy oxidation and subsequent conversion into innocuous water adducts, degradation products and/or oligomers.

Finally, the quercetin OH-3 group has been shown to play a crucial role from two viewpoints: it allows the formation of *p*-quinonoid compounds, quickly converted into solvent adducts which still react with one-electron oxidants, thus prolonging the antioxidant activity; in its deprotonated form, it is highly effective in stabilizing radicals, thus allowing autoxidation to proceed under relatively mild alkaline conditions.

Experimental

Materials

Quercetin, rutin, DPPH (Sigma-Aldrich), CAN (Fluka) and Bu′OK (Acros) of the highest quality available (95–99%) were used without purification. 3-Methylquercetin was a kind gift from Professors B. Voirin and M. Jay (Université Claude Bernard-Lyon I).

Absorption spectra

Spectra were recorded on a Hewlett-Packard 8453 diode-array spectrometer equipped with a magnetically stirred quartz cell (optical pathlength: 1 cm). The temperature in the cell was kept at 25 °C by means of a water-thermostatted bath.

Kinetic experiments

All experiments were monitored by UV–visible spectroscopy.

Oxidation by CAN. To 2 cm^3 of a freshly prepared 5×10^{-5} mol dm^{-3} solution of flavonoid in DMF placed in the spectrometer cell were successively added 50 mm^3 of a Bu′OK solution in DMF (typical Bu′OK : flavonoid ratio in the cell = 0–3) and 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of CAN in acetonitrile.

Oxidation by DPPH. To 2 cm^3 of a freshly prepared 2×10^{-4} mol dm^{-3} solution of DPPH in DMF or MeOH placed in the spectrometer cell were added 50 to 400 mm^3 of a freshly prepared 10^{-3} mol dm^{-3} solution of flavonoid in the same solvent.

Autoxidation in DMSO. To 1.9 cm^3 of DMSO placed in the spectrometer cell were successively added 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of flavonoid in DMSO and 100 mm^3 of a 1.8×10^{-2} mol dm^{-3} solution of Bu′OK in DMSO.

Autoxidation in aqueous solutions. 50 mm^3 of a freshly prepared 2×10^{-3} mol dm^{-3} solution of quercetin in methanol were diluted into 2 cm^3 of a 5×10^{-2} mol dm^{-3} $NaHCO_3$ or glycine buffer (pH 8.6–11.2 adjusted by addition of 2 mol dm^{-3} NaOH without dilution, 0.2 mol dm^{-3} ionic strength adjusted by NaCl) placed in the spectrometer cell.

NMR experiments

NMR experiments were carried out on a Bruker apparatus (300 MHz, 27 °C).

Rutin quinone. A 10^{-2} mol dm^{-3} solution of rutin in CD_3OD was added with two equivalents of DPPH just before recording the ^{1}H-NMR spectrum. δ(ppm) = 7.81 (broad d, J = 10.3 Hz, H-6′), 6.48 (d, J = 10.3 Hz, H-5′), 6.40 (broad s, H-8), 6.24 (broad s, H-6), 5.48 (broad s, H-2′), 4.9 (H-1Glc, partially masked by the water peak), 4.59 (broad s, H-1Rha), 3.85 (broad d, J = 11.0 Hz, H-6Glc), 3.65 (broad s, H-2Rha), 3.60–3.15 (H-2Glc, H-3Glc, H-4Glc, H-5Glc, H-6′Glc, H-3Rha, H-4Rha, H-5Rha), 1.02 (d, J = 5.9 Hz, Me Rha).

Rutin quinone–benzenesulfinate adduct. $PhSO_2Na$ (1 equiv.) and DPPH (2 equiv.) were successively added to a 2×10^{-2} mol dm^{-3} solution of rutin in MeOH. After stirring for a few minutes, the adduct was precipitated from the reaction mixture upon addition of CH_2Cl_2, washed with CH_2Cl_2 and dried.

^{1}H-NMR spectrum (CD_3OD). δ(ppm) = 7.76 (d, J = 7.4 Hz, Ph, H-*ortho*), 7.61 (*t*, J = 7.4 Hz, Ph, H-*para*), 7.53 (*t*, J = 7.4, Ph, H-*meta*), 7.49 (s, H-2′), 6.95 (s, H-5′), 6.14 (d, J = 2.2 Hz, H-8), 5.76 (d, J = 2.2 Hz, H-6), 4.9–4.8 (H-1Glc, masked by the water peak), 4.68 (broad s, H-1Rha), 3.84 (broad d, J = 12.5 Hz, H-6Glc), 3.80 (broad s, H-2Rha), 3.65–3.20 (H-2Glc, H-3Glc, H-4Glc, H-5Glc, H-6′Glc, H-3Rha, H-4Rha, H-5Rha), 1.24 (d, J = 6.6 Hz, Me Rha).

^{13}C-NMR spectrum (CD_3OD, tentative attributions from DEPT and comparison with rutin[25]). δ(ppm) = 178.0 (C-4), 169.9 (C-7), 161.9 (C-5), 158.8 (C-9), 157.9 (C-4′), 154.9 (C-3′), 150.9 (C-2), 143.7 (C-1, $PhSO_2$), 136.3 (C-3), 132.9 (C-4, $PhSO_2$), 129.1 (C-2, C-6, $PhSO_2$), 127.0 (C-3, C-5, $PhSO_2$), 126.4 (C-1′), 124.2 (C-6′), 119.4 (C-5′), 116.5 (C-2′), 103.8 (C-10), 101.4 (C6, C-1Glc), 100.7 (C-1Rha), 95.4 (C-8), 77.1 (C-3Glc), 76.1 (C-5Glc), 74.2 (C-2Glc), 73.2 (C-4Rha), 71.2, 71.1 (C-2Rha, C-3Rha), 69.9 (C-4Glc), 68.8 (C-5Rha), 66.6 (C-6Glc), 17.0 (C-6Rha).

IR (KBr pellet): ν(cm^{-1}) = 1651.0 (C=O), 1614.2 (C=C), 1299.9, 1083.6 (S=O).

Mass (FAB, negative mode): m/z = 749.2 ($M - H^+$).

Data analysis

The curve-fittings of absorbance *vs*. time plots were carried out on a Pentium 120 PC using the *Scientist* program (MicroMath, Salt Lake City, UT, USA). Beer's law and sets of differential kinetic equations (*see text*) with initial conditions on concentrations (*see Kinetic experiments in Experimental*) were input in the model. Curve-fittings were achieved through least square regression and yielded optimized values for the parameters (kinetic rate constants, molar absorption coefficients). Standard deviations are reported.

Semi-empirical quantum mechanics calculations

Semi-empirical quantum mechanics calculations were run *in vacuum* on a Pentium 90 PC using the HyperChem program (Autodesk, Sausalito, CA, USA) with the AM1 parametrization. Allowance was made for spin interactions in radicals (unrestricted Hartree–Fock method).

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