Free Rad. Res., Vol. 29, pp. 339–350
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Two-electron Electrochemical Oxidation of Quercetin and Kaempferol Changes Only the Flavonoid C-ring

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Accepted by Prof. H. Sies

(Received 24 March 1998)

Bulk electrolysis of the antioxidant flavonoids quercetin and kaempferol in acetonitrile both yield a single oxidation product in two-electron processes. The oxidation products are more polar than their parent compounds, with an increased molecular weight of 16 g/mol, and were identified as 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone and 2-(4-hydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone for quercetin and kaempferol, respectively. Two-electron oxidation of the parent flavonoid is suggested to yield a 3,4-flavandione with unchanged substitution pattern in the A- and B-ring, which may rearrange to form the substituted 3(2H)-benzofuranone through the chalcan-trione ring-chain tautomer. The acidity of the 3-OH group is suggested to determine the fate of the flavonoid phenoxyl radical, originally formed by one-electron oxidation, as no well-defined oxidation product of luteolin (lacking the 3-OH group) could be isolated despite rather similar half-peak potentials: $E_{p/2} = 0.97$ V, 0.98 V and 1.17 V *vs.* NHE for quercetin, kaempferol and luteolin, respectively, as measured by cyclic voltammetry in acetonitrile.

Keywords: Quercetin, kaempferol, electrochemistry, oxidation product, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone, 2-(4-hydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone

INTRODUCTION

The fact that a diet rich in fresh fruits and vegetables has been associated with a decreased risk of cardiovascular diseases and cancer has in recent years led to an increasing interest in the natural compounds present in these foods.[1] Especially the flavonoids have gained increased attention due to numerous reports of beneficial properties, including their antioxidant activity.[2] Flavonoids have, as other phenolic antioxidants, primarily been regarded as free radical terminators serving as antioxidants either by donation of

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a hydrogen atom or an electron.[3] This kind of antioxidant action means, that the flavonoid itself is oxidised. In general, ortho and para diols are easily oxidised to ortho- and para-quinones, and this is usually also considered to be the fate of flavonoids with a catechol (1,2-dihydroxybenzene) structure in the B-ring[3] (Figure 1). Despite this, flavonoid quinones have, to the best of our knowledge, never been isolated in amounts that allowed unambiguous assignment of the chemical structure by ^{1}H and ^{13}C NMR spectroscopy. The immense amount of literature concerned with the antioxidant activity of flavonoids reveals a rather ambiguous situation. Absolute as well as relative efficiency seems to vary from one assay to another, and sometimes even prooxidant effects are found. The metabolic fate of flavonoids in humans, including the risk of mutagenic effects, is also rather controversial. Identification of the oxidation products of potential antioxidants may therefore provide deeper insight into the mechanism of their antioxidant action, and may form the basis for new biomarkers for the antioxidant action of flavonoids. The purpose of this study was to identify oxidation products of quercetin and kaempferol, two of the most abundant flavonoids, using UV-vis, MS, ^{1}H and ^{13}C NMR spectroscopy. The oxidation was performed electrochemically by bulk electrolysis.

MATERIALS AND METHODS

Chemicals

Acetonitrile, MeCN, and methanol, MeOH, was from Fischer Scientific (Leicestershire, UK) and of analytical (HPLC) grade. Water was purified through a Millipore Q-plus purification train (Millipore, MA 01757, USA). Quercetin dihydrate, anthraquinone, ferrocene and lithium perchlorate, $LiClO_4$, were from Aldrich Chemical Co. (Steinheim, Germany). Kaempferol and luteolin were from Apin Chemicals Ltd. (Abingdon, UK). Formic acid was from Merck (Darmstadt, FRG).

Reduction Potentials of Phenoxyl Radicals

Experiments were carried out in MeCN/0.10 M $LiClO_4$ at 25.0 ± 0.1°C with a BAS CV-50W voltammetric analyser (Bioanalytical Systems Inc., West Lafayette, IN, USA). Cyclic voltammograms, with a scan-rate of 100 mV/s, were acquired with a 3.0 mm diameter glassy carbon working electrode (BAS MF-2012) *vs.* a non-aqueous reference electrode (BAS MF-2062) containing the solvent MeCN/0.1 M $LiClO_4$, and with a platinum wire auxiliary electrode (BAS MW-1032). All solutions were purged thoroughly, while stirring, for 10 min with argon before recording the voltammograms, and then provided with a blanket of argon, while measuring. The argon was led through a bottle containing MeCN/0.10 M $LiClO_4$ in order to assure a constant volume in the electrochemical cell. The electrode was polished before each scan with a BAS PK-4 (part MF-2060) polishing kit, and subsequently rinsed by ultrasound to remove residual abrasive particles. The IR compensation was performed automatically at 0.0 V *vs.* the non-aqueous reference electrode immediately before each scan. The reduction potentials of the flavonoid phenoxyl radicals are reported as half-peak potentials, $E_{p/2}$, i.e. the potential where $i = \frac{1}{2} i_p$. The reduction potentials *vs.* NHE were calculated using the first reversible wave of anthraquinone as an internal standard with a reduction potential of −0.53 V *vs.* NHE. This potential was measured relative to the reduction potential of the ferrocenium/ferrocene couple defined as +0.55 V *vs.* NHE in MeCN/0.20 M $LiClO_4$.[4]

Bulk Electrolysis

Bulk electrolysis was performed at room temperature in a BAS MF-1056 bulk electrolysis cell at a potential 200 mV more positive than the half-peak potential determined by cyclic voltammetry.

The flavonoid was dissolved in MeCN/0.10 M $LiClO_4$ to achieve a concentration of 0.30 mM and 69 ml of this solution, corresponding to 20.7 µmol, which according to Faraday's law of electrolysis would require 2.0 C to be oxidised in a one-electron oxidation, was then purged thoroughly in the cell with argon for 10 min. Purging was maintained throughout the electrolysis and the argon was led through a bottle containing MeCN/0.10 M $LiClO_4$. The current was measured in sample intervals of 1 s, and when the current reached 1% of the initial current, the bulk electrolysis was defined as being 'complete'. A sample of 500 µl was taken before electrolysis, after 1.0, 2.0 and 3.0 C, and after complete electrolysis (approximately 4 C). A 400 µl aliquot of each sample was diluted with 1.6 ml MeCN/0.10 M $LiClO_4$ and immediately transferred to a 10.0 mm Hellma quartz cell (Hellma, Müllheim/Baden, Germany). UV-vis spectra were recorded at 25°C using a HP 8453 UV-vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA, USA) with a temperature controlled cell-holder. The undiluted samples were further analysed by HPLC.

Chromatographic Separation

HPLC was performed on a Hewlett-Packard 1100 system (Waldbronn, Germany) with a diode array detector using a Purospher RP-18 column (4 × 250 mM, 5 µm, Hewlett-Packard) with simultaneous detection at 250, 270, 290, 350 and 380 nm. An amount of 20 µl of the electrolysed sample was injected, and the column temperature was maintained constantly at 35°C. The column was eluted with water containing 1% formic acid, HCOOH, v/v in a linear gradient with 25–30% MeCN in 20 min, followed by 30–40% MeCN from 20 to 25 min, 40–60% MeCN from 25 to 28 min, 60–100% MeCN from 28 to 30 min and 100% MeCN from 30 to 35 min. After complete electrolysis, the acetonitrile was evaporated at room temperature under reduced pressure, and the dried material dissolved in 10 ml H_2O (1% HCOOH) and loaded onto a 2 g C18 Mega Bond Elut® cartridge (Varian, Harbor City, USA) for liquid chromatography. The cartridge was eluted with 2 × 10 ml H_2O (1% HCOOH), which effectively removed the supporting electrolyte, $LiClO_4$. It was further eluted with 10 × 10 ml H_2O (1% HCOOH) containing 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 per cent MeOH, and finally with 2 × 10 ml MeOH. All samples were analysed by HPLC. The oxidation product from quercetin typically eluted with 25% MeOH, whereas the oxidation product from kaempferol typically eluted with 35% MeOH.

LC-MS

The HPLC system consisted of a Waters 717 autoinjector, 616 pump, and 996 PDA detector (Waters, Milfort, USA) with a fixed 20 µl injection loop, connected to a Supelcosil RP C18 column (4.6 × 250 mm, 5 µm, Supelco, Bellefonte, USA) protected by a guard column (Supelco Guard LC-18). The mobile phase consisted of 30% methanol/70% water with 1% formic acid (A) and 100% methanol (B). The gradient changed from 0% to 86% B in 50 min at a flow rate of 1 ml/min. UV spectra were recorded from 220 to 450 nm at a rate of 1.00 spectra per second and a resolution of 1.2 nm. Mass spectrometry was performed on a VG Platform II quadrupole instrument (Micromass, Chesire, UK) using the APcI (Atmospheric Pressure chemical Ionisation) inlet. The probe and ion source parameters were: source temperature 150°C, probe temperature 450°C, cone voltage −30 V and corona discharge 1.6–1.9 kV. Negative ion mass spectra were acquired from mass 120 to 450 at a scan rate of one scan per second. The HPLC was connected to the probe of the mass spectrometer via the UV cell outlet using PEEK tubing.

^1H and ^{13}C NMR spectroscopy

^1H, ^{13}C NMR and two-dimensional ^1H–^{13}C correlated NMR spectra were obtained in

DMSO-d_6, as well as in DMSO-d_6 with D_2O, with a Bruker AMX 400WB 400 MHz spectrometer (Rheinstetten, FRG).

RESULTS

The chemical structure and the numbering of the carbon atoms of the investigated flavonoids are shown in Figure 1. From cyclic voltammetry in MeCN/0.10 M $LiClO_4$, half-peak potentials of 0.97, 0.98 and 1.17 V *vs.* NHE were derived for quercetin, kaempferol and luteolin, respectively, which should be compared to 0.29, 0.39 and 0.41 V *vs.* NHE in aqueous solution with pH = 7.4 and ionic strength 0.16 (NaCl).[5] The optical changes during bulk electrolysis, are shown in Figure 2 for the three flavonoids, and it is seen, that the oxidation results in products which have maximum absorption at lower wavelength than the parent compound, indicating decreased conjugation. The HPLC results corresponding to the optical spectra in Figure 2 are shown in Table I. If all of the flavonoid had been oxidised in a two-electron oxidation, this would require 4.0 C, whereas the actual charge consumed is 3.8 ± 0.1 C for quercetin, and 3.7 ± 0.1 C for both

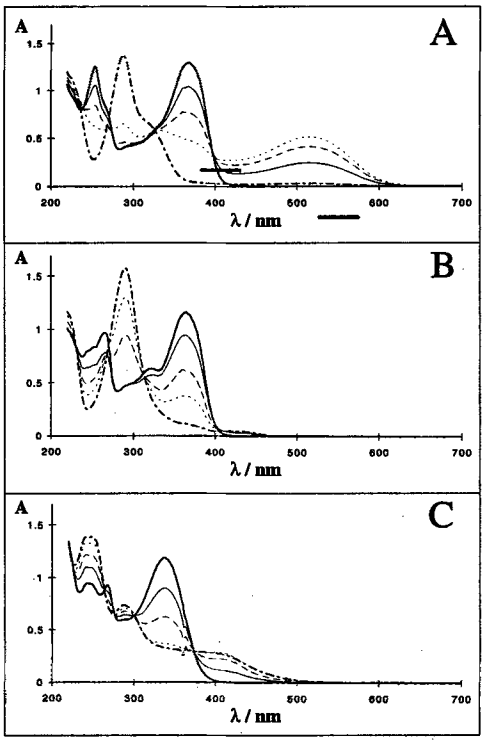


FIGURE 2 Optical changes during electrochemical oxidation of an 0.30 mM solution of A: quercetin, B: kaempferol, C: luteolin in MeCN/0.10 M $LiClO_4$. Samples were diluted 5 times to a concentration of 60 μM before recording. Spectrum before electrolysis: (——); spectrum after 1.0 C: (——); spectrum after 2.0 C: (— —); spectrum after 3.0 C: (----); spectrum after complete electrolysis: (——).

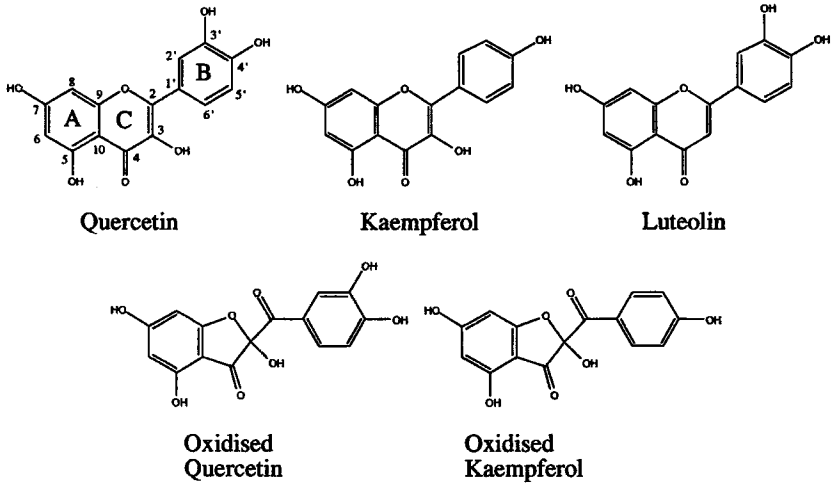


FIGURE 1 Chemical structures and carbon atom numbering for the investigated flavonoids and the isolated oxidation products. Quercetin and luteolin both have a catechol (1,2-dihydroxybenzene) structure in the B-ring.

TABLE I HPLC samples from bulk electrolysis. Quercetin and kaempferol were detected at 380 nm and their oxidation products were detected at 290 nm. Luteolin was detected at 350 nm. The given areas are mean values from two subsequent HPLC injections. Complete electrolysis corresponds to 3.8 ± 0.1 C for quercetin, and 3.7 ± 0.1 C for both kaempferol and luteolin. The given percentages of the oxidation products were calculated from the standard curve obtained from a solution prepared from each of the isolated oxidation products

	Before electrolysis	1.0 C	2.0 C	3.0 C	Complete electrolysis
Quercetin					
Area in HPLC	2849	1915	1254	496	0
% found	100	67	44	17	0
Quercetin-ox.					
Area in HPLC	0	1380	2631	3777	4304
% found	0	32	60	85	97
Kaempferol					
Area in HPLC	2547	1741	1109	440	246
% found	100	68	44	17	10
Kaempferol-ox.					
Area in HPLC	0	1210	2422	3643	3932
% found	0	27	54	81	87
Luteolin					
Area in HPLC	2972	2487	1855	1273	1188
% found	100	84	62	43	40

TABLE II ^{1}H NMR signal assignments for quercetin, kaempferol and their oxidation products in DMSO-d_6

Carbon number*: Flavonoid	3	5	6	8	2′	3′	5′	6′
Quercetin	OH	OH 12.51	6.21 (d) $J_{H6-H8} = 1.5$ Hz	6.43 (d)	7.70 (d) $J_{H2'-H6'} = 1.8$ Hz	OH	6.90 (d) $J_{H5'-H6'} = 8.5$ Hz	7.56 (dd)
Quercetin-ox.	—	OH	5.96 (d) $J_{H6-H8} = 1.7$ Hz	5.90 (d)	7.56 (d) $J_{H2'-H6'} = 2.0$ Hz	OH	6.80 (d) $J_{H2'-H6'} = 8.2$ Hz	7.53 (dd)
Kaempferol	OH	OH 12.50	6.21 (d) $J_{H6-H8} = 2.0$ Hz	6.46 (d)	8.07 (dd)	6.94 (dd)	6.94 (dd) $J_{H5'-H6'} = 9.0$ Hz	8.07 (dd)
Kaempferol-ox.	—	OH	5.96 (d) $J_{H6-H8} = 1.8$ Hz	5.91 (d)	8.03 (dd)	6.84 (dd)	6.84 (dd) $J_{H5'-H6'} = 8.9$ Hz	8.03 (dd)

*See Figure 1 for numbering.

kaempferol and luteolin. Only one oxidation product was observed by HPLC during bulk electrolysis of quercetin. The product was eluted on the column after 5.5 min, whereas quercetin was eluted after 18.2 min, and the oxidation product is thus significantly more polar than the parent compound. Likewise, electrolysis of kaempferol showed only one product by HPLC, which was eluted after 7.3 min, whereas kaempferol was eluted after 26.5 min, which also means a large change in polarity. Mass spectrometry of the purified oxidation products revealed a mass of 318 g/mol for the oxidation product of quercetin and 302 g/mol for the oxidation product of kaempferol which for both compounds is 16 g/mol more than their parent compounds. The obtained ^{1}H NMR and ^{13}C NMR data for quercetin, kaempferol and their oxidation products are shown in Tables II and III, respectively. The chemical shift values of quercetin and kaempferol corresponds to what has been found by others.[6,7] The structure of the isolated oxidation products are shown in Figure 1. Both compounds were obtained as a light

TABLE III ^{13}C NMR signal assignments for quercetin, kaempferol and their oxidation products in DMSO-d_6

Carbon*	Quercetin	Quercetin-ox.	Kaempferol	Kaempferol-ox.	$\delta_{quercetin} - \delta_{kaempferol}$	$\delta_{quercetin\text{-}ox.} - \delta_{kaempferol\text{-}ox}$	$\delta_{quercetin} - \delta_{quercetin\text{-}ox}$	$\delta_{kaempferol} - \delta_{kaempferol\text{-}ox.}$
2	146.7	104.5	146.7	104.6	0.0	−0.1	42.2	42.1
3	135.6	189.8	135.6	189.7	0.0	0.1	−54.2	−54.1
4	175.8	190.2	175.8	190.2	0.0	0.0	−14.4	−14.4
5	160.6	168.4	160.6	168.5	0.0	−0.1	−7.8	−7.9
6	98.1	96.5	98.1	96.5	0.0	0.0	1.6	1.6
7	163.8	171.8	163.8	171.8	0.0	0.0	−8.0	−8.0
8	93.3	90.3	93.4	90.3	−0.1	0.0	3.0	3.1
9	156.1	158.5	156.1	158.5	0.0	0.0	−2.4	−2.4
10	102.9	100.4	103.0	100.4	−0.1	0.0	2.5	2.6
1′	121.9	124.9	121.6	124.7	0.3	0.2	−3.0	−3.1
2′	115.0	117.3	129.5	133.0	−14.5	−15.7	−2.3	−3.5
3′	145.0	144.7	115.4	114.9	29.6	29.8	0.3	0.5
4′	147.6	151.3	159.1	162.5	−11.5	−11.2	−3.7	−3.4
5′	115.5	114.8	115.4	114.9	0.1	−0.1	0.7	0.5
6′	119.9	123.7	129.5	133.0	−9.6	−9.3	−3.8	−3.5

*See Figure 1 for numbering.

yellow powder and $\lambda_{max}(EtOH) = 293\,nm$ ($\varepsilon = 21900\,M^{-1}\,cm^{-1}$) was found for the oxidation product of quercetin with a shoulder at 321 nm ($\varepsilon = 17400\,M^{-1}\,cm^{-1}$), whereas $\lambda_{max}(EtOH) = 296\,nm$ ($\varepsilon = 19900\,M^{-1}\,cm^{-1}$) was found for the oxidation product of kaempferol. Luteolin was clearly consumed during electrolysis as seen in Table I but no oxidation product was detected by HPLC. It is furthermore seen from Table I, that almost half of the luteolin is present in the HPLC samples after complete electrolysis.

DISCUSSION

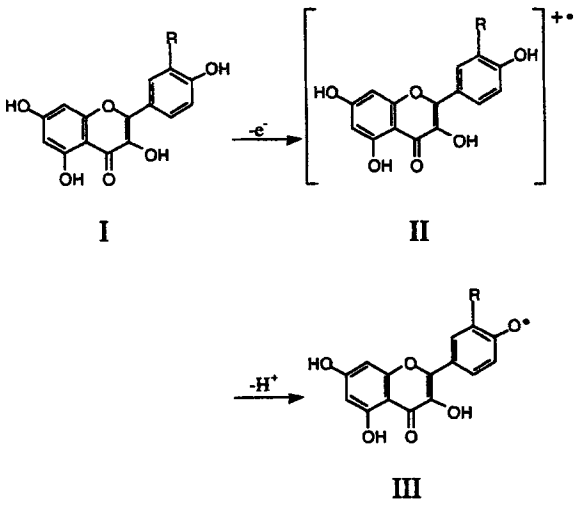
Several oxidation products of quercetin and kaempferol have been described.[8–19] But with the exception of the work by Miller and Schreier,[13,14] none of these reports provide ^{13}C NMR spectral data, which are essential to make an unequivocal assignment of the chemical structure. Remarkably, no oxidation products have, to the best of our knowledge, been reported for luteolin, which could suggest that the presence of the 3-OH group is crucial in determining the oxidation mechanism. Frey-Schröder and Barz (1979) thus reported that 3,4′-dihydroxyflavones, but not flavonol 3-glycosides, were oxidised by horseradish peroxidase.[10] This is in accordance with Loth and Diedrich (1968) who made the general observation, that oxidation with potassium iodate in a mixture of methanol, glacial acetic acid and dioxan led to strongly red coloured solutions for all the investigated flavonols with a catechol pattern in the B-ring, whereas flavones lacking the 3-OH group or flavonols with a 3-methoxy group, were much harder to oxidise giving yellow solutions.[8]

Chemical oxidation of flavonoids is complex due to the varying redox properties,[5] which means that different oxidising agents may be needed for individual compounds. Moreover, the mechanism of oxidation seems to vary with the oxidising agent, and mechanisms described for specific oxidising agents may be irrelevant for their biological or technical antioxidant actions. It is difficult to control chemical oxidation, since few oxidising agents are specific, and they may subsequently be able to react further with the oxidation products. In the investigations by Miller and Schreier,[13,14] oxidation with peroxidase thus resulted in identification of four reaction products for kaempferol as a substrate, and eight reaction products were identified following

oxidation of quercetin. Most of the problems related to lack of control of the oxidation process can be avoided with bulk electrolysis, since a more gentle oxidation can be performed exactly at the desired potential in order to ensure a common mechanism, and the oxidation can be stopped at any time to avoid secondary processes.

The oxidation of quercetin produces intermediates which result in a strongly red coloured solution ($\lambda_{max} = 515$ nm) as seen in Figure 2A. The final two-electron oxidation product is, however, colourless ($\lambda_{max} = 290$ nm). With regard to kaempferol, no long lived intermediates absorbing at higher wavelength are observed as may be seen in Figure 2B. The final two-electron oxidation product is, as for quercetin, colourless ($\lambda_{max} = 290$ nm). Inspection of Tables II and III reveal that quercetin and kaempferol have experienced very similar changes during the oxidation. From Table II it is seen that H6 and H8 in the A-ring have experienced the largest change in chemical shift, whereas the chemical shifts for the hydrogen atoms in ring B are almost unaffected, which indicates an unchanged substitution pattern in the B-ring. Comparison of the two-dimensional ^{1}H–^{13}C correlated NMR spectra of the parent compounds and their oxidation products furthermore show, that the remaining two hydrogen atoms are present in the A-ring with unchanged substitution pattern (data not shown). Unambiguous assignment of the H6 and H8 protons in the oxidation products is a necessity for the assignment of the corresponding carbon signals in the two-dimensional ^{1}H–^{13}C correlated NMR spectrum. This has previously caused some confusion in the literature, but it is now generally accepted, that introduction of a 5-OH group to the flavone skeleton produces a chemical shift for C6, which is 3–5 ppm higher than for C8.[6] This is assumed also to be valid for the oxidation products, and a reversal of the shift order for H6 and H8 in the oxidation products compared to the parent compounds is thus observed, *cf.* Table II. Comparison of the ^{13}C NMR data for the flavonoids and their oxidation products in Table III primarily shows two major changes: (i) two carbonyl groups in the oxidation product compared to one in the parent compound. The higher chemical shift values (e.g. 189.8 and 190.2 ppm in oxidised quercetin compared to 175.8 ppm in quercetin) indicate the absence of the 2,3-double bond in the C-ring. (ii) A significantly changed chemical shift for C2 (from 146.7 ppm in quercetin to 104.5 ppm in oxidised quercetin), furthermore indicates a saturated carbon in the C-ring. Comparison with the measured molecular mass, leads to identification of 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone as the oxidation product of quercetin and to the identification of 2-(4-hydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone as the oxidation product of kaempferol, see Figure 1.

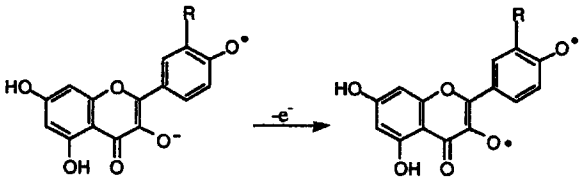
The mechanism can be accounted for as follows. Initial oxidation of quercetin, I (R = OH), or kaempferol, I (R = H), will produce the transient radical cation, II, which by analogy with other cation radicals rapidly loses a proton (typically $pK_a < 0$) to form the neutral phenoxyl radical III.[20]



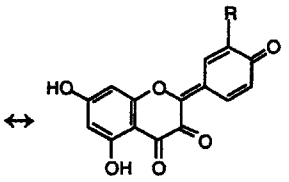
I II III

Formally this corresponds to donation of a hydrogen atom by the antioxidant, or in aqueous

solution to loss of a proton with subsequent electron transfer. Jovanovic *et al.* (1996)[21] have recently demonstrated by comparison with model compounds for the A- and B-ring, that oxidation of flavonoids with a catechol structure will solely take place in the B-ring. No B-ring monosubstituted compounds were investigated by Jovanovic *et al.*, but for hesperidin, which has an ortho-methoxyphenol-like B-ring, oxidation was also concluded to take place in the B-ring. Agrawal and Schneider (1983) studied the deprotonation induced ^{13}C NMR shifts for apigenin (4′,5,7-trihydroxyflavone) and naringenin (4′,5,7-trihydroxyflavanone) and found the 7-OH group to be the most acidic.[22] However, in the case of kaempferol we tend to believe that the 4′-OH group is the more acidic in analogy to quercetin. Loss of a proton from 4′-OH leads to the neutral phenoxyl radical, III. The radical III (R = OH) was recently found[21] to have $pK_{a1} = 4.2$, which in comparison with the 3,4-dihydroxycinnamate radical was assigned to the proton of the other hydroxy group in the B-ring. The pK_{a1} for III (R = OH) is thus significantly lower than $pK_{a1} = 6.74$ for quercetin,[23] as found for many other phenolic compounds,[23] and also for the ubiquitous antioxidant ascorbate with $pK_a = -0.45$ for the ascorbate radical compared to $pK_{a1} = 4.2$ for ascorbic acid.[24] We believe that deprotonation of 3-OH is more likely to occur, since the B-ring radical III (R = OH) is assumed to be stabilised by intramolecular hydrogen bonding due to the ortho-substitution in the B-ring. Loss of a proton from 3-OH in III will result in the anion radical IV, which is assumed to be strongly stabilised by intramolecular hydrogen bonding when R = OH, due to the ortho-substitution in the B-ring, as well as by intramolecular hydrogen bonding between the negatively charged oxygen in the 3-position and H6′, since this will stabilise a highly favoured six-ring configuration, resulting in planarity of the entire molecule. A subsequent one-electron oxidation will result in VI:



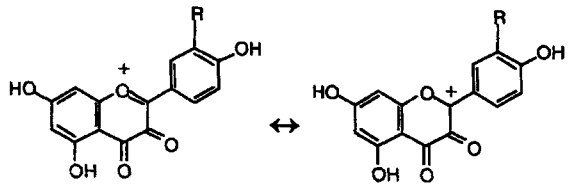
IV V



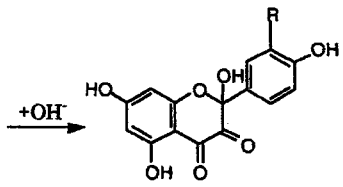
VI

In the case of quercetin, the neutral phenoxyl radical, III (R = OH), will most likely disproportionate to the corresponding ortho-quinone and quercetin, and it should be noted, that VI (R = OH) is a tautomer of this ortho-quinone which is assumed to be planar due to hydrogen bonding between H6′ and 3-OH, leading to a highly conjugated structure which accounts for the characteristic red colour, as generally found for ortho-quinones.[25] Since the phenoxyl radical of kaempferol III (R = H) can not disproportionate to a quinoid structure, loss of the 3-OH proton to produce the anion radical IV (R = H) is expected to proceed much faster than for quercetin and without the formation of a red intermediate. The structure VI is very similar to the quinoidal base of an anthocyanin, and is as such expected to participate in an acid–base equilibrium with the flavylium cation VII, which in principle should be possible to isolate from a completely dry MeCN solution. It is, however, difficult to assure complete dryness, and since commercially available quercetin is supplied as a dihydrate, and water always will be present in any biologically relevant assay, no special precautions were taken to avoid trace amounts of water. Complete hydration will result in the 3,4-flavandione, IX, which in analogy to

anthocyanins may be described as a pseudobase or a hemiketal:

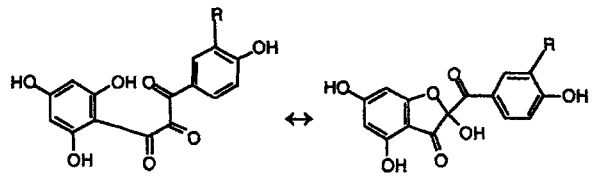


VII VIII



IX

In similarity with anthocyanins, a ring-chain tautomeric equilibrium resulting in the chalcan-trione, X, may exist, which subsequently may lead to formation of the substituted 3(2H)-benzofuranone, XI:



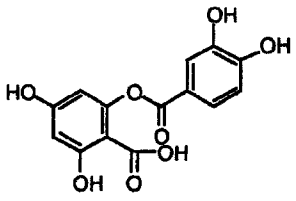
X XI

It is not easy to distinguish between the 3,4-flavandione IX and the substituted 3(2H)-benzo-furanone XI from the available spectroscopic data, but the ring opened chalcan-trione X does clearly not exist in DMSO-d_6, since this would make H6 and H8 chemically equivalent and also the carbon atom pairs C6/C8, and C5/C9, respectively, which is not the case as seen in Tables II and III. It is, however, noteworthy, that the characteristic sharp signal around 12–13 ppm due to the strong hydrogen bond between 5-OH and 4-CO, which is found in all flavanones, flavones and flavonols with a 5-OH group,[26] including quercetin and kaempferol as seen from Table II, is absent in the oxidation products. This hydrogen bond is expected to be equally pronounced in a 3,4-flavandione like IX, and the absence of such a signal thus strongly suggest that the substituted 3(2H)-benzofuranone XI is the isolated oxidation product. Concerning the stoichiometry of the overall oxidation, some remarks seems appropriate. Inspection of Table II shows that after 2.0 C where all the quercetin present on an average has experienced one-electron oxidation, 44% quercetin and 60% oxidation product is found in the HPLC sample. This means that the oxidation product also is generated when the second electron is not removed from IV (R = OH) by the electrode. The anion radical IV (R = OH) is thus assumed to disproportionate to the quinoid structure VI (R = OH) and the dianion of quercetin. This dianion will undergo fast protonation to yield quercetin due to the higher pK_a value of quercetin compared with its phenoxyl radical. The fact that the sum of quercetin and its oxidation product sometimes exceeds 100% must be ascribed to the experimental uncertainty, however, since neither quercetin nor kaempferol is completely converted to their respective oxidation products, this suggests that a few per cent of the generated phenoxyl radicals decay by other reaction mechanisms, e.g. by dimerisation. As for quercetin, interruption of the electrolysis of kaempferol will lead to disproportionation of the anion radical IV (R = H) to yield IX (R = H) and kaempferol dianion.

Since the acidity of the 3-OH group seems crucial for the fate of the antioxidant radicals of quercetin and kaempferol, we also investigated the oxidation of luteolin which lacks this group (Figure 1). The optical changes are shown in (Figure 2C). Clearly, intermediates absorbing at higher wavelengths are formed, probably due to B-ring ortho-quinones, however, we were not able to detect any products by HPLC, and

surprisingly high amounts of luteolin were found in the HPLC samples even after 'complete' electrolysis.

The most frequently reported oxidation product of quercetin[11,16,17,19] is a so-called depside or phenolic carboxylic acid ester:



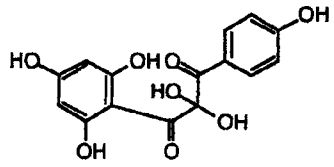
XII

It is noteworthy, that none of the investigations provided ^{13}C NMR data, since the most striking difference between this molecular structure and XI, is the number of carbon atoms (14 *vs.* 15). Quercetin quinone, the ortho-quinone tautomer of VI, has also been reported,[14] but no ^{13}C NMR data were provided. Utaka and Takeda[12] suggested XI (R = OH) as product from the oxidation of quercetin with $CuCl_2$ in dimethylformamide, in a 28% yield but reported no NMR data. Miller and Schreier[14] found eight oxidation products from the reaction between quercetin and peroxidase/H_2O_2 but the major oxidation product was a dimer, and none of the other products were similar to XI.

The isolated oxidation products of quercetin and kaempferol contain four and three hydroxyl groups respectively, and both may thus still be able to work as antioxidants. The dramatic change in polarity is noteworthy, since this means that the oxidation products may be able to display antioxidant actions in more polar environments compared to the parent compound. Furthermore, the oxidation products may seek towards a more polar environment, which may increase the probability of being regenerated by interactions with other antioxidants such as ascorbate. The isolated products were completely stable under the applied experimental conditions, and exposure to oxygen did not cause any degradation, a fact which suggests that they could serve as biomarkers for antioxidant action of the parent compounds *in vivo*.

The final hydration of the flavylium cation VII calls for a word of caution, since other nucleophiles may be able to react likewise. Smith[27] found that oxidation of flavonol and 4′-methoxyflavonol with periodic acid gave similar oxidation products (i.e. a 3,4-flavandione or the corresponding substituted 3(2H)-benzofuranone) with an increase in molecular weight of 16 g/mol, but when the oxidation was performed in methanol,[28] a methoxy group rather than a hydroxy group was found in the 2 position. Utaka and Takeda[12] similarly found that oxidation of quercetin and flavonol with $CuCl_2$ in methanol or ethanol gave the corresponding 2-alkoxyflavan-3,4-diones. The presence of alcohols may thus be crucial for the fate of the antioxidant, since the introduction of an alkoxy group in the 2-position, to yield a ketal, excludes the possibility of ring opening, which only is possible with the hemiketal.

None of the oxidation products reported for kaempferol in the literature[13,15,16] is structurally identical to the product identified in the present study although the major oxidation product isolated by Miller and Schreier[13] was 2,2-dihydroxy-1-(2,4,6-trihydroxyphenyl)-3-(4-hydroxyphenyl)-1,3-propandione, XIII, which is easily obtained from hydration of the C3 carbonyl group in X (R = H):



XIII

The similarity between X (R = H) and XIII clearly suggests that the open chain tautomer exists in aqueous solution, and apparently also

in the acetone-d_6 used as solvent for NMR spectroscopy by Miller and Schreier (1985), a fact which further confirms that the results from investigations of antioxidant reactions is strongly dependent on the reaction medium. The presence of alcohols or water may thus be crucial for the fate of the antioxidant, since acid- as well as base-catalysed hemiketal formation of X is possible in alcoholic solutions and in water. Acid-catalysis may for alcohols further result in the more stable ketal at C3. Since *gem*-diols like XIII generally are unstable, and easily dehydrate to the corresponding ketone, it seems that the oxidation product of kaempferol identified in the present study in principle is identical to the major oxidation product isolated by Miller and Schreier (1985) following enzymatic oxidation.[13]

Acknowledgement

This research was conducted as part of the frame programme "Natural Antioxidants from Plants" (Danish Veterinary and Food Administration) sponsored by the Danish Research Councils under the FØTEK 2 programme in co-operation with LMC – Center for Advanced Food Studies.

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