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Oxidation of flavonols with Cu(II), Fe(II) and Fe(III) in aqueous media

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Reactions of flavonols, 3-methoxyflavones, and flavones with Cu^{2+}, Fe^{3+}, and Fe^{2+} in acidic aqueous solutions with three different organic modifiers are investigated. Complexation may be followed by an oxidation of flavonols, yielding additional peaks in HPLC chromatograms of the reaction mixtures. The products are synthesized with high yields and isolated in mg amounts. They are characterized as 2-(hydroxybenzoyl)-2-hydroxybenzofuran-3(2H)-ones, using two-dimensional NMR techniques and PBI-NCI and -EI-MS. The electrochemical behaviour of the compounds is further investigated with cyclic voltammetry.

Introduction

The antioxidant and chelating properties of flavonoids (Fig. 1 and Table 1) are held responsible for the positive impact of these polyphenols on human health,[1] especially in the prevention of cancer, coronary heart disease and neurodegenerative diseases.[2] While inhibiting damage to fatty acids[3] and low density lipoproteins in transition metal-dependent and -independent oxidation *in vitro*[4] the flavonols might be oxidized themselves. For a better insight into their fate during metabolism emphasis has to be put on studies of their interactions with prooxidative compounds found in cell systems, *i.e.* enzymes and transition metal cations.

Different enzymes have been reported to catalyze the oxidation of flavonoids, leading to a variety of reaction products. Attention was especially paid to the investigation of quercetinase,[6] peroxidase[7] and polyphenoloxidase.[8]

Several authors have reported the chelation of Cu^{2+} and Fe^{3+} by various polyhydroxy-compounds, proposing the 3,4-dihydroxy moiety of the catechol rings, the 3-hydroxy-4-keto groups and the 5-hydroxy-4-keto function as possible binding sites.[9] Although there have been attempts to detect those complexes in plant material,[9b,10] there is no strong evidence for their actual existence. The possibility of a redox reaction following the coordinative binding of the cation was not discussed in those papers.

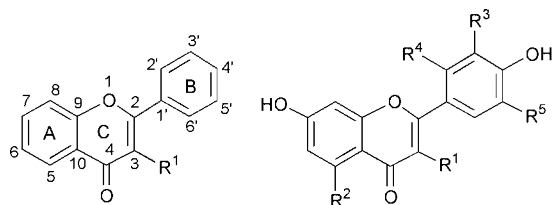

Fig. 1 Structures and numbering of the investigated flavonoids.

Table 1

	R^1		R^1	R^2	R^3	R^4	R^5
Flavonol	OH	Quercetin	OH	OH	OH	H	H
Flav. 3-methyl ether	OCH_3	Kaempferol	OH	OH	H	H	H
		Morin	OH	OH	H	OH	H
		Fisetin	OH	H	OH	H	H
		Myricetin	OH	OH	OH	H	OH
		Luteolin	H	OH	OH	H	H
		Q-3-methyl ether	OCH_3	OH	OH	H	H

The copper(II)-catalyzed oxidation of quercetin and flavonol in anhydrous alcohols was reported by Utaka and Takeda.[11] They proposed the addition of an ethoxy- or methoxy-group at C-2 leading to a 2-alkoxyflavan-3,4-dione though the NMR data were not given. Brown *et al.*[12] examined the interactions of flavonoids with Cu^{2+} in buffered aqueous solutions, proposing the abstraction of 2 e^- and 2 H^+ from the hydroxy-groups at C-3 and C-4′ for both quercetin and kaempferol, but no spectroscopic evidence for the product was given. Murari *et al.*[13] reported the oxidation of quercetin in alkaline hexacyanoferrate(III) solution, yielding mainly catellagic acid and coupling products of catechuic acid and quercetin.

Recently Viborg Jørgensen *et al.*[14] reported the electrochemical oxidation of quercetin and kaempferol. After bulk electrolysis in acetonitrile they isolated one distinct product for each of the flavonols, which was characterized by UV/Vis, HPLC, APCI-MS and NMR measurements. They proposed the products to be substituted benzofuranones which are derived by rearrangement of chalcantriones from the initially formed 2-hydroxybenzopyran-3,4-diones (Fig. 2). The latter compound has been described as an oxidation product of quercetin in plant material by Igarashi *et al.*[15] They succeeded in the preparation of 2,5,7,3′,4′-pentahydroxyflavan-3,4-dione (yield: 0.5–1% only) through reaction of quercetin with an enzyme solution or a combination of irradiation and bubbling with oxygen at 80 °C. Hirose *et al.*[16] isolated the same compound from onions, while Purev and Pospisil[17] suggested the flavandione as an intermediate in the degradation pathway of quercetin in cell suspension cultures of *Nicotiana tabacum*, but gave no spectroscopic evidence for its actual existence.

Though the ^1H and ^{13}C NMR data provided in these publications are identical, different structures were proposed. The mass spectrometric methods used provided information about the molecular mass of the respective compounds, but lacked further structural information. In addition the role of prooxidative cations was not discussed.

Recently Dangles *et al.*[5] investigated the periodate oxidation of quercetin, rutin, isoquercitrin and flavonol 3-methyl ether in methanol and in aqueous buffers (pH 5.5 to 9.0) in the presence or absence of bovine serum albumin. They describe the formation of a quinoid structure after abstraction of 2 e^-, followed

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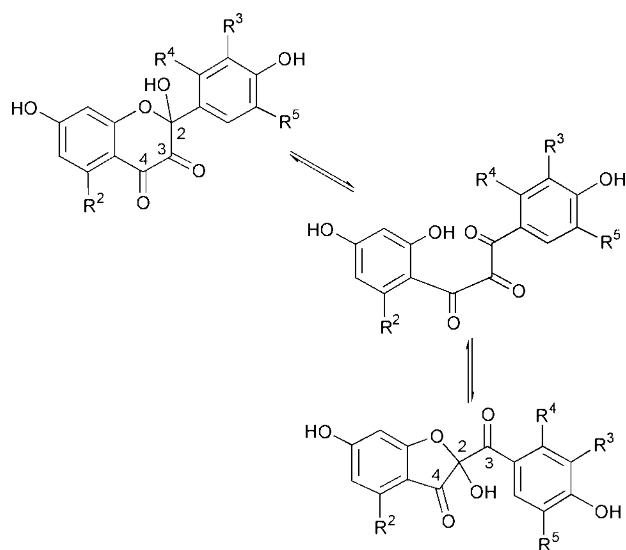

Fig. 2 Reaction products of metal-catalysed oxidation of flavonols. Equilibrium between the initially formed 2-(hydroxyphenyl)-2-hydroxybenzopyran-3,4-dione and the resulting 2-(hydroxybenzoyl)-2-hydroxybenzofuran-3(2*H*)-one (for residues R see Fig. 1 and Table 1) (Note: numbering of C-atoms does not follow IUPAC rules, but was adapted from the numbering used in the literature for comparison of NMR results).

by addition of solvent molecules (either methanol or water) on C-2 and C-3. Other oxidizing agents led to a stepwise abstraction of electrons from quercetin as well, but no attempts were made to isolate further degradation products.

As both redox reaction and complexation are strongly dependent on the conditions in the matrix and those chosen for measurement (*i.e.* oxidising agent, pH value, solvent system, redox potential, coligands), it is difficult to investigate the actual reactions in food matrices and biological samples. This complexity very often demands a knowledge and final judicious choice of combinations of different separation and detection techniques.[18]

In this work the interactions between six different flavonols and the 3-methyl ethers of quercetin and flavonol with Cu^{2+}, Fe^{3+} and Fe^{2+} in different solvent systems with high water contents are investigated. For the most abundant flavonols quercetin and kaempferol starting materials and products are separated by HPLC and the results are compared to those achieved with bulk electrolysis. The molecular structure and electrochemical behaviour of the oxidation products are elucidated with UV/Vis, one- and two-dimensional NMR, MS, and cyclic voltammetry (CV).

Results and discussion

Course of the reaction

The interactions of the flavonoids with Fe^{3+}, Fe^{2+} and Cu^{2+} in 70/30 (v/v) mixtures of aqueous acetic acid (20 g dm^{-3}) and an organic modifier were assessed with UV/Vis spectroscopy. The shift of absorption maxima was seen as an indication of either complexation or redox reaction.

Fe^{3+} is known for its high affinity for ligands with an α-hydroxy-keto moiety in aromatic systems. From 3-hydroxypyridin-4-one derivatives, which are used as therapeutic ferric iron chelators, it is known that complexation *via* this functional group leads to a strong bathochromic shift.[19] On addition of Fe^{3+} to an acetonitrile containing solution of flavonol an absorption shift (370 nm→405 nm) is observed as well, yielding a green solution whose colour is stable for several days with no further change of the spectrum. Upon hydroxy-substitution of the B-ring, as present in all other investigated flavonols, a similar spectral shift is observed. Thus initially the complexation takes place in the same manner. But after a short time the colour vanishes and an increase in absorption at 293 nm is observed. The resulting spectra are identical to those published by Viborg Jørgensen *et al.*[14] for the electrochemical oxidation products of quercetin and kaempferol respectively. The presence of the free 3-OH group is both crucial for complexation and redox reaction, as neither luteolin nor flavonol 3-methyl ether and quercetin 3-methyl ether showed any spectral change on interaction with ferric iron.

The above described reactions were also investigated with methanol and ethanol as organic modifiers. The finally achieved spectra were identical in all cases, and no differences in reaction speed could be noticed. The influence of the solute could only be seen in the different colours of the intermediate complexes.

As expected, Fe^{2+} causes no immediate change of the UV/Vis spectra of the flavonoids, though it is able to act catalytically. 24 h of stirring of a solution containing equimolar amounts of Fe^{2+} and fisetin qualitatively led to the same spectral shifts as described above, but only a small amount of the hydroxyflavonol had reacted. This clearly indicates that the presence of iron in either oxidation state may lead to a tranformation of the flavonols due to the possibility of oxidation of Fe^{2+} to Fe^{3+} through molecular oxygen.

The coordinative binding of Cu^{2+} to flavonol has been described to be strongly dependent on coligands and solutes. In contrast to Fe^{3+} no bathochromic shift as indication for complexation could be observed under the conditions we had chosen. Nevertheless, for the substituted flavonols, reactions similar to those with Fe^{3+} yielding identical spectra were measured. Similar spectra were published by Wedepohl and Schwedt[9a] for the reaction of quercetin with Cu^{2+}. They interpreted the rise in absorption at about 290 nm and the decrease at 370 nm as indication of a stable complex. In contradiction to that assumption we proved the irreversibility of the reaction with cation exchange. The full removal of Cu^{2+} from the solution (the copper content of the solution as measured with AAS was <2% of the added amount) did not reinstate the spectrum of the free flavonol, but the absorption at $\lambda = 293$ nm remained unaltered. For this reason a redox reaction must have taken place after complexation.

Substitution of acetonitrile with alcohols significantly slowed down this reaction (faster in EtOH than in MeOH) and a yellow colouration was observed intermediately, indicating a complexation. An increase of absorbance at 293 nm nevertheless could be observed, showing that the formation of identical products is likely.

Electrochemical oxidation of quercetin (but not kaempferol) in acetonitrile is reported to produce strongly red coloured intermediates ($\lambda_{max} = 515$ nm). An identical colouring can be observed when Cu^{2+} is used as oxidant with quercetin, fisetin and myricetin, *i.e.* if a catechol structure is present in the B-ring. The presence of acetonitrile, water and Cu^{2+} is crucial for this reaction, as neither in pure acetonitrile, nor in alcoholic solution, nor with either ferric or ferrous iron was a similar spectral change observed. The purple colour vanishes during a time period of about 15 minutes, depending on the water content of the solution. The more water is present in the mixtures, the less stable is the coloured intermediate. From these results it clearly can be concluded that water plays a vital role in the reaction. Since water is the most important solvent in biological matrices and prooxidants like Cu^{2+} and Fe^{3+}/Fe^{2+} are found ubiquitously, the measurements conducted here are closer to natural systems than those reported by Viborg Jørgensen *et al.*[14] Therefore it can be derived from our results that the oxidation of flavonols described here is likely to occur in biological systems.

The presence of three isosbestic points at 245, 271 and 332 nm in the finally achieved spectra of oxidized quercetin suggests that only one absorbent product is formed. It is noteworthy that the same spectral data were given for the reaction

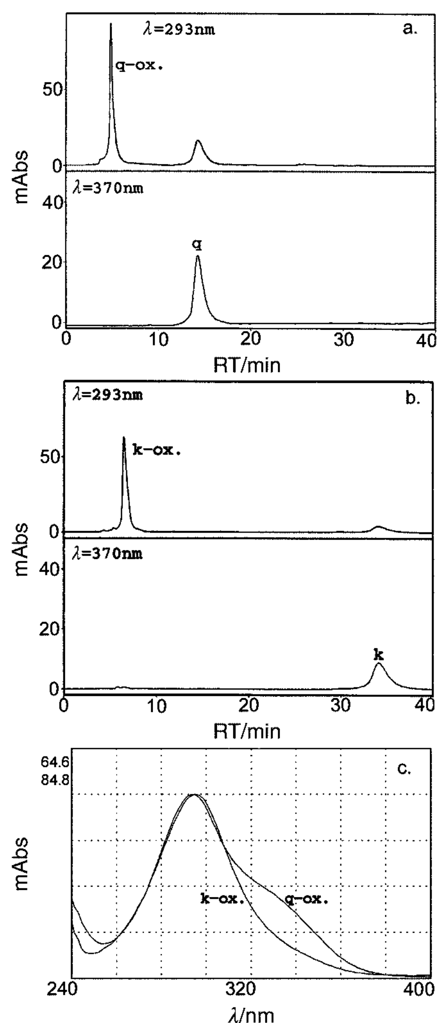

Fig. 3 HPLC chromatograms of reaction mixtures of (a) quercetin and (b) kaempferol with Cu^{2+} (molar ratio 1:1, respectively); eluent: acetonitrile–aqueous acetic acid (20 g dm^{-3}) (30/70, v/v), flow: 0.7 cm^3 min^{-1}, column: polystyrenedivinylbenzene (PS/DVB), DAD detection with two traces (293 nm for oxidized flavonols, 370 nm for flavonols); (c) peak spectra for the compounds eluting at 5.2 and 6.4 min.

of quercetin with polyphenoloxidase,[8] making it likely that the compounds are identical.

Chromatography of reaction mixtures

HPLC was used to confirm that exactly one corresponding oxidation product was formed from each hydroxyflavonol. For reasons of feasibility the reaction mixtures were injected onto the column without prior removal of the cations. To avoid adsorption of cations on a silica based stationary phase[10c,20] a polystyrenedivinylbenzene (PS/DVB) material was used. Preliminary studies had shown that cations injected onto this column all elute within the void volume. With this system four peaks were detected using a photodiode-array detector (DAD), corresponding to quercetin, kaempferol and their two oxidation products (Fig. 3). An increase of the amount of the added cation resulted in an increase of the peak areas for the first two compounds, while those of the free hydroxyflavonols decreased. The UV/Vis spectra of the first two eluting peaks are identical to those of the solutions of the respective hydroxyflavonol with a 5:1 molar surplus of Cu^{2+} or Fe^{3+}.

Though in isocratic mode the resolution of the oxidation products is poor, it is obvious that they can easily be separated from quercetin and kaempferol. They elute shortly after the void volume and the retention times show that their polarity is significantly higher than that of the hydroxyflavonols. The advantage of the method described is that a fast analysis can be performed without the need for removal of cations, which allows chromatography of a variety of cation-containing reaction mixtures. No decrease in column performance or change of retention behaviour could be observed, since no cations were able to bind to the stationary phase.

Structural properties of oxidized flavonols

NMR spectrometry. Data from UV/Vis and HPLC-DAD measurements suggest that the oxidation products of metal catalyzed and electrochemical reactions are identical, a fact which was verified with NMR.

The ^{13}C and ^{1}H data are in good agreement with those published by Igarashi *et al.*[15] (quercetin oxidized enzymatically) and Viborg Jørgensen *et al.*[14] (quercetin and kaempferol oxidized electrochemically).

A unique structure validation based on the one-dimensional NMR data is difficult.[23] Viborg Jørgensen *et al.* see the absence of a sharp signal around 12–13 ppm, which would have been due to a strong hydrogen bond between OH-5 and CO-4, as an indication of the existence of the benzoylbenzofuran-3-one instead of the phenylbenzopyran-3,4-dione as described by Igarashi *et al.* (Fig. 2). This assumption is so far confirmed by our observation that in the case of morin a distinct signal is seen at 11.9 ppm, which can easily be explained by hydrogen bonding between OH-2′ and CO-3 of the substituted benzoylbenzofuran-3-one. Since the presence or absence of signals for hydrogen bonds depends on the chosen solvent, only two-dimensional NMR techniques are suitable for an explicit decision for one of the proposed structures.

Chemical shifts of the five aromatic protons were verified with HH-COSY. Subsequently the chemical shifts of the respective C-atoms 2′, 5′, 6′, 6 and 8 could be determined with HMQC. Discrimination between the pyranoid and furanoid forms was finally achieved with HMBC (Fig. 4), which reveals coupling between carbon atoms and protons *via* 2 or 3 (C-H protons, O-H protons), or 4 bonds (C-H protons only). Four results (shown as encircled regions in Fig. 4) clearly indicate that the substituted benzoylbenzofuran-3-one is present:

1. OH-2 (8.6 ppm), identified by its coupling with C-2, shows a coupling with CO-3 and CO-4 with similar intensity and
2. C-3 couples to H-5′, which both are unlikely for the pyranoid ring.
3. No signal is seen for the coupling between OH-2 and C-1′, and
4. C-2 reveals no coupling with H-2′ and H-6′, which both would be expected for the pyranoid form.

Furthermore in the literature cited above there are some differences in the signal assignments for the oxidized quercetin, which could be unequivocally resolved with the HMBC results (Table 2).

For the reaction products of fisetin, morin and myricetin ^{1}H- and ^{13}C-signals could be assigned in the same manner. As it is unlikely that the equilibrium (Fig. 2) depends on the substitution of the A- and B-ring of the respective compounds, it can be concluded that the courses of reaction are identical for all investigated flavonols. In the case of myricetin, a mixture of at least two products was achieved, owing to the easier oxidizability of the three hydroxy groups in the B-ring.

Mass spectroscopy. To date no selective detection method for the analysis of the oxidized flavonols has been described. The strong hypsochromic shift of the absorption maxima during the oxidation demands UV detection around 290 nm. This loss of selectivity—compared to the detection of the flavonols themselves—makes the application of mass spectrometric techniques necessary. As the molecular masses of the investigated flavonols and their oxidation products are all in the range of 286 to 334 g mol^{-1} with differences of 16 units, detection of the

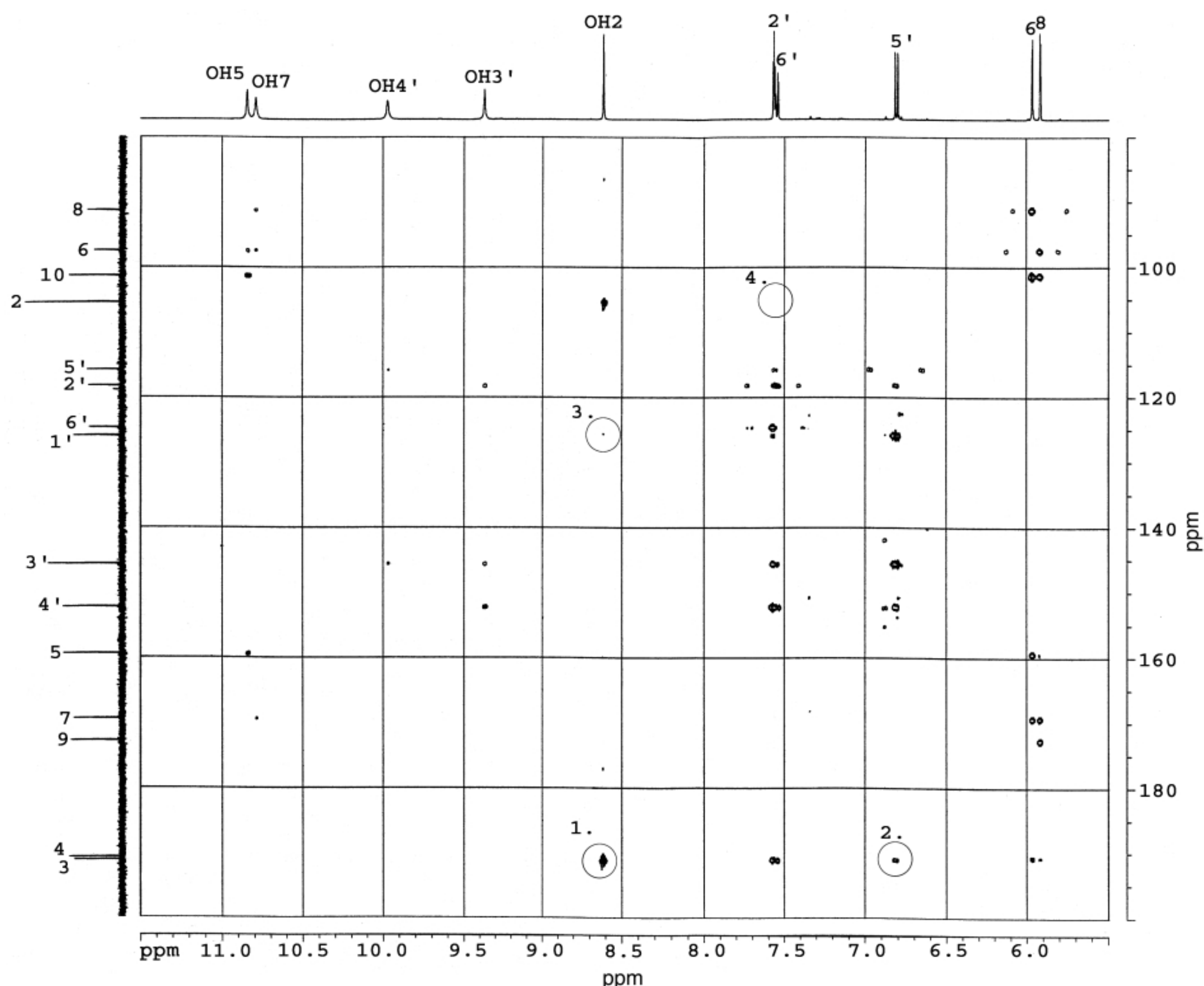

Fig. 4 HMBC-NMR of oxidized quercetin (left trace: C-nuclei, upper trace: protons); encircled regions 1–4 are explained as main clues in the text.

Table 2 ^{13}C-NMR chemical shifts for the oxidized flavonols quercetin (q), kaempferol (k), morin (m) and fisetin (f) in CD_3CN

C atom	Chemical shift/ppm			
	q-ox	k-ox	m-ox	f-ox
2	103.8	102.0	104.5	103.7
3	192.1	192.5	194.2	193.8
4	190.4	190.7	191.5	190.4
5	159.3[a]	163.8	159.3	127.9
6	98.3	98.6	98.2	113.6
7	170.1[a]	170.3	170.1	168.5
8	93.0	93.5	92.7	100.1
9	173.0[a]	173.2	172.6	174.2
10	103.8	104.3	101.5	112.3
1′	125.8	125.9	110.8	125.8
2′	117.3	133.9	166.5	117.3
3′	145.4[a]	116.7	103.9	145.4
4′	152.3[a]	159.4	167.8	152.2
5′	116.1	116.7	109.6	116.1
6′	125.1	133.9	135.5	125.1

[a] Signal assignment confirmed with HMBC differing from literature (for numbering see Fig. 1 and 2).

Table 3 Most prominent fragments in EI mass spectra of flavonols and oxidized flavonols (new fragments are emphasized) (Note: additional fragments for morin are due to OH-2′)

Compd	M/g mol^{-1}	Fragments [m/z]
q	302	302, 153, 137, 109
q-ox	318	<u>154</u>, 153, 137, 109
k	286	286, 153, 121, 93
k-ox	302	153, <u>138</u>, 121, 93
m	302	302, 285, 153, 137, 121
m-ox	318	<u>154</u>, 153, 137, <u>136</u>, <u>126</u>, 121
f	286	286, 137, 121, 120, 109
f-ox	302	<u>154</u>, 137, 121, 120, 109

molecular ion alone, as achieved with APCI- or ESI-interfaces in combination with quadrupole MS,[14] is not sufficient for post-chromatographic confirmation of the compounds. Therefore we studied the mass spectrometric behaviour of the compounds with negative chemical ionization (NCI) and electron impact ionization (EI) after sample introduction *via* a particle beam interface (PBI).

Determination of the molecular masses of the oxidation products with NCI confirmed the increase of 16 mass units relative to the respective flavonols. [M$^-$] was the base peak in all cases and no significant fragmentation was observed, while the strong fragmentation in EI mode allowed structure assignment.

Table 3 shows a comparison of fragments found for flavonols and oxidized flavonols. For all investigated flavonols EI produces spectra as described in the literature,[21] with [M$^+$] as base peak and fragments with relative intensities between 10% and 20%.

In contrast, no [M$^+$] is observed for the oxidized flavonols as a result of the loss of aromaticity of the C-ring. The new fragments characteristic of the oxidation products are those with m/z 154 (dihydroxy substitution in the B-ring) and m/z 138 (monohydroxy substitution in the B-ring), corresponding to (di)hydroxybenzoic acid. From the present data it cannot be decided whether these fragments emanate from benzofuranones (rearrangement of OH-2, new bond to C-3) or benzopyrandiones (scission beween (1) O-1 and C-9 and (2) C-2 and C-3). However, they may be used as diagnostic masses for detection of oxidized flavonols.

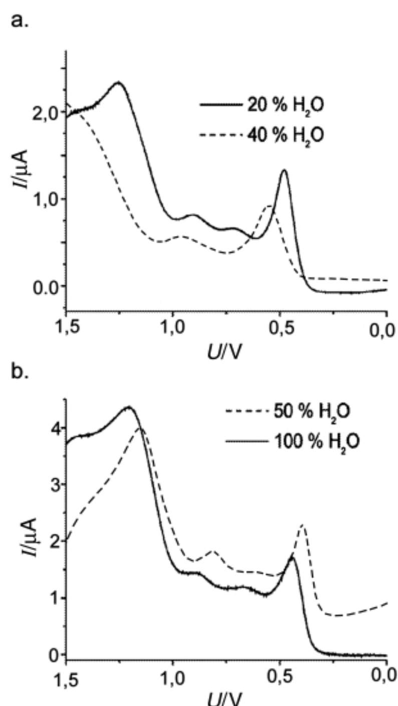

Fig. 5 Cyclic voltammograms (50 mV s^{-1}) of 20 μg cm^{-3} quercetin under variation of the content of water and organic solvent component. Only forward scans. (a) Measurements in supporting electrolyte consisting of acetonitrile and aqueous acetic acid (20 g dm^{-3}), water content indicated as H_2O. (b) Measurements in supporting electrolyte consisting of Britton–Robinson buffer in mixture with methanol according to Hendrickson *et al.*[22]

With this, it was checked whether the isolated compounds characterized in flow injection mode are in fact the same as measured within solution with UV/Vis and HPLC/DAD. As low flow rates are recommended for the PBI, a 2 mm id HPLC column at 0.2 cm^3 min^{-1} was chosen with the same eluent as for HPLC/DAD measurement. The eluting peaks were monitored with a multi-channel UV detector (293 nm, 370 nm) and the mass spectrometer with selected ion monitoring (*m*/*z* 154, 153, 138, 137, 109, 93). No differences in retention times and characteristic masses between the isolated compounds and a solution of the respective hydroxyflavonol and Cu^{2+} (molar ratio 1:1, copper removed by ion-exchange prior to injection) were found.

Cyclic voltammetry. Cyclic voltammograms of quercetin show three or four oxidative waves,[14,22] depending on the composition of the eluent. Water contents higher than 50% and acetonitrile contents lower than 50% yield four waves (Fig. 5a). The oxidation potentials are +0.48 V, +0.70 V, +0.90 V and +1.25 V. In agreement with reported measurements the first oxidation step is reversible. To exclude the possibility that the additional oxidation step is dependent on interactions between above chosen electrolyte components and quercetin, voltammetric measurements were repeated in Britton–Robinson buffer[22] varying methanol content. It was found that the additional peak appeared as well at 10% methanol and less (Fig. 5b). The dependency of this additional signal on the polarity of the electrolyte may be explained with the ratio of intra- and inter-molecular H-bonding taking place either between OH-5 and CO-4 (low water content) or between OH-5 and water molecules (high water content).

In order to associate oxidation potentials with reactive groups in the structure of quercetin and of its oxidation product both these compounds and luteolin were measured with cyclic voltammetry (Fig. 6). For the interpretation of CV measurements of the benzofuranone the first oxidation potential is compared with those of quercetin and luteolin. The bicycle (A- and C-ring) can be considered as a substituent of the phenyl ring affecting the oxidation potential of OH-3′ and OH-4′ due to electron push or pull. A strong electron attracting effect by the substituent should shift the oxidation potential to higher values. The order of electron withdrawing effect of the bicycles and the oxidation potentials for the phenylic OH-groups is: quercetin (+0.47 V) < luteolin (+0.56 V) < benzofuranone (+0.69 V). Hydrodynamic voltammograms of five oxidized flavonols are presented in another publication.[24]

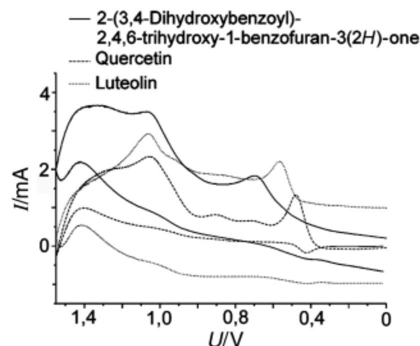

Fig. 6 Cyclic voltammograms of quercetin, oxidized quercetin and luteolin in a mixture of acetonitrile and aqueous acetic acid (20 g dm^{-3}) (20/80, v/v).

Conclusions

The present results show that flavonols may be oxidized in the presence of Cu^{2+} and Fe^{3+}, yielding 2-(hydroxybenzoyl)-2-hydroxybenzofuran-3(2*H*)-ones. Though the solvents used have a strong impact on reaction speed, the resulting compounds were equal in all cases.

The HPLC method can be taken as a basis for further investigation of oxidized flavonols, as it is suitable for fast and easy separation of compounds found in cation-containing reaction mixtures. Mass spectrometric studies of the compounds for the first time provided information about their characteristic fragmentation, which can be used for their selective detection after chromatography.

Finally, the investigation of the electrochemical behaviour of the compounds gave information about their oxidation potentials under different measuring conditions.

It is likely that the compounds reported here are to be found in biological systems. The reported result may be taken as a starting point for further investigation of stable compounds derived from oxidation of antioxidants.

Experimental

Reagents and solutions

Acetonitrile (Roth, Karlsruhe, Germany) and methanol (Merck, Darmstadt, Germany) were of HPLC grade, diethyl ether (Merck), hydrochloric acid 25% (Merck) and acetic acid 100% (Merck) were of analytical grade, ethanol was of technical grade and distilled before use. Water was of ultrapure quality (18.5 MΩ) as delivered by a Milli-Q water purification system (Waters, Milford, USA).

Quercetin, morin, fisetin and luteolin were obtained from Sigma (Deisenhofen, Germany), kaempferol and myricetin from Fluka (Deisenhofen, Germany) and flavonol from Avocado Research Chemicals (Heysham, UK). 3-Methyl ethers of quercetin and flavonol were prepared in our department and their structures verified by NMR- and MS-measurements. Stock solutions of the phenols with a concentration of 500 μg cm^{-3} were prepared in either acetonitrile, methanol or ethanol.

Aqueous Cu(II) (Baker, Phillipsburg, USA) and Fe(III) (Riedel de Haen, Seelze, Germany) standard solutions for AAS (1000 μg cm^{-3}) were used as stock solutions. A solution of 50.09

mg $Fe(NH_4)_2(SO_4)_2$ (Merck, analytical grade) in 10 cm^3 acetic acid (20 g dm^{-3}) was freshly prepared before use.

The working standard solutions (100 µg cm^{-3}) of all the compounds were prepared by diluting the stock solutions with a mixture of the respective organic modifier and aqueous acetic acid 20 g dm^{-3} (pH = 2.5) (30/70, v/v). The solutions were stored at 4 °C in polyethylene bottles to avoid interactions of cations with glass surfaces.

$CuCl_2 \cdot 2H_2O$ (Merck) and $Fe_2(SO_4)_3 \cdot H_2O$ (Riedel de Haen) for preparation of the oxidation products were of analytical grade. Lewatit S100 (Bayer, Leverkusen, Germany) was used as strong cation exchanger. Na_2SO_4 was of analytical grade and dried for 2 h at 500 °C.

Instrumentation

UV/Vis spectroscopy. UV/Vis spectra in the range from 240 nm to 600 nm were recorded with a Uvikon Spectrophotometer 930 (Kontron, Neufahrn, Germany). Double beam measurement was carried out in 5 mm quartz cuvettes with the respective solute as reference.

Atomic absorption spectroscopy. The copper contents of the solutions before and after ion-exchange were measured with a Philips PU 9100X flame atomic absorption spectrometer (Pye Unicam, Kassel, Germany) at a wavelength of 328.4 nm.

HPLC/DAD. The chromatographic system consisted of a Maxi-Star K-1000 HPLC-pump with a sample injection valve equipped with a 20 mm^3 loop (both Knauer, Berlin, Germany). A Nucleogel RP 100 5/250 analytical column (250 × 4 mm id, 5 µm) (Macherey Nagel, Düren, Germany) was used with an eluent containing acetonitrile and aqueous acetic acid 20 g dm^{-3} (28/72, v/v) at a flow rate of 0.7 cm^3 min^{-1}.

Eluting substances were detected with a Shimadzu SPD-M10AVP photodiode-array detector in a range from 240 nm to 600 nm. Data were stored and evaluated using CLASS-M10A-Software (Shimadzu, Kyoto, Japan).

Cyclic voltammetry (CV). A µAutolab (Eco Chemie, Utrecht, Netherlands) was used as potentiostat controlled by GPES 4.4 software (Eco chemie). A glassy carbon disc electrode with 2 mm diameter was used and a saturated Ag/AgCl (3 mol dm^{-3} KCl) was used as reference electrode (both Metrohm, Herisau, Switzerland). Measurements were carried out in a 694 VA stand (Metrohm) with 10 cm^3 sample volume. A 20 g dm^{-3} aqueous solution of acetic acid was mixed with acetonitrile in various concentration ratios as supporting electrolyte. Further a solution of 0.05 mol dm^{-3} sodium phosphate buffer (pH 7) containing 0.5 mol dm^{-3} potassium nitrate was mixed with methanol in various concentration ratios. Solutions were purged with nitrogen before measurement. Stair case ramps were defined by the step potential (U_{step} = 1.1 mV) and the scan rate (U/t = 50 mV s^{-1}). Additional parameters were chosen as follows: start potential = 0 V, first vertex potential = +1.6 V, second vertex potential = 0 V. Cycles were measured twice to detect electrochemical products generated in the first cycle at the electrode. All measurements were background corrected.

HPLC- and flow injection-PBI/MS. For mass spectrometric measurements after ion-exchange the solutions were introduced *via* an injection valve (Rheodyne, Cotati, USA) fitted with a 20 mm^3 sample loop onto a Superspher 100 RP18 analytical column (125 × 2 mm id, 5 µm) (Merck, Darmstadt, Germany). The eluent, an acetonitrile–aqueous acetic acid (20 g dm^{-3}) mixture (30/70, v/v), was delivered at a flow rate of 0.2 cm^3 min^{-1} by a HPLC pump model 616 equipped with a controller model 600S (Waters, Milford, USA). Continuous sparging with helium at a rate of 10 cm^3 min^{-1} was used. Eluting compounds were monitored with a variable wavelength detector model 490E (Waters, Milford, USA) in maxplot mode at 293 nm and 370 nm simultaneously. For direct injection of samples without chromatographic separation (flow injection mode) a second (5 mm^3) sample loop (Rheodyne, Cotati, USA) was mounted between UV detector and mass spectrometer.

The eluent was introduced *via* a particle beam interface (40 °C, He pressure 600 kPa) into the mass spectrometer model SSQ710 (Finnigan, Bremen, Germany). The analytes were ionized both in EI mode (70 eV, 250 °C) and using methane as reactand gas for NCI (*ca.* 8000 mTorr, 70 eV, 200 °C). In flow injection mode ions were recorded in a full scan from 100 to 350 *m/z* in 0.5 s. Selected ion monitoring (SIM) with a scan time of 0.1 s was chosen for detection of compounds after chromatographic separation because of its higher sensitivity.

Data recording and treatment was done with Finnigan ICIS 8.3.0 software on a DEC alpha station.

NMR spectrometry. One-dimensional NMR spectra were recorded with a Bruker AM 300 in CD_3CN with tetramethylsilane as internal standard. ^{13}C NMR spectra were measured at a frequency of 75 MHz. The position of the signals was taken from the broad band decoupled spectra, the multiplicity from the DEPT spectra. ^{1}H spectra were recorded at a frequency of 300 MHz. Two-dimensional HH-COSY, HMQC and HMBC measurements were done in $CDCl_3$ with a Bruker Avance 500 at 500 MHz and 125 MHz respectively.

Procedures

Reaction of flavonols with cations. To 400 mm^3 of the polyphenol solution (100 µg cm^{-3}) the appropriate amount of metal ion solution (100 µg cm^{-3}) was added, whereat the concentrations of the cations were varied in molar ratios of cation to ligand from 1 : 16 up to 5 : 1. The mixtures were made up to a volume of 2 cm^3 in plastic Eppendorf caps. With all the following measurements no interferences of compounds from the plastic material were observed.

To investigate whether molecular oxygen is crucial for the course of the reaction the solutions were purged with nitrogen for 30 minutes before and after mixing. Since the results of the subsequent photometric and chromatographic investigations showed no differences between samples treated with or without nitrogen, the purging was considered not to be necessary.

Preparation of the oxidation products for MS and NMR. With regard to the available amount 0.1 mmol or 0.05 mmol of the respective polyphenol were weighed into a 100 cm^3 round bottomed flask and diluted by adding 30 cm^3 acetonitrile and 30 cm^3 hydrochloric acid 0.01 mol dm^{-3} at 40 °C. 83.3 mg (0.5 mmol) or 41.7 mg (0.25 mmol) $CuCl_2 \cdot 2H_2O$ or 50.0 mg (0.12 mmol) $Fe_2(SO_4)_3 \cdot H_2O$ were added, and the solutions were stirred at ambient temperature for 24 h. The decrease of the UV absorbance at the maxima of the flavonols (370 nm) was monitored. Subsequently metal ions were removed through ion-exchange. Therefore the strong cation exchanger was filled into a glass column (10 mm id) about 5 cm in height and pretreated by washing with 10 cm^3 of HCl (1 mol dm^{-3}) followed by water until the eluting liquid reached neutral pH. The sample solution was introduced into the column and eluted into a separating funnel (250 cm^3) at a flow rate of about 3 cm^3 min^{-1}, followed by washing of the ion-exchanger with 10 cm^3 water. The reaction products were extracted three times with 30 cm^3 diethyl ether each, the combined phases of ether and acetonitrile dried over Na_2SO_4 and filtered through a folded filter into a 250 cm^3 round bottomed flask. The solvent was rotary evaporated at 40 °C to a volume of about 10 cm^3. The remaining solution was transferred quantitatively into a 25 cm^3 tapered flask. Again the solvent was removed through rotary evaporation at 40 °C, the last traces being removed through overnight lyophilization.

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