Free Rad. Res., Vol. 28, pp. 335–351
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Flavonoid Deactivation of Ferrylmyoglobin in Relation to Ease of Oxidation as Determined by Cyclic Voltammetry

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Accepted by Prof. H. Sies

(Received 31 October 1997; In revised form 12 January 1998)

Fourteen flavonoid aglycones, and the flavonoid glycoside rutin, with redox potentials ranging from 0.20 (myricetin) to 0.83 V (chrysin) vs. NHE, as determined by cyclic voltammetry at 23°C in aqueous 50 mM phosphate, ionic strength 0.16 (NaCl) with pH = 7.4 and compared with redox potentials determined for four cinnamic acid derivatives, were all found to reduce ferrylmyoglobin, MbFe(IV)=O, to metmyoglobin, MbFe(III). Reaction stoichiometry depends strongly on the number of hydroxyl groups in the flavonoid B-ring. All compounds with 3′,4′-dihydroxy substitution reduce 2 equivalents of MbFe(IV)=O, whereas naringenin, hesperitin and kaempferol, with one hydroxyl group in the B-ring, reduce with a one-to-one stoichiometry. As studied spectrophotometrically under pseudo-first-order conditions with flavonoids in excess, rutin and apigenin react with MbFe(IV)=O with very similar and moderately high activation enthalpies of $\Delta H^{\ddagger}_{298} = 69 \pm 1\,\mathrm{kJ\,mol^{-1}}$ and $\Delta H^{\ddagger}_{298} = 65 \pm 3\,\mathrm{kJ\,mol^{-1}}$, respectively, and with positive activation entropies of $\Delta S^{\ddagger}_{298} = 23 \pm 4\,\mathrm{J\,mol^{-1}\,K^{-1}}$ and $\Delta S^{\ddagger}_{298} = 13 \pm 9\,\mathrm{J\,mol^{-1}\,K^{-1}}$, respectively, in agreement with outer-sphere electron transfer as rate determining. For the fifteen plant polyphenols only qualitative relations exist between redox potential and rate constants rather than a linear free energy relationship ($r^2 = 0.503$), and especially the flavone apigenin was found more efficient as reducing agent. For the flavanones, a linear relation ($r^2 = 0.971$) indicate that, in the absence of a 2,3 double bond, removal of the 4-carbonyl group or addition of a 3-hydroxy group only has minor effect on reactivity. The flavonols are the most efficient reducing agents, effectively reducing MbFe(IV)=O to MbFe(III) and establishing a steady state distribution between the flavonol and MbFe(III) and oxymyoglobin, $MbFe(II)O_2$. Oxidised flavonols reduces MbFe(III) to $MbFe(II)O_2$ very efficiently and much faster than the parent flavonol.

Keywords: Ferrylmyoglobin, hypervalent iron, flavonoids, redox potentials, cyclic voltammetry, linear free energy relationship

INTRODUCTION

Epidemiological studies indicate that diets rich in vegetables and fruits seem to have positive health effects and to yield protection against certain chronic diseases such as arteriosclerosis

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and cancer.[1,2] In an effort to understand these observations, increasing attention has been paid to the antioxidative properties of different groups of non-nutritive components in vegetables and fruits. Especially the role of flavonoids and carotenoids as antioxidants have been the subject of renewed interest. More than 4000 flavonoids are presently known,[3] but the major interest has been devoted to those which occur in the human diet in amounts comparable to the better characterised antioxidants vitamin C, vitamin E and β-carotene. Currently, the estimated daily intake in Denmark per capita of flavonoids is 26 mg aglycone.[4] In nature flavonoids primarily occur as glycosides, and it is usually assumed that they are cleaved to the corresponding aglycone in the intestinal tract, and that this cleavage is essential for further absorption.[5] In a recent study, however, surprisingly high concentrations of especially quercetin glycosides were found in human plasma obtained from normal individuals on a non-supplemented diet.[6] Numerous studies have focused on the flavonoids as antioxidants[7–12] and in many of these investigations attempts have been made to establish structure–activity relationships, following the original proposal by Bors *et al.*[12] This has proved successful in several cases for a large number of flavonoids using specific assays. Notably, the order reverses for some compounds, when different screening assays are compared and many explanations have been offered, such as differences in solubility, lipophilicity and metal chelation properties. On the other hand, it seems always to be assumed, that flavonoids exert their antioxidative action by a common mechanism depending on the hydrogen atom donating properties of the phenolic groups. Moreover, this mechanism also accounts for the observation that flavonoids, which display excellent antioxidant activity, under some conditions are prooxidants in the presence of Cu^{2+}.[11]

Hypervalent forms of the heme pigments haemoglobin and myoglobin, detected under ischaemic conditions in cardiac muscle[13] have been shown to be powerful initiators of lipid oxidation[14] and of protein cross-linking.[15] The hypervalent muscle heme protein ferryl-myoglobin has been shown to oxidise cinnamic acid derivatives,[16] and we undertook the present investigation in order to explore whether it is reasonable to assume that flavonoids act as antioxidants by electron donation to heme derived prooxidants parallel to the more well-established hydrogen atom donation to lipid derived free radicals. Flavonoids were selected according to two criteria: (i) each should be commonly occurring in a typical diet, and (ii) they should together provide a large span in electron donating properties. The structure of the fifteen compounds selected may be seen in Figure 1, and the combined electrochemical and reaction kinetic investigation of the electron donating properties of these compounds should provide data for a discussion of structure–activity relationships. A verification of a linear

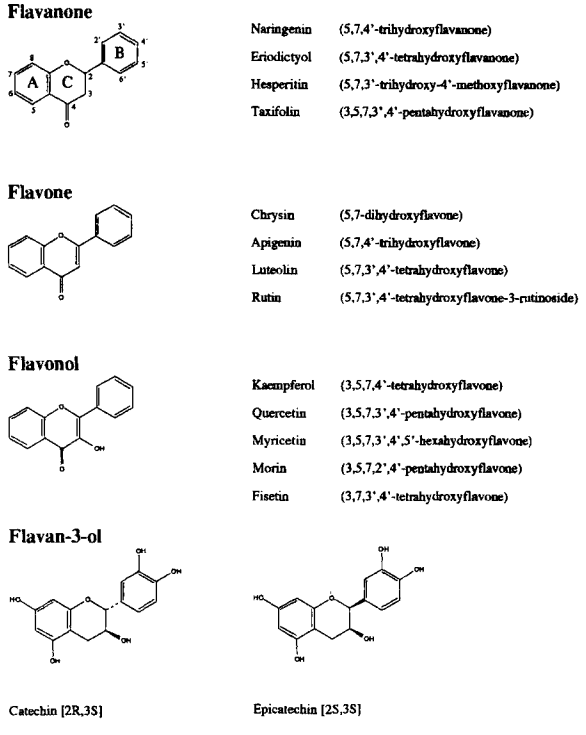

FIGURE 1 Flavonoid structures.

free energy relationship would empirically be a strong indication of a common reaction mechanism in deactivation of ferrylmyoglobin by flavonoids with a large span in redox potentials.[17] Large deviations from linearity, although less informative, could indicate different reaction mechanisms.

MATERIALS AND METHODS

Chemicals

Metmyoglobin, MbFe(III), (horse heart, type III) and bovine liver catalase (41 000 units/mg protein, 140 mg protein/ml, used as received) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). MbFe(III) was (10 mg/ml in 50.0 mM phosphate, pH = 7.4, ionic strength 0.16 with NaCl) chromatographically purified on a Sephadex G50 column (40 × 2.5 cm). H_2O_2 (35%) was from Riedel-de Haën (Seelze, Germany). Tetrabutylammonium tetrafluoroborate (99%), $TBABF_4$, quercetin dihydrate (98%), rutin trihydrate, fisetin, naringenin, caffeic acid (97%), *trans*-cinnamic acid (99%), Trolox® (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid) (97%) and anthraquinone (97%) from Aldrich Chemical Co. (Steinheim, Germany). Kaempferol, chrysin, myricetin, apigenin, luteolin and (−)-epicatechin were from Apin Chemicals Ltd. (Abingdon, UK), and morin, (+)-catechin hydrate, (±)-taxifolin, hesperitin, chlorogenic acid (> 95%), ferulic acid and *p*-coumaric acid were from Sigma Chemical Co. (St. Louis, MO, USA). Eriodictyol was from Roth (Karlsruhe, Germany), ascorbic acid (99.7%), *all-rac*-α-tocopherol and potassium hexacyanoferrate(III) (99%) from Merck (Darmstadt, Germany) and aluminium oxide (alumina) was from BDH Chemicals Ltd. (Poole, England). All these chemicals were used without further purification. Analytical grade dimethyl sulphoxide, DMSO, and dimethylformamide, DMF, were from Labscan Ltd. (Dublin, Ireland) and water was purified through a Millipore Q-plus purification train (Millipore, MA 01757, USA).

Redox Potentials

Cyclic voltammograms, with a scan-rate of 100 mV/s, were acquired with a BAS CV-50 W voltammetric analyser (Bioanalytical Systems Inc., W. Lafayette, IN, USA) at 23 ± 1°C both for aqueous and DMF solutions, with a 3.0 mm diameter glassy carbon working electrode (BAS MF-2012, polished before each scan with a BAS PK-4 polishing kit, and rinsed by sonification) vs. a Ag/AgCl reference electrode (BAS MF-2063), and with a platinum wire auxiliary electrode (BAS MW-1032) under a blanket of argon. Cinnamic acid, its derivatives, ascorbic acid and Trolox® dissolved (2.00 mM) in 50 mM phosphate, pH = 7.4, ionic strength 0.16 with NaCl, was purged, while stirring, for 10 min with argon before recording the voltammograms. As for the flavonoids, 4.88 ml 50 mM phosphate, pH = 7.4, ionic strength 0.16 with NaCl, was purged for 10 min with argon, and then 125 µl of a 20 mM flavonoid solution in DMSO was added, to yield 0.50 mM (except for quercetin, kaempferol and apigenin where 0.20 mM solutions and for chrysin where an 0.10 mM solution were analysed) and 2.5% (v/v) DMSO. Compensation for the voltage drop iR_s (due to solution resistance) was performed electronically with the BAS CV-50 W analyser at a potential where no Faradaic processes occurred, i.e. at −0.2 V vs. Ag/AgCl (0 V vs. NHE), at 0.8 V for potassium hexacyanoferrate(III) and at 0 V vs. a non-aqueous reference electrode (BAS MF-2062) containing DMF/0.1 M $TBABF_4$, for DMF solutions. The voltammograms used to determine the redox potentials were acquired by sweeping the potential linearly from −0.2 V vs. Ag/AgCl to 0.2 V after the peak potential, and vice versa in the reverse scan, following exploratory scans to +1.0 V. The redox potentials were calculated from anodic and cathodic peak potentials, as half-wave potentials, $E_{1/2} = (E_{pa} + E_{pc})/2$, for the reversible and quasi-reversible

systems, while for irreversible systems, the potential was determined as half-peak potentials, $E_{p/2}$, where current is half the anodic peak current. Potentials vs. NHE were calculated by addition of 197 mV to the value obtained versus the Ag/AgCl reference electrode.[18] For the measurements in DMF/0.1 M $TBABF_4$, the solvent with supporting electrolyte was dried through a column of activated alumina just before use, and potentials calculated using the first reversible wave of anthraquinone as internal standard with −0.800 V vs. SCE (−0.559 V vs. NHE[18]).

Reaction Stoichiometry

Flavonoid solutions in DMSO were made the day used, but were found (UV–Vis spectroscopy) stable for at least 3 months in a refrigerator. Concentrations of MbFe(III) and H_2O_2 were determined spectrophotometrically ($\varepsilon_{525} = 7700\,M^{-1}\,cm^{-1}$[19] and $\varepsilon_{240} = 39.4\,M^{-1}\,cm^{-1}$[20] respectively). Solutions of MbFe(IV)=O were synthesised by reacting 5.00 ml of 220 μM MbFe(III) with 0.500 ml of 8.63 mM H_2O_2 (ratio of 1 : 3.9) for 3 min followed by addition of 10 μl catalase, stored on ice and identified spectrophotometrically (HP 8453 UV-vis diode array, Hewlett-Packard Co., Palo Alto, CA, USA). In experiments designed to establish reaction stoichiometry, 2.94 ml of MbFe(IV)=O was added, immediately after synthesis, to a 10.00 mm quartz cuvette (Hellma, Müllheim/Baden, Germany) in a thermostatted cell-holder with magnetic stirring at 20.0°C, and decay of absorbance at 580 nm monitored. Temperature equilibrium was allowed to be established for 10 min, and the observed decay reflected autoreduction of MbFe(IV)=O to MbFe(III).[19] Initial concentration of MbFe(IV)=O was 140 ± 5 μM, as calculated from spectral data, using the Whitburn equations,[21] corresponding to 70% conversion from MbFe(III), as expected from the MbFe(III)/H_2O_2 ratio of 1 : 4,[17] decreasing to 125 ± 10 μM after 10 min of autoreduction in agreement with autoreduction rate.[19] To the MbFe(IV)=O solution was then 4 × 15 μl of a 6.00 mM flavonoid DMSO solution added with constant stirring, i.e. 120 μM flavonoid in reaction mixture. Absorption spectra were recorded following each of the four additions, and when two additions were sufficient for conversion of MbFe(IV)=O to MbFe(III), a stoichiometric factor of 2 assigned, which was further confirmed in experiments between the same amount of MbFe(IV)=O and a single addition of 30 μl of 6.00 mM flavonoid. For compounds were 4 additions were found necessary for a complete conversion of MbFe(IV)=O to MbFe(III), a stoichiometric factor 1 was assigned. Again it was confirmed that a single addition of 60 μl of 6.00 mM flavonoid did yield conversion of MbFe(IV)=O to MbFe(III), and equally important, that no MbFe(II)O_2 was formed.

Reaction Kinetics

MbFe(IV)=O solution (0.500 ml) was diluted 10 times by 4.50 ml buffer, and 2.90 ml was transferred to the cuvette, and a temperature equilibrium allowed to be established for 5 min (10.0 or 25.0°C). The total myoglobin concentration was 20 μM with a MbFe(IV)=O concentration between 12 and 14 μM as determined from spectral data, depending on the temperature.[19] Reduction of MbFe(IV)=O by flavonoid was followed under pseudo-first-order conditions after addition of 100 μl of 6.0 mM flavonoid, using decay in absorption at 580 nm. Exponential decays were observed in agreement with first-order kinetics, and pseudo-first-order rate constants were calculated using non-linear regression analysis. The decay observed by addition of DMSO alone could not be distinguished from autoreduction, confirming that DMSO did not influence the reaction. Due to solubility problems, the reaction with apigenin was studied in a solution with 6–7 μM MbFe(IV)=O after addition of 100 μl of a 3 mM apigenin DMSO solution. At 10°C the low solubility of apigenin in water hampered the

kinetic investigation, and the rate constant at 10°C was estimated using the Arrhenius relationship. The solubility of chrysin in water is low, and the reaction with MbFe(IV)=O slow, and it was not followed to completion at either 10 or 25°C. Myricetin, quercetin, fisetin and kaempferol all converted MbFe(IV)=O to a mixture of MbFe(III) and $MbFe(II)O_2$ with flavonoid in excess, and concentrations of MbFe(III) and $MbFe(II)O_2$ were calculated using the extinction coefficient $\varepsilon_{580} = 3200\,M^{-1}\,cm^{-1}$ for MbFe(III), and $\varepsilon_{580} = 14400\,M^{-1}\,cm^{-1}$ for $MbFe(II)O_2$,[14] respectively, and

$$[MbFe(II)O_2] = \left(A_{580} - c_0 \cdot \varepsilon_{580}^{MbFe(III)}\right) \Big/ \left(\varepsilon_{580}^{MbFe(II)O_2} - \varepsilon_{580}^{MbFe(III)}\right).$$

Since any $MbFe(II)O_2$ formed during reduction of MbFe(IV)=O will be converted to MbFe(III) in a slow autoxidation reaction and since MbFe(III) may be converted to $MbFe(II)O_2$ by the flavonoids, an upper limit for formation of $MbFe(II)O_2$ during the direct reduction of MbFe(IV)=O was estimated for all flavonoids. The absorbance minimum at 580 nm with respect to time corresponds to the maximal concentration of $MbFe(II)O_2$ formed directly from MbFe(IV)=O, which, with the exception of myricetin, quercetin, fisetin and kaempferol, was found less than 4%, and to have minor influence on the observed pseudo-first-order rate constants k_{obs}, for reduction of MbFe(IV)=O to MbFe(III). For myricetin, quercetin, fisetin and kaempferol, only the absorbance change for the time elapsed to reach the absorbance minimum was used to calculate k_{obs}, (vide infra). The Pro/Kineticist software (Applied Photophysics Ltd., London, UK) using numerical integration was used to determine the rate constant, k_1, for direct reduction of MbFe(IV)=O to MbFe(III) by the flavonoids, correcting for subsequent reactions such as further reduction of MbFe(III) to $MbFe(II)O_2$ by oxidised flavonoids.

RESULTS

Three types of results were obtained in the present study under similar experimental conditions in aqueous solution with pH = 7.4 (50 mM phosphate buffer) adjusted to ionic strength 0.16 with NaCl: (i) redox potentials for fourteen flavonoid aglycones and for the glycoside rutin (see Figure 3) in comparison with five cinnamic acid derivatives and α-tocopherol, Trolox® and ascorbate; (ii) reaction stoichiometries for reaction between MbFe(IV)=O and flavonoids; and (iii) kinetic data for reduction of MbFe(IV)=O by the flavonoids.

Electrochemical Characterisation

Voltammograms for cinnamic acid derivatives are shown in Figure 2. Cinnamic acid could not be oxidised in the aqueous medium and it should be

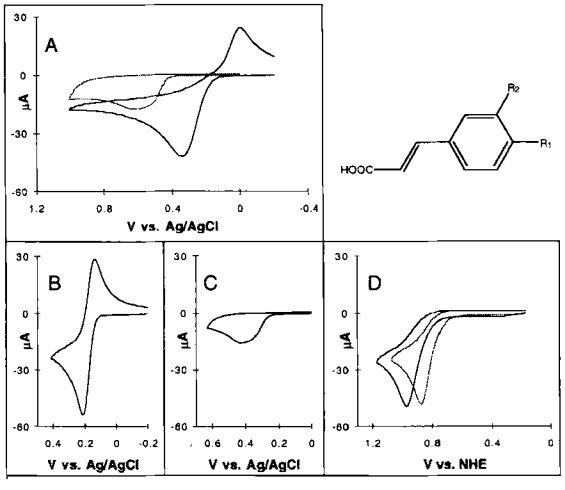

FIGURE 2 Cyclic voltammograms of cinnamic acid derivatives obtained at 23°C for 2.00 mM solutions in 50 mM phosphate buffer, pH = 7.4, ionic strength 0.16 adjusted with NaCl vs. a Ag/AgCl reference electrode at a scan rate of 100 mV/s. A: *p*-coumaric acid (R_1=OH, R_2=H), irreversible, dotted line; and caffeic acid (R_1=OH, R_2=OH), quasi-reversible, full line. B: Chlorogenic acid (R_1=OH, R_2=OH and esterified with 3-OH of quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid)), reversible. C: Ferulic acid (R_1=OH, R_2=OCH_3), irreversible. D: Cyclic voltammograms for a 2.00 mM solution of Trolox® (full line) and *all-rac*-α-tocopherol (dotted line) in DMF/0.1 M $TBABF_4$ vs. NHE.

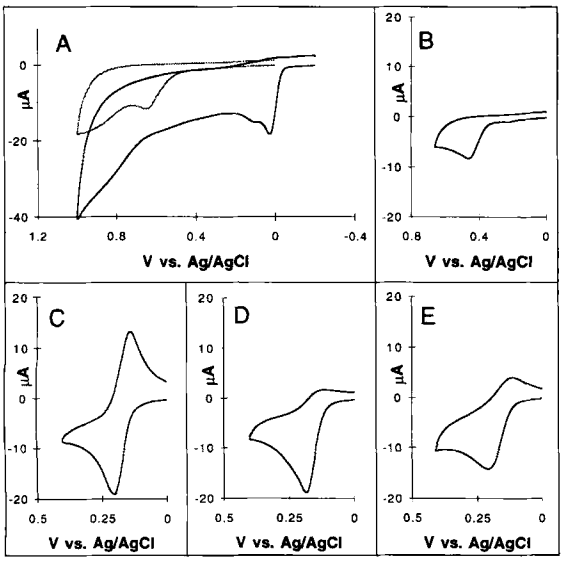

FIGURE 3 Cyclic voltammograms of flavonoids obtained at 23°C for a 0.50 mM solution in 50 mM phosphate buffer, pH = 7.4, ionic strength 0.16 adjusted with NaCl vs. a Ag/AgCl reference electrode. A: Myricetin, quasi reversible (full line), and naringenin, irreversible (dotted line). B: Hesperitin (irreversible). C: (±)Taxifolin (reversible). D: Morin (quasi-reversible). E: (+)-Catechin (quasi-reversible).

noted that oxidation of chlorogenic acid and caffeic acid, which are the more reducing, occurs as reversible and quasi-reversible processes, respectively, while *p*-coumaric and ferulic acid which are less reducing, are oxidised in an irreversible process. Also, the oxidation of Trolox® and ascorbate in aqueous solution and of Trolox® and *all-rac*-α-tocopherol in DMF was irreversible processes. These differences in reversibility at the electrode should be considered in relation to the normalised anodic peak current ($i_{pa}/c_{reducing\ agent}$) given in Table I, and which typically is higher for the reversible processes. From Figure 2 it is thus seen that the anodic peak currents for the dihydroxy compounds chlorogenic and caffeic acid are at least twice as high as for the monohydroxy compounds. Comparison with the cathodic peak current for a 2.0 mM solution of the one-electron reduction of potassium hexacyanoferrate(III) known to be fully reversible ($i_{pa} = 20\,\mu A$, i.e. $i_{pa}/c_{reducing\ agent} = 0.010\,\mu A\,l\,mol^{-1}$, results not shown) indicate, that the voltammograms of the dihydroxy compounds corresponds to two-electron oxidations, and to one-electron oxidations for the monohydroxy compounds (see Figure 2). It was characteristic for the four cinnamic acid derivatives, that they only displayed one voltammetric curve in the potential window investigated (−0.2 to 1.0 V vs. Ag/AgCl, i.e. 0 to 0.8 V vs. NHE), as seen in Figure 2A. Since the water soluble vitamin E analogue, Trolox®, is a common reference compound for quantification of antioxidant activity, we also investigated the voltammetric behaviour of Trolox® in aqueous solution and in the aprotic solvent DMF for comparison with α-tocopherol (Figure 2D). The potentials in DMF for Trolox® and α-tocopherol (Table I), differed by 90 mV, which is more than normally assumed.[22] For the flavonoids similar electrochemical characterisation is collected in Table II and cyclic voltammograms shown in Figure 3. Again it is seen, that the reducing agents with the highest normalised anodic peak current tend to undergo reversible processes. Redox potentials have previously been determined for several flavonoids under varying conditions.[7,9,23,25–27] As may be seen from Table III, there is generally good agreement between the electrochemically determined potentials considering the slightly differing solution and temperature conditions, in contrast to the potentials determined from pulse radiolysis experiments,[7,26,27] which tend to deviate non-systematically from potentials determined electrochemically. However, it should be noted that the high pH conditions used for pulse radiolysis entail an uncertain extrapolation procedure to neutral pH,[9,26,28] and redox potentials should preferably be reported without extrapolation.[26] Redox potentials for the most commonly occurring flavonoids (Table II) determined by cyclic voltammetry under identical conditions span the rather large potential range of 0.63 V, which is much larger than the potential range of 0.30 V for the cinnamic acid derivatives (Table I). Moreover,

TABLE I Redox potentials vs. NHE together with normalised anodic peak current ($i_{pa}/c_{reducing\ agent}$) determined by cyclic voltammetry at 23°C for cinnamic acid derivatives, Trolox® and ascorbate, each 2.00 mM in aqueous solution pH = 7.4 (50 mM phosphate buffer and ionic strength adjusted to 0.16 with NaCl), and for Trolox® and *all-rac*-α-tocopherol (also 2.00 mM) in DMF/0.1 M $TBABF_4$. Comparison is made with ν_i, the initial rate of reduction of MbFe(IV)=O, generated from the reaction between 10 μM MbFe(III) and 15 μM H_2O_2, by 10 μM reducing agent at 37°C, from Ref. [16], and where available, with the second-order rate constant for the reducing agents at 37°C from Ref. [29]. The carotenoid crocin is included for further comparison (data at 37°C from Ref. [38])

Reducing agent	$i_{pa}/c_{reducing\ agent}$ (μA l mol^{-1})	*E*/*V* vs. NHE[a] aqueous solution	*E*/*V* vs. NHE[a] in DMF	ν_i (μM min^{-1})	*k* (l mol^{-1} s^{-1})
Cinnamic acid	—	>1.4	—	[b]	—
p-Coumaric acid	0.008	0.66 (i)	—	2.4	22.5
Ferulic acid	0.008	0.50 (i)	—	6.7	8
Chlorogenic acid	0.026	0.37 (r)	—	11.9	—
Caffeic acid	0.025	0.36 (q)	—	15.6	65
α-tocopherol	—	—	0.80 (i)	—	—
Trolox®	—	0.31 (i)	0.89 (i)	—	—
Ascorbate	—	0.22 (i)	—	—	—
Crocin	—	0.74 (i)	—	—	465

[a] The waves in the corresponding cyclic voltammograms are characterised as (i): irreversible, (q): quasi-reversible and (r): reversible, cf. Figure 2.
[b] No reaction.

while the cinnamic acid derivatives only display one wave in the potential window examined (Figure 2A), all the investigated flavonoids show at least two waves in their cyclic voltammograms (see Figure 3A), although only the first seems well defined. The anodic peak current normalised by division with flavonoid concentration also shown in Table II, do not give as clear a division into one- or two-electron oxidations as for the cinnamic acid derivatives. Although large variations should be expected due to different diffusion coefficients, more or less reversible electron transfer reactions and differences in relative stability of the intermediates formed by oxidation at the electrode, it is noteworthy, that the three flavonoids with the lowest normalised currents, hesperitin, naringenin and kaempferol, only have one free hydroxyl group in the B-ring, whereas apigenin, despite only one hydroxyl group in the B-ring, has one of the highest normalised currents.

Reaction Stoichiometries

Depending on the redox potentials of the individual flavonoids (flav) and the relative stability and reactivity of the one-electron oxidised flavonoids, flav$^{\bullet}$(–H), different reactions may be important under different concentration conditions to yield the two-electron oxidised flavonoids (flav(–2H)) or the flavonoid dimers (flav–flav):

$$\mathrm{MbFe(IV)=O + flav + H^+} \xrightarrow{k_1} \mathrm{MbFe(III) + flav^{\bullet}(-H) + H_2O} \quad (1)$$

$$\mathrm{MbFe(IV)=O + flav^{\bullet}(-H) + H^+} \xrightarrow{k_2} \mathrm{MbFe(III) + flav(-2H) + H_2O} \quad (2)$$

$$\mathrm{MbFe(III) + flav} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{MbFe(II) + flav^{\bullet}(-H) + H^+} \quad (3)$$

$$\mathrm{MbFe(III) + flav^{\bullet}(-H)} \xrightarrow{k_4} \mathrm{MbFe(II) + flav(-2H) + H^+} \quad (4)$$

$$\mathrm{MbFe(II) + O_2} \xrightarrow{\text{fast}} \mathrm{MbFe(II)O_2} \quad (5)$$

$$\mathrm{flav^{\bullet}(-H) + flav^{\bullet}(-H)} \xrightarrow{k_6} \mathrm{flav(-2H) + flav} \quad (6)$$

$$\mathrm{flav^{\bullet}(-H) + flav^{\bullet}(-H)} \xrightarrow{k_7} \mathrm{flav{-}flav.} \quad (7)$$

TABLE II Redox potentials vs. NHE together with normalised anodic peak current ($i_{pa}/c_{reducing\ agent}$) for flavonoids determined by cyclic voltammetry at 23°C in aqueous 50 mM phosphate buffer, 2.5% DMSO, pH = 7.4, ionic strength adjusted to 0.16 with NaCl, compared with pseudo-first-order rate constants determined for reduction of MbFe(IV)=O to yield MbFe(III) at pH = 7.4, ionic strength 0.16 (with NaCl) at 10.0 ± 0.2°C and at 25.0 ± 0.1°C for conditions of excess flavonoid. The stoichiometric factor, *n*, as determined by optical titration, is the number of MbFe(IV)=O reduced to MbFe(III) by each reducing agent

Reducing agent	*n*	$i_{pa}/c_{reducing\ agent}$ ($\mu A\,l\,mol^{-1}$)	*E*/*V* vs. NHE [a]	k_1 ($l\,mol^{-1}\,s^{-1}$) at 10.0°C [b]	k_1 ($l\,mol^{-1}\,s^{-1}$) at 25.0°C [b]
Myricetin	2	0.043	0.20 (q)	572 ± 30 [c]	—
Ascorbate	—	0.026	0.22 (i) [d]	16.3 ± 0.4 [e]	—
Quercetin	2	0.043	0.29 (q)	279 ± 20 [c]	—
Fisetin	2	0.032	0.30 (q)	88 ± 10 [c]	—
Trolox®	—	0.018	0.31 (i) [d]	20.4 ± 0.2	95 ± 3
Epicatechin	2	0.036	0.33 (q)	20 ± 1	93 ± 4
Morin	1	0.037	0.34 (q)	111 ± 8	—
Catechin	2	0.032	0.36 (q)	23.3 ± 0.6	119 ± 10
Eriodictyol	2	0.035	0.36 (r)	25 ± 1	112 ± 4
Taxifolin	2	0.037	0.37 (r)	17.8 ± 0.2	95 ± 1
Kaempferol	1	0.025	0.39 (q)	115 ± 10 [c]	—
Rutin	2	0.026	0.40 (q)	22.7 ± 0.3	105 ± 1
Luteolin	2	0.037	0.41 (r)	63.3 ± 0.5	—
Hesperitin	1	0.016	0.59 (i)	9.3 ± 0.1	42 ± 1
Apigenin	2	0.042	0.71 (i)	31 ± 2 [f]	125 ± 6
Naringenin	1	0.023	0.76 (i)	4.03 ± 0.01	14.5 ± 0.5
Chrysin	[g]	0.032	0.83 (i)	[g]	< 1 [g]

[a] The waves in the corresponding cyclic voltammograms are characterised as (i): irreversible, (q): quasi-reversible and (r): reversible, cf. Figure 3.
[b] Second-order rate constant calculated from pseudo-first-order rate constants, as mean of 3–6 measurements, using $k_1 = k_{obs}/[flavonoid]$, cf. Figure 6A for rutin.
[c] Derived from simulations with the Pro/Kineticist software.
[d] From Table I.
[e] A more detailed study of the reaction with ascorbate is available[35] in which this rate constant is shown to be a composite quantity.
[f] Rate constant at 10°C calculated from the Arrhenius plot, cf. Figure 6B, as the kinetic experiment could not be performed at 10°C due to low solubility.
[g] Reaction to slow to provide reliable data.

Due to a stronger driving force for reactions involving MbFe(IV)=O we will neglect the reverse reactions of Eqs. (1) and (2), as we will also neglect any two-electron reduction processes in the kinetic modelling. A reaction sequence combining reactions of Eqs. (1) and (2), or combining two reactions of Eq. (1) with the reaction of Eq. (6), yields a two-to-one MbFe(IV)=O/flav stoichiometry with metmyoglobin as reaction product (Case 1):

$$2MbFe(IV)=O + flav + 2H^+ \longrightarrow 2MbFe(III) + flav(-2H) + 2H_2O \quad (8)$$

while the formation of flavonoid dimers (or polymers) by oxidation (two reactions of Eq. (1) and one of Eq. (7)) yields a one-to-one MbFe(IV)=O/flav stoichiometry with metmyoglobin as reaction product (Case 2):

$$2MbFe(IV)=O + 2flav + 2H^+ \longrightarrow 2MbFe(III) + flav{-}flav + 2H_2O. \quad (9)$$

Further reaction of flav$^\bullet$(−H) or flav with MbFe(III) may lead to formation of MbFe(II)O_2 as seen (i) from a combination of the reactions of Eqs. (1), (4) and (5), or (ii) from a combination of the reaction of Eq. (1) with the reaction of Eq. (3)

TABLE III A comparison between redox potentials determined by cyclic voltammetry (CV) and pulse radiolysis (PR)

Method	CV	CV	CV	CV	PR	PR
Reference	(this paper)	[9]	[23]	[25]	[7,27]	[26]
pH	7.4	7.4[a]	7.5[b]	7.0[c]	7.0[d]	8.5
Temperature	23°C	n.s.[e]	25°C	n.s.[e]	20°C	22°C
Flavonoid						
Quercetin	0.29	0.27	0.30	0.28	0.33, 0.6	0.398
Fisetin	0.30	0.36	0.38	0.31	—	0.214
Taxifolin	0.37	0.39	0.39	—	—	0.083
Kaempferol	0.39	0.36	0.41	0.38	0.95	0.209
Rutin	0.40	0.42	—	—	0.6	0.275
Luteolin	0.41	0.42	0.42	0.40	—	0.299

[a] 0.050 M phosphate buffer, 2.5% (v/v) DMSO. Potentials converted from SCE to NHE by addition of 241 mV. Scan rate $\nu = 20$ mV/s.
[b] 0.10 M phosphate buffer, 2.5% (v/v) DMSO. Potentials converted from SCE to NHE by addition of 241 mV. Scan rate $\nu = 100$ mV/s except for kaempferol where $\nu = 500$ mV/s.
[c] 0.10 M phosphate buffer, 50% (v/v) methanol. Potentials converted from Ag/AgCl to NHE by addition of 197 mV. Scan rate $\nu = 200$ mV/s.
[d] Calculated from values obtained in aqueous solution at pH = 10.
[e] Conditions not specified.

and with the reactions of Eqs. (5) and (6), to yield a one-to-one MbFe(IV)=O/flav stoichiometry with oxymyoglobin as reaction product (Case 3):

$$\mathrm{MbFe(IV){=}O + flav + O_2} \longrightarrow \mathrm{MbFe(II)O_2 + flav(-2H) + H_2O}. \quad (10)$$

A one-to-two MbFe(IV)=O/flav stoichiometry to yield $MbFe(II)O_2$ is obtained by combining the reactions of Eqs. (1), (3), (5) and (7) (Case 4):

$$\mathrm{MbFe(IV){=}O + 2flav + O_2} \longrightarrow \mathrm{MbFe(II)O_2 + flav{-}flav + H_2O}. \quad (11)$$

The optical titration experiments, in which notably MbFe(IV)=O were in excess during the reactions were, designed to distinguish between Case 1 (Eq. (8)) and Case 2 (Eq. (9)), and from the examples shown in Figure 4, it is seen that kaempferol provides an example of Case 2 with a one-to-one MbFe(IV)=O/flav stoichiometry, while quercetin has a two-to-one MbFe(IV)=O/flav stoichiometry (Case 1). The stoichiometric factors found for the flavonoids may be found in Table II (except for chrysin where the reaction was too slow to be investigated). It is seen, that all compounds having a 3′,4′-dihydroxy substitution pattern in the B-ring are capable of reducing two moles of MbFe(IV)=O, whereas all of the investigated compounds, except apigenin, with only one hydroxyl group in the B-ring, deactivates MbFe(IV)=O to MbFe(III) with a one-to-one stoichiometry. Morin has two hydroxyl groups in the B-ring, but in the 2′ and 4′ position, which notably leads to a one-to-one stoichiometry. On the contrary, apigenin is capable of reducing two moles of MbFe(IV)=O, despite it only has 1 hydroxyl group in the B-ring. Of the four flavonoids reacting with a 1:1 stoichiometry, we recognise kaempferol, hesperitin and naringenin as those with the lowest normalised anodic peak current of the compounds investigated. However, the slow increase in absorbance at 580 nm seen in Figure 4B for quercetin following the initial reduction of MbFe(IV)=O corresponds to a further reduction of MbFe(III) to $MbFe(II)O_2$ (cf. Eq. (3)). Fisetin and myricetin showed similar spectral behaviour as those shown in the inset to Figure 4B, while for kaempferol (Figure 4A) the reduction although significant was even slower. This leads us to the conclusion, that while quercetin, myricetin and fisetin are examples of Case 1 for short reaction times, they provide examples were further oxidation of the two-electron oxidised flavonoids, flav(–2H) occurs.

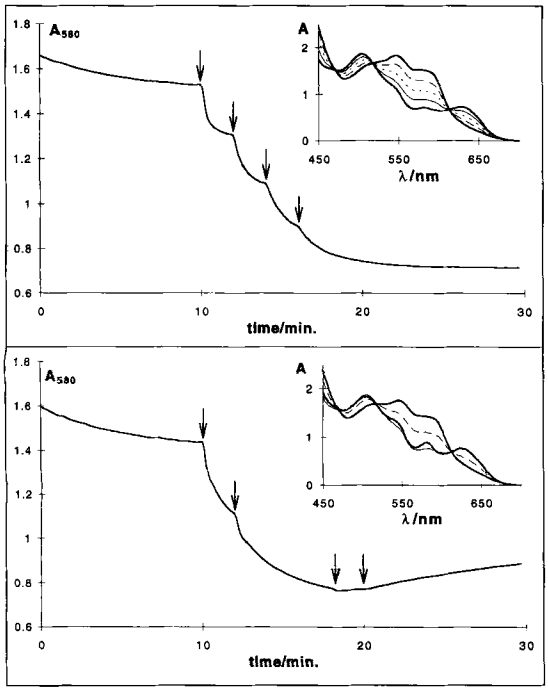

FIGURE 4 *Upper panel*: Absorbance at 580 nm during optical titration at 20.0°C of 2.94 ml of solution of 128 μM MbFe(IV)=O as calculated after 10 min of autoreduction by stepwise addition of 60 μl of a 6.00 mM kaempferol solution in DMSO at 10, 12, 14 and 16 min (as marked with an arrow) to increase the kaempferol concentration with 30 μM at each of the four additions. Inset – the absorption spectra recorded at 10, 12, 14, 16 and 30 min. *Lower panel*: Absorbance at 580 nm during optical titration at 20.0°C of 2.94 ml of 115 μM MbFe(IV)=O as calculated at 10 min of autoreduction by stepwise addition of 60 μl of a 6.00 mM quercetin solution in DMSO at 10, 12, 18 and 20 min (as marked with an arrow) to increase the quercetin concentration with 30 μM at each of the four additions. Inset – the absorption spectra recorded at 10, 12, 18 and 30 min. For both optical titrations the initial decrease in absorbance until first addition at 10 min is due to (slow) autoreduction of MbFe(IV)=O.

Further discussion of examples of Case 4 (Eq. (11)) will have to await flavonoid product analysis, and such work is in progress.

Reaction Kinetics

In contrast to the optical titrations, the reaction kinetics of reduction of MbFe(IV)=O by flavonoids were studied under conditions of large excess of flavonoid. As may be seen from Table I, in which initial rates or second-order-rate constants for reduction of MbFe(IV)=O by cinnamic acid derivatives, where available,[16,29] are compared with the redox potentials of the cinnamic acid derivatives, at least a qualitative correlation exist between initial rates and driving force. It should be noted that for none of the cinnamic acid derivatives a subsequent reduction of MbFe(III) to MbFe(II)O_2 was observed even on a longer time scale.[16] For the flavonoids a larger number of closely related compounds are available and the primary goal of the kinetic investigation was to determine k_1, the second-order-rate constant for the reaction of Eq. (1). However, due to varying stoichiometries a kinetic investigation was considered necessary for each reducing agent in order to correct for other reactions than the initial reduction. As may be seen in Figure 5 (upper panel) under the conditions of (at least) 10 times excess of flavonoid compared to MbFe(IV)=O, most of the flavonoids converted MbFe(IV)=O completely to MbFe(III) at 10°C before significant amounts of MbFe(II)O_2 was formed in much slower reactions (cf. Eq. (3)). Chrysin forms an exception as hardly any reaction occurred with MbFe(IV)=O. For the flavonoids other than flavonols, where the formation of MbFe(III) was separated in time from the formation of MbFe(II)O_2, simple first-order kinetics was observed, as seen in Figure 5 (upper panel) for luteolin and taxifolin (examples of Case 1 stoichiometry) and for hesperitin (an example of Case 2 stoichiometry). The observed pseudo-first-order rate constant, k_{obs}, was further found to depend linearly on the reducing agent concentration as shown in Figure 6A for rutin, and the second-order-rate constant of the reaction of Eq. (1) given in Table II was for all reducing agents except myricetin, quercetin, fisetin and kaempferol calculated according to the simple relationship:

$$k_1 = k_{obs}/[\text{reducing agent}]. \qquad (12)$$

For the flavonols, the further reduction of MbFe(III) to MbFe(II)O_2 was, with the exception

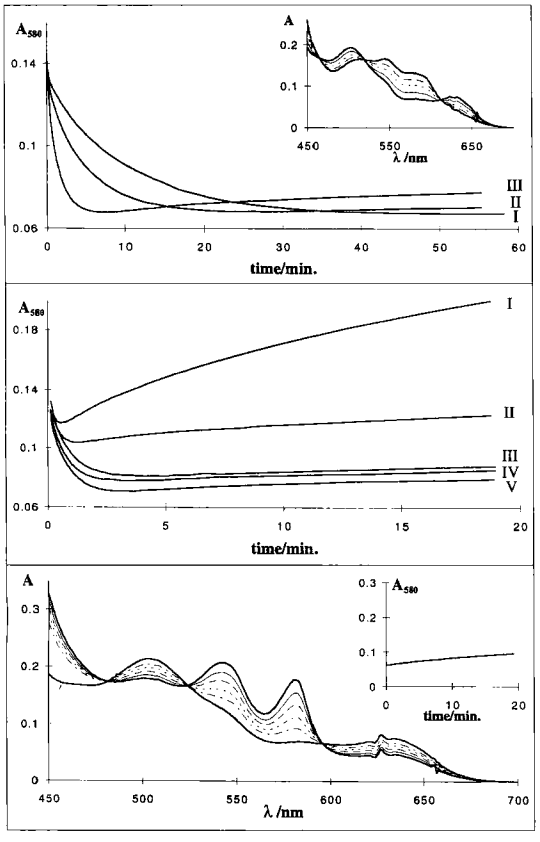

FIGURE 5 *Upper panel*: Absorbance change at 580 nm observed during reaction between MbFe(IV)=O (approximately 14 μM in aqueous solution with pH = 7.4 (50 mM phosphate buffer) at ionic strength 0.16 at 10.0°C) and I: hesperitin; II: taxifolin; and III: luteolin, each in a concentration of 200 μM. Inset – absorption spectra recorded during the reduction of MbFe(IV)=O to MbFe(III) by taxifolin at 16.2 s, 77.7 s, 2 min and 48 s, 6 min and 20 s, and at 28 min and 30 s. *Midmost panel*: Absorbance change at 580 nm, observed during the reaction between MbFe(IV)=O (approximately 14 μM in aqueous solution with pH = 7.4 (50 mM phosphate buffer) at ionic strength 0.16 at 10.0°C) and I: myricetin; II: quercetin; III: fisetin; IV: kaempferol; and V: morin; each in a concentration of 200 μM. The total myoglobin concentration is 20 μM in all experiments, and consists, in the absorbance minimum, of a mixture of MbFe(III) and MbFe(II)O_2, where the percentage of MbFe(II)O_2 has been calculated to be I: 24 ± 3; II: 17.5 ± 0.4; III: 7.2 ± 0.4; IV: 6.5 ± 0.3; and V: 2.9 ± 0.1. *Lower panel*: The absorption spectra recorded at 10.0°C during reduction of 2.94 ml of a 20.0 μM MbFe(III) solution by stepwise addition of 60 μl of a 6.00 mM quercetin solution in DMSO, resulting in partial conversion of MbFe(III) to MbFe(II)O_2. Inset – absorbance at 580 nm at 10.0°C during reduction of 2.90 ml of a 20.0 μM MbFe(III) solution by addition of 100 μl of a 6.00 mM quercetin solution in DMSO, from which an approximate second-order rate constant of 2.61 $mol^{-1} s^{-1}$ is derived. The reaction could only be followed for 20 min due to precipitation of quercetin (or quercetin reaction products).

of morin, occurring on the same time scale as the initial reduction, cf. Figure 5 (midmost panel). However, the direct reaction between the flavonols and MbFe(III) was, when investigated separately, surprisingly slow, cf. Figure 5 (lower panel). For the fastest reduction of MbFe(III) by a flavonoid, i.e. the reduction by myricetin, a second-order rate constant of 3.51 $mol^{-1} s^{-1}$ was estimated from the initial rate, corresponding to a half-life of approximately 16 min while for the second fastest, quercetin, 2.61 $mol^{-1} s^{-1}$ was estimated from the initial rate, corresponding to a half-life of approximately 22 min (cf. the inset of Figure 5 (lower panel)). Complete conversion to MbFe(II)O_2 was found for none of the flavonoids, and equilibrium between MbFe(III) and MbFe(II)O_2 seems to be established in the presence of the flavonol, and the observed rate constant is accordingly the sum of the rate constants for the forward and backward reactions of the equilibrium process. Determination of the equilibrium constant was strongly complicated by the facts, that MbFe(II)O_2 autoxidises to MbFe(III), and that flavonol precipitates from the solution after a few hours. Rather than equilibrium, the distribution between MbFe(II)O_2 and MbFe(III) can be characterised as a steady state. Calculations using the Pro/Kineticist software showed, that when using a rate constant of 3.51 $mol^{-1} s^{-1}$, as determined for myricetin, for the conversion of MbFe(III) to MbFe(II)O_2, an almost negligible amount of MbFe(II)O_2 would have been formed at the time where the absorbance minimum is reached (cf. Figure 5 (midmost panel)). The faster formation of MbFe(II)O_2 was then ascribed to reactions initiated by the conversion of MbFe(IV)=O to MbFe(III), notably the reaction of Eq. (4) in which one-electron oxidised flavonoid is reducing MbFe(III). For the four flavonols, the absorbance changes occurring until the absorbance minimum is reached, was used to calculate approximate values of k_{obs}, and from simulations based on the differential equations describing the rate for the reactions of Eqs. (1), (2), (4), (6) and (7) it was

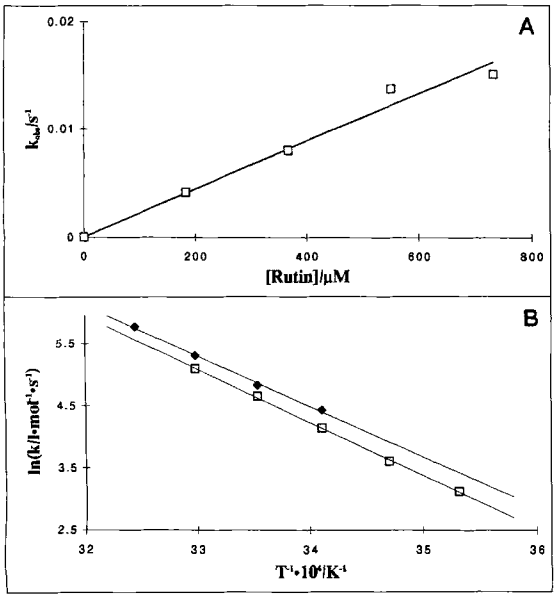

FIGURE 6 A: Pseudo-first order rate constants determined at 10°C for reduction of MbFe(IV)=O in an initial concentration of approximately 14 μM by rutin as function of rutin concentration in aqueous solution with pH = 7.4 (50 mM phosphate buffer) at ionic strength 0.16. From the linear dependence a second-order rate constant of $22 \pm 2\,l\,mol^{-1}\,s^{-1}$ is determined. B: (□) Second-order rate constants for the reaction between MbFe(IV)=O and rutin in 50 mM phosphate buffer (pH = 7.4) with ionic strength 0.16 plotted according to the Arrhenius equation. (◆) Second-order rate constants for the reaction between MbFe(IV)=O and apigenin in 50 mM phosphate buffer (pH = 7.4) with ionic strength 0.16 plotted according to the Arrhenius equation.

concluded, that the amount of $MbFe(II)O_2$ detected spectrophotometrically could not be formed without the involvement of one-electron oxidised flavonoids.

For rutin and apigenin (both simple Case 1 stoichiometry), the temperature dependence was studied in the temperature range 5–30°C, and 20–35°C, respectively, and linear Arrhenius plot was obtained for the second-order rate constants as shown in Figure 6B. Using transition state theory, the following activation parameters were derived: $\Delta H^{\ddagger}_{298} = 69 \pm 1\,kJ\,mol^{-1}$ and $\Delta S^{\ddagger}_{298} = 23 \pm 4\,J\,mol^{-1}\,K^{-1}$ for rutin; and $\Delta H^{\ddagger}_{298} = 65 \pm 3\,kJ\,mol^{-1}$ and $\Delta S^{\ddagger}_{298} = 13 \pm 9\,J\,mol^{-1}\,K^{-1}$ for apigenin.

DISCUSSION

Few mechanistic studies of flavonoids as antioxidants are available. Jovanovic *et al.*[7] studied the reaction between a number of flavonoids and the superoxide anion radical and find that flavonoids reduce the superoxide anion radical in the pH range 7–10. It is interesting to compare the superoxide reduction with the ferrylmyoglobin reduction by flavonoids since the standard reduction potential for $(O_2^{\bullet-}, 2H^+)/H_2O_2$ at pH = 7.0 of +0.94 V is very similar to the standard reduction potential $E^0 = +0.85$ V (pH = 7.4) vs. NHE for MbFe(IV)=O/MbFe(III).[30,31] For the superoxide anion radical very low activation enthalpies, $\Delta H^{\ddagger}_{298} = 9.6$ to $15.1\,kJ\,mol^{-1}$, and negative activation entropies, $\Delta S^{\ddagger}_{298} = -105$ to $-117\,J\,mol^{-1}\,K^{-1}$, were found for the reaction with rutin and Trolox® at pH = 10 and for methylgallate at pH = 7, suggesting an inner-sphere electron transfer mechanism for reduction.[7] In contrast, relatively high activation enthalpies, $\Delta H^{\ddagger}_{298} = 69 \pm 1\,kJ\,mol^{-1}$ for rutin and $\Delta H^{\ddagger}_{298} = 65 \pm 3\,kJ\,mol^{-1}$ for apigenin, respectively, were found in the present study for reduction of ferrylmyoglobin together with positive activation entropies, $\Delta S^{\ddagger}_{298} = 23 \pm 4\,J\,mol^{-1}\,K^{-1}$ for rutin and $\Delta S^{\ddagger}_{298} = 13 \pm 9\,J\,mol^{-1}\,K^{-1}$ for apigenin, respectively. Both the relatively high enthalpy and the positive entropy of activation suggest an outer-sphere electron transfer reaction mechanism for reduction of ferrylmyoglobin by flavonoids.

It should be noted, that although outer-sphere electron transfer also seems to be indicated for apigenin, the rate of reduction of ferrylmyoglobin differs significantly when apigenin is compared to other flavonoids with similar redox potentials as seen in Figure 7, in which a free energy relationship is explored by plotting $\ln(k_1)$ versus redox potential. As seen from Figure 7A, a simple linear relationship is not observed between reaction free energy and activation free energy (proportional to $\ln(k_1)$), and especially the flavone apigenin seems to be a much more

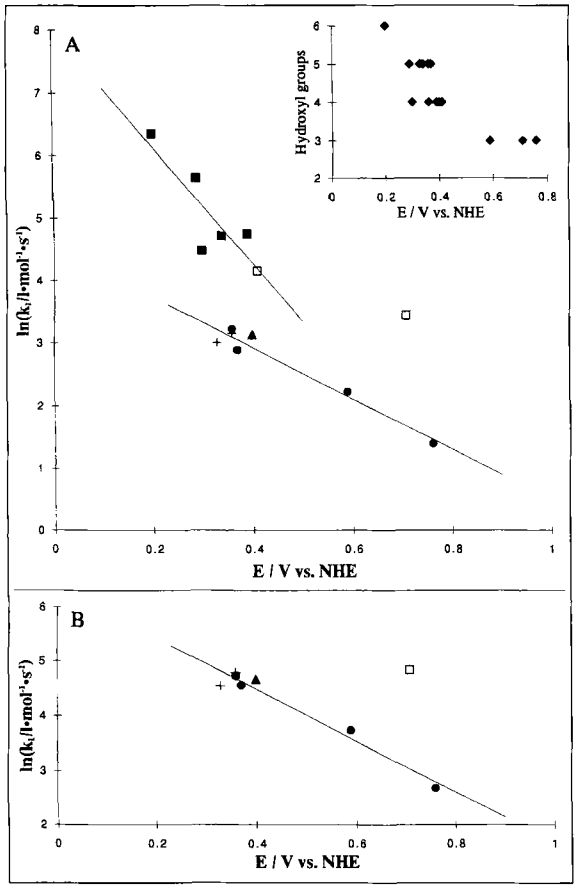

FIGURE 7 A: $\ln(k_1/l\,mol^{-1}\,s^{-1}$ where k_1 is the second-order rate constant for reduction of MbFe(IV)=O determined at 10°C, as function of redox potentials as determined by cyclic voltammetry at 23°C, cf. Table II. Flavanones (●), flavones (□), flavonols (■), flavan-3-ols (+) and rutin (▲). Inset – total number of hydroxyl groups as function of redox potentials, as determined by cyclic voltammetry at 23°C. B: $\ln(k_1/l\,mol^{-1}\,s^{-1}$ where k_1 is the second-order rate constant for reduction of MbFe(IV)=O determined at 25°C, as function of redox potentials as determined by cyclic voltammetry at 23°C, cf. Table II. Flavanones (●), flavones (□), flavan-3-ols (+) and rutin (▲).

efficient reducing agent than flavonoids with similar redox potentials. The kinetic data (Table II) further reveal that, whereas addition of a 3-hydroxyl group to the flavones apigenin or luteolin significantly raises the reactivity of the resulting compounds, kaempferol and quercetin, a similar effect is not seen when the 2,3 double bond is absent, as in the flavanones. Addition of a 3-hydroxyl group to eriodictyol, formally to convert it to taxifolin, actually lowers the reactivity, and removal of the 4-carbonyl group from taxifolin, as in catechin and epicatechin, surprisingly leads to a minor increase in reactivity. While addition of a 3-hydroxy group to luteolin to yield quercetin increases the reactivity, glycoside formation with a sugar residue, like in rutin, dramatically lowers the reactivity. The flavonols myricetin, quercetin and kaempferol exhibit high correlation between reaction free energy and activation free energy, in contrast morin and fisetin clearly behave differently. In comparison with quercetin, the lack of the 5–OH group in fisetin, lowers the reactivity despite the fact that the redox potentials are very similar, and surprisingly, k_1 is even lower for fisetin than for kaempferol. Apparently, the effect of interrupting the catechol pattern as in morin only has a minor effect on k_1, although less MbFe(II)O_2 is produced with morin as reducing agent, compared to kaempferol, as seen from Figure 5 (midmost panel).

Cao *et al.* recently found a linear relationship between the antioxidant activity of flavones, as determined by the oxygen radical absorbance capacity (ORAC) assay, and the number of hydroxyl groups, but not for the flavanones.[11] From the inset in Figure 7A, it is seen, that some relationship between the ease of oxidation and the number of hydroxyl groups exists. The rate of reduction of ferrylmyoglobin is thus controlled by the ease of oxidation of the antioxidant as is further demonstrated in Figure 7A, where a relationship is evident although the linear correlation coefficient is only $r^2 = 0.509$. It is, however, apparent that, not considering apigenin, the flavonoids can be divided into two groups: (i) The flavonols and the flavone luteolin ($r^2 = 0.749$), and (ii) the flavanones, rutin, (+)-catechin and (−)-epicatechin ($r^2 = 0.946$ at 10°C and $r^2 = 0.955$ at 25°C). The 2,3 double bond seems accordingly to be the most important single factor in determining the relation between rate of electron transfer and driving force. Candeias *et al.*[32] recently demonstrated that

the ease of oxidation of an antioxidant only partly explains the reactivity in the study of the reaction between horseradish peroxidase compound I and substituted phenols and indole-3-acetic acids. The rate of reaction was concluded to be controlled by the redox potential of substrate within each substrate class, but substrates of different structures were oxidised by compound I at very different rates, even when the driving force was the same. Both enzyme–substrate complex dissociation and reorganisation energy was considered to be important. Similar effects are also to be expected for ferrylmyoglobin, and when the reactivities of flavanones, flavones, glycosylated flavones, flavonols, and catechins as reducing agents for ferrylmyoglobin are compared, only a qualitative correlation with the redox potentials is to be expected. However, the large deviation for the reactivity of apigenin still remains to be explained as it could indicate a different reaction mechanism compared to the other flavonoids.

It is also noteworthy, that with $E^0(O_2(^1\Delta_g)/O_2^{\bullet-}) = +0.65\,V$ at pH 7.0,[30] the phenoxyl radicals of flavonoids as apigenin, naringenin and chrysin ($E_{p/2} > +0.65\,V$ vs. NHE, see Table II) should be capable of oxidising the superoxide anion radical to singlet oxygen. Singlet oxygen should on the other hand oxidise all other flavonoids in Table II to yield the superoxide anion radical and flavonoid phenoxyl radicals as primary oxidation products. However, Tournaire *et al.* find that flavanones such as eriodictyol, taxifolin and naringenin as well as the flavan-3-ol catechin are chemically inert towards singlet oxygen, while flavones, and especially flavonoles were found to react with singlet oxygen.[33] Most surprisingly, a (slow) reaction between chrysin and singlet oxygen has been observed although electron transfer can be concluded to be thermodynamically unfavourable according to the potential determined for chrysin (Table II). A [2+2] cycloaddition of singlet oxygen to the 2,3 double bond in the C-ring, with a subsequent opening of the C-ring, as concluded from the ^1H NMR spectrum was suggested, although a mechanism entailing an opening of the C-ring still seems controversial. Further characterisation of structure, stability and reactivity of the oxidised flavonoids is clearly needed prior to further discussions of free energy relationships.

Laranjinha *et al.* investigated the reduction of MbFe(IV)=O by phenolic acid derivatives of cinnamic acid.[16] Cinnamic acid itself was found not to be reactive towards MbFe(IV)=O, whereas the efficiency of the derivatives was ranked in terms of differences in the initial rate of reduction which was correlated with redox potentials, although unknown under the actual experimental conditions. Caffeic and chlorogenic acid reacted faster with MbFe(IV)=O than Trolox®, and caffeic acid again reacted faster than ascorbate. Castelluccio *et al.*[29] determined the second-order rate constant at 37°C for the reaction between three of the compounds and MbFe(IV)=O (Table I). It may be concluded from Table I, that the potentials at least quantitatively reflects the order of the initial rates for the cinnamic acid derivatives, but not the higher reactivity of caffeic acid compared to Trolox® and ascorbate, or the higher reactivity of chlorogenic acid compared to Trolox®. It is noteworthy, that the sequence chlorogenic ≈ caffeic > ferulic > *p*-coumaric acid is also found for the antioxidants effectiveness against lipid peroxyl radicals.[29] Laranjinha *et al.* further suggest that caffeic acid acts synergistically with α-tocopherol by recycling the α-tocopheroxyl radical, and find that the phenoxyl radical of *p*-coumaric acid (produced by electron-transfer between *p*-coumaric acid and ferrylmyoglobin) oxidises α-tocopherol, assuming that the standard reduction potential of α-tocopherol is identical to that for the water-soluble analogue, Trolox®.[34] The half-peak potentials, in aprotic media (Table I), obtained in the present study, differ by 90 mV between Trolox® and of α-tocopherol. If a similar difference exist between the standard reduction potentials for the oxidised form of Trolox® and α-tocopherol in aqueous systems, this would

mean that the standard reduction potentials for α-tocopherol and ascorbate would be identical, which is interesting in relation to the theory of regeneration of α-tocopherol by ascorbate in biological membranes. Notably, the value of +0.39 V for Trolox® was reported for pH = 8.5[26] in better agreement with our value of +0.31 V than the often quoted value of +0.48 V.[22] Even for a potential of α-tocopherol identical to that of Trolox®, the regeneration of α-tocopherol by caffeic acid should, however, not be thermodynamic favourable.[7,22]

Cyclic voltammetry as well as pulse radiolysis are both excellent techniques for measuring redox potentials, and should, of course, result in comparable values. However, as seen from Table III, it is difficult to establish an unambiguous antioxidant hierarchy based on reported potentials. The potentials determined by Jovanovic *et al.* at pH = 7.0 by pulse radiolysis gives consistently higher values, although the trend is similar to the electrochemically determined potentials.[7,27] The potentials determined by pulse radiolysis have been determined exclusively with Trolox® as the standard redox compound, but the redox potential for Trolox® has only been *measured* by pulse radiolysis at pH = 13.5, and the reference value of 0.48 V vs. NHE at pH = 7.0 subsequently *calculated*.[22] Such a calculation requires a detailed knowledge of pK_a values of the parent compound as well as of its phenoxyl radical, which makes a directly measured value more reliable. It is notable, that Jovanovic *et al.* in their latest investigation[27] use ascorbate as standard to determine the redox potential for quercetin, and obtain 0.33 V vs. NHE at pH = 7.0. Assuming a decrease of 60 mV per pH unit, as predicted from the Nernst equation, this latter value is within experimental uncertainty identical to our value of 0.29 V at pH = 7.4, i.e. the difference between the redox potentials at pH = 7.0 reported by Jovanovic *et al.*[7] and our measured redox potentials can be ascribed to differences in reference values. The redox potentials determined by Bors *et al.*[26] are different both in value and ordering compared to the values obtained by Jovanovic *et al.* [7,27] most likely due to the very high doses used by Bors *et al.*[26] resulting in interference from second-order radical–radical processes.[27]

Notably, most of the flavonoids in Table II react faster with MbFe(IV)=O than ascorbate. The most striking example is apigenin, which has a redox potential almost half a volt more positive than ascorbate, and clearly a change in reaction mechanism is indicated by the activation parameters, $\Delta H^{\ddagger}_{298} = 65\,\mathrm{kJ\,mol^{-1}}$ and $\Delta S^{\ddagger}_{298} = +13\,\mathrm{J\,mol^{-1}\,K^{-1}}$ for apigenin, as compared to $\Delta H^{\ddagger}_{298} = 45\,\mathrm{kJ\,mol^{-1}}$ and $\Delta S^{\ddagger}_{298} = -85\,\mathrm{J\,mol^{-1}\,K^{-1}}$ for ascorbate.[35] Also the oxidised flavonoids or cinnamic acid derivatives may in subsequent reactions be effective as antioxidants and, as may be seen from Table II, most of the investigated flavonoids reduce MbFe(IV)=O in a two-to-one stoichiometry, which means either, that both the flavonoid, and the derived phenoxyl radical is capable of reducing MbFe(IV)=O to MbFe(III), cf. Eqs. (1) and (2), or that the flavonoid is regenerated by disproportionation, cf. Eq. (6). The half-life of the phenoxyl radicals of fisetin, kaempferol, quercetin and morin has been found to be on the millisecond time scale at pH = 11.5 but less at pH = 7.4.[36,28] Antioxidants reacting with a one-to-one stoichiometry is *a priori* expected to be only half as efficient as antioxidants reacting with a two-to-one stoichiometry, a fact which should be taken into account when considering structure–activity relationships, and which at least partly, could explain why quercetin has been found 3 times better as antioxidant than kaempferol.[10] Like kaempferol, wine phenolics has recently been found to react in a one-to-one stoichiometry with ferrylmyoglobin.[37] The stoichiometric factor of two for apigenin is difficult to explain, but a high pK_a (and the pK_a value is at present unknown) might lead to a transient cation radical with high reactivity as found for the water-soluble carotenoid crocin. Crocin was found to have a redox potential of 0.74 V vs.

NHE, but despite this high redox potential it deactivates MbFe(IV)=O very effectively, and much faster than ascorbate.[38] On the other hand, the electron donating capacity is expected to increase with increasing negative charge as has been found in the reaction between the 2,6-di-tert-butyl-4-(4-methoxyphenyl)phenoxyl radical and rutin for increasing pH and negative charge.[39] However, if electron transfer precedes loss of a proton, as expected for apigenin, the resulting cation radical will be highly acidic, and proton dissociation is expected to compete with further reduction at physiological pH. It is generally assumed, that the dissociation of flavonoids occur in the sequence 7-OH > 4′-OH > 5-OH[40] with the exception of morin, where the 2′-OH group dissociates first, with the exceptional low pK_a (1) value of 3.46 and pK_a (2) = 8.1.[7] At pH = 7.4 morin molecules oxidised at the electrode during cyclic voltammetry will be double negatively charged, yielding a two-electron oxidation, whereas the one-to-one stoichiometry observed for the reaction between MbFe(IV)=O and morin suggests, that dimerisation of the radicals obtained after initial reduction of MbFe(IV)=O is more important than further reduction of MbFe(IV)=O.

Hypervalent iron in heme pigments is a powerful oxidant and flavonoids may prove to have important functions in deactivation of ferrylmyoglobin and ferrylhemoglobin during oxidative stress.[41] It is concluded, that flavonoids deactivate ferrylmyoglobin by outer-sphere electron transfer but with different reaction stoichiometries, and linear relationships seems to exist between reaction free energy and activation free energy separately within series of flavanones and flavonols. For the flavonoids without a 2,3 double bond, the reaction rate is less sensitive to changes in the redox potential of the flavonoid. One flavone, apigenin, has, however, been found to have significantly higher reactivity than expected from the redox potential and also to deactivate to ferrylmyoglobin with a 2:1 stoichiometry despite rather poor reducing properties. In contrast morin, with better reducing properties, only deactivates ferrylmyoglobin with a 1:1 stoichiometry. Such specific effects will be studied in more detail.

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.

References

[1] L. O. Dragsted, M. Strube and J. C. Larsen (1993) Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacology & Toxicology*, **72**, 116–135.
[2] J. V. Formica and W. Regelson (1995) Review of the biology of quercetin and related bioflavonoids. *Food and Chemical Toxicology*, **33**, 1061–1080.
[3] J. B. Harborne, ed. (1988) *The Flavonoids. Advances in Research Since 1980*. Chapman & Hall, London.
[4] U. Justesen, P. Knuthsen and T. Leth (1997) Determination of plant polyphenols in Danish foodstuffs by HPLC-UV and LC-MS detection. *Cancer Letters*, **114**, 165–167.
[5] J. Kühnau (1976) The flavonoids. A class of semi-essential food components: Their role in human nutrition. *World Review of Nutrition and Dietetics*, **24**, 117–191.
[6] G. Paganga and C. A. Rice-Evans (1997) The identification of flavonoids as glycosides in human plasma. *FEBS Letters*, **401**, 78–82.
[7] S. V. Jovanovic, S. Steenken, M. Tosic, B. Marjanovic and B. M. Simic (1994) Flavonoids as antioxidants. *Journal of the American Chemical Society*, **116**, 4846–4851.
[8] M. Foti, M. Piattelli, M. T. Baratta and G. Ruberto (1996) Flavonoids, coumarins and cinnamic acids as antioxidants in a micellar system. Structure–activity relationship. *Journal of Agricultural and Food Chemistry*, **44**, 497–501.
[9] S. A. B. E. van Acker, D. van den Berg, M. N. J. L. Tromp, D. H. Griffioen, W. P. van Bennekom, W. J. F. van der Vijgh and A. Bast (1996) Structural aspects of antioxidant activity of flavonoids. *Free Radical Biology & Medicine*, **20**, 331–342.
[10] C. A. Rice-Evans, N. J. Miller and G. Paganga (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology & Medicine*, **20**, 933–956.
[11] G. Cao, E. Sofic and R. L. Prior (1997) Antioxidant and prooxidant behaviour of flavonoids: structure–activity relationships. *Free Radical Biology & Medicine*, **22**, 749–760.
[12] W. Bors, W. Heller, C. Michel and M. Saran (1990) Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. *Methods in Enzymology*, **186**, 343–355.

[13] L. Eddy, A. Arduini, F. Okonski and P. Hochstein (1990) The detection of hypervalent states of myoglobin by reflectance spectrophotometry in cardiac ischemic/reperfusion injury. *FASEB Journal*, **4**, A1219 (5535).
[14] D. Galaris, A. Sevanian, E. Cadenas and P. Hochstein (1990) Ferrylmyoglobin-catalyzed linoleic acid peroxidation. *Archives of Biochemistry and Biophysics*, **281**, 163–169.
[15] H. Østdal, B. Daneshvar and L. H. Skibsted (1996) Reduction of ferrylmyoglobin by β-lactoglobulin. *Free Radical Research*, **24**, 429–438.
[16] J. Laranjinha, L. Almeida and V. Madeira (1995) Reduction of ferrylmyoglobin by dietary phenolic acid derivatives of cinnamic acid. *Free Radical Biology & Medicine*, **19**, 329–337.
[17] C. Guilivi and E. Cadenas (1994) Ferrylmyoglobin: Formation and chemical reactivity towards electron-donating compounds. *Methods in Enzymology*, **233**, 189–202.
[18] A. J. Bard and L. R. Faulkner (1980) *Electrochemical Methods*. Wiley, New York.
[19] A. Mikkelsen and L. H. Skibsted (1995) Acid-catalysed reduction of ferrylmyoglobin: Product distribution and kinetics of autoreduction and reduction by NADH. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung*, **200**, 171–177.
[20] D. P. Nelson and L. A. Kiesow (1972) Enthalpy of decomposition of hydrogen peroxide by catalase at 25°C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Analytical Biochemistry*, **49**, 474–478.
[21] K. D. Whitburn, J. J. Shieh, R. M. Sellers, M. Z. Hoffman and I. A. Taub (1982) Redox transformations in ferrimyoglobin induced by radiation-generated radicals in aqueous solution. *Journal of Biological Chemistry*, **257**, 1860–1869.
[22] S. Steenken and P. Neta (1982) One-electron redox potentials of phenols. Hydroxy- and aminophenols and related compounds of biological interest. *Journal of Physical Chemistry*, **86**, 3661–3667.
[23] W. F. Hodnick, E. B. Milosavljevic, J. H. Nelson and R. S. Pardini (1988) Electrochemistry of flavonoids. Relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids. *Biochemical Pharmacology*, **37**, 2607–2611.
[24] H. P. Hendrickson, A. D. Kaufman and C. E. Lunte (1994) Electrochemistry of catechol-containing flavonoids. *Journal of Pharmaceutical & Biomedical Analysis*, **12**, 325–334.
[25] H. P. Hendrickson, M. Sahafayen, M. A. Bell, A. D. Kaufman, M. E. Hadwiger and C. E. Lunte (1994) Relationship of flavonoid oxidation potential and effect on rat hepatic microsomal metabolism of benzene and phenol. *Journal of Pharmaceutical & Biomedical Analysis*, **12**, 335–341.
[26] W. Bors, C. Michel and S. Schikora (1995) Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: A pulse radiolysis study. *Free Radical Biology & Medicine*, **19**, 45–52.
[27] S. V. Jovanovic, S. Steenken, Y. Hara and M. G. Simic (1996) Reduction potentials of flavonoid and model phenoxyl radicals. Which ring is responsible for antioxidant activity? *Journal of the Chemical Society: Perkin Transactions*, **2**, 2497–2504.
[28] S. A. B. E. van Acker, M. J. de Groot, D. van den Berg, M. N. J. L. Tromp, G. D. den Kelder, W. J. F. van der Vijgh and A. Bast (1996) A quantum chemical explanation of the antioxidant activity of flavonoids. *Chemical Research in Toxicology*, **9**, 1305–1312.
[29] C. Castelluccio, G. Paganga, N. Melikian, G. P. Bolwell, J. Pridham, J. Sampson and C. Rice-Evans (1995) Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *FEBS Letters*, **368**, 188–192.
[30] W. H. Koppenol (1976) Reactions involving singlet oxygen and the superoxide anion. *Nature*, **262**, 420–421.
[31] P. George and D. H. Irvine (1955) A possible structure for the higher oxidation state of metmyoglobin. *Biochemical Journal*, **60**, 596–604.
[32] L. P. Candeias, L. K. Folkes and P. Wardman (1997) Factors controlling the substrate specificity of peroxidases: Kinetics and thermodynamics of the reaction of horseradish peroxidase compound I with phenol and indole-3-acetic acids. *Biochemistry*, **36**, 7081–7085.
[33] C. Tournaire, S. Croux, M. Maurette, I. Beck, M. Hocquaux, A. M. Braun and E. Oliveros (1993) Antioxidant activity of flavonoids: efficiency of singlet oxygen ($^1\Delta_g$) quenching. *Journal of Photochemistry and Photobiology B: Biology*, **19**, 205–215.
[34] J. Laranjinha, O. Vieira, V. Madeira and L. Almeida (1995) Two related phenolic antioxidants with opposite effects on Vitamin E content in low density lipoproteins oxidized by ferrylmyoglobin: Consumption vs. regeneration. *Archives of Biochemistry and Biophysics*, **323**, 373–381.
[35] M. Kröger-Ohlsen and L. H. Skibsted (1997) Kinetics and mechanism of reduction of ferrylmyoglobin by ascorbate and D-isoascorbate. *Journal of Agricultural and Food Chemistry*, **45**, 668–676.
[36] W. Bors and M. Saran (1987) Radical scavenging by flavonoid antioxidants. *Free Radical Research Communications*, **2**, 289–294.
[37] J. Kanner, E. Frankel, R. Granit, B. German and J. E. Kinsella (1994) Natural antioxidants ingrapes and wines. *Journal of Agricultural and Food Chemistry*, **42**, 64–69.
[38] L. V. Jørgensen, H. J. Andersen and L. H. Skibsted (1997) Kinetics of reduction of hypervalent iron in myoglobin by crocin in aqueous solution. *Free Radical Research*, **27**, 73–87.
[39] K. Mukai, W. Oka, K. Watanabe, Y. Egawa and S. Nagaoka. (1997) Kinetic study of free-radical-scavenging action of flavonoids in homogenous and Triton X-100 micellar solutions. *Journal of Physical Chemistry A*, **101**, 3746–3753.
[40] P. K. Agrawal and H. J. Schneider (1983) Deprotonation induced ^{13}C NMR shifts in phenols and flavonoids. *Tetrahedron Letters*, **24**, 177–180.
[41] L. N. Grindberg, E. A. Rachmilewitz and H. Newmark (1994) Protective effects of rutin against hemoglobin oxidation. *Biochemical Pharmacology*, **48**, 643–649.