Free Rad. Res., Vol. 30, pp. 207–220
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Regeneration of Phenolic Antioxidants from Phenoxyl Radicals: An ESR and Electrochemical Study of Antioxidant Hierarchy

LARS VIBORG JØRGENSEN[a,b], HELLE LINDBERG MADSEN[a], MARIANNE KAABER THOMSEN[a], LARS OVE DRAGSTED[b] and LEIF H. SKIBSTED[a,*]

[a]*Food Chemistry, Department of Dairy and Food Science, Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark;* [b]*Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark*

Accepted by Prof. H. Sies

(Received 19 June 1998; In revised form 5 October 1998)

Radicals from the flavonoids quercetin, (+)-catechin, (±)-taxifolin and luteolin, and from *all-rac*-α-tocopherol have been generated electrochemically by one-electron oxidation in deaerated dimethylformamide (DMF), and characterised by electron spin resonance spectroscopy (ESR) after spin-trapping by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Simulations of the ESR spectrum based on estimated coupling constants of the spin-trapped quercetin radical, confirmed that this antioxidant radical is oxygen-centered. The complex mixture of radicals, quinoid intermediates and stable two-electron oxidation products, were for each antioxidant allowed to react with each of the four other antioxidants, and the progression of reaction followed by ESR after addition of DMPO, and the product solution further analysed by HPLC. *All-rac*-α-tocopherol was found to be most efficient in regenerating each of the other antioxidants from their oxidation products with a regeneration index (defined as moles regenerated of the oxidised phenolic antioxidant divided with moles of *all-rac*-α-tocopherol consumed) of 0.90 ± 0.16 for quercetin, 0.48 ± 0.11 for (+)-catechin, 0.48 ± 0.06 for (±)-taxifolin and 0.50 ± 0.10 for luteolin in equimolar 1.00 mM solution. Quercetin was found to have the highest regeneration index among the flavonoids: 0.88 ± 0.13 for (+)-catechin, 0.41 ± 0.03 for (±)-taxifolin and 0.41 ± 0.02 for luteolin. The antioxidant hierarchy based on the reduction potentials determined by cyclic voltammetry under similar conditions (0.93 V for *all-rac*-α-tocopherol, 1.07 V for quercetin, 1.15 V for luteolin, 1.16 V for (+)-catechin and 1.20 V for (±)-taxifolin) is compared with the observed over-all regeneration (34% for quercetin, 34% for (+)-catechin, 52% for (±)-taxifolin and 43% for luteolin by *all-rac*-α-tocopherol).

Keywords: Flavonoids, electrolysis, reduction potentials, phenoxyl radicals, ESR, regeneration

INTRODUCTION

Flavonoids are polyphenolic compounds present in fruit and vegetables primarily as glycosides, but it is usually assumed that absorption occurs in the intestine after hydrolysis to the corresponding aglycone.[1] The aglycones derived from the

*Corresponding author. Tel.: +45 35283221. Fax: +45 35283344. E-mail: ls@kvl.dk.

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flavonoids are excellent antioxidants, and for a normal human diet their antioxidant capacity are comparable to or even superior to that of vitamin C and E.[2,3] Furthermore, the antioxidant capacity found in red wine, black currant juice and other juices, where more than a single flavonoid is present, has been found to exceed the expected capacity as calculated by addition of the contributions from the single components.[2,4,5] This observation could be explained by the presence of unidentified compounds with exceptionally good antioxidant capacity, or by synergistic interactions between the identified compounds. It seems generally accepted, that the hydrophilic ascorbate is capable of regenerating the lipophilic α-tocopherol from the α-tocopheroxyl radical in membrane interfaces, as may be understood in terms of the difference between the reduction potentials of the α-tocopheroxyl radical and the ascorbyl radical.[6] More recently it has likewise been demonstrated, that ascorbate in homogeneous solution regenerates the plant phenolic chlorogenic acid from its phenoxyl radical.[7] Flavonoids have been found to inhibit low-density lipoprotein (LDL) oxidation *in vitro*, which likewise may be due to a similar interaction between α-tocopherol as present in LDL and the more hydrophilic flavonoids.[8,9] Such free radical exchange reactions between α-tocopherol and flavonoids have, however, not been directly observed, and the purpose of the present investigation was to examine whether phenolic antioxidants can be regenerated, by other phenolic antioxidants, after controlled one-electron oxidation to yield phenoxyl radicals, and whether this can be understood on the basis of differences between the reduction potentials of their phenoxyl radicals.[10] Four flavonoid aglycones belonging to four different flavonoid subclasses and *all-rac*-α-tocopherol were investigated in homogenous solution in the aprotic solvent dimethylformamide (DMF) combining electron spin resonance (ESR) spectroscopy and bulk electrolysis. Quercetin, a flavonol, (+)-catechin, a flavan-3-ol, and luteolin, a flavone, were chosen because they are among the most abundant flavonoids found in the human diet. (±)-Taxifolin, a flavanonol or dihydroflavonol, is not important for the human diet,[11] but was chosen because the reduction potential of its phenoxyl radical recently was reported to be lower than the reduction potential for the ascorbyl radical, which makes (±)-taxifolin capable of reducing the ascorbyl radical and thereby regenerate ascorbate.[12,13]

MATERIALS AND METHODS

Chemicals

Quercetin dihydrate (98%) and analytical grade tetrabutylammonium tetrafluoroborate, $TBABF_4$, 5,5-dimethyl-1-pyrroline-N-oxide, DMPO, ferrocene and anthraquinone were from Aldrich Chemical Co. (Steinheim, Germany). Luteolin (HPLC pure) was from Apin Chemicals Ltd. (Abingdon, UK), and (+)-catechin hydrate (HPLC pure) and (±)-taxifolin (HPLC pure) were from Sigma Chemical Co. (St. Louis, MO, USA). Analytical grade formic acid and *all-rac*-α-tocopherol were from Merck (Darmstadt, Germany). Analytical grade DMF and chloroform were from Labscan Ltd. (Dublin, Ireland). Analytical grade acetonitrile, MeCN, was from Fischer Scientific (Leicestershire, UK), and water was purified through a Millipore Q-plus purification train (Millipore, Bedford, MA, USA).

Reduction Potentials of Phenoxyl Radicals

Experiments were carried out in DMF/0.10 M $TBABF_4$ at 25.0 ± 0.1°C with a BAS CV-50W voltammetric analyser (Bioanalytical Systems Inc., West Lafayette, IN, USA). Cyclic voltammograms, at a scan-rate of 100 and 500 mV/s, were acquired with a 1.6 mm diameter platinum working electrode (BAS MF-2012) vs. a non-aqueous reference electrode (BAS MF-2062) containing the solvent DMF/0.10 M $TBABF_4$ in combination with a platinum wire auxiliary electrode

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(BAS MW-1032). All solutions were purged thoroughly while stirring for 10 min with argon before recording the voltammograms, and then provided with a blanket of argon during measurements. The electrode was polished before each scan with a BAS PK-4 (part MF-2060) polishing kit, and subsequently rinsed by ultrasound to remove residual abrasive particles. Electronic compensation of the solution resistance was performed automatically at 0.0 V vs. the non-aqueous reference electrode immediately before each scan. The reduction potentials of the flavonoid phenoxyl radicals and *all-rac*-α-tocopheroxyl radical, reported as half-peak potentials, $E_{p/2}$ vs. NHE, at a scan-rate of 100 mV/s, were calculated relative to the reduction potential of the ferrocenium/ferrocene couple defined as +0.72 V vs. NHE in DMF/0.10 M $TBABF_4$.[14]

Regeneration Experiments

(a) Quantitative Chromatography Bulk electrolysis was performed at room temperature in the BAS MF-1056 bulk electrolysis cell for each flavonoid and *all-rac*-α-tocopherol at a potential 200 mV more positive than the half-peak potential determined for each compound by cyclic voltammetry. The amount of a phenolic antioxidant, P_1, necessary to achieve a concentration of 1.00 mM was dissolved in DMF/0.10 M $TBABF_4$, and 75 ml of this solution was transferred to the cell. The 75 µmol thus subject to electrolysis would, according to Faraday's law, require 7.24 C to be oxidised in a one-electron oxidation. The solution was purged thoroughly in the cell with argon for 10 min before and constantly during the electrolysis. A sample of 500 µl, S1, was withdrawn before electrolysis. After 6.0 C, the electrolysis was stopped to avoid the generation of two-electron oxidised species in the electrode process, and a sample of 500 µ1, S2, was taken from the cell. Immediately after this, 500 µl of a 150 mM argon purged solution of another phenolic antioxidant, P_2, was added to the cell. The mixture, S3, was allowed to react for 10 min while it was constantly purged with argon and subsequently analysed by HPLC. Ten µl of the 150 mM solution of P_2 was diluted with 1.49 ml DMF/0.10 M $TBABF_4$ and the resulting solution, S4, was analysed by HPLC. The amount of the phenolic antioxidant P_1 oxidised was calculated from a comparison of the HPLC chromatograms of S1 and S2, and the amount of oxidised P_1 regenerated was estimated from a similar comparison of S2 and S3. The amount of phenolic antioxidant P_2 consumed in the regeneration process was calculated from a comparison of the HPLC chromatograms of S3 and S4.

(b) ESR Spectroscopy A similar experimental design as described above was used to monitor the radical exchange directly. For each of the five phenolic antioxidants, samples of 2.00 ml was withdrawn after 6.0 C, and added to 500 µl of a 0.50 M DMPO solution in DMF which had been purged with argon. The mixture was immediately transferred to a flat quartz cell (WG-813-TMS-S, 430 µl, Wilmad Glass Company Inc., Bueno, NJ) and throughout the handling procedure access to oxygen was minimised. The reaction of the phenoxyl radical with the spin-trap to form the spin adduct was followed by ESR spectroscopy (Bruker ECS 106 spectrometer, Bruker Karlsruhe, Germany) at the following spectrometer settings: center field 3393.95 G; sweep width 90 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; conversion time 81.92 ms; time constant 163.84 ms and sweep time 83.89 s. The development of spin adducts were followed for a period of 60 min with measurements after 2, 4 and 7 min, and then every sixth minute. In the regeneration experiments monitored by ESR, a 2.00 ml sample of the electrolysed phenolic antioxidant P_1 was withdrawn and mixed with 500 µl of a 0.50 M DMPO solution and subjected to ESR spectroscopy in order to assure the presence of radicals. After 6.0 C the electrolysis was stopped, and 500 µl of a 150 mM solution purged with argon of another phenolic antioxidant, P_2, was added to the cell. The mixture was

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allowed to react for 2 min, and then a 2.00 ml sample was withdrawn and mixed with 500 µl of a 0.50 M DMPO solution in DMF which had been purged with argon. The mixture was followed by ESR spectroscopy for 60 min with measurements 2, 4 and 7 min after addition of DMPO, and then every sixth minute.

Simulations of ESR Spectra

The ESR spectra recorded for the DMPO spin adducts of the phenoxyl radicals were analysed using the software program Simfonia (Bruker Instruments Inc., Billerica, MA, USA). The estimated coupling constants were used for the simulations of ESR spectra performed with the Winsim software program (National Institute of Environmental Health Sciences, Public EPR software tools) assuming a Lorentzian lineshape and optimised via the LMB1 algorithm.

Chromatographic Separation

HPLC was performed on a Purospher RP-18 column (4 × 250 mm, 5 µm, Hewlett-Packard, Palo Alto, CA, USA) and a Hewlett-Packard 1090 system (Waldbronn, Germany) with a diode array detector using simultaneous detection at 250, 270, 290, 350 and 380 nm. Twenty µl of the sample was injected, and the column temperature was maintained constantly at 35°C. The column was eluted with water containing 1% (v/v) formic acid and MeCN in a linear gradient starting with 25–30% (v/v) MeCN in 20 min, followed by 30–40% MeCN from 20 to 25 min, 40–60% MeCN from 25 to 28 min, 60–100% MeCN from 28 to 30 min and 100% MeCN from 30 to 45 min. All calculations are based on mean values from two subsequent HPLC injections.

RESULTS AND DISCUSSION

Cyclic Voltammetry

Cyclic voltammograms of each of the four flavonoids quercetin, (+)-catechin, (±)-taxifolin and luteolin and of *all-rac*-α-tocopherol (formula shown in Figure 1) showed that the five phenolic antioxidants behaved similarly during oxidation. As may be seen in Figure 2 for *all-rac*-α-tocopherol and luteolin, oxidation of the phenol gives rise to an irreversible wave, which means a half-life less than one second for the generated radicals. On the reverse scan a new wave is further seen, which is not seen, if the scan is initiated in the negative direction. This new cathodic wave can be assigned to the generation of compounds by a reaction between the radicals formed during oxidation at the electrode, and it may further be concluded that these products are easily reduced. The common catechol structure in the B-ring makes it plausible that these compounds are ortho-quinones generated by disproportionation of two phenoxyl radicals,[24] in line with the recent finding of Jovanovic *et al.*,[25] who found that oxidation solely takes place in the B-ring for flavonoids with a catechol structure. For *all-rac*-α-tocopherol, the new wave can be ascribed to α-tocopherolquinone obtained by ring opening of the heterocyclic ring. The reduction potentials of these presumed quinoid compounds are also presented as half-peak potentials, $E_{p/2}$, in Table I and show that while *all-rac*-α-tocopherol is the strongest reducing agent among the five antioxidants and has the oxidation product which is the most difficult to reduce, the same simple relationship does not apply to the flavonoids. While the reduction potentials for the phenoxyl radicals increases along the series *all-rac*-α-tocopherol < quercetin < luteolin ~ (+)-catechin < (±)-taxifolin, the reduction potential for the more stable quinoid oxidation products increases along the series *all-rac*-α-tocopherol < (+)-catechin < (±)-taxifolin < quercetin ~ luteolin.

Regeneration Experiments

Each of the four flavonoids were electrolysed under controlled conditions corresponding to one electron per flavonoid but the oxidation was stopped after consumption of 6.0 C (one-electron

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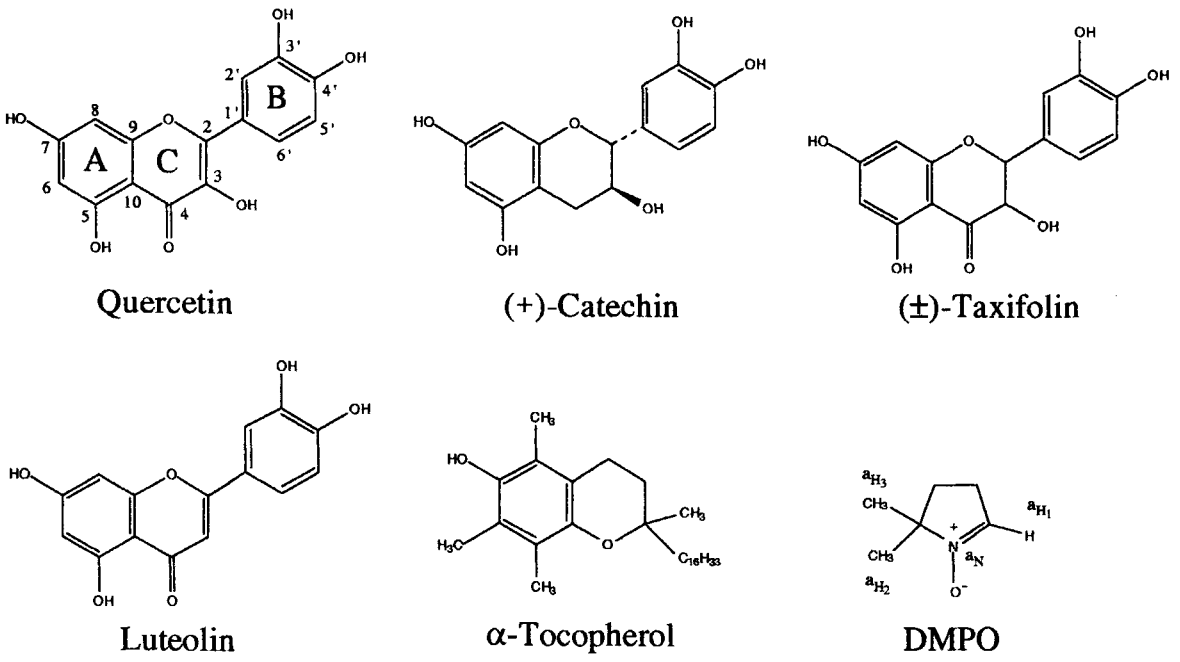

FIGURE 1 Chemical structures of flavonoids (numbering shown for quercetin), of α-tocopherol and of 5,5-dimethyl-1-pyrroline-N-oxide, DMPO. The investigated flavonoids all have a catechol (1,2-dihydroxybenzene) structure in the B-ring and differ only in the C-ring. The numbering of DMPO refers to the coupling constants in the ESR spectra.

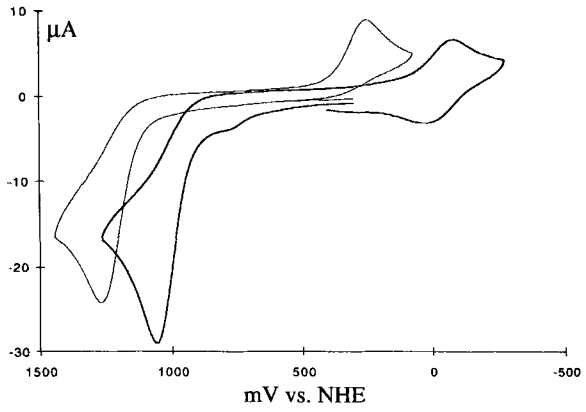

FIGURE 2 Cyclic voltammograms of a 2.00 mM solution of *all-rac*-α-tocopherol (—) and luteolin (–) in DMF/0.10 M $TBABF_4$ obtained with a scan-rate of 500 mV/s at 25°C vs. NHE. The forward scan towards a more positive potential result in a new wave on the reverse scan.

oxidation of all 75 μmol present in the solution would have required 7.24 C) corresponding to one-electron oxidation of 83% of the molecules to avoid generation of two-electron oxidised species in the electrode process. The oxidised flavonoid solutions were in separate experiments mixed with 75 μmol *all-rac*-α-tocopherol. The direct analytical data obtained by HPLC for flavonoid, oxidised flavonoid (only for quercetin, no oxidation products from (+)-catechin, (±)-taxifolin, or luteolin could be detected by HPLC), *all-rac*-α-tocopherol and oxidised *all-rac*-α-tocopherol from such experiments are presented in Table II together with calculated regeneration stoichiometries. It is seen that *all-rac*-α-tocopherol is capable of regenerating at least part of each of the oxidised flavonoids and this regeneration may be directly documented by the HPLC chromatograms as shown in Figure 3 for quercetin.

Complete electrolysis of 75 ml of a 1.00 mM *all-rac*-α-tocopherol solution in DMF/0.10 M $TBABF_4$, was found to require 2.1 ± 0.1 electrons for each *all-rac*-α-tocopherol molecule as determined by the total charge consumed during electrolysis. Only one oxidation product, identical to the one found in the regeneration experiments, with

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TABLE I Reduction potentials vs. Normal Hydrogen Electrode at 25°C for phenolic antioxidants in deaerated DMF compared with potentials obtained in deaerated aqueous pH 7.4 solution at 23°C. Reduction potentials determined for reverse scan (cathodic wave) corresponds to reduction of (rearranged) oxidation product of the antioxidant

Phenolic compound	$E_{p/2}$/V vs. NHE in DMF	$E_{1/2}$/V vs. NHE in H_2O, pH = 7.4; from Ref. [15]	$E_{p/2}$/V vs. NHE in DMF; cathodic wave
All-rac-α-tocopherol	0.93	—	−0.04
Quercetin	1.07	0.29	0.37
(+)-Catechin	1.16	0.36	0.20
(±)-Taxifolin	1.20	0.37	0.27
Luteolin	1.15	0.41	0.38

TABLE II Regeneration of flavonoids from electrochemically one-electron oxidised flavonoids by *all-rac*-α-tocopherol (P_2) as added in equimolar amount in deaerated DMF and monitored by HPLC. Area is obtained from the chromatogram by integration of absorption at 380 nm for quercetin, at 350 nm for luteolin, at 290 nm for (±)-taxifolin, *all-rac*-α-tocopherol and oxidised quercetin, and at 270 nm for (+)-catechin and oxidised *all-rac*-α-tocopherol, and is assumed to be proportional to the concentration of the antioxidant

Flavonoid (P_1)	Quercetin			(+)-Catechin			(±)-Taxifolin			Luteolin		
	Area	μmol	%	Area	μmol	%	Area	μmol	%	Area	μmol	%
P_1 (Q = 0.0 C) initial amount	11307	75[a]	100	1771	75[a]	100	11498	75[a]	100	10151	75[a]	100
P_1 (Q = 6.0 C) not oxidised[b]	5377	36	48	1127	48	64	7897	52	69	5459	40	54
P_1 oxidised (by difference)		39	52		27	36		23	31		35	46
P_{1ox} (Q = 6.0 C)[c]	4859	30	40									
P_1 after P_2 added to electrolysed P_1	7304	48	65	1379	58	78	9650	63	84	7347	54	72
P_{1ox} after P_2 added to electrolysed P_1[c]	2347	14	20									
P_1 regenerated of oxidised		13	32		11	39		11	49		14	40
P_1 unaccounted for[d]		13	15		17	22		12	16		21	28
P_2 remaining after reaction	1666	63	84	1348	55	74	1283	54	72	1200	50	67
P_2 consumed of added		12	16		20	26		21	28		25	33
P_{2ox} found	1478	12		2038	17		2446	21		2474	21	
P_2 unaccounted for[e]		0			3			0			4	
Regeneration index[f], R_i		1.04			0.55			0.55			0.56	

[a]Initial amount known from concentration of stock solution.
[b]Not oxidised after electrolysis corresponding to 1 electron/flavonoid.
[c]Oxidation product directly detected (*cf.* Ref. [16]), information only available for quercetin.
[d]P_1 unaccounted for = P_1(Q = 0.0 C) − (P_1 after P_2 added + P_{1ox} after P_2 added).
[e]P_2 unaccounted for = P_2 added − (P_2 found + P_{2ox} found).
[f]Regeneration index = mole P_1 regenerated/mole P_2 consumed.

$\lambda_{max} = 270$ nm, was detected in HPLC, and was found to appear quantitatively as *all-rac*-α-tocopherol was consumed during oxidation. A 100% conversion of *all-rac*-α-tocopherol into this single oxidation product was accordingly assumed, and the area found by HPLC was used to calculate the amount of oxidised *all-rac*-α-tocopherol generated during the regeneration experiments. Inspection of Table II justifies this approach, since the amount of oxidised *all-rac*-α-tocopherol found otherwise would exceed the amount of *all-rac*-α-tocopherol consumed during some of the regeneration experiments. Purification, spectroscopic characterisation and identification of this oxidation product of *all-rac*-α-tocopherol is in progress.

Similar experiments as those presented in Table II were conducted for all possible 20 combinations of antioxidants (in triplicate for those where regeneration was demonstrated and in duplicate for those without detectable regeneration), and the results are presented in Table III

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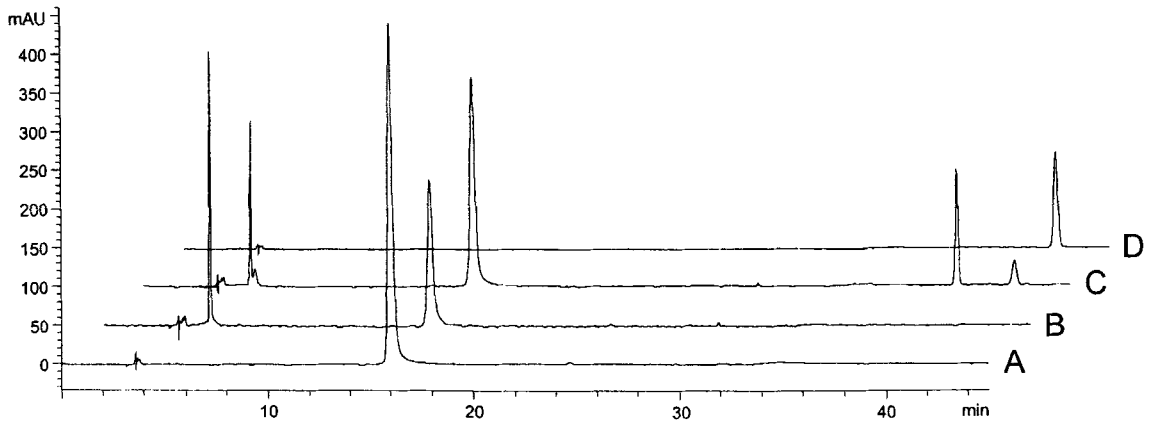

FIGURE 3 HPLC chromatograms obtained during regeneration of quercetin from oxidised solution of quercetin by *all-rac*-α-tocopherol. (A) S1, unoxidised quercetin (P_1) monitored spectrophotometrically at 270 nm, (B) S2, oxidised quercetin at 270 nm, stable oxidation product seen after 5.2 min elution, (C) S3, addition of *all-rac*-α-tocopherol to oxidised quercetin monitored at 270 nm. Quercetin is by comparison with B seen to be regenerated. Oxidised *all-rac*-α-tocopherol is seen at 39.5 min, (D) S4 *all-rac*-α-tocopherol (P_2) at 290 nm used to calculate the amount consumed in the regeneration process.

TABLE III Percentage material regenerated for equimolar concentration in DMF at 25°C of the electrochemically one-electron oxidised phenolic antioxidant P_1 after addition of the phenolic antioxidant P_2, relative to the amount consumed of P_1 during the oxidation[a]

P_1	P_2				
	α-Tocopherol[b]	Quercetin	(+)-Catechin	(±)-Taxifolin	Luteolin
α-Tocopherol		0	0	0	0
Quercetin	34 ± 3		0	0	0
(+)-Catechin	34 ± 14	32 ± 6		0	0
(±)-Taxifolin	52 ± 10	42 ± 10	24 ± 1		0
Luteolin	43 ± 6	37 ± 4	25 ± 5	16 ± 6	

[a]Each number mean of three independent experiments, except when no regeneration was detectable, where the experiments were conducted twice.
[b]Analytical details for one of the triplicate experiments for α-tocopherol presented in Table II.

as percentage regenerated antioxidant. As may be seen in Table III, a rather consistent picture is seen, as for all combinations of antioxidants where regeneration was found in one direction, the reverse reaction could not be demonstrated. The rather large standard deviations prevent the deduction of a more quantitative correlation between the percentage regenerated and the driving force calculated from the reduction potentials for the antioxidants (Table I). It is, however, noteworthy that (±)-taxifolin, with the highest phenoxyl radical reduction potential, is regenerated more efficiently than the other flavonoids both by *all-rac*-α-tocopherol and by quercetin. It is also seen that both (+)-catechin and (±)-taxifolin regenerate luteolin to a much lesser extent than *all-rac*-α-tocopherol and quercetin. Table IV shows the regeneration index, R_i, defined as the number of moles regenerated of the oxidised compound P_1, divided by the number of moles consumed of the added phenolic antioxidant P_2. While the regeneration expressed in percentage is a more practical number also including unknown "other" reactions, the index provides a fundamental parameter for the regeneration based on the molar efficiency. Again

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TABLE IV Regeneration index, defined as moles regenerated of the oxidised phenolic antioxidant P_1, after addition of the phenolic antioxidant P_2, divided by moles consumed of P_2[a] for equimolar reaction conditions in DMF at 25°C

P_1	P_2				
	α-Tocopherol[b]	Quercetin	(+)-Catechin	(±)-Taxifolin	Luteolin
α-Tocopherol		0	0	0	0
Quercetin	0.90 ± 0.16		0	0	0
(+)-Catechin	0.48 ± 0.11	0.88 ± 0.13		0	0
(±)-Taxifolin	0.48 ± 0.06	0.41 ± 0.03	0.36 ± 0.01		0
Luteolin	0.50 ± 0.10	0.41 ± 0.02	0.55 ± 0.05	0.63 ± 0.22	

[a]Each number mean of three independent experiments, except when no regeneration was detectable, where the experiments were conducted twice.
[b]Analytical details for one of the triplicate experiments for α-tocopherol presented in Table II.

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it is seen that there is no direct correlation between R_i and driving force. The observation that (±)-taxifolin may regenerate luteolin despite the ordering of the reduction potentials (Table I) shows that other subsequent reactions may be important, and in this case $E_{p/2}$ determined from the cathodic wave of the presumed ortho-quinoid compounds may provide more correct information of the thermodynamics of the reaction. It is, however, apparent that the regeneration of quercetin by *all-rac*-α-tocopherol, and the regeneration of (+)-catechin by quercetin, is far more efficient than any of the other regeneration reactions and shows close to full efficiency. Whereas it can be seen from Table III, that (+)-catechin does not regenerate any quercetin, a significant decrease of 14 ± 2% of the quercetin oxidation product was found after addition of (+)-catechin (data not shown), which means that the presence of (+)-catechin clearly has influenced the fate of intermediate oxidation products of quercetin.

Oxidation of quercetin in DMF gave 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone, which is the same oxidation product as previously isolated from oxidation of quercetin in acetonitrile.[16] It is seen in Table II, that this product and the unoxidised quercetin accounts for approximately 90% of the starting material in the regeneration experiment with *all-rac*-α-tocopherol. Although this is slightly less than found in acetonitrile, where the oxidised and unoxidised form of quercetin together accounted for almost 100%, it is very encouraging that the regeneration could be followed almost quantitatively. A 1.0 mM solution of 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone, the oxidation product of quercetin, isolated as previously described,[16] were mixed with a 1.0 mM solution of each of the five phenolic antioxidants and analysed by HPLC. None of the compounds investigated reacted with this oxidation product.

ESR Spectroscopy

The steady-state radical concentration obtained during bulk electrolysis of the phenolic compounds was expected to be relatively high,[19–23] but it was not possible to obtain any ESR spectra without the presence of a spin-trap. The spectra of the five spin adducts of the spin-trap used (DMPO) are shown in Figure 4. No spin adduct could be detected in the absence of a phenolic compound during electrolysis, which means that none of the radicals in Figure 4 are solvent radicals in accordance with the potential range normally assumed for DMF.[26] In Figure 5 is shown the ESR spectrum of oxidised luteolin spin-trapped with DMPO, and the ESR spectrum obtained after addition of quercetin to the oxidised luteolin with subsequent addition to DMPO. Comparison of the latter ESR spectrum with the ESR spectra of Figure 4 clearly shows that quercetin radicals are generated, when quercetin

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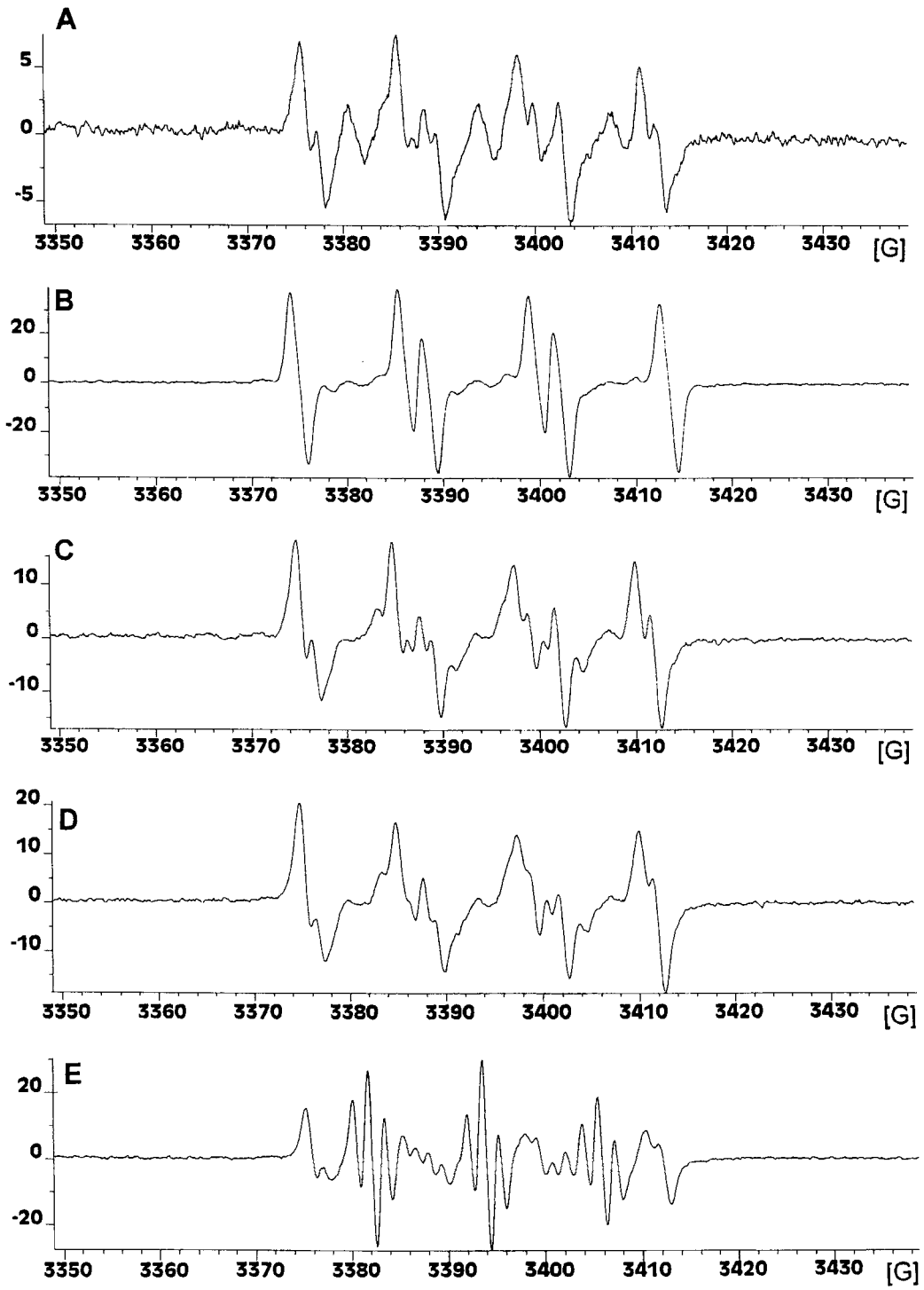

FIGURE 4 ESR spectra of the spin adducts between DMPO and electrochemically generated radicals from phenolic antioxidants in DMF/0.10 M $TBABF_4$. (A) *all-rac*-α-tocopherol, (B) quercetin, (C) (+)-catechin, (D) (±)-Taxifolin, (E) Luteolin. Peak height of ESR spectra in arbitrary units.

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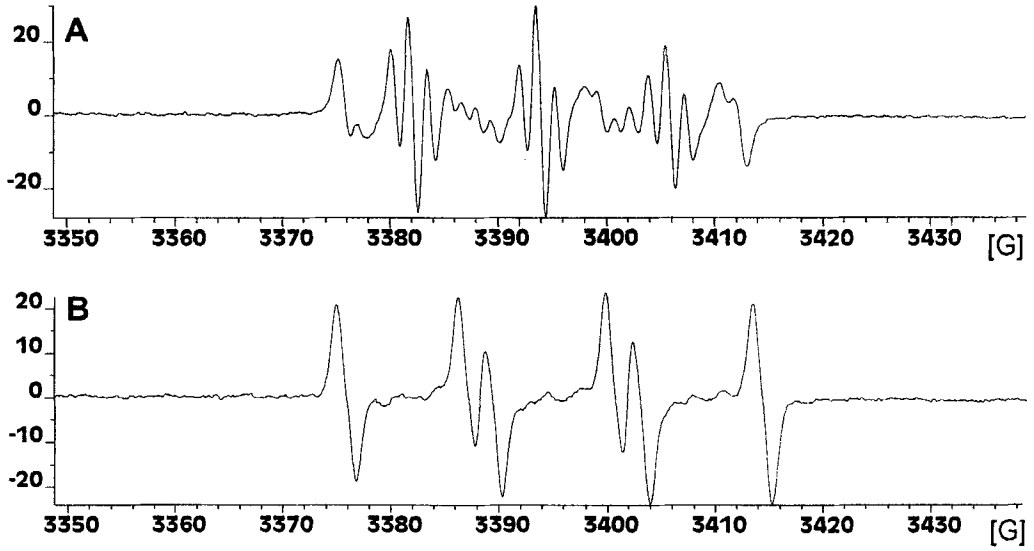

FIGURE 5 ESR spectra of spin adducts between DMPO and (A) oxidised luteolin and (B) reaction mixture of oxidised luteolin and quercetin in DMF/0.10 M $TBABF_4$. A may be seen to be identical with the spectrum in Figure 4(E). Spectrum B may be seen to be identical with the spectrum in Figure 4(B).

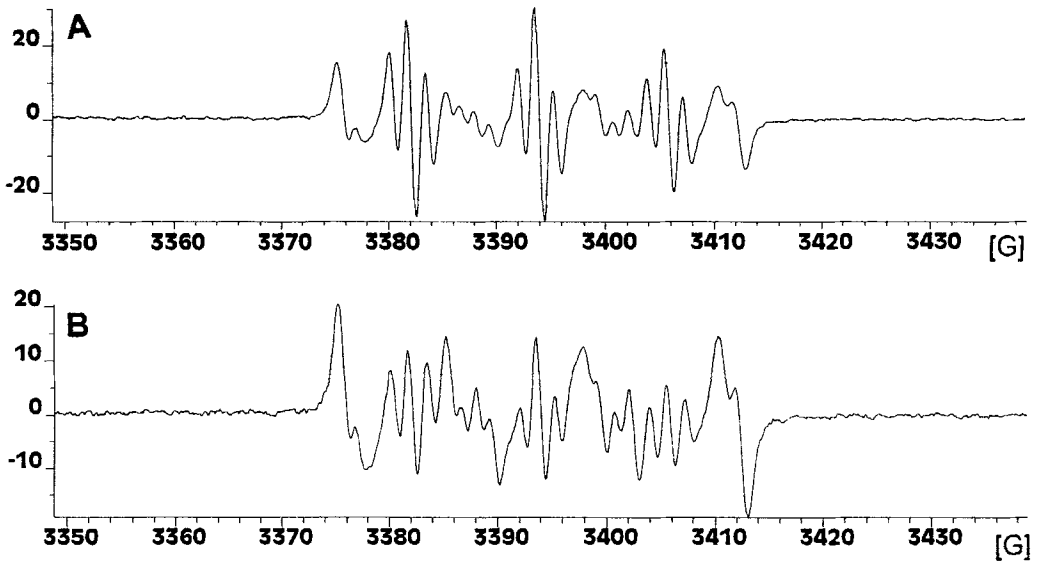

FIGURE 6 ESR spectra of spin adducts between DMPO and (A) oxidised luteolin, (B) reaction mixture of oxidised luteolin and (+)-catechin in DMF/0.10 M $TBABF_4$.

reacts with oxidised luteolin regenerating luteolin. As may be seen from the ESR spectra of Figure 6, the reaction of oxidised luteolin with (+)-catechin apparently results in a mixture of radicals for this less efficient regeneration of luteolin (Table III). The strongest ESR signal was always obtained from the spin adduct between quercetin radicals and DMPO, which made this

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stable free radical an obvious candidate for identification. The simulated spectrum using a Lorentzian lineshape in accordance with a freely rotating isotropic radical and the coupling constants directly estimated from the spectrum, is compared to the experimental spectrum in Figure 7. Inclusion of the methyl groups on DMPO in the calculation only resulted in a slightly better simulation ($r = 0.994$ compared to $r = 0.993$), whereas a realistic line width, Lw = 0.53 G compared to Lw = 0.80 G, only was found when the methyl groups were included. Since the coupling constants (see Figure 1 for numbering) found for the methyl groups, $a_{H_2}(3) = 0.29$ G and $a_{H_3}(3) = 0.62$ G, are not negligible, we believe this is a result of general validity, and suggest that the methyl groups of DMPO, in contrast to previous simulations of spin adducts with DMPO[17,18] in aerated solution, should be included at least in simulations of spectra obtained in deaerated solution. The coupling constants $a_N(1) = 13.58$ G and $a_{H_1}(1) = 11.32$ G strongly suggest that the quercetin radical is an oxygen-centered phenoxyl radical.[17] Whereas the signal obtained for spin-trapped quercetin clearly originated from a single radical, this was not the case for the four other spectra, which could not be satisfactorily simulated. The attempted simulations showed, however, that the major component of the spectra had coupling constants close to those found for quercetin. The radicals are therefore supposed to be mainly oxygen-centered.

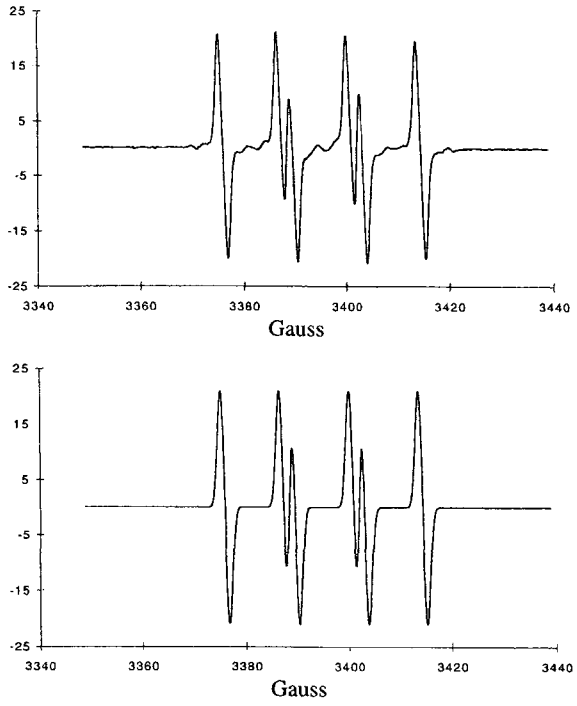

FIGURE 7 Experimental (upper panel) and simulated (lower panel) ESR spectrum of the spin adduct between DMPO and the electrochemically generated quercetin radical. The simulation of a Lorentzian line shape lead to $a_N(1) = 13.58$ G and $a_{H_1}(1) = 11.32$ G which strongly suggest that the quercetin radical is an oxygen-centered phenoxyl radical.[17] The most realistic line width, Lw = 0.53 G, was found when the methyl groups on DMPO were included in the simulation; $a_{H_2}(3) = 0.29$ G and $a_{H_3}(3) = 0.62$ G.

Rapta *et al.*[18] have oxidised galangin (3,5,7-trihydroxyflavone) electrochemically in acetonitrile, and simulated the ESR spectrum of the spin adduct with DMPO. The reported spectrum is rather different from those obtained in this study, as are the coupling constants: $a_N(1) = 14.2$ G and $a_{H_1}(1) = 21.4$ G, compared to $a_N(1) = 13.58$ G and $a_{H_1}(1) = 11.32$ G found in the present study, which suggest that the radical trapped after oxidation of galangin is carbon-centered, rather than oxygen-centered as concluded for the flavonoids of the present study.[17] Rapta *et al.* suggest that the carbon-centered radicals arise from a homolytic cleavage of the C-ring after initial two-electron oxidation.

GENERAL DISCUSSION

The steady state radical concentration generated during electrochemical oxidation was, in accordance with the short lifetime predicted from cyclic voltammetry, found to be rather low and we were not able to achieve any ESR signal without use of a spin-trap (DMPO), even when the samples were frozen immediately in liquid nitrogen. The ESR signal increased slowly with time after addition of DMPO, which suggest an equilibrium between the phenoxyl radicals and an ortho-quinone shifting towards the phenoxyl radical by the

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spin-trap:

$$HO-P-O^{\bullet} + HO-P-O^{\bullet} \rightleftharpoons O=P=O + HO-P-OH \quad (1)$$

Interactions between antioxidants and antioxidant hierarchies are often discussed on the basis of the reduction potentials of their corresponding radicals neglecting kinetic factors.[10] It has, however, been difficult to obtain consistent reduction potentials for phenolic antioxidants in aqueous media,[12,15,24,25,27] since oxidation of the phenolate anion usually is accompanied with deprotonation of the free radical formed by electron transfer requiring strict pH control.[24,25,27] Reduction potentials determined for flavonoids in aprotic media like DMF are typically more positive than those determined in water, since the parent phenol is oxidised with subsequent deprotonation of the radical cation to yield a neutral phenoxyl radical. Potentials can normally only be considered to be accurate within $\pm$ 20 mV,[28] and when potentials like those of Table I are combined for two related phenolic antioxidants interacting:

$$HO-P_1-O^{\bullet} + HO-P_2-OH \rightleftharpoons HO-P_1-OH + HO-P_2-O^{\bullet} \quad (2)$$

the uncertainties alone, even neglecting kinetic factors, makes it difficult to predict whether one antioxidant may regenerate another or vice versa. However, the potentials of Table I predicts, that *all-rac*-α-tocopherol should be able to regenerate the four flavonoids from their phenoxyl radicals, as also found in the regeneration experiments (Tables II–IV). Quercetin like *all-rac*-α-tocopherol has a potential significantly below that of the other phenolic antioxidants of Table I, and also for quercetin, the direction of reaction of Equation (2) is as predicted from the potentials. However, for (+)-catechin, (±)-taxifolin and luteolin, the ordering of the flavonoids with respect to regeneration (Table III) is not that simply predicted from the reduction potentials, but rather that predicted from the potentials determined in the cathodic wave on the reverse scan. This ambiguity in the use of the potentials stresses the importance of the method developed with direct HPLC detection of the product distribution after the reaction of Equation (2) has been allowed to reach equilibrium or at least a steady-state. The capacity of quercetin to reduce the oxidised form of the other flavonoids is further seen by the ESR regeneration experiment where quercetin was added to other oxidised flavonoids, and in which the ESR spectrum unambiguously was found to be similar to the one obtained for quercetin following direct electrolysis, see Figures 4 and 5. Moreover, electrochemical oxidation of quercetin with subsequent addition of *all-rac*-α-tocopherol did not result in the spectrum obtained directly for oxidised *all-rac*-α-tocopherol after addition of DMPO, but rather the spectrum for quercetin. According to the potentials, the *all-rac*-α-tocopherol spectrum was to be expected but a more stable adduct between DMPO and quercetin radicals seems to be formed, since the spectrum obtained following oxidation of *all-rac*-α-tocopherol did not change when quercetin was added. The consumption of *all-rac*-α-tocopherol and direct detection of its oxidation product in the HPLC analysis, when exposed to oxidised quercetin, further confirmed that *all-rac*-α-tocopherol is capable of regenerating quercetin.

It is seen from Tables II and III, that in each case less than all of the antioxidant P_1 is regenerated from the phenoxyl radical by the other antioxidant P_2 added, and furthermore seen from Table IV, that more than one molecule of P_2 is consumed for every molecule of P_1 regenerated. This suggest that the regeneration process to a certain extent is kinetically controlled and that other processes also are important. In solutions of "one-electron" oxidised flavonoids, the phenoxyl radicals corresponding to the antioxidant P_1 are in a disproportionation equilibrium (Equation (1)) with the corresponding quinone and the parent phenol as evidenced by the absence of an ESR-signal. However, free radicals may participate in

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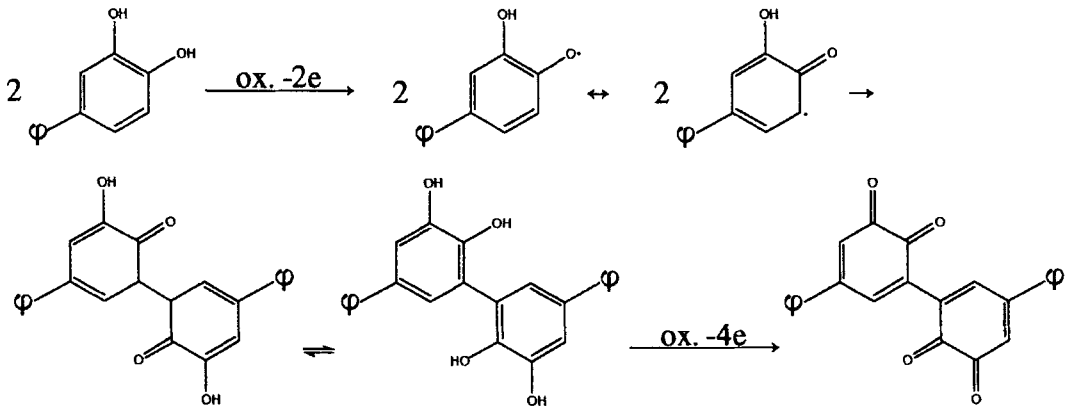

FIGURE 8 Dimerisation of two B-ring phenoxyl radicals in the ortho position. Keto-enol tautomerisation re-establishes the dihydroxy substitution, which may be further oxidised to an ortho-quinone dimer.

dimerisation or polymerisation reactions to form products from which the parent phenol cannot be regenerated, or the ortho-quinones may further react with (traces of) water like previously found for quercetin.[16] Other radicals generated in the regeneration process when the antioxidant P_2 added, may likewise react directly with the phenoxyl radical of P_1 otherwise available for regeneration although any such products could not be detected by the current HPLC-method.

If disproportionation was the only fate of the flavonoid radicals, 58% of the parent compound would be present in the sample withdrawn. Inspection of Table II reveals, that this is close to what actually is found, which clearly shows that the molecules consumed during the oxidation have experienced a net two-electron oxidation either through disproportionation or by a direct two-electron oxidation at the electrode for phenoxyl radicals more reducing than the parent compound.[15] For (±)-taxifolin, 69% of the parent compound was found after electrolysis, which suggests that the molecules consumed during the oxidation on an average have lost more than two electrons or that dimerisation competes with disproportionation. A dimer may be a better hydrogen atom donor than the parent compound since an electron-donating phenoxyl substituent in the ortho position may lower the bond dissociation energy. The initially formed dimer may thus regenerate the parent compound from its phenoxyl radical, while it is oxidised into a net three-electron oxidation per flavonoid molecule, *cf.* Figure 8.

In conclusion, regeneration of flavonoid antioxidants by *all-rac-α*-tocopherol has been followed using a combination of ESR spectroscopy and HPLC analysis. Reduction potentials for the flavonoid phenoxyl radicals may in most cases predict this regeneration, although thermodynamic control only partly accounts for the regeneration pattern. The rather short life time of the phenoxyl radicals, suggest that conproportionation of the corresponding ortho-quinone and the parent compound, prior to the regeneration reaction, is an important step in the overall regeneration, the sluggishness of which further may explain loss of antioxidant capacity during the regeneration.

Acknowledgement

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

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