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ORIGINAL PAPER

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Radical scavenging by dietary flavonoids. A kinetic study of antioxidant efficiencies

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Abstract The rate of scavenging of peroxyl radicals and of diphenylpicrylhydrazyl radicals by flavanones, flavones, flavanols and flavonols commonly occurring in foods was found, when determined by ESR spin trapping and by stopped-flow spectroscopy, respectively, to be the highest for the most reducing of the flavonoids. Among 12 flavonoids investigated a threshold potential of approximately +0.4 V seems to exist, above which the flavonoids become ineffective radical scavengers, as determined by ESR spectroscopy using 5,5-dimethyl-1-pyrroline-*N*-oxide as spin trap. Scavenging of diphenylpicrylhydrazyl radicals showed (pseudo) first order kinetics for excess of flavonoids, and the reaction half-life could also be determined for the less effective flavonoids. For kaempferol and eriodictyol, flavonoids with very similar reduction potentials for their phenoxyl radicals, rate constants for the bimolecular scavenging could be determined in methanol and had the value $(7.0 \pm 0.7) \times 10^2\ s^{-1}\ M^{-1}$ and $33 \pm 1\ s^{-1}\ M^{-1}$ at 25 °C, respectively, showing that other factors than the driving force, such as hydrophilic/lipophilic balance are important, as was further confirmed in an oxygen consumption assay based on a linoleic acid emulsion, where decreasing antioxidant efficiency followed the order: (+)-catechin > (±)-taxifolin ≥ luteolin > kaempferol > quercetin >> naringenin.

Key words Flavonoids · Radicals · Electron spin resonance · Oxygen consumption · Antioxidants

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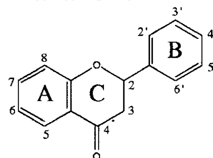
Introduction

Increasing attention is being paid to the positive health effects of flavonoids and carotenoids [1, 2], and the capacity of these non-nutritive compounds to act as antioxidants in vivo has been focused on in particular [3, 4]. Polyphenols like flavonoids can exert their antioxidative action by hydrogen atom donation to free radicals, although other mechanisms such as electron transfer and metal chelation should also be considered [5, 6]. The electron transfer antioxidant mechanism, entailing one-electron reduction of free radicals has been recently demonstrated for carotenoids and could also explain antioxidative synergism between polyphenols and carotenoids [7, 8]. Structure-activity relationships are important for industrial exploration of natural antioxidants in order to optimize selection of plant extracts for protection of processed foods. Thus, certain structural features of carotenoids like extended conjugation and absence of keto groups were found to correlate with efficient radical scavenging activity [9, 10], while for flavonoids, a correlation between ease of oxidation as determined by cyclic voltammetry, and deactivation of hypervalent heme pigments indicative of oxidative stress has been demonstrated [6]. Different antioxidants may be important at different stages of oxidative damage, and it is important to develop assays which are based on reaction kinetics and on a detailed understanding of the mechanism by which the different antioxidants are active. In order to obtain such information and to explore structure-activity relationships for antioxidative actions of flavonoids further, we undertook kinetic investigations of reactions between free radicals and the 12 flavonoids commonly occurring in foods shown in Fig. 1 which provide a large span in electron donating properties [6, 11], and compared the kinetic information with a commonly used oxygen consumption assay.

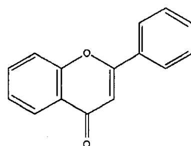
Fig. 1 Flavonoid structures

Flavanone



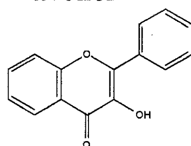
Naringenin	(5,7,4'-trihydroxyflavanone)
Eriodictyol	(5,7,3',4'-tetrahydroxyflavanone)
Hesperitin	(5,7,3'-trihydroxy-4'-methoxyflavanone)
Taxifolin	(3,5,7,3',4'-pentahydroxyflavanone)

Flavone



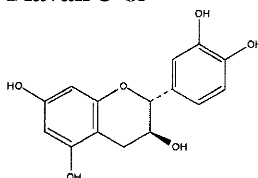
Chrysin	(5,7-dihydroxyflavone)
Luteolin	(5,7,3',4'-tetrahydroxyflavone)

Flavonol



Kaempferol	(3,5,7,4'-tetrahydroxyflavone)
Quercetin	(3,5,7,3',4'-pentahydroxyflavone)
Myricetin	(3,5,7,3',4',5'-hexahydroxyflavone)
Morin	(3,5,7,2',4'-pentahydroxyflavone)
Fisetin	(3,7,3',4'-tetrahydroxyflavone)

Flavan-3-ol

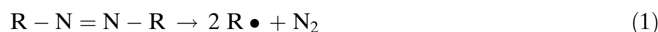


Catechin [2R,3S]

Materials and methods

Chemicals. Quercetin dihydrate, fisetin, naringenin, 2,2,6,6-tetramethylpiperidin-1-yloxyl (TEMPO), diphenylpicrylhydrazyl (DPPH), and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were from Aldrich (Steinheim, Germany). Kaempferol, chrysin, myricetin, and luteolin were from Apin (Abingdon, UK). Horseheart metmyoglobin (type III), linoleic acid, Tween 20, morin, (+)-catechin hydrate, (±)-taxifolin, and hesperetin were from Sigma (St. Louis, Mo., USA). Eriodictyol was from Roth (Karlsruhe, Germany), and 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) from WAKO (Richmond, Va., USA). These chemicals were all used without further purification. The solvents dimethyl sulphoxide (DMSO) from Merck (Darmstadt, Germany) and methanol (Lab-Scan, Dublin, Ireland) and all other chemicals were of analytical grade. Water was purified through a Q-Plus purification train (Millipore, Bedford, Mass., USA). To remove any traces of iron and other metals the phosphate buffer (5.0 mM, pH 7.4) was purified through a column packed with chelating resin (Chelex 100 based on iminodiacetic acid, from Sigma).

ESR spectroscopy. The ESR assay was based on competition between the spin trap DMPO and the actual flavonoid in scavenging of peroxyl radicals. An aqueous 0.10 M DMPO solution (5.0 mM phosphate buffer at pH 7.4) was purified on an active coal column, and the filtered solution, which was found to be ESR-silent, was stored for up to 1 week at 5 °C in the dark. The peroxyl radicals were generated through thermal cleavage of the water-soluble azo-compound AAPH in air-saturated aqueous solution:



$$R - N = N - R \rightarrow 2\,R\bullet + N_2 \quad (1)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad (2)$$

An aqueous AAPH stock solution in the same phosphate buffer was prepared daily, while 0.50 mM solutions of the actual flavonoid in DMSO were made immediately before use. Solutions of AAPH and of DMPO were mixed with DMSO (with or without flavonoid) and buffer was added to yield final concentrations of [AAPH]=10 mM, [DMPO]=5.0 mM, [flavonoid]=0.010 mM, and 2% DMSO. The reference solution had other concentrations equal but zero flavonoid concentration. Immediately after mixing, the reaction mixture was transferred to a flat quartz cell (WG-813-TMS-S, 430 µl, Wilmad Glass, Bueno, N.J., USA) intended for measurements of aqueous solutions in the thermostatically controlled cavity of a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). Oxidation was initiated by heating to 55 °C. The handling of the sample until it was placed in the heated cavity was standardized and the first measurements were taken exactly 5 min after mixing with the following spectrometer settings: center field, 3395.95 G; sweep width, 55 G; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude 1.01 G; receiver gain, 1.00×10^5; conversion time, 81.92 ms; time constant, 163.84 ms and sweep time, 83.89 s. The development of spin adducts was followed for a period of 30 min with measurements after 10, 15, 20 and 30 min in addition to the initial measurement. Twice daily the response

signal resulting from the ESR spectrometer settings was controlled by the TEMPO solution.

Stopped-flow absorption spectroscopy. An 0.16 mM solution of DPPH in methanol and a solution of flavonoid in methanol (1.6, 4.0 or 10.0 mM) were placed in each syringe of a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics, London, UK) and the reactions were followed at 25.0 °C by absorbance measurement at 516 nm, which is the wavelength with maximum difference between the radical DPPH and the reduced non-radical product of DPPH. The initial value of the absorbance, A_o, was measured for each run. The change in absorbance with time was analysed by non-linear regression analysis using Pro/Kineticist software (Applied Photophysics).

Oxygen consumption measurement. A linoleic acid oil-in-water (o/w) emulsion was made by sonification of a mixture of 25 mg Tween 20 as emulsifier, 60 mg linoleic acid and 150 µl aqueous 1.0 M NaOH and sufficient 50 mM aqueous air-saturated phosphate buffer (pH 6.8) to yield 10.0 ml. Accurately weighed amounts of flavonoid were dissolved by sonification in known volumes of this emulsion, and 100 µl of the resulting flavonoid containing linoleic acid emulsion was diluted by addition of 4.7 ml phosphate buffer (pH 6.8) and 100 µl of 0.10 M aqueous hydrochloric acid (to adjust the pH to 5.8). The oxidation was initiated by addition of 100 µl of a 0.20 mM aqueous metmyoglobin solution and 70 µl of this final emulsion, with a total volume of 5.00 ml was immediately injected into a thermostatically controlled (25.0 ± 0.1 °C) measuring cell (Chemiware, Viby J, Denmark) with no head space. The relative oxygen concentration was set to 100 % at time zero and recorded for 1 h (every 30 s). The initial linoleic acid concentration was 429 µM and metmyoglobin concentration 4 µM, while flavonoid concentration was 0.0 (reference), 9.0, 21, 86 or 429 µM.

Statistical analysis. The ESR results (I_{ESR}) reported are the average values of determination in duplicate. The results were analysed for differences between flavonoids using a one-way analysis of variance, I_{ESR} being the response variable. The results from the stopped-flow absorption spectroscopy are reported as reaction half-life ($t_{1/2}$), the reported values being the mean of four determinations. An analysis of variance was performed with $t_{1/2}$ as response variable, and the difference between the concentration for each flavonoid was determined. The analyses were performed by using the statistical software SAS ver 6.10 (1990).

Results and discussion

The scavenging activity of flavonoids towards peroxyl radicals measured by ESR was found to vary depending on the group of flavonoids. Compounds belonging to the flavonols were in general found to be very effective as peroxyl radical scavengers, while the effect of the flavonoids belonging to other groups were found to be minor. The initial rates for trapping of free radicals, d*I*/d*t*, based on the changes in relative intensities of ESR signals in the time period 5 min to 10 min were calculated for the 12 flavonoids and the reference (containing no flavonoids), and an index of the rate of scavenging of peroxyl radicals, I_{ESR}, as defined in Eq. 3 was determined for the different flavonoids and listed in Table 1.

$$I_{ESR} = dI/dt_{flavonoid}/dI/dt_{reference} \quad (3)$$

The different efficiencies of radical scavenging by the flavonoids are reflected in differences in their I_{ESR} values. A low value of I_{ESR} is thus indicative of a high scavenging activity. The statistical analyses show a significantly higher activity for the group of flavonols, while the difference between flavanones, flavones and the flavanol were only minor, although slightly higher activities were seen for the flavanol and one of the flavones. For the flavonoids not belonging to the group of flavonols, luteolin (see Fig. 1), a compound with four hydroxyl groups and extended conjugation between the C and B rings, had the highest antioxidative activity. Among the flavonols myricetin, having six hydroxyl groups, and morin, with five hydroxyl groups, were the most efficient antioxidants in this peroxyl radical scavenging assay.

The formation of spin adducts from the reaction between peroxyl radicals and DMPO in the absence of flavonols was well described by Eq. 4:

Table 1 Redox potentials of flavonoids together with an index for scavenging activity determined by ESR spin trapping and half-life for reaction with the stable DPPH radical

Flavonoid[a]		E (V[b])	I_{ESR}[c]	$t_{1/2}$ (s)		
				0.8 mM[d]	2 mM[d]	5 mM[d]
Flavanone	Eriodictyol	0.36	1.03[AB]	5.7 ± 0.3[O]	3.6 ± 0.2[P]	1.8 ± 0.1[Q]
	(±)-Taxifolin[a]	0.37	1.13[A]	14 ± 1[O]	9.8 ± 0.3[Q]	12 ± 0.1[P]
	Hesperetin	0.59	0.94[BC]	395 ± 41[O]	74 ± 4[P]	44 ± 2[P]
	Naringenin[a]	0.76	0.97[B]	> 1000	> 1000	> 1000
Flavone	Luteolin[a]	0.41	0.71[D]	6.0 ± 0.1[O]	5.7 ± 0.2[P]	4.1 ± 0.3[Q]
	Chrysin	0.83	0.95[BC]	> 1000	> 1000	> 1000
Flavanol	(+)-Catechin[a]	0.36	0.84[C]	6.3 ± 0.1[O]	5.4 ± 0.3[P]	3.8 ± 0.1[Q]
Flavonol	Myricetin	0.20	0.021[H]	2.1 ± 0.2[P]	3.7 ± 0.3[O]	4.1 ± 0.5[O]
	Quercetin[a]	0.29	0.24[EF]	1.7 ± 0.1[O]	1.1 ± 0.2[Q]	1.5 ± 0.2[P]
	Fisetin	0.30	0.29[E]	0.6 ± 0.1[O]	0.57 ± 0.01[P]	0.55 ± 0.04[P]
	Morin	0.34	0.068[HG]	0.25 ± 0.00[Q]	0.31 ± 0.01[P]	0.36 ± 0.02[O]
	Kaempferol[a]	0.39	0.15[GF]	0.66 ± 0.03[O]	0.28 ± 0.00[P]	0.155 ± 0.002[Q]

[a] Flavonoids also evaluated by the oxygen consumption assay (see Table 2)
[b] From reference [6] determined in aqueous solution at pH 7.4 at 23 °C by cyclic voltammetry
[c] As defined in Eq. 3. Figures in the column with the same superscript letter are not significantly different (P=0.05)
[d] Concentration of flavonoid. Figures in one row with the same letter are not significantly different (P=0.05). Half-lives are values for a DPPH concentration of 0.16 mM and methanol solution at 25 °C (see Fig. 3)

$$I = a + b \times \exp[(c - t) \times k_{spin}] \quad (4)$$

In which a and b are constants related to initial and final signal intensity, c is a time off-set constant correcting for the difference between the time of mixing and the time of recording the first spectrum, and k_{spin} is a pseudo first-order rate constant for formation of spin adducts. This equation also described the formation of spin adducts in the presence of flavonoids where DMPO and flavonoids compete for the peroxyl radicals generated:

$$ROO\bullet + DMPO \xrightarrow{k_1} \text{spin adduct} \quad (5)$$

$$ROO\bullet + \text{flavonoid} \xrightarrow{k_2} \text{non-radical product} \quad (6)$$

The observed rate constant for such competitive reactions, k_{spin}, determined by observation of one reaction product is, however, the sum of the rate constants for the two processes:

$$k_{spin} = k_1 + k_2 \quad (7)$$

as may be shown by integration of the rate expression [12]. As is seen from Fig. 2, the observed rate constant in the presence of the almost non-scavenging hesperetin is very similar to the rate constant observed in the absence of flavonoids (0.061 ± 0.002 min^{-1} and 0.064 ± 0.001 min^{-1}, respectively) confirming that the reaction of Eq. (6) is of minor importance for hesperetin. This is in contrast to luteolin, for which $k_{spin} = 0.079 \pm 0.005$ min^{-1} and with the value $k_1 = 0.064$ min^{-1} obtained in the reference experiment, k_2 may be estimated to be 0.015 min^{-1}. The ratio between the products formed in the reactions of Eqs. 5 and 6:

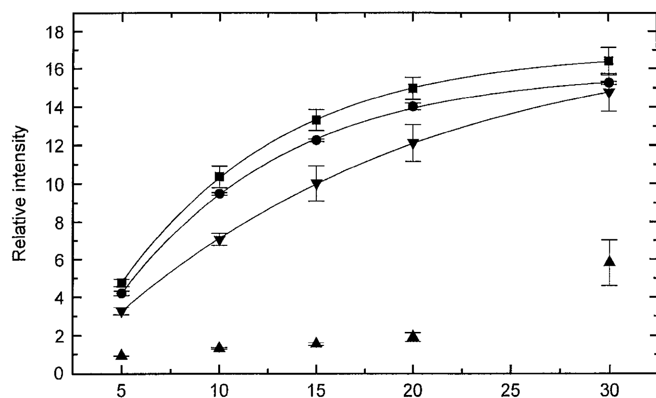


Fig. 2 Relative intensity of DMPO spin adduct obtained by ESR spectroscopy during a time period of 30 min., in an aqueous solution containing [AAPH] = 0.010 M, [DMPO] = 0.0050 M, [flavonoid] = 0.010 M and 2 % DMSO. ■ Reference (mean of three experiments), ● hesperetin (mean of two experiments), and ▾ luteolin (mean of two experiments) for which the full curve is calculated by fitting the four parameters of Eq. 4 for which $k_{spin} = 0.064 \pm 0.001$ min^{-1} for the reference, $k_{spin} = 0.0610 \pm 0.002$ min^{-1} for hesperetin and $k_{spin} = 0.079 \pm 0.005$ min^{-1} for luteolin. ▲ Morin (mean of two experiments)

$$\frac{[\text{spin-adduct}]}{[\text{non-radical product}]} = \frac{k_1}{k_1 + k_2} \quad (8)$$

is for luteolin accordingly 0.8, a value rather close to the value obtained from the initial rate (0.71, see Table 1), confirming that it is the (initial) rate of formation of spin adducts and not the observed rate constant which should be used when comparing radical scavenging efficiencies.

It should be noted that spin adducts are building up in the early stage of the assay, that later spin adducts may be oxidised by other radicals to yield non-radical products and that by the end of the assay period (30 min) a steady state level of spin adducts was observed (Fig. 2). Equation 4 gave comparable good fits for all the investigated flavonoids except compounds belonging to the class of flavonols. For this group of flavonoids rather low levels of free radicals were trapped in the beginning of the assay period and estimation of the rate constant ($k_{spin} = k_1 + k_2$) was not possible, again showing that the rate of formation of spin adducts is more accurate (Table 1). Later a small increase in free radicals was observed, and finally at the end of the observation period a rather large increase in free radicals was seen for most flavonols. The pattern with a lag period is characteristic for an efficient antioxidant. In the competition set up between DMPO and the flavonoid, the flavonoid scavenges the free radical efficiently leaving only a minor amount of the radicals left to react with DMPO. Later in the time period when some of the antioxidant had reacted with radicals a larger amount of the generated radicals were available for reaction with DMPO.

The kinetics of reaction for flavonoids were further investigated using the stable radical DPPH and stopped-flow absorption spectroscopy. Flavonoids were added in excess in each experiment to establish pseudo-first-order conditions. For some flavonoids the numerical analyses of the rate data showed good agreement between the experimental data and the absorption curve calculated from the estimated rate constant (Fig. 3A), while for other flavonoids the fit obtained by using the equation for a pseudo-first-order reaction could indicate parallel reactions of other reductants present as impurities in the flavonoid samples (Fig. 3B), which is understandable since flavonoids are isolated from plant materials. Accordingly the rate data are presented as $t_{1/2}$ in seconds for all 12 flavonoids (Table 1). The flavonols were again the most efficient scavengers of the radical with small values of $t_{1/2}$, whereas the reactions with hesperetin, and especially naringenin and chrysin were indeed very slow. A higher concentration of the flavonoid decreased $t_{1/2}$ as expected, except for the flavonols morin and myricetin where a small increase in $t_{1/2}$ was seen, an observation indicating a more complex reaction pattern and which certainly deserves further attention. However, for both kaempferol and eriodictyol a linear relationship between flavonoid concentra-

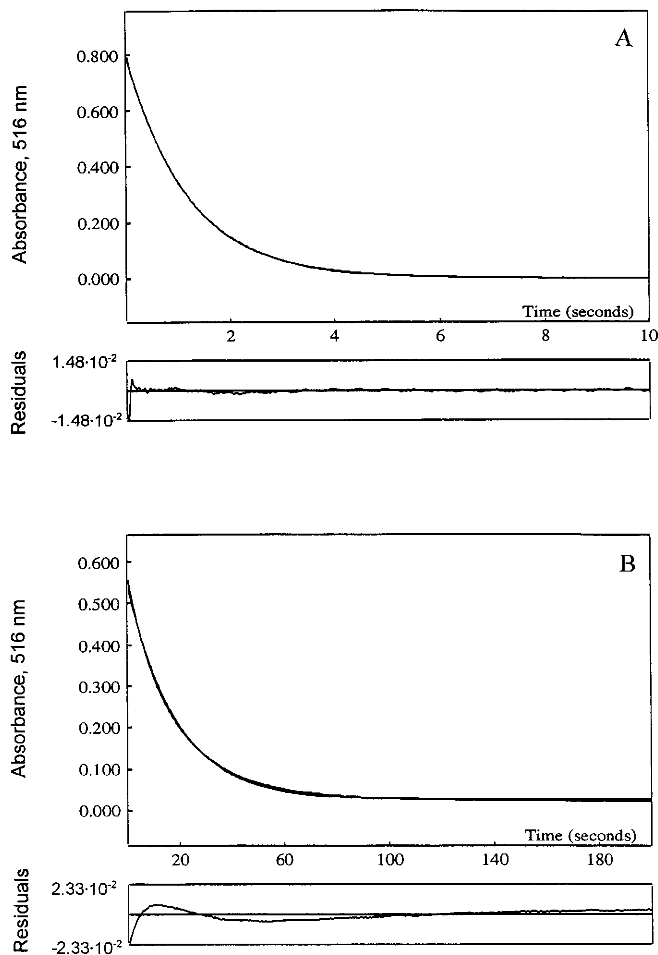


Fig. 3 A, B. Absorbance at 516 nm during reaction between 0.16 mM DPPH and (**A**) 1.6 mM kaempferol and (**B**) 1.6 mM (+)-catechin at 25.0 °C. *Lower panels* show residuals from a nonlinear regression analysis: $A(t)$=a+b exp($-k_{obs}t$), demonstrating a well-fitted equation (**A**) and a poor fitted equation (**B**)

tion and the observed first-order rate constant k_{obs} was found:

$$k_{obs} = a + k_3 \times [\text{flavonoid}] \quad (9)$$

in which k_{obs} was estimated as shown in Fig. 3 and in which the parameter a is a (non-significant) intercept. The second-order rate constants k_3 for the reaction between DPPH and the flavonol kaempferol and the flavanone eriodictyol were calculated to be 708 ± 72 s^{-1} M^{-1} and 33 ± 1 s^{-1} M^{-1}, respectively see Fig. 4. This difference in k_3 illustrates the higher activity of the flavonol kaempferol as a radical scavenger compound compared to the flavanone eriodictyol, confirming the ranking obtained in the spin trapping assay.

Oxygen consumption assays are often used to evaluate antioxidative efficiencies of antioxidants and mixtures of antioxidants in order to detect antioxidative synergism [13]. The kinetics of oxygen consumption in such emulsions in which oxidation is initiated by heme pigments or azo-compounds is complex, and often the time elapsing to reach a certain fraction of the oxygen initially present is used to compare antioxidative efficiencies, with long reaction times providing an indication of high antioxidative activity. In the present assay, linoleic acid was used as oxidation substrate and the relative oxygen concentration was recorded for 1 h (every 30 s) as a function of time in the absence (reference) and presence of each of six different flavonoids belonging to four different flavonoid subgroups, i.e. the flavanones naringenin and (±)-taxifolin, the flavone luteolin, the flavonols quercetin and kaempferol, and the flavan-3-ol (+)-catechin (Fig. 1). The assay was further developed to entail dilution of antioxidants to obtain enhanced sensitivity. At a linoleic acid/flavonoid ratio of 1:1, all the flavonoids except naringenin thus showed excellent antioxidative activity and maintained relative oxygen concentrations above 90%, whereas oxidation occurred in the reference solution depleting oxygen to below 10% within 15 min (Table 2). A relative oxygen concentration of less than 10% was reached within 30 min for the naringenin solution, which was thus clearly the least efficient of the investigated antioxidants in this assay. At a linoleic acid/flavonoid concentration of 20:1 the quercetin solution reached a relative oxygen concentration of less than 10% within 20 min, whereas the remaining four flavonoids still displayed good antioxidative activity after 1 h (relative oxygen concentration above 80%). When the amount of flavonoids was reduced to a ratio of 1:50, only the (+)-catechin solution maintained a high antioxidative activity (relative oxygen concentration above 80% after 1 h), whereas solutions containing (±)-taxifolin and luteolin reached 10% within 30 min and 25 min, respectively. The solution containing kaempferol was only slightly better than the reference at this ratio (Table 2). It is thus possible on the basis of this widely used assay to arrange the antioxidative activity of the investigated flavonoids as: (+)-catechin >(±)-taxifolin ≥ luteolin > kaempferol > quercetin naringenin.

The pioneering work of Bors et al. [14] established the value of structure-activity relationships for the antioxidative activity of natural antioxidants like plant phenols and flavonoids. Such relationships are, however, only of value for predicting antioxidative efficiencies when a large number of compounds are investigated using the same method for assessment of

Table 2 Approximate time to reach a relative oxygen concentration of 10% in the oxygen consumption assay at three different linoleic acid/flavonoid ratios

Linoleic acid/flavonoid ratio	1:1	20:1	50:1
Reference	15 min	15 min	15 min
Naringenin	30 min	15 min	15 min
Quercetin	>60 min	20 min	15 min
Kaempferol	>60 min	>60 min	17 min
Luteolin	>60 min	>60 min	25 min
(±)-Taxifolin	>60 min	>60 min	30 min
(+)-Catechin	>60 min	>60 min	>60 min

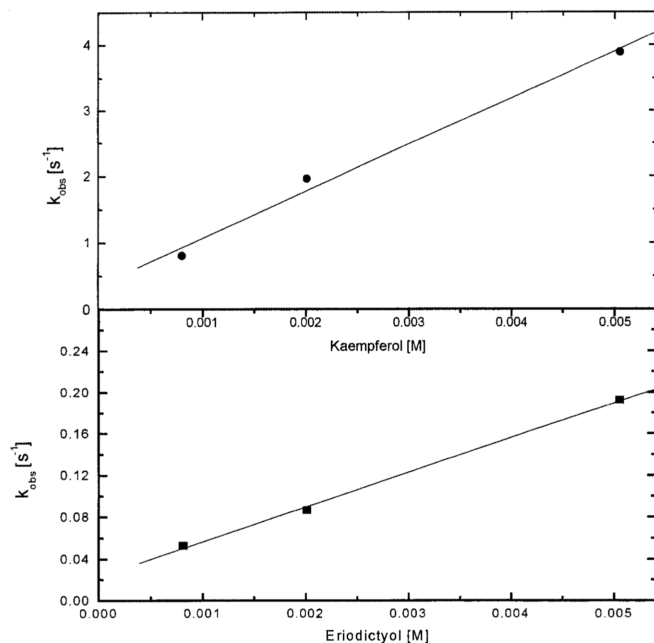


Fig. 4 Pseudo first order rate constant obtained as a function of flavonoid concentration determined for the compounds, ● kaempferol and ■ eriodictyol. The reactions with DPPH were followed at 516 nm and 25.0 °C

antioxidative activity. Many compounds have been tested by the popular TEAC [15] assay which generally seems to confirm that optimal antioxidative activity of flavonoids is governed by the three criteria originally deduced by Bors et al. [11, 14], i.e. (1) the presence of the *ortho*-dihydroxy (catechol) structure in the B-ring, (2) the presence of the 2,3-double bond in conjugation with a 4-carbonyl group, and (3) the presence of both a 3- and a 5-hydroxyl group (see Fig. 1 for numbering).

Most antioxidants react very fast with the oxidising ABTS cation radical used in the TEAC assay, however, in biologically relevant systems a wide variety of molecules can react with radicals generated enzymatically, photochemically or by metal ion catalysis. It is thus important that the antioxidant can compete efficiently with such reactions. In the ESR assay developed, such competition was established between the spin trap, DMPO, and the flavonoid in question towards the generated peroxyl radicals. The first pK_a value of most flavonoids is close to pH 7.4 [4], and the phenolate anion, which is a better electron donor than the parent compound, will therefore be the active antioxidant in this assay. As can be seen from Table 1, none of the flavanones were able to compete efficiently with the spin trap. This can partly be explained by the fact that these compounds are the least reducing antioxidants. However, (±)-taxifolin, as well as the flavone luteolin and the flavanol (+)-catechin, are of similar reducing strength as kaempferol which displays excellent antioxidative activity. This difference in reactivity is accordingly of kinetic rather than thermodynamical nature and can be ascribed to a lower free energy of activation, resulting in a higher rate constant. Similar results were previously found for the deactivation of the hypervalent meat pigment, ferrylmyoglobin by flavonoids [6]. Although the five investigated flavonols are efficient peroxyl radical scavengers, there is no simple connection between reactivity and the reduction potential of the flavonoid phenoxyl radicals determined in phosphate buffer at pH 7.4 [6], and it should be noted that the observed scavenging effect of a flavonoid reflects the combined effect of the phenolate anion and its oxidation products. The initially formed flavonoid phenoxyl radical may also be an efficient peroxyl radical scavenger but may also be trapped by DMPO; quercetin radicals are, for instance, known to form a very stable spin adduct with DMPO [16]. A potential of +0.4 V seems, however, to present a critical level above which the flavonoids are becoming ineffective as radical scavengers in this assay.

The stopped-flow experiments with DPPH took place in neat methanol. In this solvent the flavonoid is present in its non-dissociated form as would be expected in pure lipid. Chrysin, naringenin and hesperetin, being the least reducing compounds in aqueous solution, were also the least efficient antioxidants in this assay. Eriodictyol, (±)-taxifolin, luteolin and (+)-catechin which have similar reducing strengths in aqueous solution, displayed quite similar but fairly good antioxidative activities, whereas the flavonols again were the most efficient antioxidants. The results show the rather small effect on antioxidative activity of adding a 2,3-double bond (i.e. from eriodictyol to luteolin), and the introduction of a 3-hydroxyl group in eriodictyol, leading to taxifolin, actually lowered the antioxidative activity, whereas the combined effect of a 2,3-double bond and a 3-hydroxyl group (i.e. eriodictyol to quercetin) improved the antioxidative activity significantly. It is, however, noteworthy that at flavonoid concentrations of 2 mM and 5 mM, the antioxidative hierarchy of the flavonols is the opposite of their reducing strength in aqueous solution, i.e. myricetin, which is the strongest reducing agent in aqueous solution is the least efficient antioxidant among the flavonols in methanolic solution. This strongly suggests that the phenolate anion is not involved in the reaction in methanol, since phenolic antioxidants presumably work by direct hydrogen atom donation in solvents like methanol and lipids [17].

Both electron transfer and hydrogen atom donation might be expected to occur in an o/w emulsion depending on the position of the flavonoid. Accordingly, half of the compounds were investigated in the oxygen consumption assay. Naringenin, being a poor electron donor as seen by its reduction potential and a poor hydrogen atom donor as seen from the $t_{1/2}$ values, was not surprisingly found to be the least efficient antioxidant in the oxygen consumption assay. The

nonpolar flavonoids luteolin, quercetin and kaempferol were expected to be dissolved in the oil droplets of the emulsion, which should be a favourable position to halt lipid autoxidation, since oxidation is most likely to occur in the oil–air interface. They were nevertheless found to be less efficient than the two most polar flavonoids, (+)-catechin and (±)-taxifolin, and the flavone luteolin was notably better than the two flavonols. The most polar flavonoids were expected to be diluted in the aqueous phase of the o/w emulsion leading to a lower effective concentration compared to the nonpolar flavonoids in the oil–air interface, but this homogenous distribution is apparently the most efficient in this assay where oxidation is initiated by the water-soluble prooxidant metmyoglobin. This clearly shows that polarity and state of dissociation play a major role in the observed antioxidative activity of a flavonoid; (+)-catechin and (±)-taxifolin were thus found to be poor antioxidants in the ESR assay, medium in the DPPH assay and the best in the oxygen consumption assay.

In conclusion, three different and complementary methods have been used to evaluate the antioxidative activities of different flavonoids. Two of the methods were based on reaction kinetics and showed that radical scavenging of two different types of radicals can be described as bimolecular reactions, the third method being of practical use in food formulation. Radical scavenging is only efficient below a threshold value for reducing capacity of +0.4 V, but kinetic factors as well as hydrophilic/lipophilic balance are also important for the antioxidative activity displayed.

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