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The antioxidant properties of theaflavins and their gallate esters – radical scavengers or metal chelators?

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Abstract The antioxidant properties of theaflavins and their gallate esters were studied by investigating their abilities to scavenge free radicals in the aqueous and lipophilic phases. The total relative antioxidant activities in the aqueous phase were assessed by measuring their direct $ABTS^{\bullet+}$ radical scavenging abilities, and by their efficacies in inhibiting the degradation of deoxyribose induced by iron. The propensities for enhancing the resistance of LDL to oxidation mediated by Cu^{2+} were also measured. The results show that the hierarchy of reactivity of these compounds as antioxidants is: theaflavin digallate > 3′-monogallate=3-monogallate > theaflavin. Spectroscopic studies show that all the compounds chelate iron and copper; enhanced absorbance in the visible region is observed in the case of the iron-digallate complex, but not with copper.

Key words: Theaflavin; Black tea; Metal chelator; Hydroxyl radical scavenger; Total antioxidant activity; Oxidized LDL

1. Introduction

A number of studies in vivo and in vitro have investigated the effects of green and black teas and their polyphenolic constituents as antioxidants and in in vivo models of risk of cardiovascular diseases and cancers or markers of lipid metabolism. Green tea consumption has been associated with lowered cardiovascular risk through decreased serum cholesterol and triglyceride, increased HDL and a decreased atherogenic index [1]. In vivo animal studies demonstrate that green tea stimulates hepatic UDP-glucuronyl transferase activity [2] and provides protection against kidney lipid peroxidation, whereas black tea-fed rats subsequently showed enhanced protection against peroxidation of liver [3]. Theaflavins also possess in vitro antioxidative properties against lipid peroxidation in erythrocyte membranes and microsomes [4] and suppress mutagenic effects induced by hydrogen peroxide, the gallic acid moiety of the theaflavins being essential for this activity [4]. Epigallocatechin gallate, one of the major green tea polyphenolic constituents, suppresses superoxide radical and hydrogen peroxide production by tumour promoter-activated human neutrophils [5].

Studies on the constituent catechin and catechin-gallate esters of green tea have reported that they are more effective antioxidants in vitro than vitamin C [6], with a sequence of reactivity against aqueous phase radicals, in the form of the $ABTS^{\bullet+}$ radical cation, of: Epicatechin gallate (ECG) ≈ epigallocatechin gallate (EGCG) > epigallocatechin (EGC) > epicatechin (EC) ≈ catechin (C), consistent with the number and arrangement of phenolic hydroxyl groups [7].

Their abilities to enhance the resistance of low density lipoprotein to oxidation and to prolong the efficacy of α-tocopherol within the LDL [6] is in the sequence

EGCG = ECG ≈ EC = C > EGC > GA

and this is consistent with their partition coefficients.

Theaflavins are formed during the manufacture of black and oolong teas from the enzymic oxidation of the flavanols, catechin and gallocatechins, etc. by polyphenol oxidase. The reaction involves the oxidation of the B rings to the quinones, followed by a Michael addition of the gallocatechin quinone to the catechin quinone, prior to carbonyl addition across the ring and subsequent decarboxylation [8]. In this study the antioxidant properties of theaflavins and their gallate esters (Fig. 1) are studied by investigating their abilities to scavenge free radicals in the aqueous and lipophilic phases.

2. Materials and methods

Theaflavin, theaflavin 3-monogallate, theaflavin 3′-monogallate and theaflavin digallate were gifts of Unilever Research. The purities of the compounds were theaflavin 98%, theaflavin 3-monogallate 96%, theaflavin 3′-monogallate 90% (of the other 10%, 90% is theaflavin 3-monogallate) and theaflavin digallate 87%, the sample also containing 13% theaflavin 3′-monogallate. The compounds are reported to be stable in the solid state. In most organic solvents, they are stable for up to 24 h in the dark; in low pH aqueous solution (pH < 4) they are stable for several days, but at higher pH (> 6) they will degrade rapidly. The compounds were stored at 4°C in the dark until use; stock solutions were prepared by dissolution in ultrapure (18 mΩ) water at a concentration of 5 mmol/l. The dilutions of the theaflavin stock solutions were prepared daily and the concentrations and spectral profiles checked spectrophotometrically between 200 and 800 nm in a Beckman DU 7500 Diode Array spectrophotometer.

2.1. Total antioxidant activity assay

The Trolox equivalent antioxidant capacity (TEAC) of theaflavins was estimated using the ferryl myoglobin/ABTS method for total antioxidant activity [9]. This technique measures the relative ability of antioxidant substances to scavenge the ABTS radical cation ($ABTS^{\bullet+}$) generated in the aqueous phase, compared with standard amounts of the synthetic antioxidant Trolox (6-hydroxy-2,5,8-tetramethyl chroman-2-carboxylic acid, Hoffman-La Roche), the water-soluble vitamin E analogue. The basic principle is the reduction of the blue-green $ABTS^{\bullet+}$ radical by electron- or hydrogen-donating antioxidants, which is measured by suppression of its characteristic long wave absorption spectrum with maxima at 645, 734 and 815 nm. $ABTS^{\bullet+}$ is generated through the peroxidatic action of metmyoglobin in the presence of ABTS, using metmyoglobin (2.5 μM), H_2O_2 (75 μM) and ABTS (150 μM) (final concentrations). Antioxidant compounds reduce $ABTS^{\bullet+}$, as detected by the suppression of the absorption of $ABTS^{\bullet+}$, to an extent and on a time scale dependent on the antioxidant capacity of the substance under investigation. The three different dilutions of each compound were freshly prepared daily and analysed in triplicate (i.e. 9 determinations) on separate days ($n = 3$). The TEAC was calculated for each dilution and the mean value (± the standard deviation) of all the results derived: the TEAC is defined as the millimolar concentration of a Trolox solution having the antiox-

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idant capacity equivalent to a 1.0 mM solution of the substance under investigation.

The interaction of the antioxidant under investigation with the $ABTS^{\bullet+}$ radical cation in this assay is substantiated by the following findings: that the standard curve with Trolox is linear [9,10]; that the reactivities of phenolics with ferryl myoglobin itself in comparison with the ferryl myoglobin-derived $ABTS^{\bullet+}$ radical cation have shown slower rates of reaction of the phenolics with the former [11]; and that the interaction of flavonoids with pre-formed $ABTS^{\bullet+}$ through a decolorisation assay (in the absence of ferryl myoglobin as described in [12]) and isoflavonoids with pre-formed $ABTS^{\bullet+}$ [13] gives comparable results with the ABTS/ferryl myoglobin assay.

2.2. Deoxyribose assay

The ability of the theaflavins to inhibit the iron-induced decomposition of deoxyribose was assessed [14,15]. Solutions of ferrous ammonium sulphate (1 mM) and 2-deoxyribose (10 mM) were prepared in ultra pure water (18 mΩ). The final reaction mixtures consisted of 0.8 mM deoxyribose, 10 mM phosphate-buffered saline (pH 7.4) and various concentrations of the tea polyphenols (from the original 5 mM stock solution) ranging from 0 to 100 μM; the reaction was initiated by addition of ferrous ammonium sulphate (final concentration 40 μM). Six controls were also included containing (final concentrations) 0.8 mM deoxyribose in PBS, 40 μM ferrous ammonium sulphate in PBS, a combination of the latter, 100 μM tea polyphenol in PBS, 100 μM tea polyphenol with 0.8 mM deoxyribose, 100 μM tea polyphenol with 40 μM ferrous ammonium sulphate. The solutions were incubated for 1 h at 37°C during which time the deoxyribose is degraded on exposure to hydroxyl radicals generated via the Fenton reaction; the degradation is suppressed to an extent dependent on the radical scavenging and/or iron-chelating properties of the polyphenols.

The radical-mediated decomposition of the deoxyribose to malonyldialdehyde (MDA) is assessed by reaction with thiobarbituric acid in acid solution. 1 ml of 1% (w/v) thiobarbituric acid (TBA) is added to the solution followed by 1 ml of 2.8% (w/v) trichloroacetic acid. After heating for 10 mins at 100°C and cooling, the absorbance of the pink chromophore is measured at 532 nm.

2.3. Spectroscopic studies

The stock solutions of theaflavins were diluted to 40 μM in phosphate buffer (pH 7.4) and combined with 40 μM copper (sulphate) or 40 μM iron (sulphate) derived from stock solutions of 0.5 mM initial concentrations. After 1 min at ambient temperature, spectra were measured on the Beckman diode array spectrophotometer at 30°C.

2.4. Inhibition of LDL oxidation

Low density lipoproteins were isolated from plasma using the modified method of Chung et al. [16]. Concentration of LDL protein was estimated according to Markwell et al. [17]. The efficacy of the different compounds in protecting LDL against oxidation was estimated using standard procedures [7,18] by incubating LDL (125 μg/ml LDL protein) with 3 μM copper for 4 h at 37°C in the presence or absence of the various compounds. The reaction was stopped by adding 20 μM butylated hydroxytoluene (BHT) (final concentration).

LDL oxidation was measured by monitoring changes both in the surface charge of apolipoprotein B_{100} and in decomposition products of lipid peroxidation. The modification in the surface charge of apolipoprotein B_{100} was measured as relative electrophoretic mobility (REM), which is the ratio of the electrophoretic mobility of the treated sample to that of native LDL, using the Beckman Paragon LIPO electrophoresis kit. Lipid peroxidation was measured by determining the breakdown products of lipid peroxides using the thiobarbituric acid assay [19]: 0.5 ml of trichloroacetic acid (TCA) and 0.5 ml of thiobarbituric acid (0.75% in 0.1 N hydrochloric acid) (TCA), prepared daily, were added to 0.5 ml of the samples. The mixture was heated for 20 mins at 90°C and, after cooling, centrifuged at 3000 rpm for 15 mins. The absorbance was measured at 532 nm.

3. Results and discussion

The total antioxidant potentials or Trolox equivalent antioxidant activities of the theaflavins were determined by the $ABTS^{\bullet+}$ assay, relative to that of Trolox. The results (Table 1) show that the effectiveness of theaflavin as an antioxidant is increased by esterification with gallate and is further enhanced as the digallate ester. This can be predicted from previous studies on the catechins and catechin-gallate esters [6] showing that, in the case of the flavanols, increasing numbers of hydroxyl groups as *ortho*-diphenolics or triphenolics, as with incorporation of gallate esters or in the gallocatechins, progressively augments the antioxidant activities of these polyphenols against radicals generated in the aqueous phase.

The relative extents of free radical-mediated inhibition of deoxyribose degradation will give an indication of hydroxyl radical scavenging potential and/or iron chelating propensity. The effects as a function of theaflavin concentration are shown in Fig. 2. At a concentration equimolar with iron in the assay (40 μM), there is only approx. 6% inhibition for theaflavin, whereas for theaflavin 3-monogallate and theaflavin 3′-monogallate the extent of inhibition is between 50 and 60% suggesting that theaflavin is relatively a weaker inhibitor of deoxyribose degradation than the monogallate esters. The digallate shows 43% inhibition at 20 μM, a level at which there is minimal response from the monogallates and the theaflavin itself. At the concentrations applied here, hydroxyl radical scavenging by the compounds is unlikely since they are competing with 0.8 mM deoxyribose; iron chelation is the most likely explanation. That the iron-binding is more likely with the gallate moieties rather than the theaflavin hydroxyl groups is suggested by the concentration of the compounds giving 50% inhibition, being 20 μM for the digallate, and approximately twice this for each of the monogallates. Theaflavin itself is approx. 4-times less efficacious than the digallate, at 85–90 μM. Applying a hydroxyl radical scavenger, that is not an iron chelator, ethanol, at equivalent concentrations shows no inhibition of deoxyribose degradation. It has been suggested [20] that using iron(II) to initiate the deoxy-

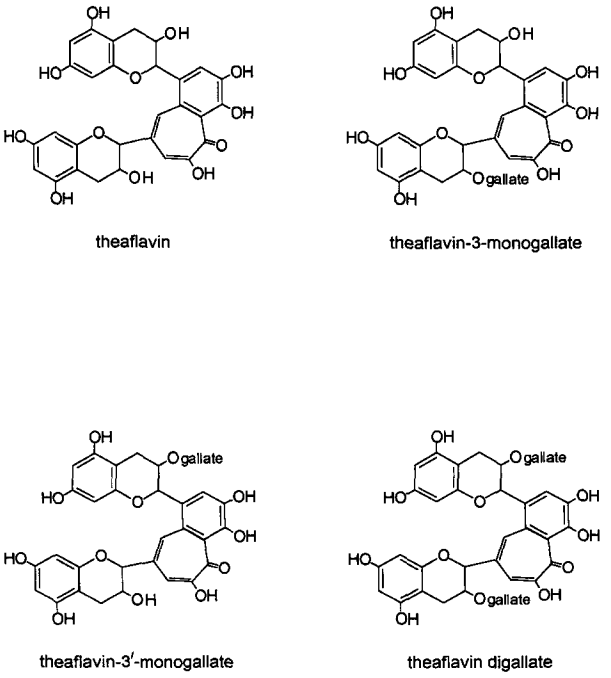


Fig. 1. Structures of theaflavin and the gallate esters.

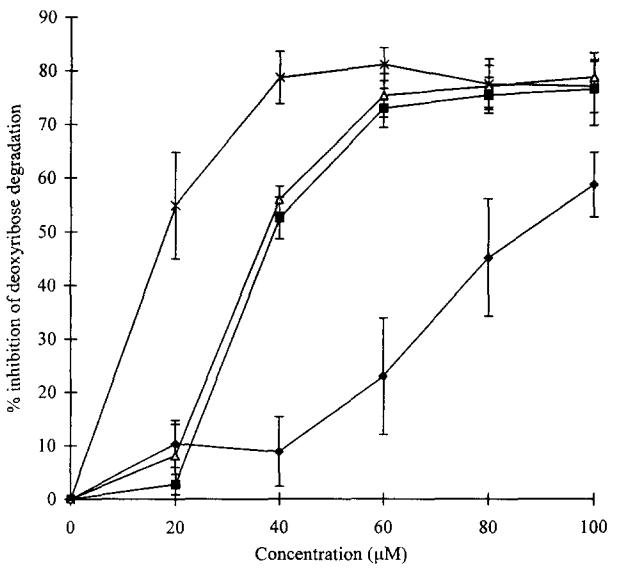


Fig. 2. Concentration dependence of the percentage inhibition of deoxyribose degradation by theaflavin (♦), theaflavin 3-monogallate (■), theaflavin 3′-monogallate (△) and theaflavin 3,3′-digallate (×) ($n=5$ or 6). Final concentrations: iron(II) = 40 μM, deoxyribose 0.8 mM.

ribose reaction might be the origin of many of the pro-oxidant effects of phenols; if the phenol reduced iron(III) to iron(II), it could continue the degradation reactions and give artificially low inhibition of deoxyribose degradation. This was tested by replacement of iron(II) in the deoxyribose assay by iron(III). The presence of the phenols did not modulate the lack of effect of iron(III) to promote deoxyribose degradation.

In order to clarify the ability of the theaflavin compounds to bind iron and copper, their interactions (both 40 μM final concentration) were studied spectroscopically. A violet complex was observed with all the theaflavin compounds in combination with iron resulting in a new peak in the visible region of the spectrum (Table 2). The peak absorbances were progressively blue-shifted and enhanced with increasing incorporation of gallate moieties into the theaflavins. Specifically, for theaflavin in the presence of iron a new peak appeared at 550 nm with an absorbance of 0.13 relative to that in the absence of iron, 0.015. With theaflavin 3-monogallate and iron, the peak absorbance of the complex occurred at 524 nm, with increased mean absorbance of 0.13. The absorbance of the theaflavin 3′-monogallate iron complex at 530 nm was more pronounced with a mean increased absorbance of 0.15. With theaflavin 3,3′-digallate, the peak absorbance occurred at 511 nm with a mean increase in absorbance of 0.18.

On addition of copper ions to the theaflavins an orange/purple complex was formed. The peak of the theaflavin-copper complex occurred at 538 nm where the mean enhanced absorbance of the new peak was 0.080. With theaflavin 3-monogallate, the increase in peak absorbance of the copper complex at 521 nm was 0.11 ($n=3$). Again a difference was observed between theaflavin 3-monogallate and theaflavin 3′-gallate in that the peak absorption of the complex occurred at 514 nm with an absorbance increase of 0.08. With theaflavin 3,3′-digallate the peak of the copper complex occurred at 494 nm with a mean absorbance enhancement of 0.10. The binding of copper to theaflavins and their gallate esters is demonstrated by the shift in the peak absorbance, however, the comparison of the gallate esters with the theaflavin itself suggests that the gallate moiety does not enhance the complexing of copper.

Overall the spectral results show enhanced association of the gallate esters with iron, as judged by the intensity of the iron-polyphenol complexes in the visible region, the digallate giving a higher absorbance than the monogallates, which display a peak at a higher intensity than theaflavin itself. The absorbance of new spectral peaks in the 500–550 nm region on interaction with copper are much the same for all four theaflavins, the gallate components making no difference. This suggests that iron may be chelating mainly through the gallic acid component of the molecules whereas the copper might chelate with the hydroxyl groups on the polyphenolic rings of the basic theaflavin molecule, independently of the gallate group. This is substantiated by studies with gallic acid showing no coloured complex formation with copper in the visible region and minimal change in the spectrum, in contrast with the iron-gallate complex formed. The violet complexes with iron(II) salts are not observed on substitution of iron(II) with iron(III), nor is the clear peak observed in the visible region.

The concentration dependence of the antioxidant ability of theaflavins against LDL oxidation was investigated by studying the enhancement of the resistance of LDL to oxidation by interacting LDL (125 μg LDL protein/ml) with copper (3 μM) final concentration at pH 7.4. The order of effectiveness of the four theaflavins to inhibit LDL oxidation was assessed by measurement of breakdown products of lipid peroxidation, the thiobarbituric acid assay, and the modification of the surface charge on the apolipoprotein B_{100}, by electrophoresis.

The results of their abilities to inhibit LDL oxidation are shown as the concentrations (μM) for 50% inhibition of lipid peroxidation and altered surface charge (Table 1) indicating the sequence of reactivity as: theaflavin 3,3′-digallate > theaflavin 3-monogallate ≈ theaflavin 3′-monogallate > theaflavin.

While the gallate esters show small differences in their reactivities, theaflavin is very distinctively less efficacious in scavenging peroxyl radicals. This may relate not only to its lower antioxidant activity in terms of content of phenolic hydroxyl groups, but also to its partitioning properties and accessibility to the peroxyl radicals. It is unlikely that the differences can be accounted for on the basis of differential affinities of theaflavin and its gallate esters for binding copper, since the results described earlier show their very similar extents of complex formation in this respect. Thus, these com-

Table 1
Antioxidant activities of the theaflavin and the gallate esters against $ABTS^{\cdot+}$ radicals in the aqueous phase (TEAC) and in inhibiting the oxidation of LDL

	TEAC (mM)	*C*50% (μM)
Theaflavin	2.94 ± 0.08	5.5 ± 0.3
Theaflavin 3-monogallate	4.65 ± 0.16	2.8 ± 0.0
Theaflavin 3′-monogallate	4.78 ± 0.19	3.4 ± 0.2
Theaflavin 3,3′-digallate	6.18 ± 0.43	2.5 ± 0.2

TEAC ± S.D., n=6, *C*50% ± S.D., n=3–4. LDL (125 μg LDL protein/ml) was oxidised with 3 μM Cu^{2+} for 4 h at 37°C. The reaction was stopped with 20 μM BHT (all final concentrations).

Table 2
Spectroscopic studies of the copper- and iron-chelating properties of the theaflavins

Theaflavin		+Cu^{2+}		+Fe^{2+}	
λ (nm)	A_λ	λ (nm)	A_λ	λ (nm)	A_λ
Theaflavin					
267	0.63 ± 0.07	274	0.71 ± 0.03	272	0.80 ± 0.06
368	0.21 ± 0.03	398	0.24 ± 0.024	390sh	0.26 ± 0.04
456	0.091 ± 0.01				
538	0.021 ± 0.004	538	0.10 ± 0.009		
550	0.015 ± 0.004			550	0.13 ± 0.02
Theaflavin 3-monogallate					
270	0.78 ± 0.03	279	0.86 ± 0.04	277	0.83 ± 0.11
366	0.20 ± 0.004	310	0.79 ± 0.04	303sh	0.77 ± 0.11
453	0.091 ± 0.003	404sh	0.30 ± 0.006	393	0.25 ± 0.025
521	0.034 ± 0.002	521	0.14 ± 0.005		
524	0.033 ± 0.003			524	0.16 ± 0.01
Theaflavin 3′-monogallate					
271	0.84 ± 0.014	277	0.82 ± 0.02	279	0.82 ± 0.03
368	0.22 ± 0.004	312	0.73 ± 0.02	304	0.80 ± 0.02
454	0.091 ± 0.004	404	0.25 ± 0.15	392sh	0.26 ± 0.004
514	0.044 ± 0.003	514	0.12 ± 0.014		
530	0.032 ± 0.003			530	0.18 ± 0.008
Theaflavin 3,3′-digallate					
272	1.23 ± 0.03	275	1.154 ± 0.06	279	1.18 ± 0.05
368	0.261 ± 0.006	317	1.00 ± 0.05	305	1.13 ± 0.04
455	0.115 ± 0.009			389sh	0.38 ± 0.007
494	0.072 ± 0.02	494	0.17 ± 0.01		
511	0.066 ± 0.01			511	0.25 ± 0.017

Concentrations of theaflavins and metal ions 40 μM (pH 7.4) (absorbance values mean ± S.D., $n = 3$).

pounds are significantly less efficacious than the monomeric catechins and catechin-gallate esters in enhancing the resistance of LDL to oxidation [6], but this may relate to accessibility to the peroxyl radicals as well as their antioxidant properties. This is consistent with the comparison made by Miura et al. [21] studying oxidation of porcine LDL in which they noted that theaflavin was a weaker antioxidant than EGCG.

Several reports have contributed to the growing debate concerning transition metal chelation by polyhydroxy compounds. Primarily, discussion has centred on the flavonols, and for copper chelation the importance of the 3-hydroxy, 4-keto function in the C ring with the 5-OH group in the A ring [22,23]. Earlier work of Thompson and Williams [24] showed that the chelation stability constant for copper is higher for the 5-OH group of the flavones than the 3-OH group due to the higher stability of the 6-membered chelate ring. The catechol structure plays a key role in iron chelation and the 3-OH, 4-keto moiety also functions as a chelation site [25]. The work described here concerns structures containing a 3,4-dihydroxy structure and the trihydroxygallate structures.

The results show that the hierarchy of the reactivities of the theaflavin compounds as antioxidants against radicals generated in the aqueous phase is the same as their sequence of effectiveness in inhibiting the oxidative degradation of deoxyribose and in inhibiting Cu^{2+}-mediated LDL oxidation. All the compounds chelate copper, the presence of gallate in the structures having no effect on the absorbance of the metal-polyphenol complex, whereas the additional gallate content apparently allows further complexation of iron with the polyphenol, as indicated by the enhanced absorbance for the digallate.

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References

[1] Imai, K. and Nakachi, K. (1995) Br. Med. J. 310, 693–696.
[2] Buabbai, A., Clifford, M.N. and Joannides, C. (1995) Food Chem. Toxicol. 33, 27–30.
[3] Sano, M., Takahashi, Y. and Yoshino, K. (1995) Biol. Pharm. Bull. 18, 1006–1008.
[4] Shiraki, M., Hara, Y., Osawa, T., Kumon, H., Nakayama, T. and Kawakishi, S. (1994) Mutat. Res. 323, 29–34.
[5] Troll, W., Lim, J.S. and Frenkel, K. (1994) A.C.S. Symp. Ser. 547, 116–121.
[6] Salah, N., Miller, N.J., Paganga, G., Tijburg, L., Bolwell, G.P. and Rice-Evans, C. (1995) Arch. Biochem. Biophys. 322, 339–346.
[7] Rice-Evans, C., Bruckdorfer, K.R., Leake, D. and Diplock, A.T. (1996) Free Radical Res., in press.
[8] Balentine, D. (1992) in: Phenolic Compounds in Food and their Effects on Health (Chi-Tang, H., Lee, C.Y., Huang M.-T. eds.) Am. Chem. Soc., Washington, DC, pp. 102–117.
[9] Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V. and Milner, A. (1993) Clin. Sci. 84, 407–412.
[10] Miller, N. and Rice-Evans, C. (1996) Redox Rep. 2, 161–171.
[11] Castelluccio, C., Paganga, G., Melikian, N., Bolwell, G.P., Pridham, J., Sampson, J. and Rice-Evans, C. (1995) FEBS Lett. 368, 188–192.
[12] Miller, N.J., Sampson, J., Candeias, L., Bramley, P. and Rice-Evans, C. (1996) FEBS Lett. 384, 240–242.
[13] Miller, N.J., Ruiz-Larrea, M.B. and Rice-Evans, C. (1996) Abstract, International Society for Free Radical Research, Barcelona, October 1–6.
[14] Halliwell, B., Grootveld, M. and Gutteridge J.M.C. (1988) Methods Biochem. Anal. 33, 59–90.

[15] Halliwell, B. and Gutteridge, J.M.C (1981) FEBS Lett. 128, 347–352.
[16] Chung, B.H., Wilkinson, T., Geer, J.C. and Segrest, J.P. (1980) Lipids 21, 284–291.
[17] Markwell, M.A., Haas, S.M., Bieber, L.L. and Tobert, N.E. (1978) Anal. Biochem. 87, 106–210.
[18] Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) Free Radical Biol. Med. 13, 341–390.
[19] Walls, R., Kumar, K.S. and Hochstein, P. (1976) Arch. Biochem. Biophys. 172, 463–468.
[20] Laughton, M.J., Halliwell, B., Evans, P.J. and Hoult J.R.S. (1989) Biochem. Pharmacol. 38, 2859–2865.
[21] Miura, S., Watanabe, J., Sano, M., Tomita, T., Osawa, T., Hara, Y. and Tomita, I. (1995) Biol. Pharm. Bull. 18, 1–4.
[22] Hudson B.J.F. and Lewis, J.I. (1983) Food Chem. 10, 47–55.
[23] Shahidi F.J.P.K and Wanasundara, P.D. (1992) Crit. Rev. Food. Sci. Nutr. 32, 67–103.
[24] Thompson, M. and Williams, C.R. (1976) Anal. Chim. Acta 85, 375–381.
[25] Van Acker, S.A.B.E, Van den Berg, D.-J., Tromp, M.N.J.L., Griffioen, D.H., Van Bennekorn, W.P., Van der Vijgh, W.J.F. and Bast, A. (1996) Free Radical Biol. Med. 20, 331–342.