Research Article

# A novel method to measure both the reductive and the radical scavenging activity in a linoleic acid model system

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The aim of this study was to develop a combined method for measuring the total antioxidant activity, the reductive and the radical scavenging activity. Linoleic acid was used as the substrate for an iron-initiated lipid peroxidation to measure the total antioxidant activity. In addition, methyl esters of linoleic acid hydroperoxides were used as substrates to measure the reductive antioxidant activity. The radical scavenging antioxidant activity was calculated by subtracting the reductive antioxidative activity from the total antioxidative activity. As representative examples, the antioxidants α-tocopherol, ascorbic acid, trans-resveratrol and L-gluthatione as well as commonly used food additives such as 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) were analyzed. The results for the novel antioxidation test showed that α-tocopherol, BHA and BHT are primarily acting as radical scavengers, whereas ascorbic acid and L-gluthatione show a strong reductive capacity. As linoleic acid as well as its hydroperoxides both are present in foods and in the organism, the test presented here can be considered representative of radical reactions occurring in food matrixes and *in vivo*. Further experiments are required to document the comprehensive applicability in foods and *in vivo*.

**Keywords:** Antioxidant test / Linoleic acid hydroperoxides / Radical scavenging activity / Reductive activity

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## 1 Introduction

There is increasing interest in the antioxidant activity of compounds present in foods. Antioxidants are believed to play an important role in the body's defense system against reactive oxygen species (ROS). Humans, like all aerobic organisms, derive most of their metabolic energy from the reduction of oxygen and are, consequently, susceptible to the damaging effects of ROS that are produced during the metabolism of oxygen. The term ROS refers to a heterogeneous group of compounds, including superoxide anions, hydroxyl radicals, and hydrogen peroxide, as well as unstable intermediates produced in the course of the peroxidation of lipids. ROS can react with nearly all biological macromolecules, such as lipids, proteins, nucleic acids, and carbohydrates [1]. The initial reaction generates a second radical, which can, in turn, react with a second macromolecule to continue the chain reaction. As protection against the deleterious effects of ROS, the human body has developed an antioxidant defense system that includes enzymatic, metal-chelating, and free radical-scavenging activities. In addition, intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system. A marginal dietary intake of antioxidants may lead to an increased production of ROS, which have been demonstrated to accelerate the aging process and are involved in the onset of many diseases, including diabetes mellitus type II, inflammatory diseases, arteriosclerosis and different types of cancer [2].

Fruits and vegetables are the most important sources of exogenous antioxidants. Their nutritional values are normally quantified by the total amount of certain components such as “total fat”, “total calories”, and “total carbohydrate”, which are labeled in the nutrition facts sheet appearing on food packages. These indexes are intended to provide useful nutritional information to consumers. Although

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**Abbreviations**: **ROS**, reactive oxygen species; **TE**, trolox equivalents; **TEAC**, Trolox equivalent antioxidant capacity

antioxidants are recognized as important phytonutrients, currently there is no "total antioxidant" as a nutritional index available for food labeling because of the lack of standard quantitation methods. One of the explanations might be that, unlike other nutrients, antioxidants are chemically diverse. Some of the most common antioxidants present in vegetables are the vitamins C and E, carotenoids, flavonoids, and thiol compounds. The chemical diversity of antioxidants makes it difficult to define one analytical method that can be used as a "golden standard" when the total antioxidant activity of one compound or even a complex food matrix has to be assessed [3]. Recently, several methods have been developed to measure "total antioxidant activity" [4], "total antioxidant capacity" [5, 6], or "total antioxidant potentials" [7, 8]. Among them, Trolox equivalent antioxidant capacity (TEAC) [9], total radical absorption potentials (TRAP) [10], ferric reducing/antioxidant power (FRAP) [11], and oxygen radical absorption capacity (ORAC) assays [12] are the representative ones. Mechanistically, these methods are based on either a single-electron transfer reaction or a hydrogen atom transfer reaction between an oxidant and a free radical. For the single-electron transfer reaction-based methods, such as FRAP and TEAC, antioxidants are oxidized by oxidants such as Fe (II) or stable radicals such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS radical cation). As a result, a single electron is transferred from the antioxidant molecule to the oxidant. The change of absorbance of either antioxidant or oxidant is measured and the absorbance value is used as the quantitation for the reducing capability of the antioxidant.

Methods based on hydrogen atom transfer reactions, such as ORAC and TRAP, utilize a radical initiator to generate a peroxyl radical that preferentially abstracts a hydrogen atom from the antioxidant. As a result, the reaction between the peroxyl radical and the target molecule probe is retarded or inhibited.

Depending on their chemical structure, some antioxidants such as vitamin C (ascorbic acid) posses a distinct potential for undergoing reductive, hydrogen atom transfer (HAT) reactions, which can be termed "reductive activity". Another group of antioxidants, such as vitamin E isomers (tocopherols), react in radical scavenging single-electron transfer (SET) modes, which can be termed as "radical scavenging activity". The relative importance of these two mechanisms depends on the target radicals and the environment. When the total antioxidant activity of antioxidants is compared based on data analyzed by the different test systems mentioned above, the results very often differ substantially, depending on the assay applied. Moreover, no analytical method is available by which the reductive and the radical scavenging activity can be measured in the same system, following comparable reaction kinetics.

In this report, for the first time, a method is described by which the reductive activity and the radical scavenging activity are measured in one test system based on the well-known iron-catalyzed oxidation of linoleic acid. This model system may be representative for oxidations occurring in cellular membranes in which polyunsaturated fatty acids are predominantly prone to ROS-attacks. The basis for this novel method is the antioxidant test system described by Bright *et al.* [13]. The principle is an inhibitory effect on the peroxidation of linoleic acid by a given antioxidant. The antioxidant first inhibits the formation of linoleic acid hydroperoxides by scavenging hydroxyl radicals and, in turn, inhibits the formation of the color indicator methylene blue by reduction of the linoleic acid hydroperoxide to the linoleic acid hydroxide (Fig. 1). In one of our previous studies, this test was used to identify the key antioxidant in bread crust [14]. However, only the total antioxidant activity was measured at that time. In the present study, the main objective was to modify the test system of Bright *et al.* [13] in such a way that the radical scavenging, the reductive activity and the total antioxidant activity of a given antioxidant can be measured. For this purpose, methyl esters of linoleic acid hydroperoxides were chemically synthesised and applied in the modified test in order to determine the reductive activity. The total antioxidant activity of some of the most studied antioxidants such as the vitamins E and C was measured by applying the conventional method of Bright *et al.* [13], the reductive activity was analyzed in the same test by replacing linoleic acid by methyl esters of linoleic acid hydroperoxides, and the radical scavenging activity was then calculated by subtracting the value obtained for the reductive activity from that obtained for the total antioxidant activity.

## 2 Materials and methods

### 2.1 Chemicals

The following compounds were obtained commercially: ascorbic acid, hydrogen peroxide, Fe(II) sulfate, EDTA, DMF, methanol, ethanol, isopropanol, n-hexane, ACN and deutero-chloroform from Merck (Darmstadt, Germany). DTT, L-glutathione, and Tween 20 were obtained from Serva (Heidelberg, Germany). Catechin, hemoglobin, linoleic acid, linoleic acid methyl ester, trans-resveratrol and Triton X-100 were purchased from Sigma (Deisenhofen, Germany). Benzoyl leucomethylene blue, trolox and 2,6-di-*tert*-butyl-p-cresol were bought from Sigma/Aldrich and DL-α-tocopherol from Riedel-de Haen (Deisenhofen, Germany). All solvents were of gradient quality.

### 2.2 Preparation of the linoleic acid hydroperoxides

For the autoxidation of linoleic acid, its methyl ester (500 mg) was covered with oxygen and incubated at 40°C for 72 h in the dark. Afterwards, the batch was dissolved in

 

1 mL methanol and used for further clean-up steps. To separate the unreacted linoleic acid methyl ester from the generated hydroperoxides, a preparative TLC with silica gel was performed (PSC-plates 20 6 20 cm Silica gel 60 $F_{254}$, 0.5 mm; Merck). As mobile solvent, a mixture of diethyl ether and hexane (3:2 v%) was used. The band of the monohydroperoxide was isolated and extracted with methanol. The batch was then centrifuged at 4000 rpm, 10°C (Jouan GR 4–12, Unterhaching, Germany) and the collected supernatants were evaporated by $N_2$ flow. The residue with a recovery of 40% was dissolved in methanol and stored at –30°C for further steps. The purity of this solution was controlled by RP-HPLC analysis monthly and the solution was stable for about 6 months.

### 2.3 Characterization of methyl esters of linoleic acid hydroperoxides

The characterization of the methyl esters of linoleic acid hydroperoxides was performed by LC/MS, NMR and comparison of the HPLC retention time with data from the literature after separation with NP-HPLC.

### 2.4 HPLC

The HPLC apparatus (BIO-TEK Instruments, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800), a Rheodyne injector (100-L loop) and a DAD (type 540+), monitoring the effluent in a wavelength range between 210 and 500 nm. NP-separations were performed dependent on the conditions reported by Chan and Levett [15]. Briefly, a stainless steel column packed with NP material (Hypersil SILICA 120 A, 5 m, Germany), either in an analytical (4.6 240 mm, 1.0 mL/min), or a semi-preparative scale (10 250 mm, 3.0 mL/min) were used for the analytical and the semi-preparative runs. For the separation, isocratic conditions were used. The solvent was 1% 2-propanole in n-hexane (both 0.1 acetic acid) and was applied as mobile phase for 20 min.

### 2.5 HPLC/MS

An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT, Bremen, Germany) electrospray in the positive mode (ESI). After injection of the sample (2–20 µL), analyses were performed using the gradient described above. Loop analysis carried out at a constant flow of 8 µL/min.

### 2.6 NMR

The $^1$H, DQF-COSY, HMQC, HMBC spectroscopy were performed on an AMX 400 spectrometer (Bruker, Rheinstetten, Germany).

### 2.7 Measurement of the antioxidant activity *in vitro*

Following a procedure reported in the literature [13], the antioxidant activity of the antioxidants selected was determined *in vitro* by measuring their inhibitory effect on linoleic acid peroxidation.

First, the linoleic acid substrate was prepared by dropwise adding linoleic acid (0.125 mL) to a mixture of de-oxygenated borate buffer (2.5 mL; 50 mmol/L; pH 9.0) and Tween 20 (0.125 mL). Then, aqueous potassium hydroxide solution (0.65 mL; 1 mol/L) was added to clarify the suspension. Thereafter, the mixture was diluted with de-oxygenated borate buffer (21.6 mL; 50 mmol/L; pH 9.0) and made up to 50 mL with de-oxygenated distilled water. An aliquot (50 µL) of a solution of the antioxidant to be analyzed (1 mmol/L in 15% aqueous ethanol) was added to a solution consisting of oxygen-saturated phosphate buffer (3 mL; 0.2 mol/L; pH 6.75), ferrous-II sulfate (100 µL; 16 mmol/L; containing 15 mmol/L EDTA) and linoleic acid substrate (1.0 mL; 8 µmol linoleic acid). After adding hydrogen peroxide solution (100 µL; 16 mmol/L), the mixture was incubated at room temperature for 10 min and an aliquot (1.0 mL) of the incubated mixture was pipetted into disposable cuvettes (1 cm id) containing a solution (2.0 mL) of the color reagent consisting of DMF (8%), Triton X-100 (1.4%), hemoglobin (56 mg/L) and benzoyl leucomethylene blue (51 mg/L) in de-oxygenated phosphate buffer (0.2 mol/L, pH 5.0). After an additional incubation time of 30 min, the absorbance was measured at $\lambda = 666$ nm against the buffer/color reagent blank.

The analysis of the measured absorbance was performed using an external seven-point-calibration curve with Trolox solutions (0,1–2 mmol/L in 15% aqueous ethanol) and the antioxidant activity of the sample was expressed as trolox equivalents (TE values).

For measuring the reductive activity, the mixture of the four synthesized isomers of the methyl esters of linoleic acid hydroperoxides were drop wise added (400 µmol dissolved in 2.5 mL methanol) to a mixture of de-oxygenated borate buffer (2.5 mL; 50 mmol/L; pH 9.0) and Tween 20 (0.125 mL). Then, the mixture was diluted with de-oxygenated borate buffer (21.6 mL; 50 mmol/L; pH 9.0) and made up to 50 mL with de-oxygenated distilled water. The further steps were accomplished as described above. The de-oxygenated solutions were prepared by constant nitrogen bubbling for 30 min.

Data are expressed as mean ± SD.

## 3 Results and discussion

Based on the original method published by Bright *et al.* [13], linoleic acid was used as the substrate for the iron-induced oxidation. The compound to be tested for its reduc-

 

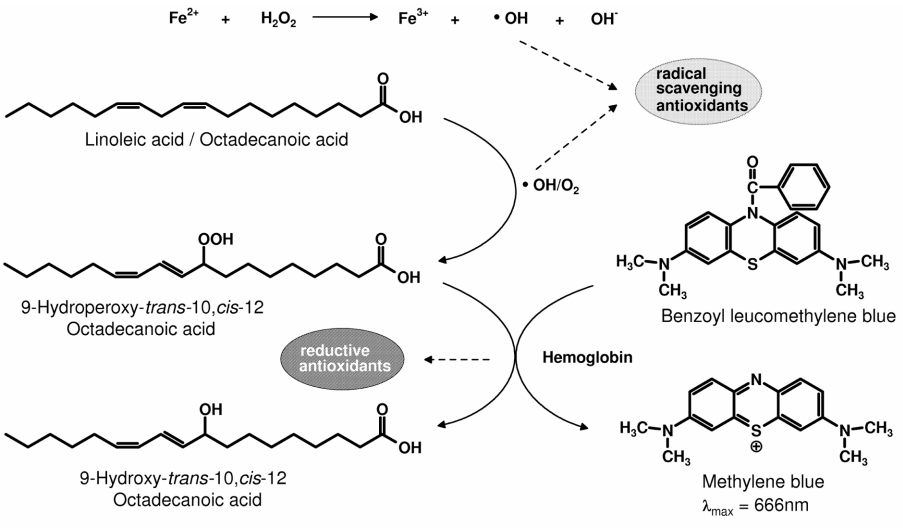


**Figure 1.** Reaction mechanism of the leucomethylene blue method.

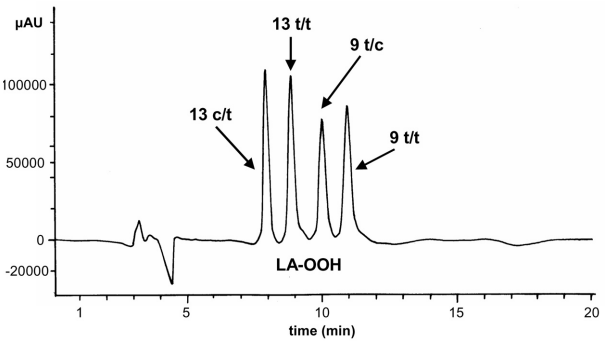


**Figure 2.** Normal phase-HPLC-chromatogram of methyl esters of linoleic acid hydroperoxides.

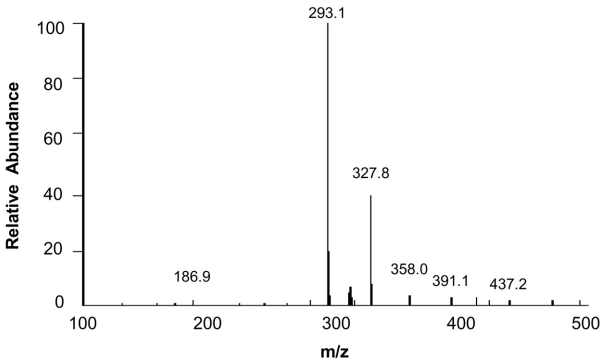


**Figure 3.** LC-ESI(+)-mass spectrum of 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate.

ing activity reacts with the hydroxyl radicals generated in the course of this Fenton-type reaction, preventing linoleic acid from being oxidized into linoleic acid hydroperoxides. This test was then extended by using methyl esters of linoleic acid hydroperoxides with which the compound to be tested reacts on the basis of its reducing activity (Fig. 1). As the methyl esters of linoleic acid hydroperoxides are commercially not available, the first step was to synthesize these substrates. For this purpose, linoleic acid methylester was autooxidized at 40°C for 72 h in the dark. The unreacted linoleic acid methyl ester were separated by preparative TLC on a silica gel. After isolation of the monohydroperoxides with a yield of about 40%, a characterization of the oxidation products was performed in order to prove the correct structure for the use in the reductive antioxidation test. The individual isomer oxidation products were separated by NP-HPLC (Fig. 2) following the method described by Chan and Levett [15]. The four different isomers identified according to the HPLC-retention times published by Chan and Levett [15] were 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate, 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate, 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate and 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate. As Chan and Levett [15] did not elucidate the structure of the LA-OOH isomers, in the present study, the molecular masses of these compounds were determined by LC/MS in the positive mode, using electrospray as the ionization method. As result, four identical fragmentation patterns with a molecular mass peak of 327.8 *m/z* and another fragment of 293.1 *m/z*, indicating the elimination of a hydroperoxide group, were obtained (Fig. 3). To verify the structure of the monohydroperoxides for the use for the reductive antioxidation test, a characterization by NMR-spectrometry was also performed (data not shown).

Having the synthesized methyl esters of linoleic acid hydroperoxides, the reductive activity was analyzed by replacing linoleic acid as oxidation substrate in the antioxidant test published by Lindenmeier [14]. Any given antioxidant exhibiting reducing activity reacts with the methyl esters of linoleic acid hydroperoxides (Fig. 1) to form the respective hydroxides. Prior to the use of a well-known antioxidant, the conversion of linoleic acid into linoleic acid hydroperoxides was determined.

Assuming that all of the linoleic acid is being oxidized in the absence of an antioxidant, the absorption of methylene

 

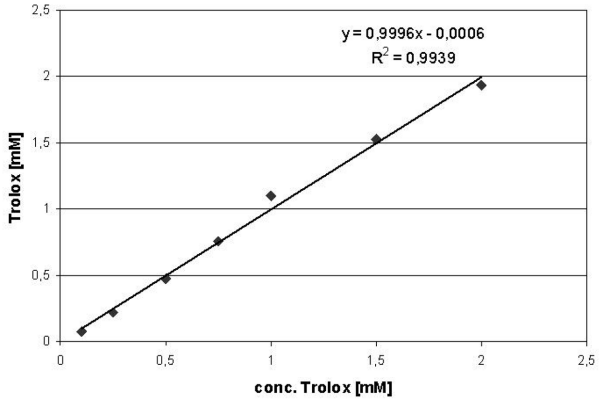


**Figure 4.** Recovery of trolox in the antioxidant test.

**Table 1.** Total antioxidant activity of selected antioxidants (*n* =3, mean ± SD)

| Antioxidant | | Total antioxidant activity trolox equivalents [mmol] |
|---|---|---|
| Trolox | [1 mM] | 1.01 ± 0.01 |
| *Tert*-Butyl-p-hydroxyanisol (BHA) | [1 mM] | 1.39 ± 0.08 |
| 2,6-Di-*tert*-butyl-p-cresol (BHT) | [1 mM] | 1.08 ± 0.03 |
| Trans-resveratrol | [1 mM] | 1.16 ± 0.19 |
| α-Tocopherol | [1 mM] | 0.89 ± 0.11 |
| Catechin | [1 mM] | 1.71 ± 0.17 |
| DTT | [1 mM] | 0.55 ± 0.10 |
| L-Glutathione | [1 mM] | 0.46 ± 0.06 |
| Ascorbic acid | [35 mM] | 1.61 ± 0.15 |

blue (Fig. 1) should be equal to that when the methyl esters of linoleic acid hydroperoxides are used as substrates. Indeed, this result was achieved using equimolar concentrations (8 µmol) of linoleic acid and its hydroperoxides.

Based on these results, well-known antioxidants were analyzed for their total antioxidant and their reductive activity. The radical scavenging activity was calculated as the difference between the total antioxidant and the reductive activity.

First, the linearity and the day-to-day precision of the test system was investigated using linoleic acid and the monohydroperoxides mixture as oxidation substrate and trolox as an antioxidant (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The results for the linoleic acid are shown in Fig. 4. The linearity of the test system for concentrations between 0.1 and 2 mM trolox equivalents (TE-values) was $r^2 = 0.9939$. The linear equation with a slope of 0.9996x and an y-intercept of −0.0006 confirmed the accuracy of the test. The day-to-day variance was below 5%.

The total antioxidant activity of selected well-known antioxidants was analyzed using linoleic acid as oxidation substrate (Table 1). With the exception of ascorbic acid, of which a concentration of 35 mM was used, each compound

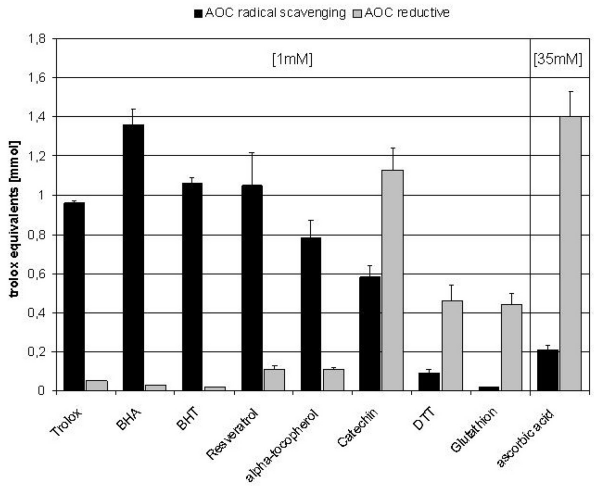


**Figure 5.** Radical scavenging and reductive activity of selected antioxidants.

was analyzed at a concentration of 1 mM. As a result, mean TE values of 0.89 and 1.01 were analyzed for α-tocopherol and trolox, respectively. Among the antioxidants tested, the highest mean TE value with 1.71 was determined for the polyphenol catechin, whereas for the tri-stilbene trans-resveratrol, a mean TE value of 1.16 was determined. The synthetic antioxidants 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) showed also high total antioxidant activities with mean TE values of 1.39 and 1.08, respectively. For the reductive agents DTT and L-glutathione (GSH), substantially lower mean TE values of 0.55 and 0.46, respectively, were obtained. With a mean TE value of 1.61 achieved by a concentration 35 times higher than all the other compounds tested at, the lowest total antioxidant activity was demonstrated for ascorbic acid.

The following experiments were carried out with the same antioxidants in the respective concentrations but with the use of methyl esters of linoleic acid hydroperoxides replacing linoleic acid. The results for this reductive activity and the calculated radical scavenging activity (TE value$_{\text{total antioxidant activity}}$ − TE value$_{\text{reductive activity}}$ = TE value$_{\text{radical scavenging activity}}$) of the selected antioxidants are displayed in Fig. 5. As the most powerful radical scavenging antioxidants trolox, BHA, BHT, trans-resveratrol and α-tocopherol were identified with mean TE values of 0.96, 1.36, 1.06, 1.05 and 0.78, respectively. For these compounds, the reductive antioxidant activity was analyzed at 0.11 TE and lower, which means that the total antioxidant activity for these compounds could be explained by their potent radical scavenging activity. These results can be explained by the chemical structure, which comprises at least one aromatic ring system that is able to stabilize radicals. The high radical scavenging activity for α-tocopherol was in line with the results reported by Sawa *et al.* [16]. These authors demonstrated that α-tocopherol has a strong ability to scavenge alkylper-

oxyl radicals. Pietta *et al.* [17] measured the total antioxidative activity of vitamin E with the TEAC antioxidation test. As a result, a TEAC value of 1.00 ± 0.08 mM was reported. This result is in line with the TE-value of 0.89 ± 0.11 obtained for the total antioxidative activity of vitamin E in this work.

For catechin, the high total antioxidant activity can be explained with high TE values analyzed for its radical scavenging (0.58 TE) and its reductive (1.13 TE) activity. DTT, GSH and ascorbic acid were identified as powerful reductive antioxidants, with mean TE values of 0.46, 0.44 and 1.4, respectively. For these three compounds, the radical scavenging activity was negligible.

## 4 Concluding remarks

Most of the commonly applied tests for measuring the antioxidant activity use synthetic radicals of high molecular weights such as ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt), DPPH (2.2-diphenyl-1-picrylhydrazyl) or DMPD (N,N-dimethyl-1,4-phenylendiamine) and APPH (2,2k-azobis-2-amidinopropane) or AMVN 2,2′-azobis-(2,4-dimethylvaleronitrile). These radicals are structurally far away from those formed in a biological system or in a food matrix. The herein presented test system is suitable for the determination of the total antioxidant activity, the reductive and the radical scavenging activity in an experimental setting that may be representative for various food matrices and for *in vivo* compartments such as cell membranes.

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