Eur Food Res Technol (2003) 216:166–173
DOI 10.1007/s00217-002-0635-6

ORIGINAL PAPER

Ramona Lichtenthäler · Friedhelm Marx
Oliver Maria Kind

Determination of antioxidative capacities using an enhanced total oxidant scavenging capacity (TOSC) assay

Received: 5 August 2002 / Revised: 25 September 2002 / Published online: 20 November 2002
© Springer-Verlag 2002

Abstract The antioxidant capacities of nine standard compounds (ascorbic acid, benzoic acid, (+)-catechin, cyanidin-3-glucoside, cyanidin-3-rutinoside, (–)-epicatechin, protocatechuic acid, Trolox and uric acid) towards the three reactive oxygen species (ROS) peroxyl radicals, hydroxyl radicals and peroxynitrite were tested with the total oxidant scavenging capacity (TOSC) assay. The time course of ethylene formation from the reaction of the ROS with α-keto-γ-methiolbutyric acid (KMBA) was monitored by automated headspace GC. By this automation and by optimisation of the preparation and storage of the assay solutions, the time- and labour-consumption of the method could be minimised. Based on the experimental data, the relation between concentration and inhibition rate was pointed out by calculating concentrations that corresponded to TOSC values of 20%, 50% and 80%, respectively. Furthermore, the compounds were classified by their reaction mode as fast-acting antioxidants, retardants or pro-oxidants.

Keywords TOSC assay · Oxidant scavenging capacity · ROS · Peroxyl radicals · Hydroxyl radicals · Peroxynitrite · Fast-acting antioxidants · Retardants

Introduction

Reactive oxygen species (ROS) are continuously formed in several metabolic pathways of aerobic organisms such as electron transport chains and active phagocytosis or as intermediates during various enzyme driven reactions [1, 2]. The main ROS resulting from these processes include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), peroxyl radical (ROO·), alkoxyl radical (RO·), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) [1, 3]. Normally, the oxidative damage they can cause to macromolecules such as DNA, proteins and lipids is prevented by a series of specially adapted enzyme systems (e.g. superoxide dismutase and catalase) and several both water and lipid soluble non-protein compounds (e.g. uric acid and tocopherols) [4, 5]. Two types of ROS scavengers can be distinguished by their reaction mode: fast-acting antioxidant and retardants (see Fig. 1). Fast-acting antioxidants are able to delay the formation or to inhibit the reaction of ROS as long as they are present. When they have been exhausted, the reaction returns to its uninhibited rate. Therefore, fast-acting antioxidants affect a lag-time of reaction and its length depends on the concentration of the antioxidant. Retardants react too slowly with ROS to cause a lag-time. Depending on their concentration, they are able to slow the rate of reaction more or less effectively but cannot stop it completely [6].

The protection offered by all these ROS scavengers is limited and several external factors like exposure to

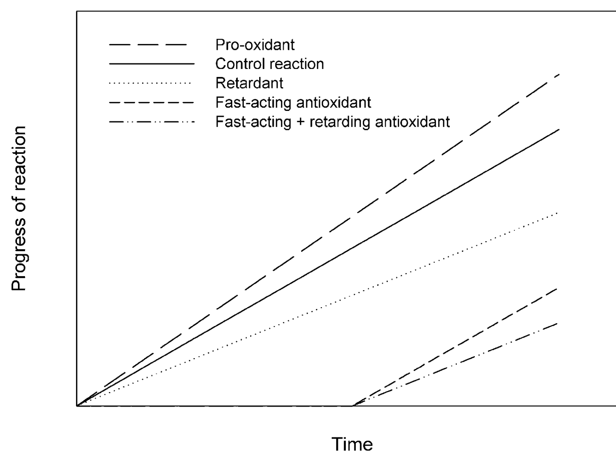

Fig. 1 Differentiation between fast-acting antioxidants, retardants and prooxidants

R. Lichtenthäler · F. Marx (✉)
Institute of Food Science and Food Chemistry,
University of Bonn, Endenicher Allee 11-13, 53115 Bonn,
Germany
e-mail: f.marx@uni-bonn.de
Tel.: +49-228-733713, Fax: +49-228-733757

O.M. Kind
Institute of Physics, University of Bonn, Nussallee 12,
53115 Bonn, Germany

environmental pollutants or cigarette smoke can enforce the internal formation of ROS. If the ROS formation exceeds the antioxidant capacity of the biological system, oxidative stress results [1, 2]. In the consequence, dietary antioxidants can play an important role in the prevention of illnesses like cancer or cardiovascular disease or in the ageing process [1, 7, 8]. Furthermore, there is an increasing demand for "natural" antioxidants to protect food from ROS caused deterioration, in particular from lipid peroxidation [8].

Numerous methodologies for measuring antioxidant capacities have been developed up to now and many approaches have been made to compare their results. The following demands on in vitro assays are common to most review articles: at least two different methods should be used, because it is absolutely possible for an antioxidant to succeed in one assay, to fail in another or to act even pro-oxidatively in a third one [9, 10]. ROS should be used for testing potential antioxidants that are relevant to processes in vivo or in food [10]. Obviously (but often neglected), the compounds should be assayed at concentrations achievable in the food matrix or in vivo [10, 11]. Ideally, the assay should be suitable for pure solutions as well as complex biological tissues and both water- and lipid-soluble compounds should be applicable.

The total oxidant scavenging capacity (TOSC) assay developed by the working groups of Regoli and Winston [12, 13, 14] is a rather new in vitro method that accomplishes, in contrast to most other assays, all these claims: the test can be applied to pure antioxidant solutions as well as complex biological samples like fluids and tissues [12, 13]. It is described in literature as suitable for detecting both water- and lipid-soluble antioxidants and compounds can be examined down to the lower µM range [13, 15]. Three different ROS with an important potential to damage biological tissues are used within this assay: peroxyl radicals, hydroxyl radicals and peroxynitrite. Peroxyl radicals are generated by the thermal homolysis of 2,2′-azobis(2-methylpropionamidine) dichloride (ABAP); hydroxyl radicals are formed during the iron plus ascorbate driven Fenton reaction; and peroxynitrite is produced by the decomposition of 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1). The TOSC assay is based upon the ethylene yielding reaction of these compounds with α-keto-γ-methiolbutyric acid (KMBA). The time course of ethylene formation is monitored by repeated gas chromatographic analysis of aliquots from the headspace of the reaction vessels. An antioxidant is characterised by its capacity to inhibit the ethylene production by the respective ROS compared to an uninhibited control reaction. The oxidisable substrate KMBA is kept at a constant concentration and assay conditions are applied which lead to an equivalent ethylene yield by all applied ROS. Thereby, the effectiveness of antioxidants against the three different oxidants can be compared under similar conditions [12, 13]. If desired, even other ROS could be applied in combination with KMBA because of its unique property to react with different oxidants, e.g. hypochlorous acid (HOCl) [16, 17] or alkoxyl radicals [18]. From the results, fast-acting antioxidants and retardants can be distinguished [12, 13] and even pro-oxidants can be detected [15]. Furthermore, research can be performed with a common GC system.

So far, most published TOSC studies deal with oxidative stress of marine organisms like scallops [19], mussels [20], sponges [21], penguins [22] and flounders [23] as biomarkers for aquatic environmental pollutants. Outside of this area, only few publications concerning the TOSC assay can be found: Dugas et al. studied the structure–activity relationship of flavonoids [14], Leung et al. analysed the oxyradical scavenging ability of linoleic acids isomers [15] and Eberhardt et al. compared the antioxidant activity of fresh apples to that of ascorbic acid [24]. However, all of these latter authors applied only peroxyl radicals to their surveys.

Up to now, drawbacks of the TOSC assay are the need for frequent manual GC injections and the short shelf-life of the test solutions (Regoli, personal communication). The aim of our work was to make the assay less labour and time consuming and easier to handle. Some more substances from other compound classes were tested in addition to standard compounds that had already been assayed by Regoli et al. [12]. This was to compare on the one hand the results for our modified assay with those of the original method and on the other hand to broaden the application field of the assay. We advanced the data evaluation to get more detailed information from the experimental data.

Material and methods

Materials

UHQ (ultra high quality) water was used for all solutions. Diethylenetriaminepentaacetic acid (DTPA), 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), α-keto-γ-methiolbutyric acid (KMBA), (+)-catechin and (–)-epicatechin were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2,2′-Azobis(2-methylpropionamidine) dichloride (ABAP), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferric chloride hexahydrate, ethylenediaminetetraacetic acid (EDTA) and uric acid were obtained from Acros Organics (Geel, Belgium). Ascorbic acid was from Kraemer & Martin (Sankt Augustin, Germany). Benzoic and protocatechuic acid were purchased from Merck (Darmstadt, Germany). Cyanidin-3-glucoside (kuromanin) chloride and cyanidin-3-rutinoside (keracyanin) chloride were from Extrasynthese (Genay, France).

TOSC assay

Solutions

All glassware including the reaction vessels was washed with UHQ water thoroughly.

TOSC solutions

Solution 1: A 0.25 mM KMBA plus 0.1 mM DTPA solution was prepared by dissolving the compounds jointly in a 100 mM potassium phosphate buffer at pH 7.4.

Solution 2: A joint 2.25 µM $FeCl_3$, 4.5 µM EDTA and 0.25 mM KMBA solution was produced. For this purpose, $FeCl_3$ and EDTA were each dissolved separately as a 225 µM solution in UHQ water and a 450 µM solution in the same buffer as for solution 1, respectively. From each of these solutions 1 mL was poured in a joint 100-mL graduated flask. KMBA was added and the flask was filled up with buffer.

Solution 3: A 200 mM ABAP solution was made with the same buffer as for solution 1.

Solution 4: A 1.8 mM solution of ascorbic acid was prepared in UHQ water.

Solution 5: A 0.8 mM SIN-1 solution was made with UHQ water.

The solutions 1 and 2 were divided in portions of 800 µL and filled in septum-sealed 10-mL headspace vials; the solutions 3, 4 and 5, respectively, were put into septum-capped 1-mL GC in portions of 1 mL. The solutions were stable at –18 °C for at least 3 months.

Standard compounds

Benzoic acid, Trolox and uric acid were dissolved in 100 mM potassium phosphate buffer at pH 7.4. Ascorbic acid, (+)-catechin, (–)-epicatechin, protocatechuic acid, cyanidin-3-glucoside and cyanidin-3-rutinoside were dissolved in UHQ water.

The concentrations of standard compounds are always referred to final assay conditions, which account for a tenth of the added concentrations.

Assay conditions

Peroxyl radicals

For a run of six staggered samples, six vials of solution 1 and one vial of solution 3 were defrosted. For the control reaction, 100 µL of UHQ water was injected directly through the septum of each headspace vial. For the antioxidant reaction, 100 µL of sample solution was used instead. The reaction vessels were brought to 37 °C in the incubator unit of a CombiPAL autosampler (CTC Analytics AG) and were agitated there automatically every 55 s for 5 s. The reaction of the first sample was started by injecting 100 µL of solution 3 directly through the septum of the headspace vials; to the following samples the reaction starter was charged at intervals of 2 min.

Hydroxyl radicals

For a run of six staggered samples, six vials with solution 2 and one vial of solution 4 were defrosted. The further procedure was in accordance with peroxyl radicals.

Peroxynitrite

For a run of six staggered samples, six vials of solution 1 and one vial of solution 5 were defrosted. The further procedure was the same as for peroxyl radicals.

Gas chromatographic analysis of ethylene

Beginning immediately after the addition of the reaction starter, aliquots of 100 µL were taken every 12 min over a period of 60 min from the headspace of each reaction vessel. The sampling was done by a CombiPAL autosampler with a 1-mL headspace syringe, which was heated up to 37 °C. The analysis of ethylene was carried out with a Shimadzu GC-17A gas chromatograph and a Chrompack PoraPLOT Q column (25 m×0.53 mm×20 µm). Oven, injection and FID temperatures were 80 °C, 100 °C and 220 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 15 mL/min in splitless mode. Under these conditions, ethylene had a retention time of 2.2 minutes. The peak integration was performed with the software EZChrom Elite v2.8 from Scientific Software.

Quantification of TOSC values

Experimental TOSC values

The kinetic curve that best fits the experimental GC data for ethylene production over a period of 60 minutes and the area beneath it were calculated mathematically. TOSC values are quantified by comparing the areas for control (CA) and sample reaction (SA) in accordance to Eq. 1.

$$\mathrm{TOSC} = 100 - \left(\frac{\int \mathrm{SA}}{\int \mathrm{CA}} \times 100 \right) \quad (1)$$

A sample with no ROS scavenging capacity receives a TOSC value of 0%, because it has the same area under the curve (AUC) as the control reaction. A compound that suppresses the ethylene formation entirely possesses an AUC of 0 and thereby a TOSC value of 100% [12, 13]. A pro-oxidant obtains a negative TOSC value because of an AUC greater than that of the control reaction [15].

Curve fits and quantification of experimental TOSC values were performed with the data analysis software Root v3.02/07. It can be downloaded free of charge at http://root.cern.ch/

Calculated TOSC values

Dose–response curves were fitted through the experimental TOSC values and the corresponding concentrations. Based on the resulting equations, concentrations were calculated, that matched TOSC values of 20%, 50% and 80%, respectively.

Curve fits and TOSC calculations were accomplished with the software TableCurve 2D v5.1 from SYSTAT Software Incorporation.

Results and discussion

Assay modifications

Handling of TOSC solutions

Compared to the original method instructions (Regoli, personal communication), the handling of solutions was simplified: as far as possible, compounds were diluted jointly instead of adding them one after the other. For example, in the case of hydroxyl radicals, a combined solution of KMBA, Fe^{3+} and EDTA in buffer was prepared instead of adding successively buffer, Fe^{3+}/EDTA and KMBA to the vial. The volumes for control and sample reaction were standardised to utilise the same prepared vials for both kinds of reaction. The use of UHQ water for the preparation of all solutions and the thoroughly washing of glassware in combination with deep freezing of the TOSC solutions in small portions obviated a daily preparation of solutions and extended their shelf-life to at least three months. Thereby, the labour and time consumption of assay preparations could be minimised.

The most important difference to the original assay solutions was, that we added DTPA not only to the per-

oxynitrite but also to the peroxyl assay. This was to avoid the formation of hydroxyl radicals in a side reaction because of possible contamination with iron or copper. Although such contamination can be diminished by faithful working with UHQ water, they cannot be eliminated completely. For the control reaction, the ethylene formation is not affected by this alteration, but, for example, ascorbic acid showed much higher and less varying antioxidant capacities towards peroxyl radicals if DTPA is added to the assay.

Assay conditions

In some TOSC publications, measurements are carried out at 35 °C [12, 21] and in the others at 39 °C [13, 14]. Though the rate of radical generation is twofold higher at 39 °C than at 35 °C, it was proven, that the TOSC values are not affected by this changing [13]. Therefore, we decided to use 37 °C instead, because this runs the assay at conditions that are present within the human body.

Optimisation of GC conditions

To ensure a sufficient separation of ethylene from other volatile compounds, GC analysis were performed initially under usual separation conditions, i.e. a carrier gas flow rate of 5 mL/min and a column temperature of 50 °C. Under these conditions, the retention time of the ethylene peak was approx. 7.9 min, and it was well separated from two other small peaks of early eluting volatile components from laboratory air. Therefore, there was no necessity to tune the GC conditions for best separation performance as described by Pham-Tuan et al. [25]. On the contrary, it was possible to speed up the GC analysis by using a higher carrier gas flow rate (15 mL/min) and a higher column temperature (80 °C) without loss of accuracy.

Automation of ethylene measurement

The automation of ethylene measurement with a Combi-PAL autosampler features great advantages towards manual GC injections: after the adding of the reaction starter, no more manual work has to be done during the further course of the assay so a lot of time can be spent for other labour. By staggering the starting time of six samples, a satisfying throughput of samples could be obtained. Because the TOSC assay is a kinetic study, an exact timing of sample getting is extremely important and this can, of course, be much better performed by an autosampler than by a human. Last but not least, the reproducibility of an autosampler in taking and injecting samples outmatches the accuracy of manual work.

TOSC values

Standard compounds were tested against the three different ROS in at least five different concentrations and analyses were carried out in quadruplicate. The concentrations were chosen in order to cover the TOSC range as complete as possible in each case. Therefore, the standard compounds had to be assayed in a more or less varying concentration range. Hence, a direct comparison of the resulting experimental TOSC values remains difficult. This crux is not specific for the TOSC method but for all assays that analyse compounds at differing concentrations. Other researchers have tried to solve this problem by extra- or interpolating their experimental data to a single concentration vs. inhibition point (e.g. results for 1 µM or 1 mM solutions [14, 21] or for the concentration that corresponds to an inhibition rate of 50% [26]). Often, a linear correlation between concentration and inhibition rate is thereby assumed; this approach is also frequently applied, when only a single concentration of a compound is tested.

Instead, we decided to calculate concentrations for three representative spots of the concentration vs. inhibition rate curve to reduce on the one hand the amount of data and, nevertheless, to give on the other hand a concise review of the complete course. Therefore, the concentrations that correspond to TOSC values of 20%, 50% and 80%, respectively, were computed.

Peroxyl radicals

The calculated concentrations and the correlation of the curve fits for peroxyl radicals can be found in Table 1;

Table 1 Calculated concentrations of antioxidants (µM) for different TOSC values and correlation for peroxyl radicals and peroxynitrite (referred to final assay conditions)

	Peroxyl radical				Peroxynitrite			
	TOSC (%)			Curve fit r^2	TOSC (%)			Curve fit r^2
	20	50	80		20	50	80	
Catechin	0.4	1.8	4.6	0.9999	0.6	5.4	55.4	1.0000
Epicatechin	0.6	2.0	4.7	0.9970	0.7	4.7	56.7	0.9997
Cyanidin-3-glucoside	0.9	2.3	4.5	0.9996	2.4	10.5	53.4	1.0000
Cyanidin-3-rutinoside	0.9	2.4	4.8	0.9994	2.5	9.2	57.4	0.9999
Protocatechuic acid	1.4	4.0	9.1	0.9999	1.3	10.8	78.0	0.9997
Trolox	1.5	7.1	16.7	0.9971	3.8	9.7	27.7	0.9984
Uric acid	2.4	8.1	17.2	0.9985	3.7	8.3	22.3	0.9999
Ascorbic acid	4.5	10.5	19.4	0.9999	3.9	10.6	32.9	1.0000

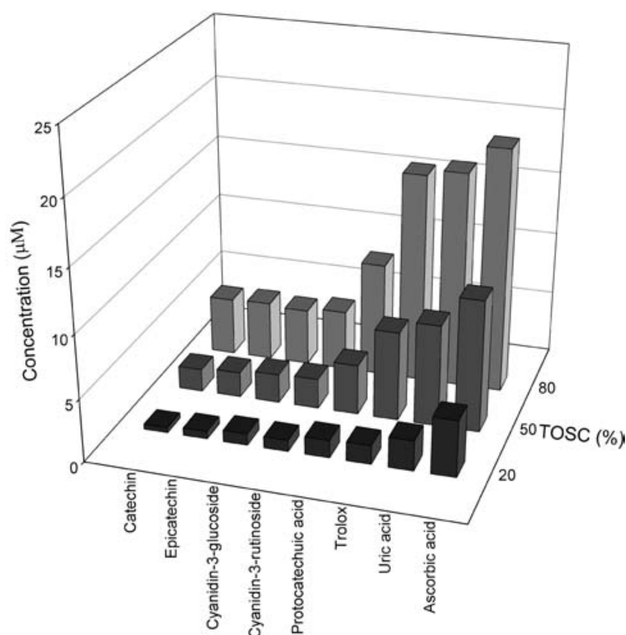

Fig. 2 Overview of scavenging capacities against peroxyl radicals (referred to final assay conditions)

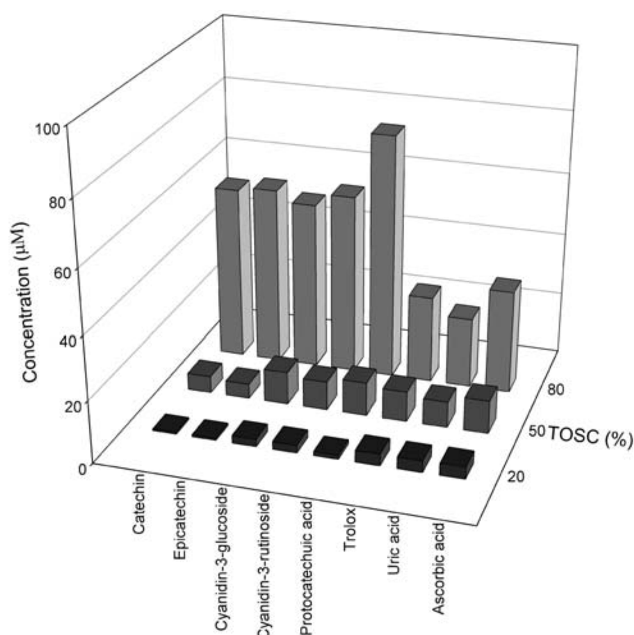

Fig. 3 Overview of scavenging capacities against peroxynitrite (referred to final assay conditions)

additionally, they are visualised in Fig. 2. Benzoic acid is excluded from this survey and classified as unsuitable, because even very high concentrations (10,000 μM) showed very low scavenging capacities against peroxyl radicals (TOSC of about 20%). Assaying higher concentrations of benzoic acid would be irrelevant, because they do not appear naturally or as food additives.

The first thing that catches the eye for all other compounds is the throughout non-linear relation between concentration and inhibition. Secondly, a continuous trend can be observed over the whole concentration range: the compounds with the highest antioxidant capacity at a TOSC of 20% are also the most active ones at a TOSC of 50% and 80%, respectively. The antioxidant capacity of compounds for peroxyl radicals can from there be recapitulated as: catechin=epicatechin=cyanidin-3-glucoside=cyanidin-3-rutinoside >protocatechuic acid >Trolox=uric acid>ascorbic acid. By quantitative comparison, catechin possesses an antioxidant capacity towards peroxyl radicals about 2-times higher than protocatechuic acid, 4-times higher than Trolox and uric acid and about 5-times higher than ascorbic acid.

Peroxynitrite

The calculated results for peroxynitrite can as well be found in Table 1 and the proportions are charted in Fig. 3. Again, benzoic acid is shut out, because a comparatively high concentration of 10,000 μM showed a dissatisfying slight effectiveness against peroxynitrite (TOSC lower than 50%).

For all other assayed standard compounds a very distinctive non-linear relation between concentration and inhibition rate could be detected: throughout, a sharp increase of inhibition capacity can be found at low concentrations, whereas the antioxidant capacity of high concentrations approaches asymptotically a certain limit of inhibition. Surprisingly, no persistent trend of antioxidant capacity can be seen over the concentration range: the compounds with the highest scavenging capacities at a TOSC of 20% (catechin, epicatechin and protocatechuic acid) are counted among those substances with the lowest scavenging activity at a TOSC of 80%; for Trolox, ascorbic acid and uric acid, it is the opposite way round. In contrast, the corresponding concentrations to a TOSC of 50% are widely alike for all compounds. Therefore, it is not possible to state an absolute rank order for peroxynitrite.

This fact is very indicative for the potential inaccuracy, when only one concentration vs. inhibition spot is quoted.

Hydroxyl radicals

None of the tested compounds showed significant antioxidant capacities towards hydroxyl radicals in a relevant concentration range. Only for benzoic acid, a certain activity towards this ROS could be detected, even though at the upper limit of concentrations representative for an occurrence in food. Therefore, Table 2 mirrors only calculated concentrations for benzoic acid and hydroxyl radicals at a TOSC of 20%, 50% and 80%, respectively.

In Fig. 4, the scavenging capacity of the other compounds is shown at a final assay concentration of 50 μM (which corresponds to an added concentration of 500 μM). It can be seen that there are no great differ-

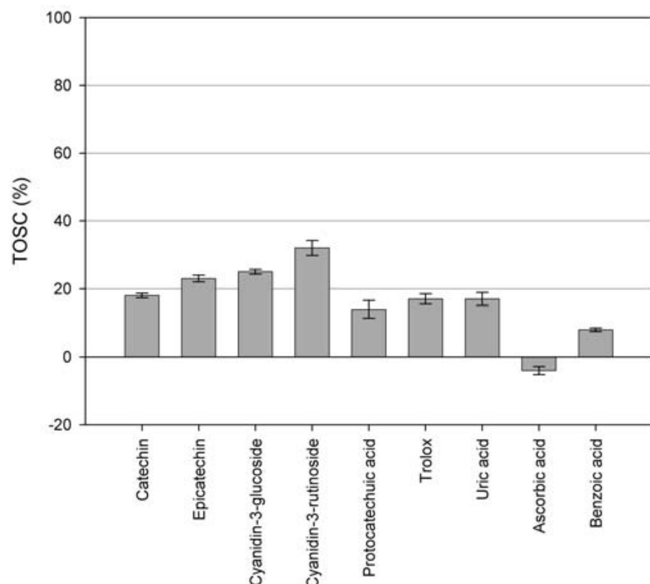

Fig. 4 TOSC for hydroxyl radicals and 50 µM solutions ±standard error of mean

Table 2 Calculated concentrations of benzoic acid (µM) for different TOSC values and correlation for hydroxyl radicals (referred to final assay conditions)

	Hydroxyl radicals			
	TOSC (%)			Curve fit r^2
	20	50	80	
Benzoic acid	104	623	2260	1.0000

ences between their (poor) scavenging capacities. The sole exception is ascorbic acid, which demonstrated even pro-oxidative features at this concentration. At a concentration level ten times higher than the herein presented, at least a low antioxidant capacity of ascorbic acid could be detected (TOSC of about 25%). Therefore, all compounds including benzoic and ascorbic acid are rated below as low active compounds towards hydroxyl radicals.

Comparison of TOSC values towards the three different ROS

The results for ascorbic acid, benzoic acid, uric acid and Trolox are in accordance with those published by Regoli et al. [12]. Apart from benzoic acid, the scavenging capacity for peroxyl radicals and peroxynitrite is relatively alike for all compounds at low inhibition rates (TOSC of 20%). For the group of Trolox, uric acid and ascorbic acid it is also comparable for both ROS at a TOSC of 50%. All other compounds require the twice to fivefold concentration for peroxynitrite than they need for peroxyl radicals. Differences grow even larger at a TOSC of 80%: most compounds need concentrations at an average ten times higher level for peroxynitrite than for peroxyl radicals; for Trolox, ascorbic and uric acid it is just twice the concentration.

Though benzoic acid is a weak scavenger of all three ROS, it features one noteworthiness: in opposition to all other tested compounds, it demonstrates its best scavenging capacities towards hydroxyl radicals.

Comparison of TOSC results with TEAC values from literature

The TEAC (Trolox equivalent antioxidant capacity) assay is one of the most commonly used in vitro antioxidant methods. Therefore, we attempted to compare the results of our examination with TEAC values from literature. Such a comparison turned out to be problematical, because at least four modifications of the TEAC assay are currently in use, and it is proven that they yield different results [9]. Furthermore, different researchers measured different concentrations (e.g. 1 mM solutions or 100 mg/L) [27, 28]. Direct comparison of these results would assume a linear correlation between concentration and inhibition rate over the whole concentration range. Our results for peroxyl radicals and peroxynitrite indicate clearly, that reality can differ a lot. Furthermore, it was recently proven, that TEAC values of some compounds like the flavonoids depend as well significantly on the measured concentration [29]. Therefore, we suggest assumption of a non-linear relation between concentration and inhibition rate until it is proven that black is white.

A blue sheet of TEAC values from different scientists is therefore mostly not recommendable. One author can be found [27, 30] who analysed at least seven of our nine standard compounds with the same TEAC version. These results can be simplified to the following order of antioxidant capacities: cyanidin-3-rutinoside>cyanidin 3-glucoside=epicatechin=catechin>protocatechuic acid> ascorbic acid=Trolox. That finding is mostly in accordance to our results for peroxyl radicals. Yet a comparison to hydroxyl radicals is not possible, because no standard compound showed sufficient scavenging features towards this ROS in a relevant concentration range. A comparison with peroxynitrite is neither feasible, since the sequence of antioxidant capacities for this ROS depends largely on the respective concentration. However, other authors indicate TEAC sequences varying from that one shown above [28, 29].

Classification of standard compounds by reaction mode

An AUC technique considers the kinetics of antioxidants and should thereby be superior to assays that measure just either an inhibition rate at a fixed time or the lag-phase of reaction [31]. In the case of the TEAC assay, for example, it could recently be shown, that TEAC values of some compounds like the flavonoids do not only

Table 3 Classification of standard compounds by reaction mode

	Peroxyl radical		Peroxynitrite		Hydroxyl radical	
	Fast-acting antioxidant	Retardant	Fast-acting antioxidant	Retardant	Fast-acting antioxidant	Retardant
Cyanidin-3-glycoside Cyanidin-3-rutinoside	+	+	–	+	–	Low activity
Trolox Uric acid	+	–	–	+	–	Low activity
Ascorbic acid	+	–	–	+	–	Pro-oxidant/low activity
Catechin Epicatechin Protocatechuic acid	–	+	–	+	–	Low activity
Benzoic acid	–	Low activity	–	Low activity	–	Low activity

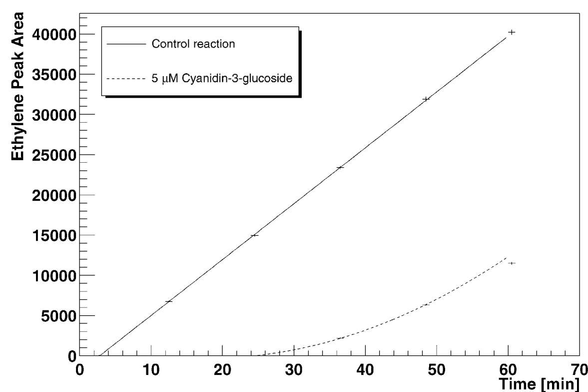

Fig. 5 Scavenging of peroxyl radicals by cyanidin-3-glucoside during the TOSC assay

differ significantly with the concentration of the compounds but also with the measuring time that was used. Therefore, the authors strongly recommended performing kinetic (TEAC) studies with different concentrations of compounds [32].

The TOSC assay is based upon such kinetic studies. Thereby, we were not only able to determine a concentration depending inhibition rate, but also to distinguish between different antioxidant types by their time course of ethylene formation (see Table 3).

The survey reveals, that only few fast-acting antioxidants can be found among the considered compounds. Furthermore, this behaviour can only be observed towards peroxyl radicals. Most compounds act as retardants against the three ROS, even if the scavenging capacity against hydroxyl radicals has to be classified as low for all compounds. Depending on its concentration, ascorbic acid can act as a pro-oxidant or a retardant of hydroxyl radicals; benzoic acid is unsuitable for all three ROS, at least in a relevant concentration range. It is very notable, that the two anthocyanins turned out to be as well fast-acting antioxidants as, at a later period of the reaction, retardants towards peroxyl radicals (see Fig. 5).

To our knowledge, such a phenomenon has not yet been described in literature. We suppose that a decomposition product or a second functional group of the anthocyanins comes into play as soon as the initial molecule has been exhausted.

So, none of the herein presented compounds offers a complete protection against hydroxyl radicals and peroxynitrite in a relevant concentration range. This is not very surprising, because both ROS are extremely reactive with very high rate constants [10]. Therefore, most antioxidants act too slowly to suppress them entirely. Only the much slower reacting peroxyl radicals [10] can be scavenged completely by a couple of compounds (cyanidin-3-glucoside, cyanidin-3-rutinoside, Trolox, ascorbic acid and uric acid).

Conclusions

The labour- and time-consumption of the TOSC assay could be minimised. Taking nine food relevant substances from different compound classes as examples, the varying reactivities of antioxidants towards three naturally occurring ROS could be demonstrated, and different reaction types could be classified. In addition, the results indicate clearly, that antioxidant capacities are often non-linear related to the respective concentrations. We therefore confirm that the TOSC assay is a most suitable testing method for ROS scavenging capacities.

Abbreviations used

AC	area of control reaction
AUC	area under the curve
DTPA	diethylenetriaminepentaacetic acid
KMBA	α-keto-γ-methiolbutyric acid
ROS	reactive oxygen species
SA	area of sample reaction
TOSC	total oxidant scavenging capacity
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid
UHQ	ultra high quality

Acknowledgement This work was supported by Unilever Bestfoods, Germany.

References

1. Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W, Vina-Ribes J (1998) Brit J Nutr 80 (Suppl. 1):S77
2. Turrens JF (1999) http://www.antioxidants.com.ar/12/Art005.htm
3. Radi R (1996) Methods Enzymol 269:354
4. Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine, 2nd edn. Clarendon Press, Oxford
5. Abuja PI (1999) FEBS Lett 446:305
6. Pryor WA, Cornicelli JA, Devall LJ, Tait B, Trivedi BK, Witiak DT, Wu M (1993) J Org Chem 58:3521
7. Lachance PA (1996) Prev Med 25:46
8. Aruoma OI, Spencer JPE, Warren D, Jenner P, Butler J, Halliwell B (1997) Food Chem 60:149
9. Schlesier K, Harwat M, Böhm V, Bitsch R (2002) Free Radic Res 36:177
10. Halliwell B, Aeschbach J, Löliger J, Aruoma OI (1995) Food Chem Toxic 33:601
11. Halliwell B (1995) Biochem Pharmacol 49:1341
12. Regoli F, Winston W (1999) Toxicol Appl Pharm 156:96
13. Winston GW, Regoli F, Dugas AJ Jr, Fong JH, Blanchard KA (1998) Free Radical Biol Med 24:480
14. Dugas AJ, Castaneda-Acosta J, Bonin GC, Price KL, Fischer NH, Winston GW (2000) J Nat Prod 63:327
15. Leung YH, Liu RH (2000) J Agric Food Chem 48:5469
16. Lawrence G, Greenwald RA (1985) Handbook of methods for oxygen radical research. CRC Press, Boca Raton
17. Kruedener S, Schempp H, Elstner EF (1995) Free Rad Biol Med 19:141
18. Pryor W, Jin X, Squadrito G (1994) Proc Natl Acad Sci 91:11173
19. Regoli F, Nigro M, Bompadre S, Winston GW (2000) Aquat Toxicol 49:13
20. Regoli F, Winston GW, Mastrangelo V, Principato G, Bompadre S (1998) Chemosphere 37:2773
21. Regoli F, Cerrano C, Chierici E, Bompadre S, Bavestrello G (2000) Mar Biol 137:453
22. Corsolini S, Nigro M, Olmastroni S, Focardi S, Regoli F (2001) Polar Biol 24:365
23. Winzer K, Winston GW, Becker W, Van Noorden CJF, Koehler A (2001) Aquat Toxicol 52:143
24. Eberhardt MV, Lee CY, Liu RH (2000) Nature 405:903
25. Pham-Tuan H, Vercammen J, Devos C, Sandra P (2000) J Chromatogr A 868:249
26. Dekker M, Verkerk A, van der Sluis AA, Khokhar S, Jongen WMF (1999) Toxicol in vitro 13:797
27. Miller NJ, Rice-Evans CA (1997) Food Chem 60:331
28. Rechner A, Patz CD, Dietrich H (1997) Flüss Obst 64:62
29. Kim DO, Lee KW, Lee, HJ, Lee CY (2002) J Agric Food Chem 50:3713
30. Rice-Evans CA, Miller NJ, Paganga G (1996) Free Rad Biol Med 20:933
31. Prior RL, Cao G (1999) Free Radical Biol Med 27:1173
32. Mira L, Silva M, Rocha R, Manso CF (1999) Redox Rep 4:69