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Assessment of Antioxidant Activity by Using Different *In Vitro* Methods

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In this study, six common tests for measuring antioxidant activity were evaluated by comparing four antioxidants and applying them to beverages (tea and juices): Trolox equivalent antioxidant capacity assay (TEAC I-III assay), Total radical-trapping antioxidant parameter assay (TRAP assay), 2,2-diphenyl-*l*-picrylhydrazyl assay (DPPH assay), *N*,*N*-dimethyl-*p*-phenylendiamine assay (DMPD assay), Photochemiluminescence assay (PCL assay) and Ferric reducing ability of plasma assay (FRAP assay). The antioxidants included gallic acid representing the group of polyphenols, uric acid as the main antioxidant in human plasma, ascorbic acid as a vitamin widely spread in fruits and Trolox® as water soluble vitamin E analogue. The six methods presented can be divided into two groups depending on the oxidising reagent. Five methods use organic radical producers (TEAC I-III, TRAP, DPPH, DMPD, PCL) and one method works with metal ions for oxidation (FRAP). Another difference between these tests is the reaction procedure. Three assays use the delay in oxidation and determine the lag phase as parameter for the antioxidant activity (TEAC I, TRAP, PCL). They determine the delay of radical generation as well as the ability to scavenge the radical. In contrast, the assays TEAC II and III, DPPH, DMPD and FRAP analyse the ability to reduce the radical cation (TEAC II and III, DPPH, DMPD) or the ferric ion (FRAP). The three tests acting by radical reduction use preformed radicals and determine the decrease in absorbance while the FRAP assay measures the formed ferrous ions by increased absorbance. Gallic acid was the strongest antioxidant in all tests with exception of the DMPD assay. In contrast, uric acid and ascorbic acid showed low activity in some assays. Most of the assays determine the antioxidant activity in the micromolar range needing minutes to hours. Only one assay (PCL) is able to analyse the antioxidant activity in the nanomolar range. Black currant juice showed highest antioxidant activity in all tests compared to tea, apple juice and tomato juice. Despite these differences, results of these *in vitro* assays give an idea of the protective efficacy of secondary plant products. It is strongly recommended to use at least two methods due to the differences between the test systems investigated.

Keywords: Antioxidant activity; TEAC; DPPH; DMPD; FRAP; TRAP

INTRODUCTION

Several epidemiological studies suggest the importance of a high consumption of secondary plant products—widely distributed in fruits and vegetables—in reducing the incidence of degenerative diseases like cancer and arteriosclerosis.[1] These substances possess a high antioxidative potential and are counterparts to oxidative stress. In the last years many analytical methods have been developed to determine the antioxidant activity in all kinds of matrices such as plasma, beverages, vegetables and fruits. After intervention studies with tea, fruits and vegetables, the antioxidant potential of plasma as well as the antioxidant activity of food and beverages was analysed in various studies.[2–7] These assays measure the ability to reduce pro-oxidants and use different radicals or metal ions as oxidants.

The aim of this study was to evaluate the comparative response of six common tests by using

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four antioxidants. These substances included gallic acid representing the group of polyphenols, uric acid as the main antioxidant in human plasma, ascorbic acid as a vitamin widely spread in fruits and Trolox® as water soluble vitamin E analogue. In addition, some beverages were analysed using the evaluated test systems.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Special reagents were ABTS (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma no. A 1888, Sigma-Aldrich, Taufkirchen, Germany), Myoglobin (Sigma no. M 1882), ABAP (2,2′-azo-bis(2-amidinopropane) hydrochloride) (Polysciences, Warrington, USA), R-Phycoerythrin (Sigma no. P 0159), DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma no. D 9132), DMPD (*N*,*N*-dimethyl-*p*-phenylendiamine dihydrochloride) (Fluka no. 07767, Sigma-Aldrich, Taufkirchen, Germany), 2,4-dinitrophenylhydrazine (Fluka no. 42210), ACW-kit (ACW = integral antioxidant capacity of water soluble substances) (Analytik Jena AG no. 400.801, Analytik Jena AG, Jena, Germany), Folin–Ciocalteu's phenol reagent (Fluka no. 47641), TPTZ (2,4,6-tripyridyl-*s*-triazine) (Sigma no. S 1253). Ascorbic acid ($c_{(\text{stock-solution})} = 5.68\,\text{mmol}\,l^{-1}$ m-H_3PO_4), gallic acid ($c_{(\text{stock-solution})} = 0.568$ or $1.116\,\text{mmol}\,l^{-1}$), Trolox® ((*S*)-(−)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (Aldrich no. 39,192-1, Sigma-Aldrich, Taufkirchen, Germany) ($c_{(\text{stock-solution})} = 2.5\,\text{mmol}\,l^{-1}$) and uric acid ($c_{(\text{stock-solution})} = 6.1$ or $9.13\,\text{mmol}\,l^{-1}$) were used as antioxidants. These stock solutions were stored at −30°C until analysis and were diluted with water just before measuring.

Beverages

For estimating the applicability of the tests on antioxidant activity for food samples, some beverages were used: two green teas, two black teas—each one Darjeeling and one Assam—, two apple juices (AJ_a and AJ_b), one black currant juice (BCJ), one black currant nectar (BCN) and two tomato juices (TJ_a and TJ_b). The tea extracts were prepared by brewing 1.3 g in 100 ml boiling distilled water for 3 min. The tea samples were cooled immediately on ice after brewing and stored at −30°C until analysis. The juices were also stored at −30°C until analysis. All beverages were diluted with water just before measuring. The tomato juices and apple juices were filtered through a membrane filter (0.2 μm). The vitamin C content of all beverages was analysed photometrically by using 2,4-dinitrophenylhydrazine.[8] The total phenolic content of beverages was determined by using the Folin–Ciocalteu method.[9] The analyses of the beverages on their antioxidant activity, total phenolic content and ascorbic acid content were done in triplicate.

Equipment

Measurements were done in disposable cuvettes or fluorescence cuvettes or microplates or reaction tubes using a spectrophotometer model Uvidec-610 (Jasco, Groβ-Umstadt, Germany), a fluorometer model TD 700 (GAT, Bremerhaven, Germany), a microplate reader model anthos ht2 (Anthos, Krefeld, Germany) and a photochem® (Analytik Jena AG, Jena, Germany). The following methods were used as originally described, only modified slightly in some cases: TEAC assay,[10–12] TRAP assay,[13,14] DPPH assay,[15] DMPD assay,[16] PCL assay,[17] FRAP assay.[18,19]

METHODS

Determination of Ascorbic Acid in Beverages[8]

This assay is based on the reaction of dehydroascorbic acid and dinitrophenylhydrazine to a coloured product, which was measured at 520 nm. In this assay, 200 μl of the diluted beverage were mixed with 300 μl trichloracetic acid. After centrifugation 300 μl of the upper layer were mixed with 100 μl dinitrophenylhydrazine reagent and heated for 1 h at 60°C. After cooling down in an ice bath, 400 μl sulfuric acid were added and mixed vigorously. After 20 min in the dark, the samples were measured photometrically at 520 nm.

Determination of Total Phenolics by Using the Folin–Ciocalteu Method[9]

This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation a green–blue complex is measurable at 750 nm. Two hundred microlitre of the diluted beverage were mixed with 1 ml Folin–Ciocalteu reagent (Fluka, 1:10 diluted) and 800 μl of water containing $Na_2CO_3{\cdot}10H_2O$ ($202.5\,\text{g}\,l^{-1}$). Absorbance at 750 nm was measured after 2 h reaction time. Gallic acid monohydrate was used as standard and the total phenolic content is expressed as gallic acid equivalents in [mg/100 ml] for the beverages.

TEAC Assay With ABTS and Metmyoglobin (= TEAC I)[10]

Antioxidant activity was analysed by using the Trolox equivalent antioxidant capacity (TEAC) assay.

This test is based on the oxidation of ABTS in the presence of H_2O_2 and metmyoglobin to the radical cation $ABTS^{\bullet+}$ (blue–green colour), which is photometrically measured at 734 nm. Dependent on the concentration of radical trapping substances oxidation is delayed. All solutions were prepared in phosphate buffered saline (PBS), pH 7.4. Stock solutions of antioxidants were diluted with water. Absorbance was recorded continuously. After formation of the radical cation $ABTS^{\bullet+}$, an increase of absorbance was registered. The antioxidant potential of the four antioxidants was checked by measuring the lag phase at different concentrations.

TEAC With MnO_2 (= TEAC II)[11]

Antioxidant activity was determined following a procedure similar to that of Miller *et al.* (1996).[11] The $ABTS^{\bullet+}$ radical cation was prepared by filtering a solution of ABTS (in PBS) through manganese dioxide powder. Excess manganese dioxide was removed from the filtrate by passing it through a 0.2 μm syringe filter. This solution was diluted in 5 mM PBS pH 7.4, adjusted to an absorbance of 0.700 ± 0.020 at 734 nm and pre-incubated at room temperature prior to use for 2 h.

One millilitre of the $ABTS^{\bullet+}$ solution and 200 μl of the solution of antioxidants (diluted with water) were vortexed for 30 s in reaction tubes, which were then centrifuged for 60 s at 10,000 rpm. The absorbance (734 nm) of the lower phase (phase separation is only achieved with organic solutions of antioxidants) was taken exactly 2 min after initiation of mixing. PBS blanks were run in each assay. The antioxidant activity of the four substances was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\%\ \text{antioxidant activity} = ((E_{(ABTS^{\bullet+})} - E_{(\text{Standard})})/E_{(ABTS^{\bullet+})}) \times 100$$

where E is the extinction.

TEAC Assay With ABTS and $K_2O_8S_2$ (= TEAC III)[12]

This test is based on the reduction of the ABTS radical cation by antioxidants. The ABTS radical cation was prepared by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture has to remain for 12–24 h until the reaction is complete and the absorbance is stable. For measurements, the $ABTS^{\bullet+}$ solution was diluted with water for the hydrophilic assay and with ethanol for the lipophilic one to an absorbance of 0.700 ± 0.020 at 734 nm. Stock solutions for the four substances were diluted with distilled water. For the photometric assay 1 ml of the $ABTS^{\bullet+}$ solution and 100 μl antioxidant solution were mixed for 45 s and measured immediately after 1 min at 734 nm. The antioxidant activity of the four substances was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\%\ \text{antioxidant activity} = ((E_{(ABTS^{\bullet+})} - E_{(\text{Standard})})/E_{(ABTS^{\bullet+})}) \times 100.$$

TRAP Assay[13,14]

The TRAP assay is defined as total radical-trapping antioxidant parameter. The fluorescence of R-Phycoerythrin (excitation $\lambda = 495$ nm and emission $\lambda = 575$ nm) is quenched by ABAP as radical generator at 37°C. Seven hundred and fifty microlitre R-Phycoerythrin (100 μl suspension in 100 ml buffer saline (pH 7.0)), 250 μl buffer saline (pH 7.0), 50 μl antioxidant solution were added in fluorescence cuvettes, mixed and maintained at 37°C for 10 min. The oxidation reaction was started by adding 26 mM ABAB working solution to the cuvettes. The decay of R-Phycoerythrin was monitored every 5 min. Antioxidants inhibit this decomposition and thus delay the decrease of fluorescence. Measurement is stopped when the fluorescence signal decreases regularly. The antioxidative potential of the four substances was evaluated by measuring the lag phase.[14] In an earlier study, Wayner *et al.*[13] determined the delay in oxygen consumption. This method was interfered by proteins and sample dilution.

DPPH Assay[15]

In the DPPH assay antioxidants reduce the free radical 2,2-diphenyl-1-picrylhydrazyl, which has an absorption maximum at 515 nm. The radical solution is prepared by dissolving 2.4 mg $DPPH^{\bullet}$ in 100 ml methanol. For the photometric assay 1.95 ml $DPPH^{\bullet}$ solution and 50 μl antioxidant solution were mixed. At first, the extinction of the disposable cuvette with 1.95 ml $DPPH^{\bullet}$ was measured as blank, then the antioxidant solution was added and mixed. The reaction was measured after 2/3/4/5/10 min and then in intervals of 5 min until $\Delta E = 0.003\ \text{min}^{-1}$. The antioxidative activity was calculated by determining the decrease in absorbance at different concentrations by using the equation already explained at the TEAC II and III assay (using $E_{(HDPPH\bullet)}$).

DMPD Assay[16]

The principle of this assay is based on the reduction of the purple radical cation $DMPD^{\bullet+}$ (*N*,*N*-dimethyl-*p*-phenylendiamine). A 100 mM DMPD solution was

prepared by dissolving 209 mg DMPD in 10 ml distilled water. One millilitre of this solution was added to 100 ml 0.1 M acetate buffer (pH 5.25). Adding 0.2 ml of a 0.05 M ferric chloride solution resulted in the purple radical cation $DMPD^{\bullet+}$, which was measured at 505 nm and equilibrated to an absorbance of 0.900 ± 0.100. The DMPD radical cation was stable up to 12 h. One microlitre of $DMPD^{\bullet+}$ solution and 50 µl antioxidant solution were mixed continuously for 10 min at 25°C. After mixing, the absorbance of this solution was taken at 505 nm. The antioxidative potential of the four substances was evaluated as shown for the DPPH assay.

PCL Assay[17]

In the PCL assay (Photochemiluminescence) the photochemical generation of free radicals is combined with the sensitive detection by using chemiluminescence. This reaction is induced by optical excitation of a photosensitiser *S* which results in the generation of the superoxide radical $O_2^{\bullet-}$:[15]

$$S + h\nu + O_2 \rightarrow [S^* O_2] \rightarrow S^{\bullet+} + O_2^{\bullet-}$$

The free radicals are visualised with a chemiluminescent detection reagent. Luminol works as photosensitiser as well as oxygen radical detection reagent. This reaction takes place in the Photochem®. The four antioxidants were measured with the ACW kit. 1.5 ml reagent one (buffer solution pH 10.5), 1 ml reagent two (water), 25 µl reagent three (photosensitiser) and 10 µl standard solution were mixed and measured. These are standardised conditions, so the results are comparable to other assays. The antioxidant potential was assayed by means of the lag phase at different concentrations.

FRAP Assay[18,19]

The FRAP assay "Ferric reducing ability of plasma" as described by Benzie and Strain (1996)[18] was used with minor modification.[19] The reaction was carried out in a microtiter plate. The antioxidative activity of the standards was estimated by using the increase in absorbance caused by the generated ferrous ions. 30 µl H_2O and 10 µl antioxidant solution were pipetted in a microtiter plate, 200 µl FRAP-solution were added, mixed for 10 s and the absorbance was taken after 8 min.

Comparison of Six *In Vitro* Assays

For comparing these six tests for measuring antioxidant activity, the lag phase was evaluated in the TEAC I, TRAP and PCL assay, % antioxidant activity (equation explained already) was used for comparing TEAC II, TEAC III, DPPH, DMPD, the concentration of Fe^{2+} in $mmol\,l^{-1}$ was evaluated in the FRAP assay.

Statistical Analysis

All results presented in this study are the average of at least three measurements. Means ± standard deviations are shown in figures.

RESULTS

The six methods presented can be classified into two groups depending on the oxidising reagent. Five

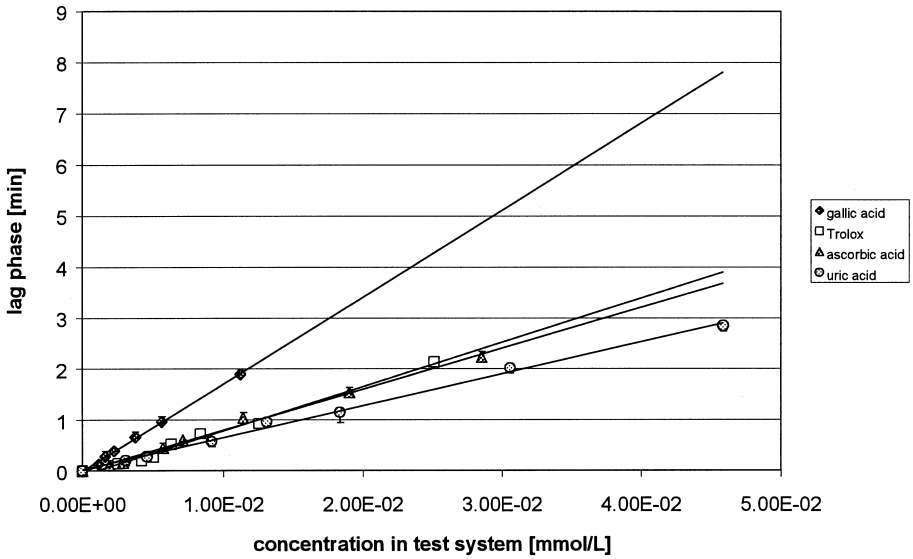

FIGURE 1 Lag phases of the four standard antioxidants Trolox, gallic acid, uric acid and ascorbic acid with different concentrations in the TEAC I assay.

methods use radicals (TEAC I–III, TRAP, DPPH, DMPD, PCL) and one method works employing metal ions (FRAP) for oxidation. Another difference between these tests is the reaction procedure. Three assays use the delay of oxidation and determine the lag phase as parameter for the antioxidant activity (TEAC I, TRAP, PCL). They determine the delay in radical generation as well as the ability to scavenge the radical. In contrast, the TEAC assay II and III, the DPPH assay, the DMPD assay and the FRAP assay analyse only the ability to reduce the radical cation (TEAC II and III, DPPH, DMPD) or the ferric ion (FRAP). The three tests acting by radical reduction use preformed radicals and determine the decrease in absorbance while the FRAP assay measures the increased absorbance of the formed ferrous ions.

Antioxidant activity is a sum parameter including all effective antioxidants. In this study four antioxidants—ascorbic acid, gallic acid, Trolox® and uric acid—were used to get better information of their ability to react in the different assays. As recently reported for three test systems,[20] the results for these six assays were not comparable, too. Whereas gallic acid was the strongest antioxidant in each test with exception of the DMPD assay, differences were observed for the other antioxidants. In addition, the concentrations and measurement times differed extremely.

Appraisal of the Antioxidant Capacity by Using the Lag Phases

In three tests (TEAC I, TRAP, PCL) the antioxidant potential of the substances was assessed by using the lag phases. Figure 1 shows the TEAC I assay with ABTS and metmyoglobin.

Micromolar concentrations resulted in lag phases of some minutes. Gallic acid was the strongest antioxidant in this test followed by the other substances reacting in comparable concentrations. Handling the TEAC I assay is rather simple. Using the TRAP assay, the concentrations in the test system are in the same range but measuring the lag phases needs around 1 h. PCL is more sensitive than the TEAC I and the TRAP assay. The lag phases of the PCL assay resemble that of the TEAC test. Figure 2 shows the lag phases of uric acid in PCL, TEAC I and TRAP assay.

The concentrations of uric acid are comparable in the TEAC I and TRAP assay. But the lag phases in the TRAP assay are longer than in the TEAC I assay. The lag phases of TEAC I and PCL assay are in a comparable range of some minutes. The PCL assay is more sensitive than the TEAC I and the TRAP assay.

When regarding the differences within the ranking of antioxidants, ascorbic acid showed low antioxidant activity in the TRAP assay, while in the TEAC assay its activity was comparable to Trolox® and uric acid. Uric acid was less active in the PCL assay compared to Trolox® and ascorbic acid.

Evaluation of Antioxidant Activity by Using the FRAP Assay

The FRAP assay showed comparable sensitivity with a concentration range of 0–20 $\mu mol\,l^{-1}$, calculated in the test system. By using microplates, a lot of samples can be analysed within a short time. Thus, the FRAP assay is one of the most rapid tests and very useful for routine analyses. The limitation, however, is the non-physiologically low pH value (3.5) used.[21] Figure 3 shows the antioxidant activity

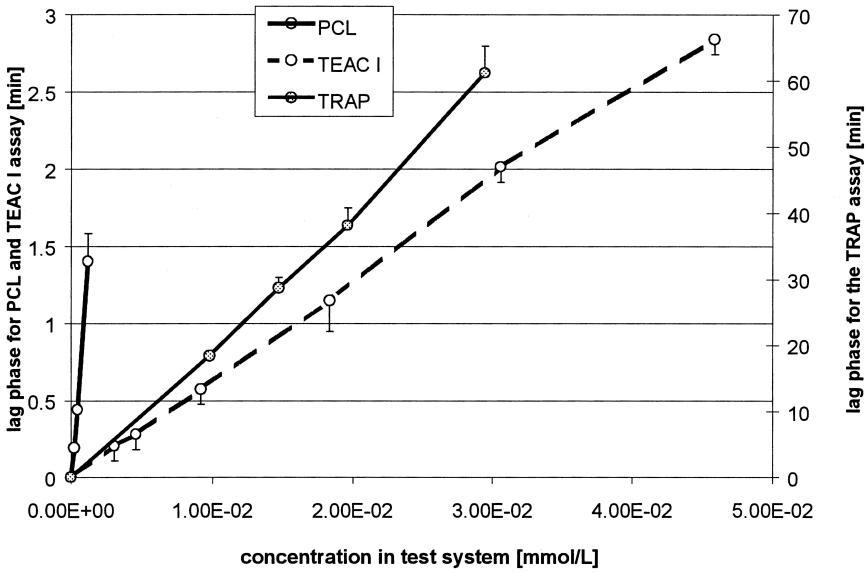

FIGURE 2 Lag phases of uric acid with different concentrations in the PCL, TEAC I and the TRAP assay.

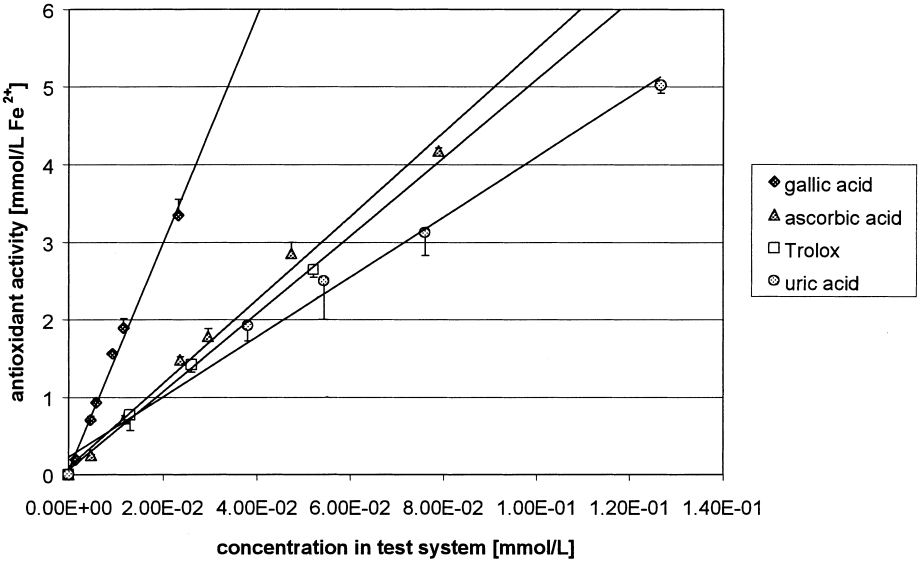

FIGURE 3 Antioxidant activity (expressed as mmol l^{-1} Fe^{2+}) of the four standard antioxidants Trolox, gallic acid, uric acid and ascorbic acid with different concentrations in the FRAP assay.

expressed as mmol l^{-1} Fe^{2+} for the four antioxidative substances investigated.

In this test gallic acid was the strongest antioxidant as well. Ascorbic acid, Trolox and uric acid responded comparably.

Evaluation of Antioxidant Activity by Reducing the Radical

Four of the *in vitro* tests (TEAC II, TEAC III, DPPH and DMPD) analysing only the reduction of the generated radical by antioxidants were compared by evaluating the antioxidant activity of hydrophilic antioxidants. The TEAC II+III and the DPPH assay can be used for lipophilic antioxidants, too by dissolving the radicals in an organic solvent like methanol. Testing only hydrophilic antioxidants, uric acid, the main antioxidant in human plasma, responded very differently in TEAC II+III, DPPH and DMPD assay. Figure 4 illustrates the behaviour of uric acid in these assays.

In the DMPD test, uric acid showed no antioxidant activity and a very low one in the DPPH assay with concentrations in the test system ranging from 0.015 to 0.076 mmol l^{-1}. Different solvent systems, like water for TEAC III (hydrophilic) and ethanol for TEAC III (lipophilic), led to different antioxidant activity, probably caused by different pH values. The pH value for the TEAC III—hydrophilic version—is 7.4 and for the TEAC III (lipophilic) 5.0. Van den Berg *et al.* (1999)[22] showed different antioxidant activity for β-carotene by using different solvents. The different response of uric acid in these test systems can be of advantage when testing the antioxidant activity in human plasma samples after ingestion of plant foods. Studies focussing on this problem are currently under work in our laboratory.

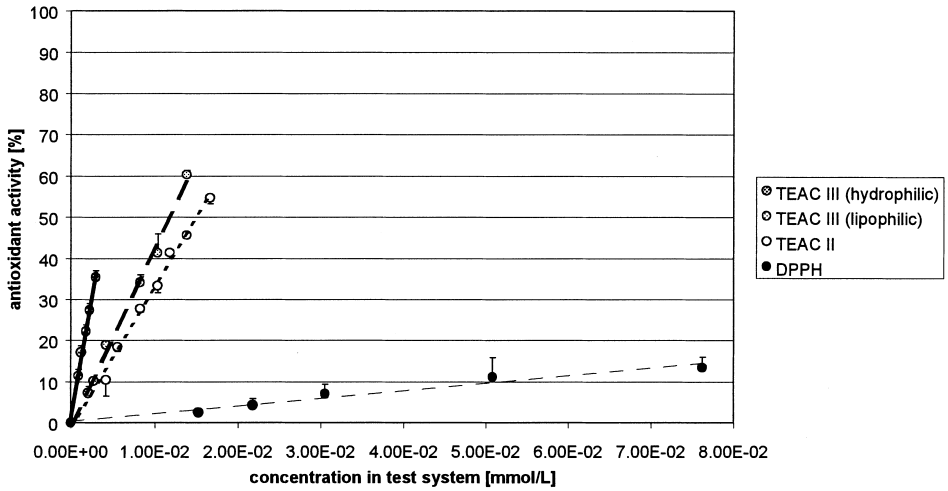

FIGURE 4 Antioxidant activity of uric acid with different concentrations in the DPPH, TEAC II and TEAC III (lipophilic and hydrophilic version) assay.

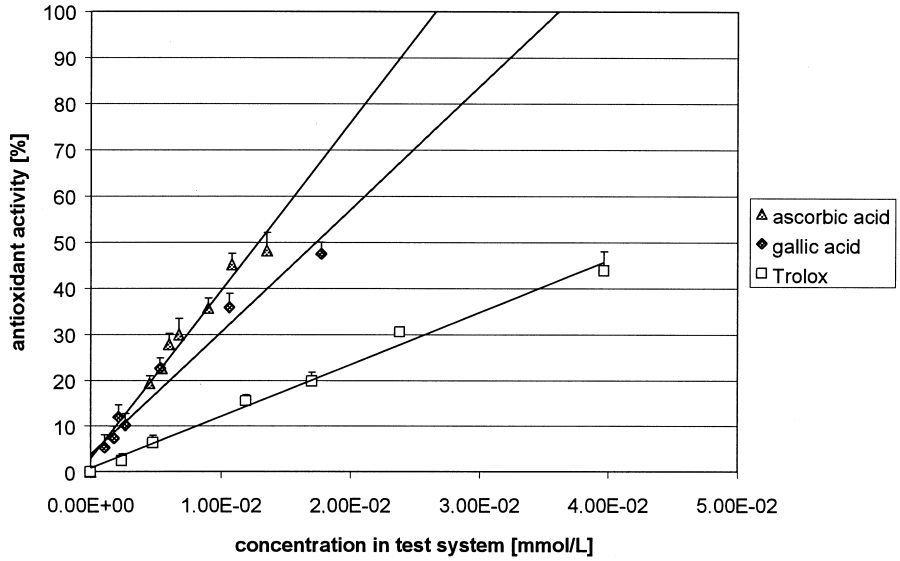

FIGURE 5 Antioxidant activity of three standard antioxidants Trolox, gallic acid and ascorbic acid with different concentrations in the DMPD assay.

Figures 5 and 6 show the antioxidant behaviour of the other antioxidants in the DMPD and the DPPH assay.

Regarding the preparation procedure and the sensitivity of the DMPD and DPPH assay, both are comparable. In the DMPD assay Trolox and ascorbic acid showed different kinetics. Ascorbic acid scavenges immediately the DMPD radical, whereas Trolox needs some minutes to do so, which was also observed by Fogliano *et al.* (1999)[16] The DMPD assay was the only test where gallic acid did not respond as the strongest antioxidant. Ascorbic acid was more efficient than gallic acid in this test. In the DPPH assay, similar concentrations of ascorbic acid and Trolox showed comparable antioxidant activity. Gallic acid was the strongest antioxidant in the DPPH assay. Brand-Williams *et al.* (1995)[15] described three types of reaction kinetics. Ascorbic acid reaches a steady state after 1 min and Trolox needs some minutes. The authors found a very slow response for guaiacol. In the present study, uric acid responded comparably.

Comparison Between TEAC I, TEAC II and TEAC III

The most often used TEAC assay now exists in three versions. TEAC I only allows to measure hydrophilic antioxidants. TEAC II usually is an assay for lipophilic antioxidants like carotenoids and tocopherols. TEAC III enables measurement of both kinds of antioxidants by changing the solvent. TEAC I measures the ability of delaying the radical formation as well as that of scavenging the radical

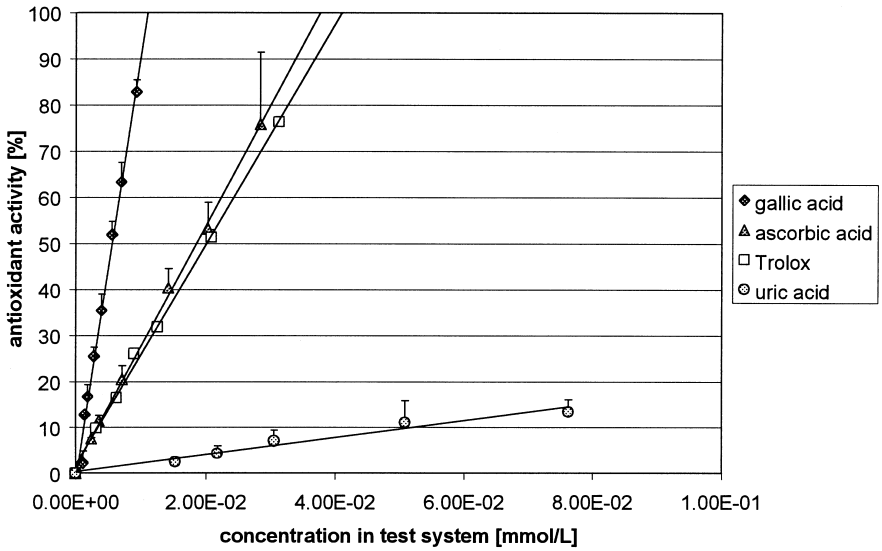

FIGURE 6 Antioxidant activity of the four standard antioxidants Trolox, gallic acid, uric acid and ascorbic acid with different concentrations in the DPPH assay.

TABLE I Trolox equivalent antioxidant capacities (TEAC) [mmol l^{-1}] of the four antioxidants Trolox, gallic acid, uric acid and ascorbic acid

Assay/antioxidant	Trolox	Gallic acid	Uric acid	Ascorbic acid
TEAC I	1.00	2.24 ± 0.27	0.86 ± 0.13	1.06 ± 0.09
TEAC II	1.00	4.35 ± 0.22	0.86 ± 0.11	0.99 ± 0.09
TEAC III hydrophilic	1.00	3.83 ± 0.10	0.83 ± 0.06	0.31 ± 0.03
TEAC III lipophilic	1.00	3.35 ± 0.32	0.99 ± 0.17	1.14 ± 0.06

whereas the other two use preformed radical cations determining only the scavenging ability of antioxidants. Table I shows the TEAC values of the four antioxidants in the TEAC I, II and TEAC III (hydrophilic and lipophilic version).

In these four versions of the TEAC assay the TEAC value of Trolox is 1.00. Gallic acid responded in all assays as strongest antioxidant. But the TEAC value analysed in TEAC I was lower, whereas the TEAC values of TEAC II, and TEAC III (hydrophilic and lipophilic version) showed comparable antioxidant activity. Uric acid responded in all four versions of the TEAC assay in a comparable range, also described by Re *et al.* (1999).[12] The TEAC values of ascorbic acid did not differ in TEAC I, TEAC II and TEAC III (lipophilic version). But the TEAC value analysed in TEAC III (hydrophilic version) was lower than in the three others.

Evaluation of The *In Vitro* Assays for Measuring Antioxidant Activity by Using Beverages

The antioxidant activity of beverages was assessed by using assays measuring the ability of reducing radical cations (TEAC II and TEAC III, DPPH and DMPD). Additionally, the antioxidant activity was determined by using the FRAP and the PCL assay. In all tests, black currant juice showed the strongest antioxidant activity and had also the highest total phenolic content and the highest amount of ascorbic acid. Table II illustrates the amount of ascorbic acid and the total phenolic content expressed as gallic acid equivalents in the beverages.

TABLE II Amounts of ascorbic acid [mg l^{-1}] and gallic acid equivalents GAE [mg 100 ml^{-1}] in beverages

Beverages	Ascorbic acid [mg l^{-1}]	GAE [mg 100 ml^{-1}]
BCJ	2408.1 ± 37.5	519.8 ± 4.5
BCN	458.2 ± 7.9	112.2 ± 0.5
AJ_a	311.7 ± 4.7	74.9 ± 1.1
AJ_b	75.6 ± 3.3	49.2 ± 0.3
TJ_a	83.8 ± 3.9	25.6 ± 0.1
TJ_b	75.4 ± 2.9	19.9 ± 0.5
Green Darjeeling	59.5 ± 0.5	53.2 ± 2.3
Green Assam	58.3 ± 0.7	49.2 ± 6.6
Black Darjeeling	32.5 ± 0.2	46.9 ± 5.1
Black Assam	37.6 ± 1.9	41.1 ± 6.1

(BCJ: black currant juice; BCN: black currant nectar; AJ: apple juice (AJ_a and AJ_b); TJ: tomato juice (TJ_a and TJ_b)).

The juices had higher amounts of ascorbic acid than the tea extracts. Tomato juices had the lowest total phenolic content compared to the other beverages. The tea extracts were in a comparable range for amounts of ascorbic acid and for the total phenolic content. Table III shows the antioxidant activity and the contribution of ascorbic acid to the TEAC values of beverages in TEAC II, TEAC III—hydrophilic and lipophilic version, DMPD and DPPH assay.

Black currant juice had the strongest antioxidant activity in all tests followed by black currant nectar with exception of the TEAC III—lipophilic version. The different assays resulted in different antioxidant activity and the ranking differed also from assay to assay. Only antioxidant activity analysed by the hydrophilic and lipophilic version of TEAC III and the TEAC II was in a comparable range for the tea extracts and the black currant juice and nectar, and for one apple juice. The two tomato juices resulted in different antioxidant activities by using TEAC II, TEAC III—hydrophilic and lipophilic version. The DMPD and DPPH assay resulted in lower antioxidant activity than the three versions of TEAC. Only for black currant juice and nectar, as well as for apple juice and tomato juice, the antioxidant activity analysed by DMPD was high, probably due to the high content of ascorbic acid in these beverages. Ascorbic acid was the strongest antioxidant in the DMPD assay. This high response of ascorbic acid reflects the contribution of ascorbic acid to the TEAC value analysed by the DMPD assay. Even at low contents of ascorbic acid in teas, the relative contribution of ascorbic acid to the TEAC value is around 34.0%, comparable to that in black currant juice (35.2%). The higher antioxidant activity of the black currant juice is caused by a high amount of polyphenols. In the other assays, the relative contribution of ascorbic acid to the TEAC values is very low (2.7%) in teas. The response of polyphenols is strong. In these assays, the tea extracts had comparable antioxidant activity in all tests, in the same range for the three versions of TEAC and in the same range for DMPD and DPPH. The comparison of these assays can be troublesome, but they can be used to present a ranking of these beverages within each assay. Figure 7 shows the antioxidant activity of beverages by using the FRAP assay and the PCL assay.

TABLE III Antioxidant activity expressed as TEAC [mmol l^{-1}] and contribution of ascorbic acid to TEAC expressed as $TEAC_{ascorbic\ acid}$ [mmol l^{-1}] of beverages in TEAC II, TEAC III hydrophilic and lipophilic version, DPPH and DMPD assay

		TEAC II	TEAC III hydrophilic	TEAC III lipophilic	DPPH	DMPD
BCJ	TEAC	54.70	38.33	49.28	130.28	138.85
	$TEAC_{ascorbic\ acid}$	13.54	4.22	15.59	14.08	48.81
BCN	TEAC	13.13	6.44	8.58	55.83	49.90
	$TEAC_{ascorbic\ acid}$	2.58	0.81	2.97	2.68	9.29
Green Assam	TEAC	7.56	4.41	9.69	16.33	3.31
	$TEAC_{ascorbic\ acid}$	0.33	0.10	0.38	0.34	1.18
Black Assam	TEAC	7.43	3.88	7.50	16.82	2.35
	$TEAC_{ascorbic\ acid}$	0.21	0.07	0.24	0.22	0.76
Black Darjeeling	TEAC	6.67	5.76	7.70	16.72	2.05
	$TEAC_{ascorbic\ acid}$	0.18	0.06	0.21	0.19	0.66
Green Darjeeling	TEAC	6.76	5.11	7.82	17.02	3.35
	$TEAC_{ascorbic\ acid}$	0.33	0.10	0.39	0.35	1.21
AJ_a	TEAC	5.11	4.36	3.02	6.86	8.59
	$TEAC_{ascorbic\ acid}$	1.75	0.55	2.02	1.82	6.32
AJ_b	TEAC	3.63	2.64	4.91	8.40	8.35
	$TEAC_{ascorbic\ acid}$	0.43	0.13	0.49	0.44	1.53
TJ_a	TEAC	4.79	0.68	1.33	1.39	8.81
	$TEAC_{ascorbic\ acid}$	0.50	0.15	0.54	0.49	1.70
TJ_b	TEAC	2.15	0.49	1.10	1.46	7.70
	$TEAC_{ascorbic\ acid}$	0.42	0.13	0.49	0.44	1.53

(BCJ: black currant juice; BCN: black currant nectar; AJ: apple juice (AJ_a and AJ_b); TJ: tomato juice (TJ_a and TJ_b)).

In the FRAP assay also, black currant juice and nectar responded more strongly than the other beverages. Due to the low activity of ascorbic acid in this test, the apple juices and tomato juices had only an activity in the range of the tea extracts in contrary to the DMPD assay. The tea extracts showed antioxidant activity in a comparable range. In the PCL assay, black currant juice responded stronger than the other beverages, too and the tea extracts showed antioxidant activity comparable to the black currant nectar in contrast to the FRAP assay with higher activity for the black currant nectar. The correlation between the FRAP and PCL assay was weak ($r = 0.813$) and might be caused by a different response of antioxidants from tea, like catechins for example. The correlation between the TEAC II, TEAC III (hydrophilic and lipophilic), DMPD and DPPH assay was strong for the TEAC II and TEAC III (hydrophilic version) and DPPH assay, whereas the correlation between total phenolic content and TEAC III (lipophilic version) ($r = 0.976$) and with DMPD ($r = 0.972$) was weaker. In the DMPD assay, gallic acid was not the strongest antioxidant.

DISCUSSION

The six *in vitro* test systems for analysing the antioxidant activity showed different results when using four prominent standard antioxidants. Gallic acid was the strongest antioxidant in all tests except

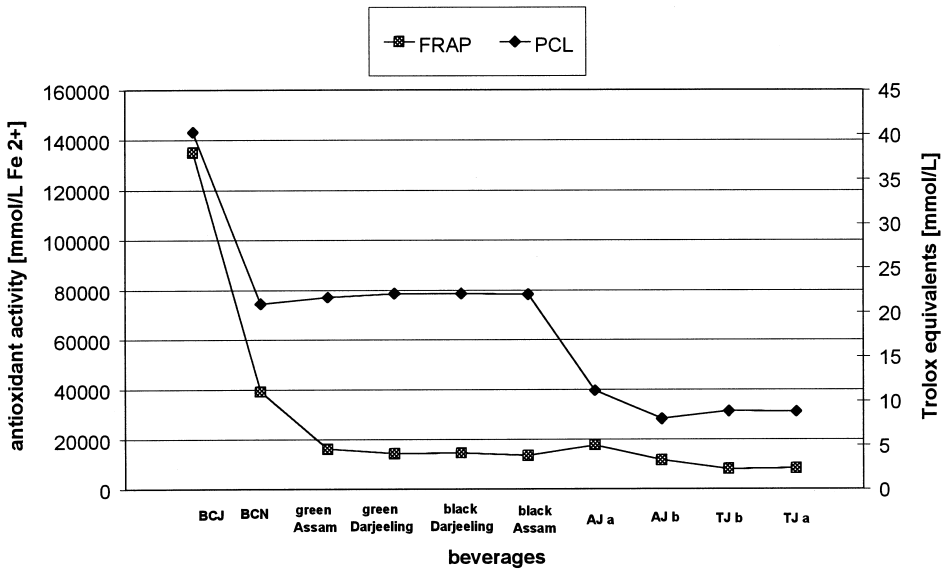

FIGURE 7 Antioxidant activity (expressed in mmol l^{-1} Fe^{2+} and in Trolox equivalents in mmol l^{-1}) of beverages in the FRAP and PCL assay (BCJ: black currant juice, BCN: black currant nectar; AJ: apple juice (AJ_a and AJ_b); TJ: tomato juice (TJ_a and TJ_b)).

the DMPD assay. In contrast, the response of uric acid or ascorbic acid was weak in some methods. Strube *et al.* (1997)[23] found that the "pre-addition" assay characterised by adding antioxidants before generation of the radical as shown for TEAC I might result in an overestimation of the antioxidant capacity because substances interfere with the formation of the radical. In this study, the TEAC values of uric acid were comparable in all tests as also described by Re *et al.* (1999).[12] Ascorbic acid showed a lower response in the TEAC III hydrophilic version than in the TEAC I, II, and III lipophilic version. For gallic acid, the response in TEAC I was two times lower than in the TEAC II and TEAC III. This shows that TEAC values analysed by TEAC I were not overestimated, coinciding with.[12] Other assays like FRAP, DPPH, DMPD and PCL are done in non-physiological pH values. Thus, it is difficult to transfer the results of these assays to the physiological environment of the human body. The often cited ORAC assay[21] is based on the principle of the TRAP assay presented in this study. Cao *et al.* (1993)[24] used an area under the curve technique for determining antioxidant activity as ORAC-value and took into account the responding time of antioxidants. In the present study, the antioxidant activity was evaluated by lag phase or by decrease in absorbance to be able to compare these different test systems. Van den Berg *et al.* (1999)[22] described another modification of the TEAC assay. The ABTS radical anion was pre-generated by ABAP, already known as radical generator in the TRAP assay. This assay was used for hydrophilic and lipophilic antioxidants like TEAC III.

For a further comparative estimation of these assays, the antioxidant activity of several beverages was assessed. It was comparable when analysed by the three versions of TEAC. In all tests, black currant juice was the strongest antioxidant. But the ranking of the other beverages differed from assay to assay. Van den Berg *et al.* 1999[22] described that the application of these assays to fruit juices was complicated. However, it is possible to use a ranking in every assay for comparing beverages. A strong correlation was found for the total phenolic content and the FRAP assay[25] and also for the total phenolic content and the other assays of antioxidant activity. There was also a strong correlation between the three versions of TEAC and the DPPH ($r = 0.999$). In contrary, DMPD showed a lower correlation to the DPPH ($r = 0.960$), to the TEAC II ($r = 0.966$), to the TEAC III (hydrophilic) ($r = 0.946$) and to the TEAC III (lipophilic) ($r = 0.927$). The weaker correlations are probably caused by the higher antioxidant activity of ascorbic acid compared to its antioxidant activity in the three versions of TEAC.

Most of the assays determine the antioxidant activity in the micromolar range needing minutes to hours. Only one assay (PCL) is able to analyse the antioxidant activity in the nanomolar range within minutes. Only one radical (superoxide radical) used in the PCL assay is also occurring in the human body. The LDL oxidation, another *in vitro* assay, is based on the autoxidation of isolated LDL particles.[26,27] This test is also very sensitive by analysing the antioxidant activity in a nanomolar range, but the reaction procedure is time-consuming (data not shown).[20] So the LDL oxidation is not useful for routine analysis. Thus, it is not possible to transfer the results from these tests to processes in the human organism with exception of the PCL test. However, results of these *in vitro* assays give an idea of the protective efficacy of secondary plant products. Due to the differences between the test systems investigated it is strongly recommended to use at least two methods depending on the antioxidant potential expected and perhaps on the origin of substances.

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