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Comparing antioxidative food additives and secondary plant products – use of different assays

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Abstract

Twelve food additives and six secondary plant products were analysed on their antioxidant activity by using three different test systems (Trolox equivalent antioxidant capacity, photochemiluminescence, ferric reducing antioxidant power). The results differed depending on the assay. All the food additives showed antioxidant activities comparable to the calibration substance Trolox. In contrast, the secondary plant products had an up to 16 times higher antioxidant potential. This might present a good reason for the food industry to use natural antioxidants instead of synthetic ones to get storage stability for processed food items – which, according to recent surveys, is in the interest of consumers.

Keywords: Food additives; Antioxidant activity; Trolox equivalent antioxidant capacity; Photochemiluminescence; Ferric reducing antioxidant power

1. Introduction

Lipid peroxidation is one reaction in food products which leads to off-flavour and other quality losses (e.g. changes in colour and texture) with large economical relevance (Kanner, 1994). To stabilize products, the food industry uses food additives with antioxidant activity. Typical antioxidants are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) as well as ascorbic acid and their derivatives. During the last decade, the consumer asked more and more for natural antioxidants instead of synthetic ones. Secondary plant products, widely known for their health promoting effects, have then been evaluated also for their use as food ingredients with stabilizing effects (Britt, Gomaa, Gray, & Booren, 1998; Madsen & Bertelsen, 1995; Madsen, Andersen, Christiansen, Brockhoff, & Bertelsen, 1996). Now, a variety of analytical methods exists to determine the antioxidant activity in all kind of matrices. The use of more than one assay has been strongly recommended. An overview on most of these assays determining the antioxidant potential is given by Böhm and Schlesier (2004).

The aim of the present investigation was, on the one hand, to compare different regularly used food additives regarding their antioxidant activity by using more than one assay system. There are only scarce investigations on natural antioxidants which are asked for by the consumer more and more. Thus, the results of the food additives were then compared to those of some exemplarily selected, prominent secondary plant products, which were also tested by using

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the same assays, to check their antioxidative potential.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade. Special reagents were ABTS (2,2′-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)) (Sigma NO. A 1888, Sigma–Aldrich, Taufkirchen, Germany), Myoglobin (Sigma NO. M 1882), ACL kit (ACL, integral antioxidant capacity of lipophilic substances) (Analytik Jena AG NO. 400.803, Analytik Jena AG, Jena, Germany), ACW kit (ACW, integral antioxidant capacity of water soluble substances) (Analytik Jena AG NO. 400.801), TPTZ (2,4,6-tripyridyl-*s*-triazine) (Sigma NO. T 1253), Trolox® ((*S*)-(−)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich NO. 39192-1, Sigma–Aldrich, Taufkirchen, Germany). As samples, the following compounds were analysed on their antioxidant activity: (a) food additives: ascorbic acid (⩾99%, Merck NO. 500074, Merck, Darmstadt, Germany), isoascorbic acid (⩾99%, Fluka NO. 58320, Sigma–Aldrich, Taufkirchen, Germany), calcium ascorbate (⩾99%, Fluka NO. 11138), sodium ascorbate (⩾99%, Fluka NO. 11140), BHA (⩾98%, Fluka NO. 20021), BHT (⩾99%, Sigma NO. B 1378), propyl gallate (Sigma NO. P-3130), octyl gallate (⩾99%, Fluka NO. 48700), α-tocopherol (⩾95%, Calbiochem NO. 613424, Merck, Darmstadt, Germany), β-tocopherol (⩾95%, Calbiochem NO. 613424), γ-tocopherol (⩾ 95%, Calbiochem NO. 613424), δ-tocopherol (⩾95%, Calbiochem NO. 613424); (b) secondary plant products: caffeic acid (⩾99%, Sigma NO. C 0625), (±)-catechin (Sigma NO. C 1788), eugenol (⩾99%, Riedel-de Haën NO. 35995, Sigma–Aldrich, Taufkirchen, Germany), gallic acid (⩾98%, Fluka NO. 48630), quercetin (Extrasynthese NO. 1135 S, Extrasynthese, Genay, France) as one of the most investigated polyphenols, and rosmarinic acid (⩾97%, Fluka NO. 44699). For the hydrophilic compounds aqueous solutions (1 mmol/L) were prepared and used undiluted or diluted up to 1:50 for the measurements. The tocopherols were dissolved at concentrations of approx. 2.5 mmol/L in *n*-hexane and diluted 1:20–1:200 for analysis. The other lipophilic substances were dissolved at concentrations of around 1 mmol/L in ethanol and diluted up to 1:50 for the determinations.

2.2. Equipment

Measurements were done in disposable cuvettes or microplates or reaction tubes using a spectrophotometer model Uvidec-610 (Jasco, Groß-Umstadt, 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 in some cases slightly modified: Trolox equivalent antioxidant capacity (TEAC) assay (Böhm, Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993; Miller, Sampson, Candeias, Bramley, & Rice-Evans, 1996), photochemiluminescence (PCL) assay (Popov & Lewin, 1999), Ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996; Bub et al., 2000).

2.3. TEAC assay with ABTS and Metmyoglobin (=TEAC I) (Miller et al. 1993)

Antioxidant activity was analysed by using the 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 distilled water. Absorbance was recorded continuously. After formation of the radical cation $ABTS^{\bullet+}$, an increase of absorbance was registered. The antioxidant potential of the substances was checked by measuring the lag phase.

2.4. TEAC with MnO_2 (=TEAC II) (Böhm et al., 2002; Miller et al., 1996)

Antioxidant activity was determined following a procedure similar to that of Miller, Sampson et al. (1996) slightly modified (Böhm et al., 2002). The $ABTS^{\bullet+}$ radical cation was generated 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 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 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 was taken exactly 2 min after initiation of mixing. Solvent blanks were run in each assay. The antioxidant activity of the substances was calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$\%\text{antioxidant activity} = [(E\ (ABTS^{\bullet+}) - E\ (\text{Standard}))/E\ (ABTS^{\bullet+})] * 100 \quad (E = \text{extinction}).$$

2.5. PCL assay (Popov & Lewin, 1999)

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

$$S + h\nu + O_2 \rightarrow [S * O_2] \rightarrow S^{\bullet +} + O_2^{\bullet -} \text{ (Popov Lewin, 1999).}$$

The free radicals are visualised with the chemiluminescent detection reagent luminol. It works as photosensitizer as well as oxygen radical detection reagent. This reaction takes place in the Photochem®. The hydrophilic antioxidants were measured with the ACW kit. 1.5 mL reagent 1 (buffer solution pH 10.5), 1 mL reagent 2 (water), 25 μL reagent 3 (photosensitizer) and 10 μL antioxidant solution were mixed and measured. The lipophilic antioxidants were measured with the ACL kit. 2.3 mL reagent 1 (methanol), 200 μL reagent 2 (buffer solution), 25 μL reagent 3 (photosensitizer) and 10 μL antioxidant 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 (ACW) or by means of the area under the curve (ACL) at different concentrations.

2.6. FRAP assay (Benzie & Strain, 1996; Bub et al., 2000)

The FRAP assay as described by Benzie and Strain (1996) was used with minor modification (Bub et al., 2000). 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. Thirty microlitre of H_2O and 10 μL antioxidant solution were pipetted in a microtiter plate, 200 μL FRAP-solution (acetate buffer, ferric chloride solution, tripyridyl-*s*-triazin (TPTZ) solution) were added, mixed for 10 s and the absorbance was taken after 8 min.

2.7. Statistical analysis

All measurements were done at least in duplicate. All results are given as mean values ± standard deviation. Differences between compounds within each assay were tested for significance by using the one-way ANOVA procedure, Tukey (SPSS for Windows, Release 10.07 (June 2000, SPSS Inc., Chicago)), using a level of significance of $p < 0.05$. The results were defined as "comparable" if $p > 0.05$.

3. Results

3.1. Hydrophilic antioxidants

The regularly used antioxidants ascorbic acid, isoascorbic acid, calcium ascorbate and sodium ascorbate were analysed on their antioxidant activity in comparison to Trolox. Within the two methods TEAC I assay and PCL assay (ACW), these substances showed Trolox equivalents (TE) in the same order of magnitude which are well correlated ($r = 0.82$). In contrast, the FRAP results (mmol ferrous ions formed/mmol) were higher for all compounds and less correlated ($r = 0.57$) to the TEAC results. Calcium ascorbate had significantly higher antioxidant activity in all three assays used. Ascorbic acid and the other derivatives showed nearly comparable results in a lower range. The difference between calcium ascorbate and the other derivatives can be explained by the different molecular structure and was as expected. Calcium ascorbate contains two molecules ascorbic acid per molecule.

The secondary plant products catechin, gallic acid, caffeic acid and rosmarinic acid resulted in different antioxidant activities (Fig. 1) depending on the test system used. The PCL (ACW) results of caffeic acid (ca) and rosmarinic acid (ra) were higher than their results in the TEAC assay and in the FRAP assay. Both compounds showed significantly higher antioxidant activity (PCL: 11.2 TE (ca) and 8.2 TE (ra)) compared to all other substances. Catechin could not be analysed in the PCL (ACW) assay for yet unknown reasons.

3.2. Lipophilic antioxidants

The results for the regularly used lipophilic tocopherols and octyl gallate as well as propyl gallate were in a comparable order of magnitude for the TEAC II assay and the PCL (ACL) assay, both results were well correlated ($r = 0.94$). BHA and BHT showed large differences between both methods. Within the group of lipophilic antioxidants, both gallates had significantly higher antioxidant activity compared to the other substances.

As seen for the hydrophilic assays, the antioxidant activities of the secondary plant products eugenol, quercetin and rosmarinic acid differed largely between both methods (Fig. 2). Rosmarinic acid was the most active compound in the PCL (ACL) assay while quercetin was the antioxidant with the highest activity in the TEAC assay, resulting in a similar TEAC value as recently reported and being higher than all the quercetin glycosides investigated there (Miller & Ruiz-Larrea, 2002).

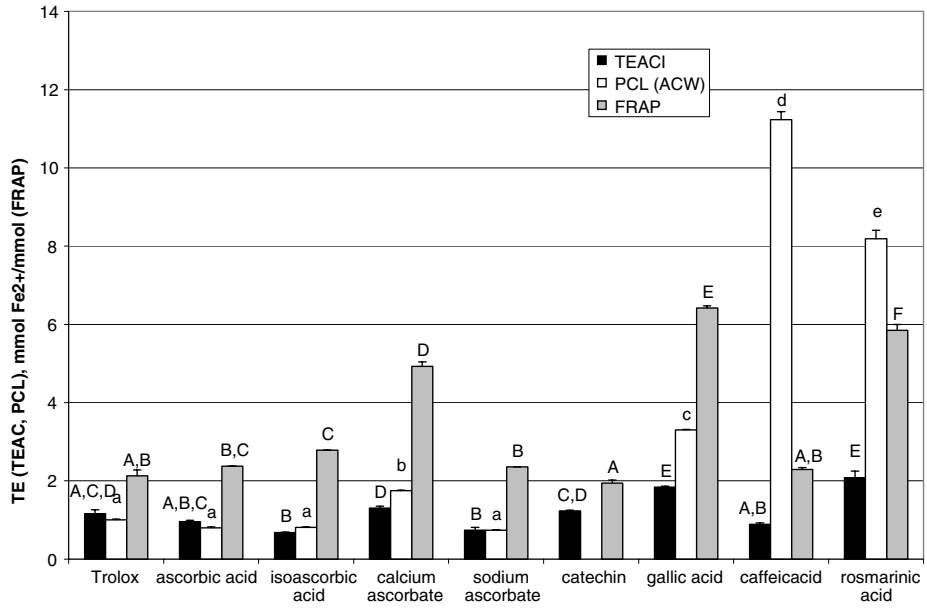

Fig. 1. Hydrophilic antioxidant activity of food additives and secondary plant products in the TEAC I, PCL (ACW) and FRAP assay. Bars for the same assay with different superscript letters are significantly different, $p < 0.05$.

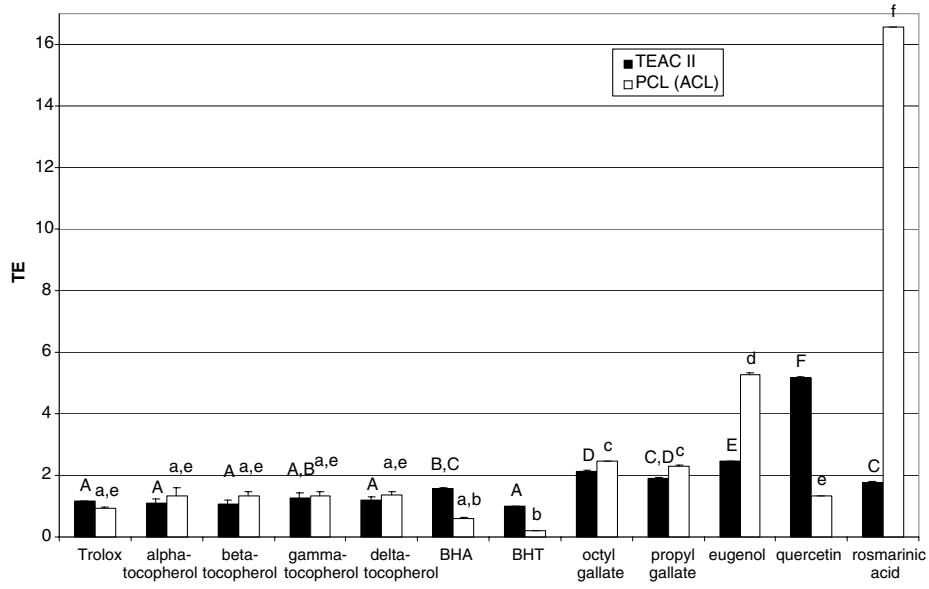

Fig. 2. Lipophilic antioxidant activity of food additives and secondary plant products in the TEAC II and PCL (ACL) assay. Bars for the same assay with different superscript letters are significantly different, $p < 0.05$.

4. Discussion

Partially good correlations were observed between the antioxidant activities of hydrophilic food additives as well as of lipophilic compounds in different test systems. The results of the secondary plant products were not correlated between the different assays, neither for hydrophilic substances nor for lipophilic molecules. These observations are comparable to those of Stupans, Kirlich, Tuck, and Hayball (2002) who determined the antioxidant activity of several natural antioxidants by using three test systems (DPPH assay, LDL oxidation test, formation of dichlorofluorescein). They concluded that activity in one test does not necessarily correlate with activity in another. Ou, Huang, Hampsch-Woodill, Flanagan, and Deemer (2002) analysed 927 vegetable samples on their antioxidant capacity (oxygen-radical absorbing capacity (ORAC) assay, FRAP assay) and observed different ranking orders for both test systems. Different ranking orders of antioxidant capacity were also shown for 34 vegetables and 30 fruits by Pellegrini et al. (2003) who used three different test systems

(TEAC, FRAP, TRAP (total radical trapping antioxidant parameter)). In contrast, Proteggente et al. (2002) showed a good correlation between results in the TEAC, ORAC and FRAP assays for 20 fruits and vegetables. Recently, Murcia et al. (2004) compared the antioxidant activity of seven dessert spices with that of three food additives (BHA, BHT, propyl gallate) by using six different methods. They showed higher antioxidant potential for some natural antioxidants (anise, cinnamon, liquorice, mint) compared to the regularly used food antioxidants as was similarly shown in our investigations. This recent publication, to our best knowledge, is the only one comparing natural antioxidants to common antioxidative food additives so far, in contrast to studies investigating fruits and vegetables. However, it investigated only spices which can be used for desserts. Our experiments used natural antioxidants with a broader spectrum of usage. Nearly all existing comparison studies on antioxidant activity clearly showed differences from test to test due to different reaction principles. Thus, it is strongly recommended and should be state of the art to use more than one assay to determine the antioxidant potential of food extracts or single compounds. However, a ranking within each assay is possible. Ongoing investigations will enlighten more in detail the reasons for the different behaviour from test to test and will lead to recommendations for using the methods.

5. Conclusions

Ascorbic acid and their derivatives as well as tocopherols, BHA and BTH, all regularly used as food additives for stabilisation purposes, showed comparable antioxidant power by using different test systems. In contrast, some natural antioxidants had an up to 16 times higher antioxidant potential. With these results, it might be possible for the food industry to use secondary plant products instead of synthetic compounds to increase the storage stability of processed food items, which might be a good alternative asked for by the consumer.

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