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A critical appraisal of the use of the antioxidant capacity (TEAC) assay in defining optimal antioxidant structures

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Abstract

In the Trolox Equivalent Antioxidant Capacity (TEAC) assay, i.e. the capacity of a compound to scavenge the ABTS radical ($ABTS^{\bullet}$), is assessed. The aim of the present study is to evaluate the applicability of the TEAC assay to predict the antioxidant effectivity of a compound. For this purpose the TEAC assay is compared with other screening assays, such as superoxide scavenging, peroxynitrite scavenging and lipid peroxidation. Of the structurally related compounds, catechol, resorcinol and hydroquinone, resorcinol has the highest TEAC. In contrast, resorcinol appears to have a much lower antioxidant activity than catechol and hydroquinone in other in vitro assays. Similar discrepancies were observed with the flavonoids, chrysin and galangin. The TEAC values of chrysin and galangin are comparable, whereas galangin appears to be a much better antioxidant in other assays. The relatively high TEAC values of chrysin and resorcinol are due to the ability of the reaction products, formed by the reaction of the parent compound with $ABTS^{\bullet}$, to further react with $ABTS^{\bullet}$. With catechol, hydroquinone and galangin, these reaction products do not react with $ABTS^{\bullet}$ and therefore make no contribution to the TEAC. The possible contribution of reaction products to the TEAC of a compound hampers the use of the TEAC assay for constructing structure–activity relationships (SAR).

Keywords: SAR; TEAC; Antioxidant; ABTS radical; Flavonoid

1. Introduction

Oxidative stress is involved in the pathogenesis of various chronic diseases, such as cardiovascular disease and cancer (Halliwell & Gutteridge, 1999). Antioxidants protect against free radicals and are therefore important in obtaining and preserving good health. In this respect, much attention has been given to the flavonoids, a class of polyphenols with strong antioxidant activities.

Within the wide range of methods used to screen antioxidants, the Trolox Equivalent Antioxidant Capacity (TEAC) assay is very popular. This assay is based on the scavenging of the relatively stable blue/green ABTS radical ($ABTS^{\bullet}$), converting it into a colourless product. The degree of this decolorization reflects the amount of $ABTS^{\bullet}$ that has been scavenged and can be determined spectrophotometrically. The TEAC value is assigned by comparing the scavenging capacity of an antioxidant to that of trolox.

The TEAC assay is evaluated using three structurally related compounds. The TEAC values of these compounds are compared to their antioxidant activities in several antioxidant assays. Based on the results, the applicability of the TEAC assay for predicting the antioxidant effectivity of a compound is discussed.

2. Materials and methods

2.1. Chemicals

2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid (trolox), resorcinol, hydroquinone, catechol, xanthine, xanthine oxidase, Nitro Blue Tetrazolium

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(NBT), ascorbic acid, solid KO_2 and dihydrorhodamine-123 were obtained from Sigma and $K_2S_2O_8$ and $FeSO_4$ were obtained from Merck. Nitrogen monoxide was obtained from AGA (Hamburg, Germany). All other chemicals were of analytical grade purity.

2.2. TEAC assay

The TEAC assay, described by van den Berg, Haenen, van den Berg, and Bast (1999) and by Re et al. (1999) has been used with minor modifications. This assay assesses the total radical scavenging capacity, based on the ability of a compound to scavenge the stable ABTS radical ($ABTS^{\bullet}$). $ABTS^{\bullet}$ was formed by adding $K_2S_2O_8$ to ABTS (Re et al., 1999). The reaction of $ABTS^{\bullet}$ with antioxidants was determined spectrophotometrically at 734 nm. The degree of the decolorization gives the antioxidant capacity, which can be converted into TEAC-values.

The $ABTS^{\bullet}$ solution was diluted with phosphate buffered saline (PBS), pH 7.4, to a final absorbance of the control of 0.7 ± 0.02 at 734 nm at 37°6C. Stock solutions of trolox, catechol, resorcinol and hydroquinone were prepared in ethanol.

2.3. Superoxide scavenging (SOS)

Superoxide ($O_2^{\bullet -}$) scavenging was tested in a competition assay in which xanthine–xanthine oxidase was used as an $O_2^{\bullet -}$ generator. Uric acid was produced in the same stoichiometry as $O_2^{\bullet -}$. The reduction of NBT by $O_2^{\bullet -}$ is monitored spectrophotometrically at 550 nm at 25 °C for 3 min. $O_2^{\bullet -}$ scavenging activity of the compounds was determined by competition of the compound with NBT for the $O_2^{\bullet -}$. The compounds tested did not affect uric acid production, determined at 290 nm, indicating that the antioxidants did not influence the $O_2^{\bullet -}$ production by inhibiting xanthine oxidase. The effectivity is expressed as the concentration of scavenger that inhibits NBT reduction for 50% (IC_{50}).

2.4. Peroxynitrite synthesis and peroxynitrite scavenging (PON)

Potassium oxoperoxonitrite(1-) (ONOOK) was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol, Kissner, and Beckman (1996). Peroxynitrite scavenging was measured by the oxidation of dihydrorhodamine-123 (DHR), as described by Kooy, Royall, Ischiropoulos, and Beckman (1994). Fluorescence measurements were performed on a SPF-500C™ spectrophotometer (SLM AMINCO) at 37 °C with excitation and emission wavelengths of, respectively, 500 and 536 nm. The effects are expressed as the concentrations capable of inhibiting fifty percent of the oxidation of DHR (IC_{50}).

2.5. Preparation of microsomes for lipid peroxidation

To obtain microsomes, livers from male Wistar rats (200–250 g) were used. After decapitation, the livers were removed and homogenized (1:2, w/v) in ice-cold sodium phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000 *g* (20 min at 4 °C). The supernatant was centrifuged at 10,000 *g* (20 min at 4 °C) and again at 65,000 *g* (60 min at 4 °C). Subsequently, the microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml) and stored at −80 °C. Before using the control microsomes, they were thawed and washed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl.

2.6. Lipid peroxidation

Microsomes (final concentration approximately 1 mg protein/ ml) were incubated in Tris–HCl/KCl (50 mM/ 150 mM, pH 7.4) at 37 °C in a shaking water bath. Ascorbic acid (0.2 mM) was neutralized with KOH before addition. Reactions were started by adding a freshly prepared $FeSO_4$ solution (10 μM). Lipid peroxidation was assayed by measuring thiobarbituric acid (TBA)-reactive material, as described previously by Haenen and Bast (1983). The reaction in an aliquot of the incubation mixture (0.3 ml) was stopped by mixing with ice-cold TBA–trichloroacetic acid (TCA)–HCl–butylhydroxytoluene (BHT) solution (2 ml). After heating (15 min at 80 °C) and centrifugation (5 min) the absorbance at 535 versus 600 nm was determined. The TBA–TCA–HCl solution was prepared by dissolving 1.68 g TCA and 41.60 mg TBA in 10 ml 0.125 M HCl. One ml BHT-solution (1.5 mg/ ml ethanol) was added to 10 ml TBA–TCA–HCl. The added test compounds did not interfere with the assay in the concentrations used. Results are expressed as means ± SEM.

3. Results

The reaction of the three isomers, catechol, resorcinol and hydroquinone, with $ABTS^{\bullet}$ was examined. The TEAC at 6 min, calculated from the experiment depicted in Fig. 1, of resorcinol was 2.49 ± 0.01 whereas the TEAC values of catechol and hydroquinone were respectively, 1.45 ± 0.02 and 1.33 ± 0.06.

In superoxide scavenging, or in the inhibition of lipid peroxidation, as well as in peroxynitrite scavenging, resorcinol appeared to have a poor antioxidant activity. In these assays hydroquinone and catechol were 10–1000 times more potent than resorcinol (Fig. 2).

A closer look at the time course of the $ABTS^{\bullet}$ consumption in the TEAC assay revealed that resorcinol induced, besides a fast reaction, a slow reaction (Fig. 1).

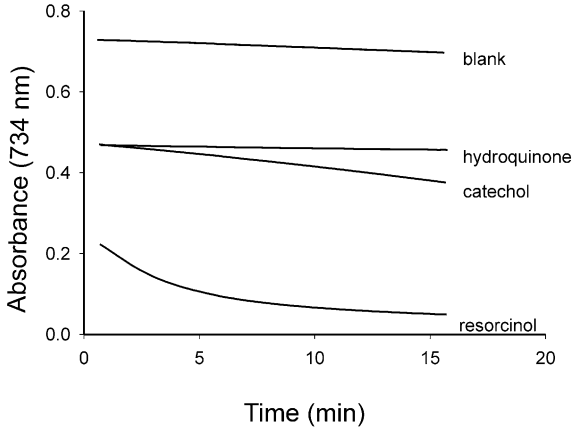

Fig. 1. The reaction of catechol, hydroquinone and resorcinol with ABTS• over time. The absorbance at 734 nm, due to ABTS•, is followed over time. The initial concentration of the tested compounds is 10 μM.

The TEAC value was determined at 6 min and, within these 6 min, the slow reaction had already made a substantial contribution. Moreover, the reaction of resorcinol with ABTS• continued after 6 min.

The slow reaction of resorcinol with ABTS• was examined in more detail by monitoring the changes in the absorbance spectrum during the reaction (Fig. 3). Resorcinol does not absorb between the 300 and 800 nm. ABTS• showed an absorbance maximum at 734 nm. Within ten seconds after adding resorcinol to the ABTS• solution, a product was detected with an absorbance maximum at 540 nm. Subsequently, this product disappeared and another product appeared with an absorbance maximum of 470 nm. In the conversion of the first product into the second one, an isosbestic point was found at 500 nm. During the formation of the second product, the ABTS• concentration also decreased, which was deduced from the reduction of the absorbance at 734 nm. The increase in absorbance in curves 0–4 of Fig. 4 at 470 nm—due to the formation of the second product—was linearly related to the decrease in absorbance at 550 nm—due to the disappearance of the first product—and to the decrease in absorbance at 734 nm—due to the disappearance of ABTS•. Based on this linearity and the presence of an isosbestic point, it was concluded that the first product, formed in the reaction of resorcinol with ABTS•, also reacted with ABTS•. This means that the TEAC of resorcinol does not reflect the antioxidant capacity of resorcinol itself, but rather is the sum of the antioxidant capacity of resorcinol and the reaction product(s).

The reactivity of the reaction products of catechol and hydroquinone with ABTS• was much lower than that of the product formed with resorcinol. The difference in reactivity of the reaction product obtained with hydroquinone and catechol compared to that obtained with resorcinol explains the high TEAC of resorcinol compared to hydroquinone and catechol.

The reaction of ABTS• with chrysin and galangin, two flavonoids that differ only in one OH group, was also followed over the course of time. In the case of chrysin, the obtained spectrum was comparable to that of resorcinol, i.e. the appearance of reaction products that were subsequently consumed in the reaction with ABTS• (data not shown). Galangin, however, showed no evidence for intermediate reaction products at all (data not shown). As reported previously, the TEAC values of the compounds at $t = 6$ min were comparable. However, the antioxidant activity, e.g. peroxynitrite scavenging (IC_{50} galangin $= 1.02 \pm 0.11$ μM; IC_{50}

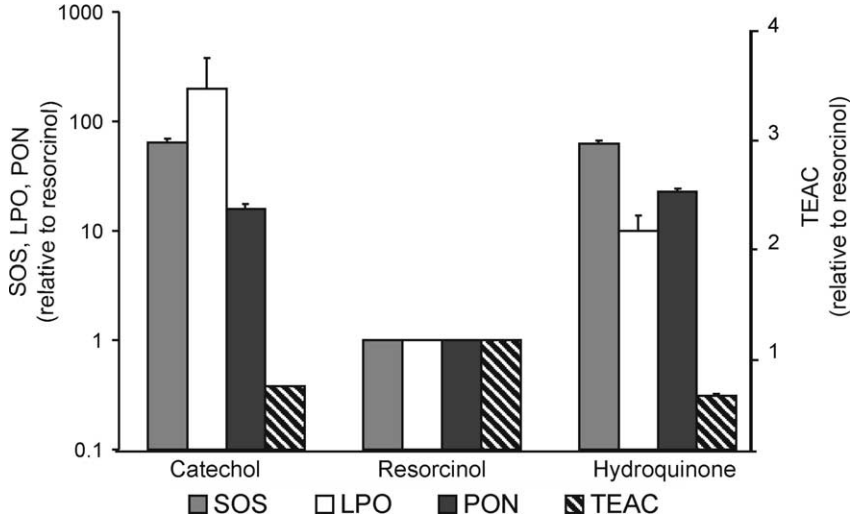

Fig. 2. The antioxidant profile of catechol, resorcinol and hydroquinone. The left *Y*-axis is for superoxide scavenging activity (IC_{50} catechol: 0.014 μM, IC_{50} resorcinol: 0.91 μM, IC_{50} hydroquinone: 0.014 μM), inhibition of lipid peroxidation (IC_{50} catechol: 10 μM, IC_{50} resorcinol: 1556 μM, IC_{50} hydroquinone: 156 μM) and peroxynitrite scavenging (IC_{50} catechol: 2 μM, IC_{50} resorcinol: 42.5 μM, IC_{50} hydroquinone: 1.89 μM). The right axis is for the TEAC-values (catechol: 1.4, resorcinol: 2.5, hydroquinone: 1.3). The activities of catechol and hydroquinone are related to those of resorcinol.

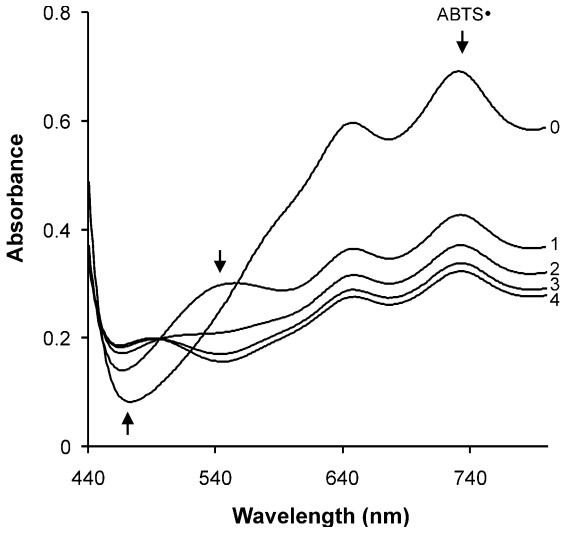

Fig. 3. Reaction of resorcinol with ABTS•, followed spectrophotometrically. Repetitive scans were made. The final concentration of resorcinol is 10 μM. Resorcinol does not absorb between 440 and 800 nm. Spectrum 0 was recorded just before the antioxidant was added, and gives the spectrum of the ABTS• solution. Spectrum 1 was recorded 10 s after the start of the reaction with the addition of ABTS•. The scan time is 30 s. The time between the consecutive spectra is 96 s.

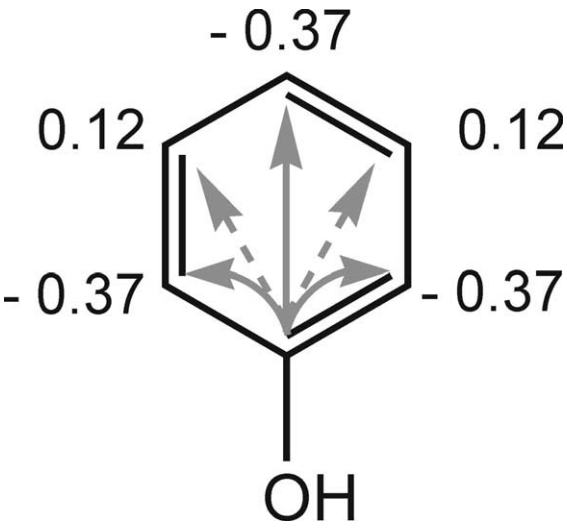

Fig. 4. The Hammet sigma values (σ) of an aromatic hydroxyl group. A hydroxyl substituent on an aromatic ring has an electron withdrawing effect on the 3 position ($\sigma = +0.12$) and an electron donating effect on the 2 and 4 position ($\sigma = -0.37$). The σ-values are from Hansch and Leo (1995).

chrysin = 295 ± 26 μM) and lipid peroxidation (IC_{50} galangin = 1.7 ± 0.12 μM; IC_{50} chrysin = 29 ± 0.95 μM) of galangin were superior to that of chrysin. Apparently the TEAC does not have to reflect the antioxidant activity and the relatively high TEAC of chrysin is caused by the contribution of reaction products to the TEAC.

4. Discussion

The applicability of the TEAC was evaluated using resorcinol, catechol and hydroquinone. Resorcinol appeared to have a much higher TEAC value than catechol and hydroquinone. This means that resorcinol can be qualified as the best antioxidant of the three compounds with the TEAC assay. This is in line with the higher TEAC of resorcylicate (3,5-dihydroxybenzoate) than protocatechuate (3,4- dihydroxybenzoate) or 2,5-dihydroxybenzoate, as reported previously (Rice-Evans, Miller, & Paganga, 1996).

Compared to phenol, the reaction of resorcinol with a radical would theoretically be reduced by the electron-withdrawing effect of the OH-group in the *meta* position. An OH-group on the *ortho* or *para* position has an electron donating effect (Hansch & Leo, 1995) (Fig. 4). This means that both catechol and hydroquinone are expected to react faster with a radical than resorcinol. Apparently, the high TEAC of resorcinol does not fit with the expected low reactivity of resorcinol.

However, in the inhibition of lipid peroxidation, the superoxide scavenging assay and in the peroxynitrite-scavenging assay, catechol and hydroquinone are much better antioxidants than resorcinol. The high TEAC value of resorcinol can be explained by the contribution of reaction products to the TEAC. In the other assays these reaction products make no contribution and the activity of the parent compound only is determined. The relatively high contribution of the reaction product of ABTS• and resorcinol is the reason why the rank order in the TEAC assay is not comparable with the rank order found in other assays.

A comparable discrepancy between TEAC and antioxidant activity is seen with the flavonoids, chrysin and galangin. The TEAC of the two compounds are comparable. The TEAC of chrysin (1.43 ± 0.07) reported in literature (Rice-Evans et al., 1996) is even higher than that of galangin (1.22 ± 0.02) (Williamson, Plumb, & Carcia-Conesa, 1999). However, the antioxidant activity of galangin is superior to that of chrysin. Apparently, the TEAC does not have to correlate with the antioxidant activity. An explanation for this discrepancy is that the TEAC assay measures the total amount of radicals scavenged over a period of time. Both the parent compound and the reaction products can contribute. Most antioxidant activity assays, however, determine the rate at which a radical is scavenged by an antioxidant. This is an activity of the parent compound.

Previously, we have found that the total amount of ABTS• scavenged by a compound correlates with the biological activity in a selected group of flavonoids (van den Berg, Haenen, van den Berg, van der Vijgh, & Bast, 2000). The rate at which the ABTS is scavenged shows a less good correlation. It is noteworthy that the number of compounds tested within this study was small and

that the correlation might have been coincidental. The present study clearly demonstrates a fundamental characteristic of the TEAC assay, i.e. the potential contribution of reaction products. The possible contribution of reaction products to the TEAC value of a compound hampers the use of the TEAC assay for the construction of SARs. In a SAR the activity has to be attributed to a single molecular structure only (Hansch & Leo, 1995). TEAC-based SARs have frequently been constructed (Lien, Ren, Bui, & Wang, 1999; Plumb, Price, & Williamson, 1999; Rice-Evans et al., 1996). When these SARs are examined in detail, inconsistencies are expected to be found.

Rudimentary SARs on the antioxidant activities of flavonoids indicate that the antioxidant activity of flavonoids depends on the number of free OH-groups (Cao, Sofic, & Prior, 1997). This is challenged by the difference in activities of catechol, resorcinol and hydroquinone.

Lien et al. (1999) have constructed a QSAR based on the number of OH substituents, also incorporating-to some extent- the location. When this QSAR is applied to myricetin and quercetin or to apigenin and kaempferol, two pairs of compounds that differ only in one OH-group (Fig. 5), myricetin has a higher predicted value than quercetin (3.55 versus 3.10) and kaempferol has a higher predicted TEAC value than apigenin (2.31 versus 1.45), according to the SAR. However, the measured TEAC value of myricetin is lower than the value of quercetin (3.10 versus 4.7) and the measured value of kampferol is lower than that of apigenin (1.34 versus 1.85). Apparently, the SAR that correlates the number of OH-moieties to the TEAC is not consistent.

Plumb et al. (1999) stated that removal of the 2,3 double bond drastically reduces the antioxidant activity, which does not hold for dihydrokaemferol compared to kaempferol (Fig. 5). Based on the TEAC values, Rice-Evans et al. (1996) reported that a criterion for maximal radical scavenging is "the combination of the 5-OH group in the A ring with the 3-OH group and the 4-oxo function in the C ring with the 2,3-double bond". This aspect of the SAR does not apply for dihydrokaempferol, which lacks the 2,3-double bond, and apigenin, that lacks the 3-OH group. Apigenin does not

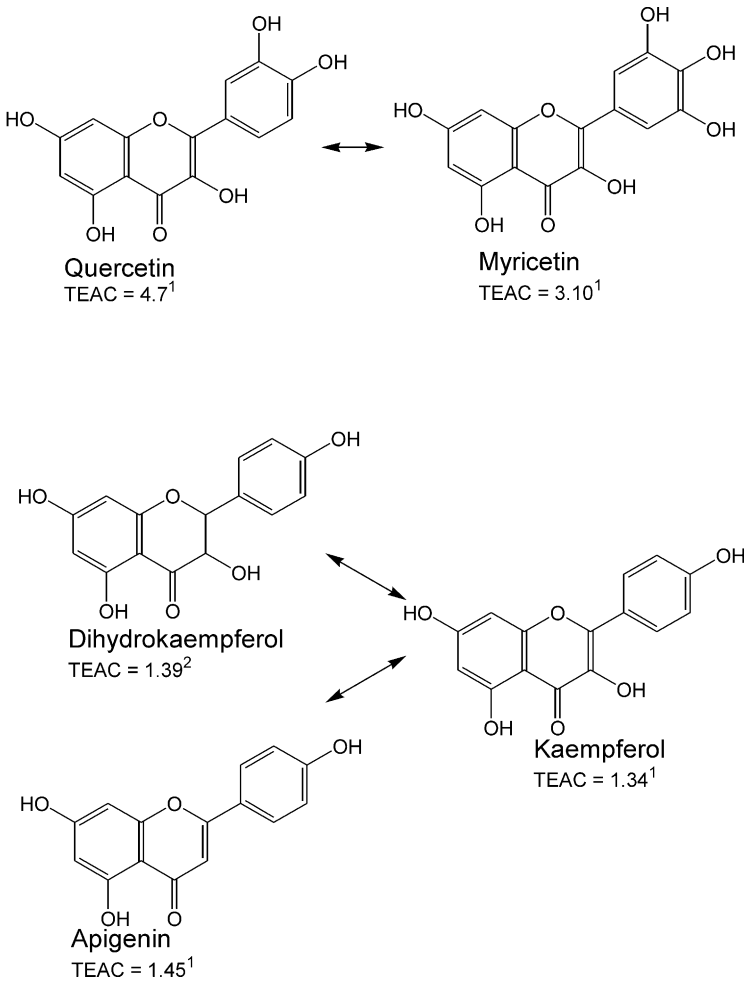

Fig. 5. Structures of several flavonoids and their TEAC values at 6 min. [1] Data are taken from Rice-Evans et al. (1996) and Lien et al. (1999). [2] Data are taken from Lien et al. (1999).

have a lower TEAC value than kaempferol. These inconsistencies illustrate the limitations of the TEAC assay in constructing SARs.

Most other screening assays have shortcomings as well. Appropriate control experiments are often needed. It should be noted that the different potencies of catechol, resorcinol and hydroquinone in the inhibition of lipid peroxidation is primarily due to differences in electron-donating effect and not to other factors, such as differences in iron chelation or lipophilicity. Moreover, none of the compounds affected xanthine oxidase in the superoxide scavenging assay.

In the case of the TEAC assay, reaction products were found to contribute and it is impossible to correct for this. In fact, a difference in TEAC value is primarily caused by a difference in reactivity of the reaction products. This makes this assay inappropriate for SARs.

The TEAC assay is, however, useful in tracking down unknown antioxidants in complex mixtures (van Overveld, Haenen, Rhemrev, Vermeiden & Bast, 2000); however, one has to be aware of possible interactions, which might result in masking (Arts, Haenen, Voss & Bast, 2001; Arts et al., 2002).

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