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Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures

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Abstract

The TEAC (Trolox equivalent antioxidant capacity) assay is based on scavenging of 2,2′-azinobis-(3- ethylbenzothiazoline-6-sulfonate) radical anions ($ABTS^{\bullet -}$). In this report we describe a modification based on pre-generation of the ABTS radical anions with a thermolabile azo compound, 2,2′-azobis- (2-amidinopropane)HCl (ABAP). This modification makes the assay less susceptible to artefacts, e.g. influence on the radical generation process. For most antioxidants tested, a biphasic reaction pattern was seen, i.e. a fast and slow scavenging rate. We evaluated application of the assay with both lipophilic and hydrophilic compounds with antioxidant capacity. Several organic solvents, compatible with water, were tested with α-tocopherol, quercetin and β-carotene. It was found that the TEACs differed in various solvents. Under standardized conditions additivity of TEACs obtained from individual antioxidants could be demonstrated. This might enable application of the assay for the identification of "unknown" antioxidants.

Keywords: ABTS; Antioxidant capacity; Free radicals; Antioxidants; TEAC

1. Introduction

It is increasingly recognized that oxidative stress is involved in the pathophysiology of various chronic diseases, such as cardiovascular disease and cancer, and that dietary antioxidants may play a role in prevention of these diseases (Halliwell & Gutteridge, 1985). Besides the antioxidant (pro-)vitamins, such as vitamin E, C and β-carotene, the number of other compounds reported to have antioxidant activity is increasing. There is a continuous search for "new" compounds and unidentified food ingredients with an antioxidant potential.

A wide range of methods are currently used to assess antioxidant capacity (Halliwell, Aeschbach, Loliger & Aruoma, 1995), for example for measurement of prevention of oxidative damage to biomolecules such as lipids or DNA and methods assessing radical scavenging. Both in vivo and in vitro assays are used and all methods have their own advantages and limitations.

Simple scavenging assays, such as the TRAP (total reactive antioxidant potential or total radical-trapping antioxidant parameter; Wayner, Burton, Ingold, Barclay & Locke, 1987) and the TEAC (Trolox equivalent antioxidant capacity) assay, have gained popularity because they enable high-throughput screening on potential antioxidant capacity. Such methods are used to assess antioxidant capacity of biological matrices, such as plasma, as well as single compounds, food components or food extracts.

The TEAC assay, originally described by Miller, Rice-Evans, Davies, Gopinathoan and Milner (1993), is based on scavenging of long-lived radical anions ($ABTS^{\bullet -}$; Scott, Chen, Bakac & Espenson, 1993). In this assay radicals are generated through the peroxidase activity of metmyoglobin in the presence of hydrogen peroxide and can easily be detected spectrophotometrically at 734 nm. In this assay antioxidants are added before the $ABTS^{\bullet -}$ formation is initiated by hydrogen peroxide, resulting in a delay in radical formation ("lag-time") which is measured. A TEAC value can be assigned to all compounds able to scavenge the $ABTS^{\bullet -}$ by comparing their scavenging capacity to that of Trolox, a water-soluble vitamin E analogue. Arnao, Cano, Hernandez-Ruiz,

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Abbreviations used: The frequently used term ABTS radical anions ($ABTS^{\bullet -}$) is usually termed ABTS radical cations in other publications.

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Garcia-Canovas and Ascosta (1996) reported a modification of the TEAC assay by introducing HRP (horseradish peroxidase) for generation of the $ABTS^{\bullet -}$. However, Strube, Haenen, van denBerg and Bast (1997) showed that these "pre-addition" assays might result in overestimation of the antioxidant capacity due to compounds interfering with the formation of the $ABTS^{\bullet -}$ and proposed a "post-addition" protocol, in which compounds were added after radical formation. In this report we describe a further refinement of the assay by pre-generating the $ABTS^{\bullet -}$ in the presence of a thermolabile azo compound, 2,2′-azobis-(2-amidinopropane) (ABAP) (Campos & Lissi, 1996). Generation of radicals before the antioxidants are added prevents interference of compounds which affect radical formation. This modification makes the assay less susceptible to artefacts and prevents overestimation of antioxidant capacity.

We also address the difference in reaction kinetics between various compounds as well as the measurement of compounds with different hydrophobicity. Because the TEAC is performed in an aqueous buffer only water-soluble compounds are measured. As foods generally contain both water- and lipid-soluble compounds with antioxidant capacity, it would be attractive to be able to use one method for screening on potential scavenging capacity. We looked into the possibility to evaluate lipid-soluble compounds in the TEAC assay by solubilizing these compounds, especially α-tocopherol and the carotenoid β-carotene, in the aqueous assay medium using suitable solvents.

2. Materials and methods

2.1. Chemicals

$ABTS^{2-}$ (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate)) was obtained as sulfonic acid from Sigma (St. Louis, MO), ABAP (2,2′-azobis-(2-amidinopropane)HCl) from Polysciences (Warrington, PA), Trolox ((+/-)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97%) from Aldrich (Milwaukee, WI), sodium-L-(+)-ascorbate from Merck (Darmstadt, Germany), quercetin, hesperidin and naringin from Fluka (Bio Chemica, Buchs, Switzerland), α-tocopherol (95%) and lycopene from Sigma, β-carotene from Merck and zeaxantin, β-cryptoxantin and narirutin from Roth (Kalsruhe, Germany). Ethanol (absolute) was purchased from J.T. Baker (Deventer, Netherlands), acetone (p.a.) from Merck and Triton X-100 (ethoxylated *p*-tert-octylphenol) from Koch-light Laboratories (Colebrook, Bucks, UK). THF (tetrahydrofuran) was obtained from Lab Scan (Dublin, Ireland) and SDS (sodium dodecyl sulfate) from Merck. All other chemicals were purchased from Merck and were all of p.a. quality.

Just before use THF was purified by distillation. A few pellets of potassium hydroxide were added to the THF before distillation.

2.2. Apparatus

A Pharmacia Ultraspec 4000 spectrophotometer with a thermostatic cuvette holder maintained at 37°C was used for all antioxidant capacity measurements.

2.3. Measurement of total antioxidant capacity

An $ABTS^{\bullet -}$ solution was prepared by mixing 2.5 mM ABAP with 20 mM $ABTS^{2-}$ stock solution in 100 mM phosphate buffer (pH 7.4), containing 150 mM NaCl (PBS). The solution was heated for 12 min at 60°C, protected from light and stored at room temperature. To check $ABTS^{\bullet -}$ formation the absorbance at 734 nm was determined (absorption had to be between 0.35 and 0.40). A fresh ABTS/ABAP stock solution was prepared every day. Because of a gradual decrease in absorbance of the ABTS/ABAP stock solution (ca 2% per hour) appropriate blanks were recorded for each measurement (the blank is the decrease in absorption of the solvent without the compound added).

For measuring antioxidant capacity 40 μl of the sample was mixed with 1960 μl of the radical solution. Absorbance was monitored at 734 nm for 6 min. The decrease in absorption at 734 nm 6 min after addition of a compound was used for calculating the TEAC. All experiments were performed at least in triplicate and in a concentration range between 1 and 10 μM (final concentrations). The antioxidants were dissolved in different solutions: ethanol, THF, acetone, SDS (final concentration in assay 0.03 mM) and TX-100 (final concentration in assay 17 mM).

2.4. Preparation of the antioxidant mixture

Single solutions of α-tocopherol, β-carotene and vitamin C were prepared in ethanol, THF and PBS, respectively. Further dilutions were made in ethanol, thus the TEAC of the single compounds and the mixture were determined in ethanol (no more than 0.04% of another solvent left).

2.5. Calculations of antioxidant capacity

A calibration curve was prepared with different concentrations of Trolox. By measuring ΔAbs over 6 min for Trolox (standard range of 0–10 μM), absorbance values were corrected for the solvent.

$$\Delta A_{\text{Trolox}} = (A_{t=0\ \text{Trolox}} - A_{t=6\ \text{min Trolox}}) - \Delta A_{\text{solvent (0–6 min)}} \quad (1)$$

The regression coefficient (*r.c.*) is calculated from the calibration curve.

$$\Delta A_{\text{Trolox}} = r.c.^{*}[\text{Trolox}] \quad (2)$$

The *r.c.* should be 0.028 per μM Trolox. To establish the TEAC for unknown compounds the $\Delta A_{\text{unknown}}$ was measured in the same way and corrected for the blank value. The TEAC of an unknown compound X gives the antioxidant capacity of that compound relative, on a molar basis, to Trolox.

$$\text{TEAC}_X = \Delta A_X/(r.c.^{*}[x]) \quad (3)$$

The TEAC of mixtures, such as a food (vegetable) extract or a drink, represents the concentration of a Trolox solution that has the same antioxidant capacity as the mixture. Usually the mixture or extract has to be diluted to have it fit in the range of the assay. Appropriate correction for the dilutions should be made.

$$\text{TEAC}_{\text{solution}} = (\Delta A_{\text{solution}}/r.c.)^{*}\text{dilution factor } (M) \quad (4)$$

3. Results

3.1. Modification of the assay

The "original" TEAC assay was modified to prevent artefacts due to interference with radical generation. Strube et al. (1997) have shown that, for example, potassium cyanide inhibits radical formation rather than scavenging $ABTS^{\bullet -}$. Such an inhibitory effect results in overestimation of the antioxidant capacity of compounds, or measuring TEACs for compounds that have actually no antioxidant potential. Therefore, $ABTS^{\bullet -}$ were pre-generated by heating $ABTS^{2-}$ with the thermolabile azo compound 2,2′-azobis-(2-amidinopropane) (ABAP). This modified protocol was evaluated by adding single compounds to the assay. Cyanide, giving an apparent TEAC in the original assay, showed no antioxidant capacity in the modified assay. Trolox reacts instantaneously with $ABTS^{\bullet -}$ (Fig. 1A). When quercetin was added to the $ABTS^{\bullet -}$, a biphasic reaction was observed (Fig. 1B). This reaction pattern implies that for the reaction of $ABTS^{\bullet -}$ with quercetin, the reduction in absorption [ΔA_X, in section calculation of antioxidant capacity (3)], and thus the TEAC, depends on the time point used to read the absorption. Although to a lesser extent, such a biphasic reaction pattern was also observed for vitamin C (Fig. 1C). This biphasic response was described as the TEAC at 10 s and 6 min respectively. The 10 s TEAC gives the "fast" reaction, whereas the TEAC at 6 min was chosen because Miller used the same time period and it also includes the greater part of the "slow" reaction.

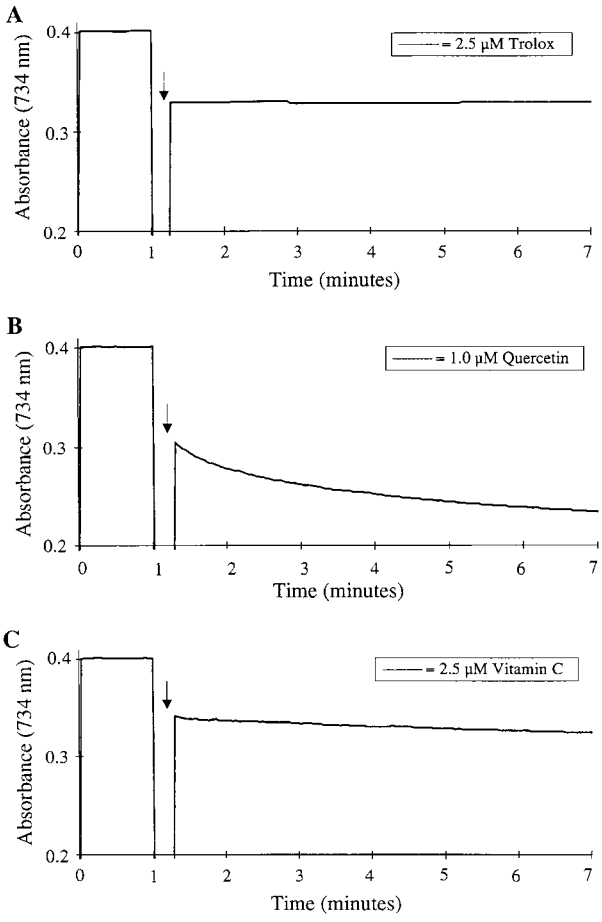

Fig. 1. Reaction of Trolox (A), quercetin (B) and vitamin C (C) with the $ABTS^{\bullet -}$. Trolox (2.5 μM final concentration) and quercetin (1 μM final concentration) dissolved in ethanol and vitamin C (2.5 μM final concentration) dissolved in PBS buffer were added at the time-point indicated by the arrow.

As shown in Fig. 2, the TEAC for quercetin is concentration-dependent: a higher concentration resulted in a lower TEAC. This was also found with flavanols such as naringin and narirutin (data not shown), but not for the other compounds tested (Table 1).

3.2. Lipophilic antioxidants

To investigate whether more lipophilic compounds could also be assessed in the modified TEAC assay, we tried to find a compatible solvent to solubilize these compounds in the aqueous assay medium. We tested several solvents, such as tetrahydrofuran (THF), acetone and Triton X-100 (TX-100). To assess the effect of these solvents, α-tocopherol and β-carotene were used as typical lipophilic compounds and quercetin as an amphiphilic compound.

As expected, the TEAC of α-tocopherol was around 1, since tocopherol has the same antioxidant moiety (the chroman ring) as Trolox, and was not affected by the solvent used to solubilize tocopherol in the aqueous assay medium (Fig. 3A).

The TEAC of quercetin, measured in the various solvents at a 1.0 μM (final) concentration, also appeared not to depend on the solvent used, except when dissolved in TX-100 (Fig. 3B). Results for β-carotene were, however, quite variable when using different solvents (Fig. 3C). With THF, β-carotene showed the highest TEAC, whereas no significant antioxidant capacity could be demonstrated if dissolved in acetone. These results show that measurement of lipid-soluble compounds in the TEAC assay can be troublesome. To overcome this solvent dependence of the TEAC, standardized procedures should be used.

The use of different solvents for solubilizing the lipid-soluble compounds in the assay did not affect the antioxidant capacity of the water-soluble antioxidants, such as vitamin C (data not shown).

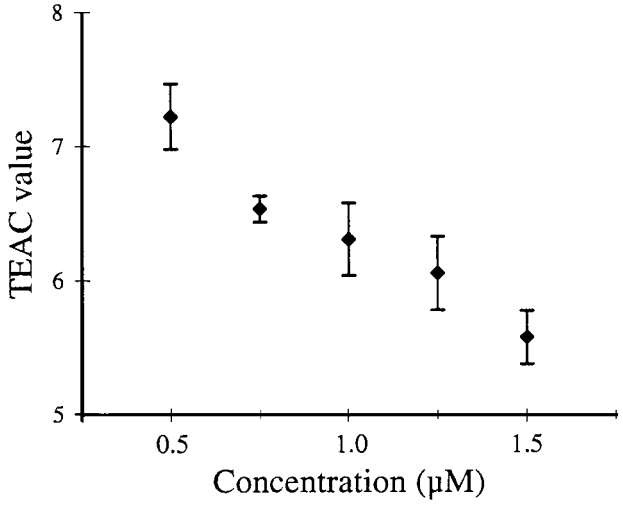

Fig. 2. The TEAC of quercetin measured after 6 min in different concentrations.

Table 1
The TEACs of various antioxidants

	Original assay	Modified assay	
	TEAC at 6 min	TEAC at 10 s	TEAC at 6 min
Trolox	1.00	1.00	1.00
α-Tocopherol	0.97 ± 0.01[f]	0.95 ± 0.02	0.97 ± 0.05
Vitamin C[b]	0.99 ± 0.04[f]	0.86 ± 0.02	1.14 ± 0.04
Dehydroascorbate[b]	0.00[g]	0.00 ± 0.01	0.25 ± 0.05
Quercetin	4.72 ± 0.10[h]	3.49 ± 0.33[e]	6.43 ± 0.27[e]
Hesperidin	1.0 ± 0.03[i]	0.16 ± 0.02[e]	1.21 ± 0.01[e]
Naringin	0.24 ± 0.02[i]	0.01 ± 0.01[e]	1.08 ± 0.01[e]
Narirutin[b]	0.8 ± 0.5[i]	0.01 ± 0.01	1.22 ± 0.03
β-Carotene	1.9 ± 0.10[cj]	0.88 ± 0.08[d]	1.61 ± 0.07[d]
Lycopene	2.9 ± 0.15[cj]	1.59 ± 0.07[d]	2.31 ± 0.07[d]
Zeaxantine	1.4 ± 0.04[cj]	0.88 ± 0.13[d]	1.70 ± 0.05[d]
β-Cryptoxantine	2.0 ± 0.02[cj]	0.95 ± 0.08[d]	1.65 ± 0.02[d]

[a] TEACs are means ± SD, $n = 3$–6. The listed compounds were dissolved in ethanol.
[b] Dissolved in PBS buffer.
[c] Dissolved in hexane/acetone.
[d] Dissolved in THF.
[e] Due to concentration dependency of the TEAC the given value was the TEAC measured at standardised final concentrations; quercetin (1 μM), naringin (5 μM) and narirutin (5 μM).
[f] Data from Miller et al. (1993).
[g] Data from Miller et al. (1995).
[h] Data from Salah et al. (1995).
[i] Data from Rice-Evans, Miller and Papaganga, (1997).
[j] Data from Miller et al. (1996).

3.3. Evaluation of antioxidant mixtures

The previous section, described measurement of TEACs for single compounds. However, the TEAC

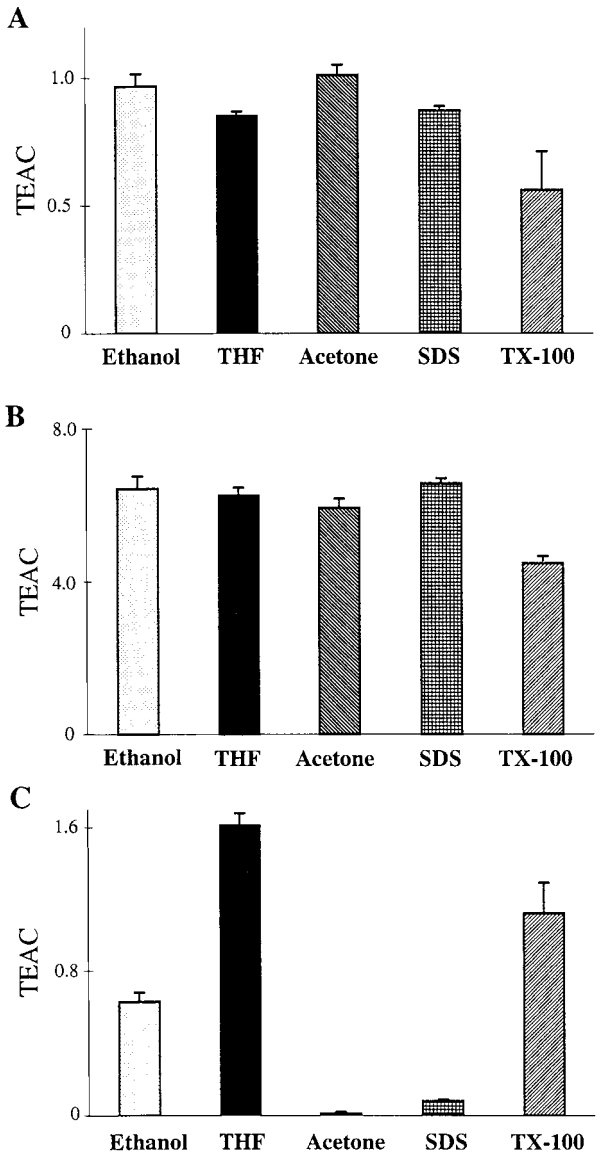

Fig. 3. The effect of different solvents on the TEAC of α-tocopherol (A) quercetin (B) and β-carotene (C). The TEAC was calculated 6 min after addition of 2.5–7.5 μM α-tocopherol, 1 μM quercetin and 1 μM β-carotene, respectively ($n = 3$).

assay is often used to assess the antioxidant capacity of mixtures of antioxidants. To evaluate the modified TEAC assay for mixtures of antioxidants, a solution of lipophilic and hydrophilic compounds was prepared in ethanol.

When using ethanol instead of THF (Table 1), the TEAC of β-carotene was 0.63 rather than 1.61. Under these conditions, the TEAC, at 6 min, for a mixture of vitamin C, α-tocopherol and β-carotene was found to be additive (Fig. 4).

The applicability of the protocol was tested for a soft drink and some fruit juices. For the TEAC of these solutions, it should be noted that a constant dilution factor was applied because of the concentration dependence of the TEAC. To standardize the procedure, "pure" drinks were diluted with PBS buffer to a 5% solution. The results are listed in Table 2.

Between the drinks, considerable differences in TEAC were found. In all the cases, the measured TEAC was higher than could be explained on the basis of vitamin C and flavanones (measured as hesperidin, naringin and narirutin) contents. Vitamin C, the main known antioxidant in the fruit juices, can largely explain the antioxidant capacity measured. The contribution of the flavanones is too small to explain the remaining antioxidant capacity. Thus, other unidentified antioxidants appear to be present as well.

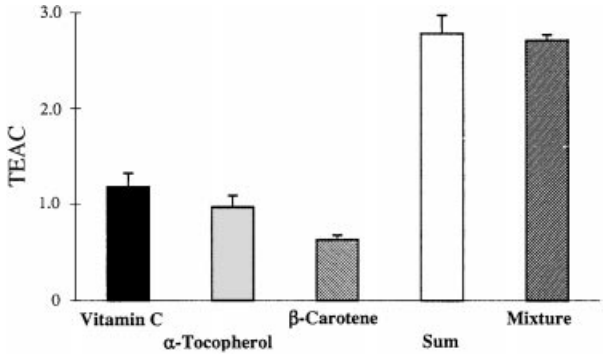

Fig. 4. The TEAC of the separate antioxidants vitamin C, α-tocopherol and β-carotene and of the mixture ($n=6$).

4. Discussion

Radical scavenging assays, such as the TEAC assay, are increasingly used for screening compounds, food products and extracts on their antioxidant capacity, but also to assess antioxidant status in biological fluids such as plasma.

In this report we describe a modification of the original TEAC assay as described by the group of Miller and Rice-Evans. In their original assay $ABTS^{\bullet-}$ were generated in the presence of the test compounds, which might result in overestimation of "true" antioxidant capacity due to interference with the radical-generating system rather then scavenging (Strube et al., 1997). We generated $ABTS^{\bullet-}$ separately before the test compound was introduced in the assay. Cyanide, which interfered in the original assay with $ABTS^{\bullet-}$ formation, indeed did not show any antioxidant capacity in the modified assay.

4.1. The modified TEAC assay

To evaluate our modified TEAC assay we determined the TEAC of some hydrophilic and lipophilic compounds with well established antioxidant values, using various assay conditions and solvents. Table 1 shows the TEACs obtained with the original and the modified assay. For some well known antioxidants such as vitamin C and quercetin we observed a biphasic reaction with a "fast" and a "slow" scavenging rate. This may be explained by the formation of reaction products that react slowly with $ABTS^{\bullet-}$ resulting in a biphasic reaction pattern. For example, if vitamin C reacts with $ABTS^{\bullet-}$, the reaction product dehydroascorbate is generated, which can be slowly autoxidized yielding vitamin C, giving the slow reaction. Also slow intramolecular rearrangement of antioxidants may cause biphasic reactions.

Table 1 shows that the "fast" TEACs of vitamin C and quercetin were slightly lower, while the "total" TEACs were higher than the TEAC reported by Miller, Diplock and Rice-Evans (1995). It should, therefore, be realized that in comparing TEACs from different

Table 2
Antioxidant capacity of some fruit/soft drinks in relation to antioxidant contents

Drinks	Vitamin C (mM) analysed	Vitamin E (mM) analysed	Hesperidin (mM) analysed	Naringin (mM) analysed	Narirutin (mM) analysed	TEAC Calculated[b]	(mM) Measured[a]
Orange juice 1	2.67	n.d.[c]	0.77	n.d.	0.12	**4.13**	**6.63**
Orange juice 2	1.11	n.d.	0.36	n.d.	0.06	**1.77**	**3.19**
Grape fruit juice	1.73	n.d.	n.d.	0.69	0.21	**2.97**	**5.57**
Soft-drink	0.91	0.06	n.d.	n.d.	n.d.	**1.10**	**1.74**

[a] The TEAC of the drinks were measured at 6 min after addition to the assay.

[b] Calculation of the theoretical TEAC is the sum of the concentrations of the single compounds multiplied by their own TEAC (determined at the concentration present in the drinks, to overcome the concentration dependency of the TEAC).

[c] n.d. Not detected (below the lower limit of quantification).

reports, the time interval used should be taken into account. Miller, and Rice-Evans (1997) also reported on a modification of their TEAC assay, including pre-formed ABTS radicals. Surprisingly, no difference was found between the TEAC at 1 min and the TEAC at 6 min after addition of vitamin C and quercetin. In contrast, Schofield and Brayanza (1996), using the same type of assay as Miller and Rice-Evans (1997) report that there is indeed a time dependency in the TEAC. Our results show that most antioxidants with $ABTS^{\bullet -}$ exhibit a slow reaction as well. Only Trolox displays a fast reaction. It can be concluded, therefore, that the TEAC of most antioxidants has to depend on the time interval used in the assay. This was indeed found in our modified assay.

Whether such a "fast" and "slow" radical scavenging mechanism also occurs in vivo is unclear. Free radical species occurring in vivo have short half-lives and react very rapidly, which might suggest that especially the fast reaction is important.

As shown in Fig. 3, quercetin exhibits a concentration-dependent response. This also holds for the flavanones naringin and narirutin (data not shown). This indicates that the TEAC of phenolic compounds like quercetin depends on the concentration used in the assay, which complicates interpretations of the assay results.

4.2. TEAC of lipophilic compounds

A practical relevant question is whether the TEAC assay can also be applied to lipophilic compounds such as α-tocopherol and β-carotene. Miller, Sampson, Candeias, Bramley and Rice-Evans (1996) have reported TEACs of carotenoids. In their procedure compounds dissolved in hexane/acetone were shaken with the ABTS radical solution. Scavenging might, in this case, occur at the interface between the aqueous and the organic phase, but whether the observed decrease in absorption is representative of total antioxidant capacity remains questionable.

In this study we compared several solvents compatible with water to solubilize lipophilic compounds in the assay mixture. Our results show that the TEAC of lipid-soluble compounds, such as β-carotene, strongly depends on the solvent used. Because of its limited solubility, β-carotene could only be measured at low concentrations (1 μM final concentration) which resulted in a small decrease in absorption and therefore a high variability.

Because of the variable TEACs found for β-carotene, the question remains: What is the "true" antioxidant capacity of β-carotene? The singlet oxygen quenching ability of β-carotene has well been established (DiMascio,Kaiser & Sies, 1989), but whether β-carotene acts in vivo as an antioxidant is still controversial (ATBC, 1994; Hennekens et al., 1994). The results of the present study show that not only the intrinsic reactivity with radicals, such as $ABTS^{\bullet -}$, is important, but that also other factors, like the solvent, play an important role in the antioxidant capacity of β-carotene.

Solubilization of lipophilic compounds, such as β-carotene, in the aqueous TEAC assay medium remains a problem; a low TEAC might reflect either limited scavenging or limited solubility.

4.3. Evaluation of the antioxidant capacity of mixtures

Under standardized conditions, the TEAC of a mixture of water and lipid-soluble compounds can be additive, i.e. equal to the sum. This might allow application of the assay for screening of mixtures, as we illustrated with some fruit juices (Table 2).

Comparing the theoretical TEAC, based on product composition, with the actually measured TEAC may indicate the presence of other, "unknown" antioxidants. However, standardization of the assay is extremely important and concentration dependence and solvent dependence should be taken into account. This approach has been reported before for apple juices. However, in that report (Miller et al., 1995), the TEAC of the individual compounds measured at 6 min were used, while the TEAC of the juice was calculated after 2 min and 50 s.

In conclusion, the modified TEAC assay described in this report is faster than the originally described assay and less sensitive to artefacts because of pre-generation of the $ABTS^{\bullet -}$ before addition of the test compounds. However, for most of the antioxidants, a biphasic reaction pattern was found and both a "fast" and a "total" TEAC are determined. Under standard conditions, lipid-soluble compounds could be measured in the assay. In the case of mixtures of lipid-soluble and water-soluble compounds, additive results could be obtained.

Application of the assay to fruit juices is complicated. In spite of these complications, we feel confident that the TEAC is a useful tool in screening the antioxidant capacity of both pure antioxidants and mixtures. For application of the assay to (solid) food products, more research is needed, especially regarding the extraction of water- and lipid-soluble antioxidants and solubilization of the latter compounds in the assay.

Based on the data presented in the present study, one should be very careful in the interpretation of the results of the TEAC assay. We believe that quantitative evaluation of antioxidant capacity using the TEAC can be troublesome or even impossible, but it can be used to provide a ranking order of antioxidants.

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