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Pitfalls in a Method for Assessment of Total Antioxidant Capacity

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A relatively simple and widely applied method for quantitating the total antioxidant capacity of body fluids and drug solutions based on the absorbance of the ABTS radical cation was evaluated. In this assay, the end-point is an antioxidant-induced decrease in absorbance at a fixed time. This decrease is used as an index of total antioxidant capacity. It is shown that Trolox, potassium cyanide and quercetin all decrease the absorbance of ABTS radical cations at a fixed time, but by different mechanisms. Trolox scavenges the ABTS radical, potassium cyanide inhibits radical formation, while quercetin acts by both mechanisms. Using this method antioxidant capacity may be overestimated, due to both a scavenger effect and an effect on the rate of ABTS oxidation. To distinguish between these effects, a post-addition assay was used in which the sample is added when the formation of radicals is stable. Using post- addition assay conditions enables discrimination between effects on radical scavenging and on the radical formation, two major mechanisms for antioxidant action. In extrapolating the results to an *in vivo* situation it should be questioned: (i) whether the peroxidase process does indeed mimic the process of radical formation *in vivo*, and (ii) whether the ABTS radicals do resemble the radical species involved in an *in vivo* situation. Results obtained in the ABTS radical-based methods should therefore be reviewed critically before the antioxidant capacity can be assessed.

Keywords: Total antioxidant capacity, Trolox, potassium cyanide, quercetin, TEAC

INTRODUCTION

There is an increasing interest in the level of antioxidants in food from nutritionists, clinicians, biochemists, food scientists and food manufacturers. Recent evidence has raised the possibility that major diseases may be prevented by the simple expedient of improving the dietary intake of antioxidants.[1] Furthermore, antioxidants are of interest because of the interaction of additives with the food matrix and maintenance of shelf-life, quality and attractiveness of the food. A broad definition of an antioxidant is "any substance that when present at low concentration compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate".[2] A still increasing number of assays to determine the antioxidant profile of single

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compounds and extracts is being developed and validated. Each assay has its own characteristics and therefore none of the assays described can give a complete picture of the antioxidant profile of a compound. Only part of the picture is revealed by any single assay. Besides measuring the rate of the reaction with an antioxidant or scavenger with a certain reactive oxygen species, it is also important to be able to measure total antioxidative capacity. Several assays have been developed for assessing this capacity.[3–10] Most of these assays could be used in plasma samples but also for evaluation of the antioxidant capacity in food. All these methods are based on inhibition of the accumulation of oxidized products.

Recently an assay based on the scavenging of ABTS radicals has been described by Miller *et al.*[7] This assay looks promising and may be used for routine screening of antioxidants. In this study the merits of the assay are evaluated.

MATERIAL AND METHODS

Materials

Myoglobin (from horse heart) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Sigma, Trolox ((+/–)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (97%)) from Aldrich, potassium iron(III) cyanide from Acros, quercetin from Fluka Bio Chemika. Potassium cyanide, hydrogen peroxide and the buffer salts sodium chloride (NaCl), sodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were all purchased from Merck.

The buffer used was a PBS buffer, which was brought to a pH of 7.4 (by addition of NaOH). The buffer contained NaCl (0.9%, w/v), Na_2HPO_4 (50 mM), KH_2PO_4 (50 mM), which were dissolved in Millipore water (15 MΩ/cm). The buffer was purged with N_2 gas, and continuously stored at 5°C. All solutions were made freshly each day. All compounds were dissolved in PBS buffer, except for Trolox and quercetin which were dissolved in ethanol (analytical reagent).

Methods

Total Antioxidant Capacity Assays

The total antioxidant capacity of the test compounds was determined according to the assay described by Miller *et al.*[7] and Rice-Evans & Miller,[11] with some modifications. Metmyoglobin was prepared from myoglobin (2.9 μM, final concentration) by the addition of potassium ferric cyanide (370 μM, final concentration). The final concentration of ABTS and H_2O_2 was 150 μM and 88 μM, respectively. The generation of $ABTS^{\bullet+}$ is measured by an increase in absorbance at 734 nm, thus the antioxidant-induced suppression of absorbance is directly related to the antioxidant capacity of the compound (solution) being tested. Absorbances were read with a Philips PU 8740 UV/VIS Scanning Spectrophotometer immediately after the addition of hydrogen peroxide. The temperature was controlled at 37°C using a circulating bath. The reaction volume was 3 ml.

The Trolox equivalent antioxidant capacity (TEAC) is defined as the concentration (mM) of Trolox having an antioxidant capacity equivalent to 1 mM of the test compound.

Inhibition Assay

Metmyoglobin, ABTS and the test compound were mixed, and the reaction was initiated by the addition of hydrogen peroxide. At a fixed time point (10 min.) the absorbance of the solution was read and compared with the blank. This assay has been termed "Inhibition assay (fixed timed point)" by Rice-Evans & Miller.[11]

Post-addition Assay

Metmyoglobin and ABTS were mixed, and the reaction was initiated by addition of hydrogen peroxide. ABTS radicals were allowed to accumu-

late until the absorbance reached ca. 0.5, then the test compound was added. The decrease in absorption caused by the test compound reflects ABTS radical scavenging capacity rather than inhibition of radical formation. The decrease in slope after addition of the test compound gives an indication of the effect of the test compound on the formation of radicals. Rice-Evans & Miller[11] described a similar assay as "decolorization assay".

RESULTS

Total Antioxidant Capacity Assays

ABTS radical type assays are based on the generation of a long-lived specific radical cation chromophore, the ABTS radical generated by the peroxidase activity of metmyoglobin. The amount of ABTS radicals is measured through determination of the absorbance at 734 nm. Scavenging of the ABTS radicals will lower the absorption. In this study, the effects of Trolox, potassium cyanide and quercetin were studied using the inhibition assay and the post-addition assay described by Miller *et al.*[7] and Rice-Evans & Miller.[11]

Inhibition Assay

Trolox, potassium cyanide and quercetin all induce a concentration-dependent decrease in amounts of ABTS radicals (Fig. 1). Both Trolox and quercetin induce a lag time in the accumulation of ABTS radicals proportional to the concentration of the test compound used, whereas in the presence of potassium cyanide no lag time in appearance of ABTS radicals is observed. The decrease in the slope illustrated in Figure 1B indicates that the potassium cyanide-induced suppression of absorbance is caused by a decrease in rate of ABTS radical formation. Quercetin also induces a concentration-dependent decrease in rate of ABTS radical formation, since the rate after the lag phase is lowered compared to that of control (Figure 1C). Trolox, however, has no effect on the reaction rate (cf. Fig. 1A). We observed that with Trolox reproducible results could be obtained, and that the length of the lag phase linearly correlates with the concentration of Trolox. With quercetin the length of the lag phase was not always proportional to the concentration. This can be explained by the dual effect of quercetin: the capacity of quercetin to scavenge ABTS radicals might be proportional to the concentration of quercetin, whereas the inhibition of radical formation is not linearly correlated to quercetin concentration. As generally observed in enzyme inhibition, a hyperbolic relation between peroxidase activity and concentration of quercetin is seen (data not shown). We also observed that there was a relatively high day-to-day variation in the length of the lag phase induced by quercetin compared with that of Trolox. The TEAC values for quercetin were 2.0–5.8 (mean ± SD: 3.6 ± 0.9; n = 34), while for other antioxidants, which only display a scavenger effect, the variations of TEAC values were within 10% (data not shown). Apparently, the TEAC value of quercetin is not well reproducible.

Post-addition Assay

Both Trolox and quercetin induce a decrease in absorbance at 734 nm, caused by scavenging of $ABTS^{\bullet+}$ (cf. Fig. 2). Addition of potassium cyanide has no effect on absorbance at 734 nm, but induces a decrease in rate of ABTS oxidation, illustrated by a decrease in the slope after the addition of potassium cyanide compared with the blank in the same time range (5–10 min in Fig. 1B). Addition of quercetin also results in a decrease in the rate of ABTS oxidation (cf. Fig. 2C). As expected from the results obtained in the inhibition assay, Trolox has no effect on rate of ABTS oxidation as illustrated by a similar slope after the addition of Trolox, when compared with the blank in Figure 1A. We observed that with Trolox reproducible results could be obtained. The TEAC value for potassium cyanide in this proce-

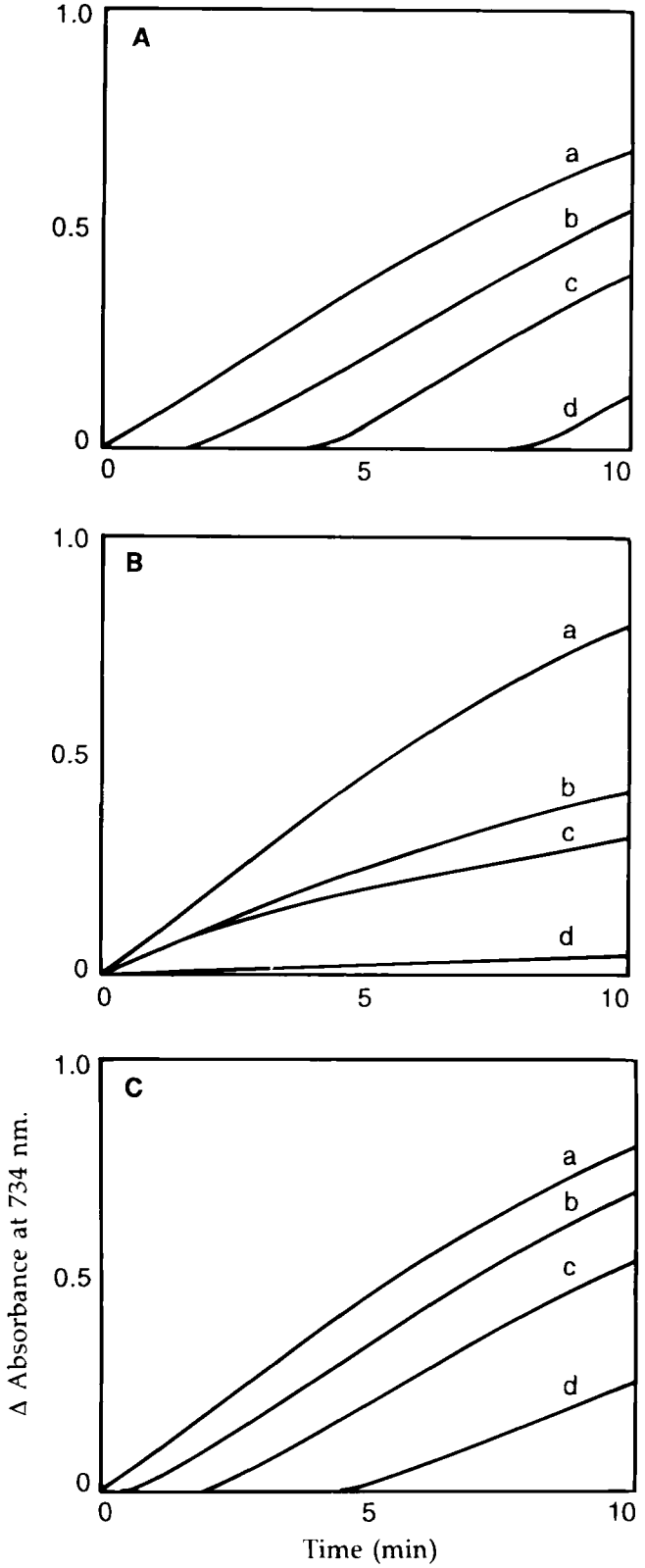

FIGURE 1 Time course of ABTS radical cation formation in the presence of myoglobin (2.9 μM), potassium iron(III) cyanide (370 μM), ABTS (150 μM) and H_2O_2 (88 μM) in 50 mM PBS buffer (pH 7.4). The reaction was followed by measuring the ΔA_{734nm} during 10 min. **A.** Trolox. a; blank (ethanol), b-d; 5, 10 and 20 μM, respectively. **B.** Potassium cyanide. a; blank (PBS buffer), b-d; 10, 20 and 100 μM, respectively. **C.** Quercetin. a; blank (ethanol), b-d; 1, 2.5 and 5 μM, respectively.

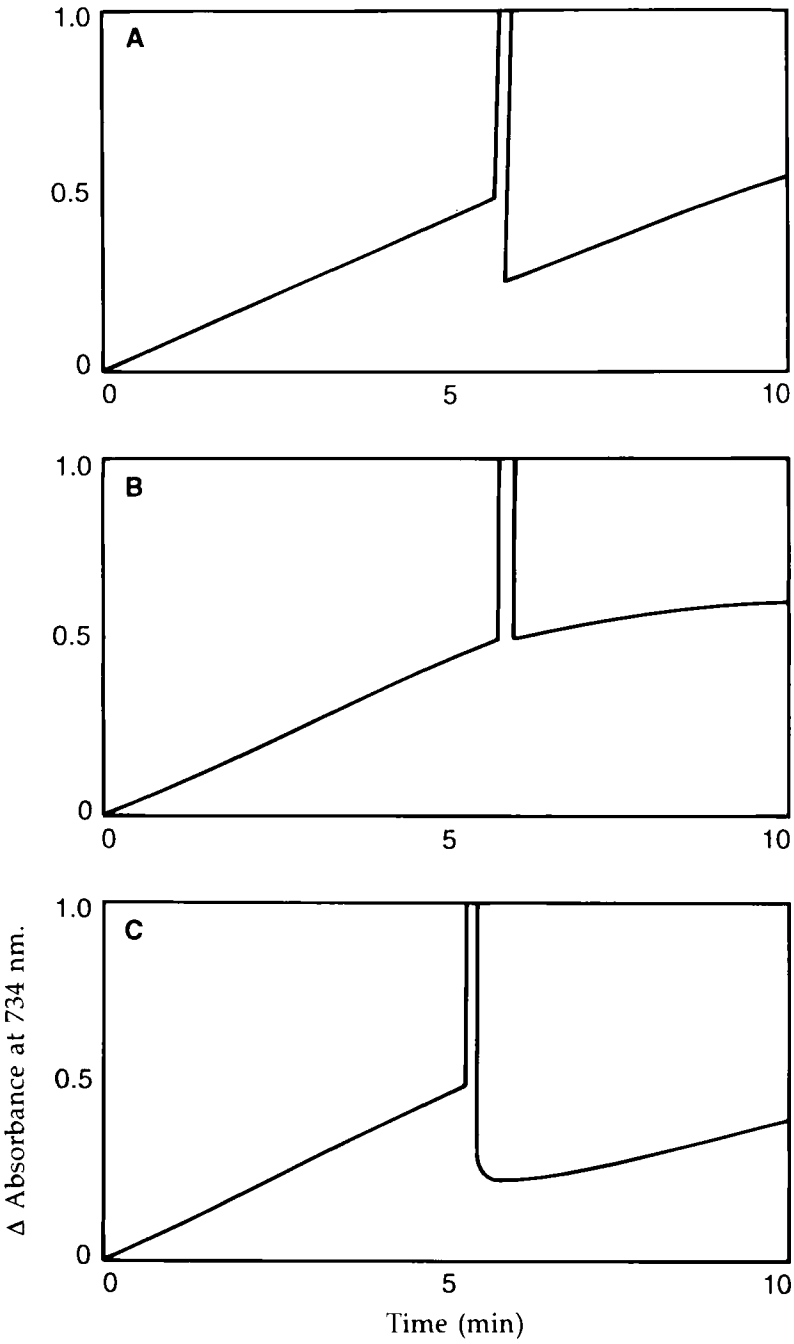

FIGURE 2 Post-addition assay. The reaction between myoglobin (2.9 μM), potassium iron(III) cyanide (370 μM), ABTS (150 μM) and H_2O_2 (88 μM) in 50 mM PBS buffer (pH 7.4) was allowed to proceed until the color was stable (ΔA_{734nm} reached ca. 0.5). The decolorization was initiated by the addition of: **(A)** Trolox (10 μM), **(B)** potassium cyanide (20 μM) or **(C)** quercetin (2.5 μM).

dure was 0. For quercetin, we again observed a relatively high day-to-day variation in reduction of absorbance at 734 nm compared with that of Trolox. The TEAC values for quercetin were 2.6–4.9 (mean ± SD: 4.0 ± 0.7; n = 8).

DISCUSSION

Miller *et al.*[7] have recently developed a method to measure antioxidant capacity, based on the scavenging by antioxidants of the radical cation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\bullet+}$). The radical is formed by the peroxidase activity of metmyoglobin with hydrogen peroxide, and has a high absorption coefficient also at higher wavelengths. Antioxidants could prevent the accumulation of $ABTS^{\bullet+}$ which is reflected in a decrease in absorption of ABTS radicals.[11]

For a correct assessment of the role of antioxidant capacity of separate compounds and food, well validated assays are needed; this assay looks promising and could be used for routine screening of antioxidants.

In the present study we found that Trolox, potassium cyanide and quercetin all reflect an antioxidant effect indicated by a decrease in absorbance. All three test compounds could therefore be labelled as antioxidant. However, several pitfalls have to be considered when the ABTS radical-based assay is used for determination of total scavenging capacity. The time course of radical formation in the presence of the compound and the length of the lag phase clearly elucidate that the antioxidant effect of the compounds investigated differed in origin. Therefore, when using this assay for measuring antioxidant capacity of test compounds or extracts, one has to consider that the assay does not distinguish between a scavenger effect and a decrease in rate of ABTS radical formation, because the absorbance is measured at a fixed time point (6 min) regardless of the time course of ABTS formation. This point has also been stressed by Arnao *et al.*[10]. We found that Trolox acts only by scavenging ABTS radicals, which should be kept in mind when compounds with potential antioxidant effects are investigated in this assay. Additionally, we found that potassium cyanide inhibits radical formation, and that quercetin displays a mix of scavenger and inhibitor effects. Using this assay as described by Miller *et al.*,[7] we also observed that the TEAC value of a compound may depend on the time point at which absorbance was measured, due to the effect of the compound on rate of radical formation. The TEAC value of quercetin measured at 6 min for example was lower than the TEAC value measured at 10 min (data not shown).

To circumvent the effect of the compounds on radical formation, we used the post-addition assay, in which the test compound was added when the ABTS radicals had already formed. The decrease in absorption caused by the test compound will then reflect ABTS radical scavenging capacity, not inhibition of radical formation. The decrease in the slope after addition of the test compound indicates whether the test compound affects radical formation. This procedure revealed indeed that Trolox is a radical scavenger, while potassium cyanide has no scavenging activity. Ascorbic acid also behaved as a radical scavenger in this procedure, with TEAC values around 1.0, both in the inhibition and in the post-addition assay (results not shown).

Recently Salah *et al.*[12] and Rice-Evans *et al.*[13] reported a TEAC value for quercetin of 4.7 using the inhibition assay. This value is slightly higher than the TEAC value of 3.6 observed in our study. Unlike our findings, they obtained a reproducible TEAC value. We assumed that the variability in the TEAC values obtained in our study may be due to a variation in quercetin-induced inhibition of peroxidase. Furthermore, we also assumed that the obtained TEAC value in the inhibition assay may be an overestimation due to both a scavenger effect and an effect on the rate of ABTS oxidation. To avoid the latter we

measured the TEAC value using the post-addition assay. Contrary to what we expected, we found a similar TEAC value as in the inhibition assay. Probably the electrochemistry of this scavenging is not straight-forward. It is expected that under the conditions applied Trolox scavenges two ABTS radicals,[14], and a TEAC-value of 4–5 means that quercetin is capable of scavenging 9–10 ABTS radicals. To our knowledge, no electrochemical oxidation potential for quercetin has been reported. Therefore, a theoretical TEAC value for quercetin can only be speculated based on the chemical structure of the compound, and a TEAC value of 4–5 may be higher than expected. This clearly stresses the necessity to further investigate the electrochemical oxidation potential for compounds with antioxidant activity/scavenging capacity.

It should be stressed that both types of reactions, the scavenging and the inhibition, could also occur *in vivo*. However, inhibition and scavenger effects observed in *in vitro* systems, such as ABTS-type assays, do not necessarily reflect the *in vivo* situation. Therefore, the physiological significance of the *in vitro* results should be confirmed in *in vivo* studies.

The *in vitro* assays could, however, be of value in screening for potential antioxidant capacity of food ingredients, as well as in the assessment of their antioxidant status. However, in extrapolating the results to an *in vivo* situation one has to ask: (i) does the peroxidase process mimic the process of radical formation *in vivo*, and (ii) do the ABTS radicals resemble the radical species involved in an *in vivo* situation.

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