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A new approach to assess the total antioxidant capacity using the TEAC assay

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Abstract

The trolox equivalent antioxidant capacity (TEAC) assay is a popular method for assessing the capacity of a compound to scavenge ABTS radicals (ABTS·). Under the conditions in which the assay is performed, the reaction between most antioxidants and ABTS· does not reach completion within the time span applied. This leads to an underestimation of the TEAC of these antioxidants. In the present study, incubations with different concentrations of ABTS· and a fixed concentration of antioxidant were performed. The decrease in ABTS· concentration in 6 min was plotted against the initial concentration of ABTS· and fitted by an exponential function. Extrapolation of the fit to an infinite excess of ABTS· gives the maximal concentration of ABTS· that can be scavenged by the antioxidant at the concentration employed. This can be used to determine the actual TEAC of antioxidants, i.e. the total antioxidant capacity.

Keywords: TEAC; Antioxidant capacity; ABTS radical

1. Introduction

The trolox equivalent antioxidant capacity (TEAC) assay (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993) is widely applied to assess the amount of radicals that can be scavenged by an antioxidant, i.e. the antioxidant capacity (Lien, Ren, Bui, & Wang, 1999; Plumb, Price, & Williamson, 1999; Rice-Evans, Miller, & Paganga, 1996). In the most recent version of this assay, an antioxidant is added to a pre-formed ABTS radical (ABTS·) solution and, after a fixed time period, the remaining ABTS· is quantified spectrophotometrically (Berg van den, Haenen, Berg van den, & Bast, 1999; Re et al., 1999; Williamson, Plumb, & Garcia-Conesa, 1999). The reduction in ABTS· concentration, induced by a certain concentration of antioxidant, is related to that of trolox and gives the TEAC value of that antioxidant. The assay is rapid, easy and correlates with the biological activity of antioxidants (Berg van den, Haenen, Berg van den, Vijgh van der, & Bast, 2000; Rezk, Haenen, Vijgh van der, & Bast, 2003).

A major problem associated with the TEAC assay is that substantial differences in reported TEAC values of an antioxidant are observed, e.g. the TEAC of quercetin varies from 3.1 (Re et al., 1999) to 6.4 (Berg van den et al., 1999). The variation cannot only be ascribed to differences in procedure. Also, with the same procedure, the TEAC of a compound may vary. With the same method, the TEAC of quercetin at a concentration of 1.5 or 1.0 μM is reported to be 5.6 or 6.4, respectively (Berg van den et al., 1999). The aim of this study is to develop a procedure to determine the true total antioxidant capacity of a compound that is independent of the concentration of the antioxidant.

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2, 5,7,8-tetramethylchroman-2-

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carboxylic acid (trolox), quercetin, chrysin, rutin, catechol and resorcinol were obtained from Sigma (St. Louis, USA) and potassium persulfate (di-potassium peroxodisulfate) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Preparation of the ABTS solution

A 7 mM solution of ABTS in milli-Q was prepared and ABTS· was formed after addition of potassium persulfate to the mixture in a final concentration of 2.45 mM. After a 12–16 h incubation at room temperature, the stock solution was diluted with PBS untill an absorbance of 0.7 (±0.02) at 734 nm was reached (Re et al., 1999).

2.3. Preparation of the antioxidant solutions

Solutions of antioxidants were prepared in ethanol. To exclude the influence of light, the solutions were prepared in the dark.

To a fixed concentration of an antioxidant, ABTS· in a variable concentration was added. The concentration of ABTS· was varied from 0 to approximately 45 μM in several different incubations. After a 6 min incubation at 37 °C, the absorbance at 734 nm was determined. The concentration of ABTS· was calculated, using a molar extinction coefficient of 1.5×10^4 M^{-1} (Re et al., 1999). The reduction in ABTS· concentration was derived from the absorbance at 734 nm of the reference (only containing ABTS·) and the incubation containing the fixed concentration of antioxidant plus the same concentration of ABTS·.

The reduction in ABTS· concentration, was plotted against the initial concentration of ABTS·. The curve was fitted according to the exponential function $y = C\left(1 - e^{(-b \cdot x)}\right)$, using Sigma Plot (version 4.01) on a conventional personal computer. In this formula, y is the reduction in ABTS· concentration, x is the initial ABTS· concentration and C is the maximal amount of ABTS· scavenged by the antioxidant at the concentration tested. In the procedure employed, the maximal initial concentration of ABTS· was 45 μM. For accurate results, the antioxidant in the concentration used, had to give a value of C between 2.5 and 25 μM. When values of C below or above these limits were obtained, the concentration of the antioxidant has to be adjusted accordingly. The TEAC was determined by dividing C by the concentration of antioxidant and by 1.9. The latter factor (1.9) is the number of molecules that can be scavenged per trolox (Arts, Haenen, Voss, & Bast, 2004). It should be noted that, in the literature, the TEAC of a compound is often incorrectly expressed as mM. As can be inferred from the procedure described above, the TEAC of a compound is a relative value and has no dimension.

3. Results

Various antioxidants behave differently in the TEAC assay. The reference compound in the TEAC assay is trolox. Trolox reacts instantaneously with ABTS· (Fig. 1). Within the time needed to put the sample into the spectrophotometer, the reaction of trolox with ABTS· is completed. Already, with a small excess of ABTS·, the total amount of ABTS· that reacts with trolox can be determined. It was found that 1.9 μmol ABTS· was consumed per μmol trolox (Arts et al., 2004). The TEAC value of trolox is independent of the concentration of trolox, as was also previously reported (Berg van den et al., 1999).

Rutin reacts more slowly than trolox with ABTS· and, within 6 min, the reaction is not completed (Fig. 1). To quantify the total amount of ABTS· that can be scavenged by rutin, the initial concentration of the antioxidant was kept constant and the initial concentration of ABTS· was varied from 0 μM up to approximately 45

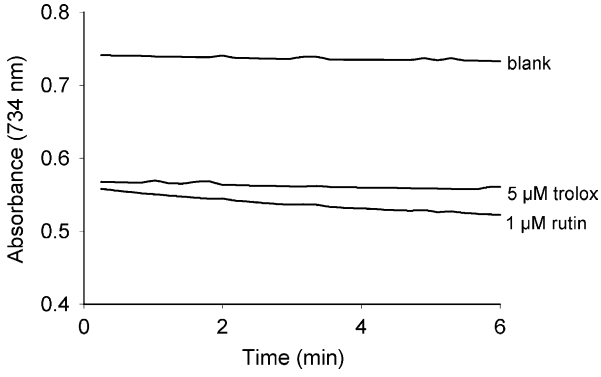

Fig. 1. Reaction of trolox and rutin with ABTS·. The absorbance at 734 nm, due to ABTS· is followed in time. The initial concentration of trolox and rutin are 5 and 1 μM, respectively.

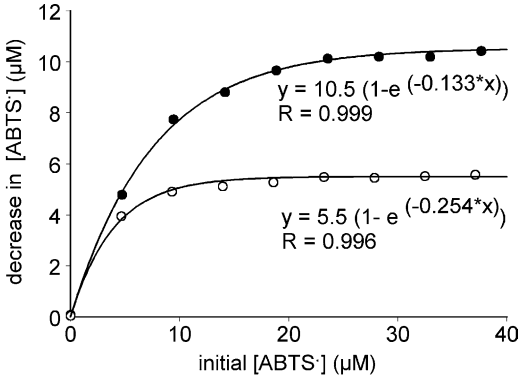

Fig. 2. Consumption of ABTS· by 0.5 μM rutin (open symbols) or 1 μM rutin (closed symbols) with a variable initial concentration of ABTS·. The data were fitted with the exponential function as described in the material and method section. This gives a TEAC of rutin of 5.53 (10.5/1.9) and 5.79 (5.5/(1.9 × 0.5)) from the data obtained with 1 and 0.5 μM, respectively.

Table 1
"New" and previously reported TEAC of several compounds

Compound	"New" TEAC	Previously reported TEAC
Quercetin	6.24 ± 0.07	3.10 ± 0.05 (Re et al., 1999)
		4.43 ± 0.02 (Williamson et al., 1999)
		4.72 ± 0.10 (Rice-Evans et al., 1996)
		4.91 ± 0.22 (Miller & Rice-Evans, 1997)
		6.43 ± 0.27 (Berg van den et al., 1999)
Rutin	5.66 ± 0.14	2.40 ± 0.06 (Rice-Evans et al., 1996)
		2.75 ± 0.05 (Benavente-Garcia, Castillo, Lorente, Ortuño, & Del Rio, 2000)
Chrysin	2.43 ± 0.17	1.43 ± 0.07 (Rice-Evans et al., 1996)
Catechol	1.42 ± 0.11	1.18 ± 0.09
		1.4 ± 0.1
Resorcinol	2.60 ± 0.20	2.33 ± 0.10
		2.49 ± 0.02

The "new" TEAC was determined according to the procedure determined in the present paper. Various concentrations of each antioxidant were tested and the TEAC is expressed as mean ± SD. The concentration range was 0.2–0.5 μM for quercetin, 0.5–1.5 μM for rutin, 1–5 μM for chrysin, 5–10 μM for catechol and 1–5 μM for resorcinol. The number of concentrations tested varied from 2 to 4. The TEAC values appeared to be independent of the concentration. The previously reported TEAC values are taken from the indicated references. Various procedures and concentrations have been used. The values of catechol and resorcinol have been obtained in our laboratory using the procedure described by Berg van den et al. (1999) at a concentration of 10 and 5 μM (first value) or 5 and 1 μM (second value), respectively.

μM ABTS·. 6 min after starting the reaction, the absorbance of the ABTS· at 734 nm was measured. The difference in the concentration of ABTS· between the reference (containing a variable amount of ABTS· and no rutin) and that of the sample (containing both the fixed concentration of rutin and the same concentration of ABTS· as the reference) was determined. This was used to determine the reduction in ABTS· concentration in the sample.

Fig. 2 shows that an increase in the initial concentration of ABTS· results in a higher ABTS· consumption. This effect decreases at higher initial concentrations of ABTS·. The curve is fitted according to an exponential function. This fit gives the total amount of ABTS· that can be scavenged by rutin at the concentration tested, i.e. "*C*" in the formula described in Section 2. Regression analysis of the value of *C*, obtained at different concentrations of rutin, plotted versus the concentration of rutin (range 0.5 to 1.5 μM), showed a linear fit ($C = 10.7 \times [\text{Rutin}]$; $R = 0.99$). This gives a TEAC value of rutin of 5.66. For accurate results, the concentration of antioxidant used, has to give a value of *C* between 2.5 and 25 μM. Table 1 shows the total antioxidant capacity values of several antioxidants according to this procedure.

4. Discussion

The TEAC assay is widely applied to assess the total amount of radicals that can be scavenged by an antioxidant, i.e. the antioxidant capacity. TEAC values reported in the literature are variable. It appears that the TEAC value largely depends on the assay conditions (Berg van den et al., 1999). The most important reason for this variation is that the reaction of an antioxidant with ABTS· usually does not reach completion within the time span applied. This results in an underestimation of the actual TEAC.

Therefore, we adjusted the assay. In the procedure employed to assess the actual TEAC, the total amount of ABTS· that can be scavenged by a certain amount of antioxidant, is calculated by extrapolating to conditions where the reaction between the antioxidant and ABTS· has reached completion, i.e. to an infinite excess of ABTS·.

The major limitation of this procedure is that the concentration range of the antioxidant, that can be used, is relatively small. A too high antioxidant concentration would need too much ABTS·, giving a too high absorbance for a reliable fit of the curve. A too low concentration of the antioxidant would result in a decrease in ABTS· absorbance too low to be measured accurately. Since the antioxidant capacity of compounds varies, the concentration range of different compounds in this assay differs. In the procedure we employed, the maximum concentration of ABTS· was 45 μM, giving an absorbance of 0.7. The minimal concentration of antioxidant in this assay that gives accurate results, has to give at least 2.5 μM reduction in the ABTS· concentration; the maximal concentration of antioxidant in the assay has to give a maximal 25 μM reduction in the ABTS· concentration.

The total antioxidant capacity represents the sum of the antioxidant capacity of the parent compound and that of the oxidation product(s) of the parent compound (Arts, Dallinga, Voss, Haenen, & Bast, 2002). The role of oxidation products of antioxidants that are formed, due to scavenging of reactive species, is becoming better understood. On the one hand, these oxidation products might be toxic (Bast & Haenen, 2002), on the other hand, a residual antioxidant activity of these oxidation

products might make a substantial contribution to the therapeutic effect of the parent compound (Arts et al., 2002). Previous procedures to determine the total antioxidant capacity by the TEAC assay give an underestimation of the TEAC. The new procedure gives the actual total antioxidant capacity and includes the potential scavenging effect of oxidation products.

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