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ARTICLE

A "Zero Sample Concentration Approach": Standardization of Methods for the Estimation of Total Antioxidant Activity by the Use of Extrapolation to Zero Sample Concentration. A Novel Standard. 1. ABTS Cation Radical Scavenging

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The study presents the general method of standardization of estimations of total antioxidant activity (TAA) by extrapolating parameters to zero sample concentration based on a pseudo-first-order kinetics model. To test its suitability, the method was applied to the ABTS cation radical scavenging assay, as the first choice. Two alternative methods of extrapolation were presented, via linear regression and numerical fitting to the dose−response profile. An extrapolation method gives the highest value of estimates, independent of sample concentration, and creates a new standard approach to the methods of TAA estimation. It is proposed to designate the modified index as "standard equivalent antioxidant capacity at zero" ($SEAC_0$) or $TEAC_0$ (for Trolox as standard). The examples of estimates for some foods and rat plasma are presented and compared with the literature method. The applicability of the extrapolation approach to the other TAA measurement methods is under evaluation.

KEYWORDS: Standardization methods; antioxidant capacity index; antioxidant activity; extrapolation method to zero sample concentration; antiradical activity; ABTS

INTRODUCTION

The antioxidant activity (AA) of foods and biological materials is estimated for screening and diagnostic purposes if detailed quantification of active constituents cannot be performed. To standardize the quantitative results, the observed effect is usually expressed as an equivalent of pure standard substance (i.e., Trolox, ascorbic acid, gallic acid, β-carotene, etc.), selected for similar chemical reactivity to the sample's active constituents. The reactivity of free radical with antioxidant can be described in general by two mechanisms, that is, electron (charge) transfer and hydrogen atom transfer (*1*, *2*). A variety of chemical reactions or properties are used in analytical methods, including free, stable, or pregenerated radical scavenging, for example, $ABTS^+$ (*3*) or DPPH (*4*, *5*), reduction of metal cationic oxidizers, for example, Fe^{3+} (*6*), Cu^{2+} (*7*), generated oxygen radicals in situ (*8*, *9*) or other radicals (*10*), β-carotene bleaching (*11*), electron spin resonance in vivo (*12*) and in vitro (*13*), spin trapping (*13*), potentiometric titration (*14*), and others (*15*). Different stabilities, kinetics, and selectivities of these reagents and a variety of potential antioxidants present in natural complex samples make these methods nonequivalent, and the correlation of AA estimates is generally not observed (*2*, *15*, *16*). Recent advances in analytical methods, especially extensive studies on the use of copper dication complexes, carried out by Apak and co-workers (*17*), are, however, promising. The methods of determination of antioxidant activity are summarized in many reviews (see, e.g., refs *1*, *2*, and *18*−*20*).

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The AA can be quantified with the use of a calibrating standard if dose−response (DR) profiles for the standard and the sample are linear or of the same shape. Linearity can be observed when the reagent has consumed the whole amount of reactive constituents of the sample or the same relative quantity of these substances has reacted with the reagent. This is observed in the reactions of some pure substances (e.g., Trolox, ascorbic acid) or their mixtures of fast kinetics relative to the measurement time. Linearity cannot be generally expected for complex samples, even containing the same constituents in different combinations (*15*, *21*). Consequently, the results of AA estimates are dependent on sample concentration, resulting in significant differences between different batches and estimations performed by using various methods.

The reaction response after the incubation period (reaction time, measurement time) can be expressed by a useful relative parameter, that is, the scavenging coefficient (antiradical efficacy, *S*), defined as a ratio of the amount of radical reagent quenched by the sample added to the initial quantity of the reagent (R_0)

$$S = (R_0 - R)/R_0 \quad (1)$$

where R is the remaining radical reagent quantity.

The DR curve is linear if the whole quantity of the active constituents of the sample has reacted with reagent. To reach a better linearity of the DR curve, higher yield of antioxidant consumption can be achieved, for example, by increasing the incubation time. However, this may be associated with increased contribution of secondary processes (*1*, *2*, *21*), disturbances

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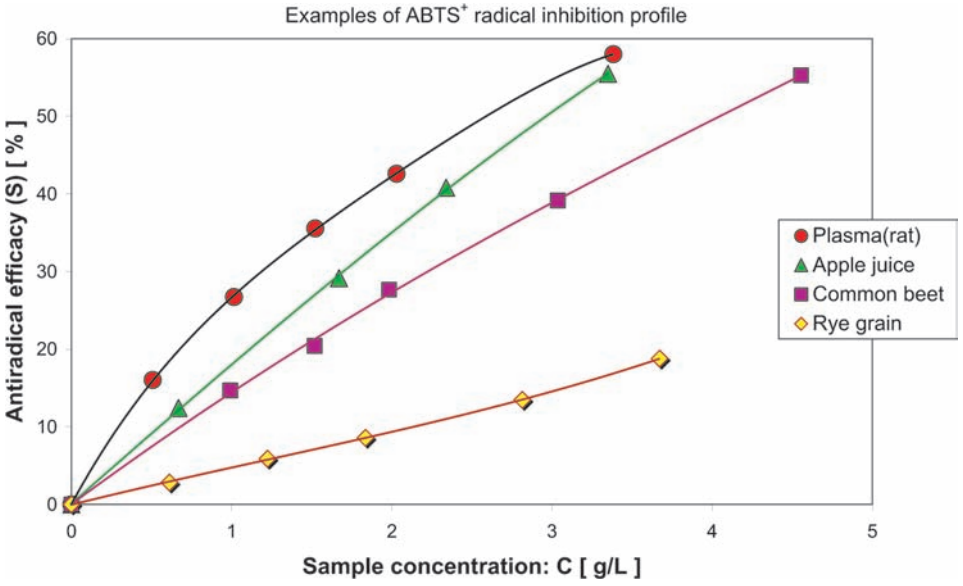

Figure 1. Examples of ABTS inhibition dose−response profiles for a variety of materials (common beet, apple juice, rye grain, rat plasma).

introduced by side processes due to natural instability of free radicals, and increase in background for spectrophotometric methods. All of the above-mentioned side processes themselves can impair the linearity of the DR profile. In biological samples, inhibition rates may be also controlled by kinetics of conformational and upper order structure rearrangement of high molecular weight substances, liberating small reactive molecules or exposing active sites, which allows for access of the reagent (e.g., thiol groups in plasma proteins). Finally, a significant increase in the measurement time is not attractive from the cost-effectiveness point of view in routine measurements.

Our studies of foods and animal tissues (*22*, *23*) and those of other authors (*15*, *24*, *25*) indicated poor DR linearity for radical scavenging, including ABTS (**Figure 1**), and problems associated with complex organic materials. If such nonlinear profiles are approximated by linear dependencies, the sample concentration range applied significantly influences the results and makes AA estimations to some extent random.

Thus, it was proposed (*23*) to standardize the AA estimation methods for such complex samples by means of the extrapolation of AA to zero sample concentration. This approach is general and not limited to any particular reagent system or method. In this virtual condition, the reagent excess over the sample is infinitely high, and a higher order kinetics may be simplified to a pseudo-first-order one and, thus, to the linearity of the reaction rate versus concentration. If all active constituents have reacted, the reagent's response should not be further dependent on the sample concentration. Moreover, a high yield of reaction of all active constituents is expected, which provides information about real total capacity of quenching the radicals via all antioxidant reaction pathways.

Generally, these conditions can be met only by extrapolation techniques, particularly for slow kinetics reactions. That approach fulfills the requirements for the estimation of concentration-independent total antioxidant capacity (TAC). As the first choice, the ABTS cationic radical reagent has been selected, because the reaction medium is similar to the physiologic aqueous conditions, that is, pH 7.4, and measurement can be performed at a temperature of 30 °C.

MATERIALS AND METHODS

Chemicals and Apparatus. Phosphate-buffered saline (PBS, pH 7.4; catalog no. P-4417) was obtained from Merck, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), and sodium persulfate were obtained from Sigma. All solvents were of pure analysis grade. Methanol and acetone were distilled before use.

Spectrophotometric measurements were performed with a UV-530 apparatus (Jasco Corp., Tokyo, Japan) equipped with a thermostatic cell holder. All reagent solutions were prepared in deionized water (18 MΩ·cm) from a double-purification system Milli-RO and Milli-Q (Millipore Corp., Billerica, MA). Sample manipulations were performed using disposable polypropylene vials and polystyrene cuvettes. Aqueous solutions were dispensed with pipettors and volatile solvents/extracts with Teflon-ended piston-rod glass syringes (Hamilton). All operations were performed in an air-conditioned room with moderate-intensity artificial wire type indirect lighting.

General Method Characteristics. ABTS radical scavenging measurements were performed according to the method reported by Re et al. (*3*) with our adaptations (*22*, *23*) described below.

Buffer. One tablet of PBS was dissolved with water in a 0.2 L volumetric flask to make the concentration 0.01 M.

Sodium persulfate reagent (0.05833 g of solid sample) was dissolved in 0.1 L of water to make a 2.45 mM solution of sodium persulfate.

ABTS Cationic Radical Reagent. An amount of 0.3841 g of ABTS solid powder sample was dissolved in 0.1 L of 2.45 mM solution of sodium persulfate and then set aside for conditioning for the next day in darkness at room temperature. Each batch of the reagent stock was tested for spectral stability before measurements. It was accepted if the absorbance decrease within measurement time with water added as a sample did not exceed ca. 1%; otherwise, the reagent stock was left for longer conditioning in darkness. The spectral background impurity of the ABTS reagent or due to decomposition products can be checked by the total inhibition test described below in the text.

On each day before analysis, a portion of ABTS stock was dissolved in PBS buffer at a 1:50 ratio. The absorbance of the reagent at 734 nm was adjusted to ca. 1.05 with PBS or ABTS stock.

Test Material Preparation. Solid material was finely powdered, and 1 g of the sample was extracted with 40 mL of 0.08 M hydrochloric acid in 80% v/v methanol for 2 h with shaking; the

solid residue was further extracted with 40 mL of 70% v/v freshly distilled acetone in water, then combined with the methanolic extract, and centrifuged. The laboratory internal test sample used in this study to test the method was a mixture of cereal grains. The sample of common beet juice was prepared manually using nonmetallic utensils, whereas fruit juices were commercial products. Wistar rat plasma was from control animals aged 4 months from other studies. All sample extracts were made in duplicate. The samples and blanks for each sample batch were stored in a refrigerator in darkness (−20 °C) until analysis.

Sample Dilutions. Liquid samples or extracts were prediluted before the measurement with sample solvent or combined solvents' mixture used for the extraction. The manually premixed diluted sample (0.6 mL), reagent diluent (0.4 mL), and reagent (2 mL) added directly into a 1 cm optical path length disposable polystyrene cuvette of 4 mL volume, covered with a cup, was incubated in thermostated air bath for 6 min, and then absorbance was measured. The sample dilution was adjusted to the requested inhibition level.

Typical predilution factors in water were 200 for rat plasma or 100−500 for fruit juices. All diluted solutions were prepared daily, directly before analysis, if possible. Reagent stocks and solvents were stored in the refrigerator (4 °C) or kept at the required temperature in darkness during the experiment course.

General Conditions. The quantitation of ABTS cation radical was based on measurement of the absorbance band decay at 734 nm after a 6 min incubation (at 30 °C) of the mixtures of sample and ABTS radical prepared at several ratios.

Preconditioned radical reagent stock solution was checked before measurements to obtain a working absorbance of 0.7 after dilution at a given ratio with the sample blank and adjusted, if necessary.

Samples were freshly prediluted with sample solvent to obtain the concentration necessary to inhibit the radical reagent roughly by about 80%. This allowed for the use the sample volumes between 0 and 1 mL for measurement to cover the range of 0−100% inhibition.

Measurement Procedure. For each sample, the reaction mixture series was prepared as follows (**Table 1**): to a series of cuvettes the sample blank or prediluted sample solution was added in increasing order of volume and adjusted to a total volume of 1 mL with adequate (sample or/and reagent) diluent. The reactive sample diluent, in particular containing acetone, should be added at the minimum volume required, due to its possible residual antioxidant activity. All cuvettes during mixture preparation were thermostated at 30 °C on the heated plate in an open box. Next, at a constant time interval synchronized with measurement throughput capability (e.g., 0.5 min for manual operation), portions of the reagent stock thermostated at 30 °C were added to the successive cuvettes, which were then covered with cups, gently mixed, and thermostated at 30 °C for an appropriate period. Absorbance was read exactly at the chosen measurement time against solvent mixture as spectral blank. For the study of the effect of reaction time, the cuvettes were placed back in a thermostat after measurement for a given period of time or left in the instrument in a thermostated automatic cuvette holder. Typically, a series of four to six successive mixtures was measured for one sample to obtain inhibition values within the range of 10−40% for the extrapolation method and extended to 60−80% for interpolation to a 50% inhibition method. For extracts in organic solvents, blank extract was similarly measured, and the background activity value calculated as for the sample was subtracted from sample estimates. The scheme in **Table 1** can be modified for the maximal sample volumes used, if necessary, up to the total volume of 1 mL. Sample concentrations were expressed for material weight (or volume) tested (extracted material, juice, plasma) versus the reaction mixture.

Table 1. Typical Reaction Mixture Preparation Scheme Used for a Sample Measurement (Volumes in Milliliters for a 4 mL Spectrophotometric Cell)

no.	sample	sample diluent	reagent diluent	radical reagent
spectral blank	0.00	1.00	2.0	0
0	0.00	1.00	0.0	2
1	0.15	0.85	0.0	2
2	0.30	0.70	0.0	2
3	0.45	0.55	0.0	2
4	0.60	0.40	0.0	2
5	1.00	0.00	0.0	2

The calibration curve was prepared similarly as described above with Trolox at the concentration in reaction mixture within in the range of 0−25 μmol/L.

For the reaction mixtures of higher background absorbance after the reaction (i.e., >0.1 absorbance at sample excess), the remaining radical reagent was inhibited in the cuvette after measurement by direct addition of an excess of colorless and fast reacting antioxidant, for example, a small volume of a concentrated solution of Trolox or ascorbic acid at the quantity necessary for total ABTS decolorization (e.g., ca. 10 μL of Trolox solution at a concentration of 10 mmol/L). The residual absorbances were subtracted from the respective reaction mixture measurements. For the ABTS method, spectral background for most samples was negligible due to high dilutions and long wavelength used. Thus, this correction may be omitted for screening studies with the use of ABTS reagent; however, such a correction can be useful for a precise study or with the use of other reagents.

Statistics. Statistical evaluations were performed with parametric tests using Statistica for Windows (StatSoft, Inc.). All extracts from solid samples were prepared in duplicate. Measurements were performed in duplicate for each extract or in triplicate for other samples. All estimations, regression, and fitting procedures were performed with preprogrammed macros in adequate modules of the above package or spreadsheet.

RESULTS

A quantitative model of dose−response (DR) was constructed assuming sample concentration (C)-dependent pseudo-first-order kinetics for radical reagent (R) decay at the sample concentration near zero. The reaction rate of sample was assumed to be the equal of and expressed by the reaction rate of reagent radical used as an antioxidant sensor. It may also be expected (but not necessarily) that the redox-active moieties in natural food products may belong to similar chemical classes with comparable reaction rate constants, which can simplify the kinetics. A lack of cross-interactions between sample species was also assumed. The kinetic test model can be then approximated by a one-exponential equation

$$S = 1 - \exp(-ktC) \quad (2)$$

where S is antiradical efficacy (quenching progress), k is total apparent pseudo-first-order rate constant for all reactive constituents, C is total sample concentration, and t is incubation time.

Trolox equivalent antioxidant capacity (TEAC) is defined for linear relationships as the ratio of the slope of the sample concentration versus inhibition coefficient curve (i.e., C vs S) for the standard (b_{std}) to the respective value for the sample (b_{sample}):

$$\mathrm{TEAC} = b_{std}/b_{sample} \quad (3)$$

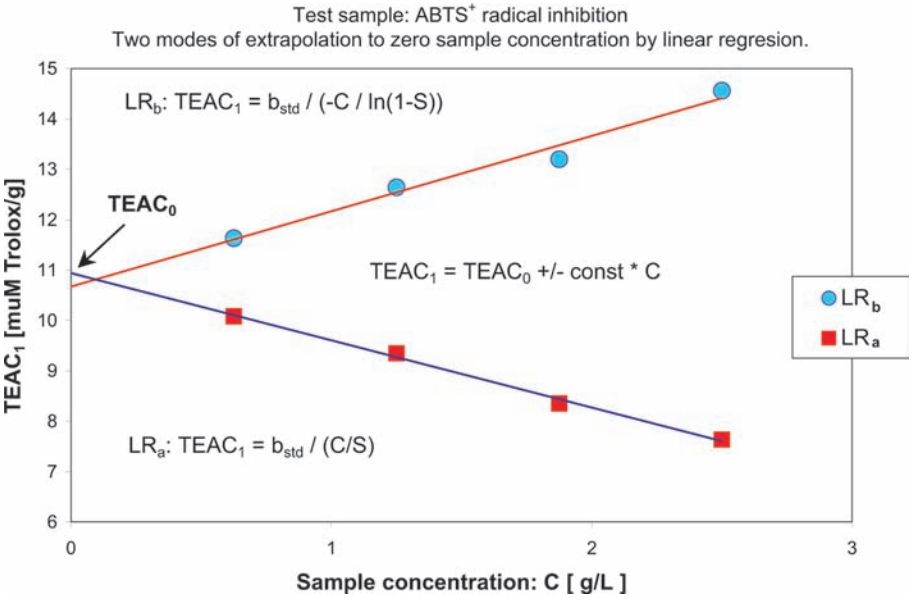

Figure 2. Illustration of the method of estimating antioxidant capacity at zero sample concentration, $TEAC_0$, by linear regression (LR): comparison of two extrapolation modes.

For small deviations from linearity, the following approximation can be made:

$$b_1 = C/S \tag{4a}$$

Parameter b_1 represents a slope of the line drawn through points (0,0) and (C,S) on a reversed DR curve. Parameter b_1 is a function of sample concentration and differs from the true slope at point (C,S) of the DR curve depending on deviation from linearity. This parameter can also be interpreted as the mean sample concentration responsible for the inhibition response of 1% (i.e., IC_1) calculated at a given point.

Generally, DR profiles are not linear as shown by examples in **Figure 1**.

If the slope is a function of sample concentration, the TEAC (eq 3) must be redefined. The true slope of the DR function at a given point can be calculated as dC/dS, where dC and dS are the differentials. This can be graphically shown as the slope of the tangent line to the DR curve. For nonlinear profiles, there is only one line crossing point $C = 0$.

Another approach is to analyze the slope of the line driven by points (C,S) and (0,0). This will be explored below.

The closest to the linearity range of the DR profile should be expected at the sample concentrations near zero, that is, at the point (0,0). Thus, the slope at (0,0) can be defined as the limit of the function 4a at zero value of concentration.

$$b_0 = \lim_{(C \to 0)}(C/S) \tag{4b}$$

For a given point (C,S) the TEAC value can be calculated from the equation

$$TEAC_1 = b_{std}/b_1 \tag{5a}$$

and at the zero sample concentration from the equation

$$TEAC_0 = b_{std}/b_0 \tag{5b}$$

where b_0 (or b_1) is the slope of a tangent line to the DR curve (C vs S) at zero (or a given concentration) and b_{std} is the slope of the calibration curve, equal to the slope of function 4a for a linear calibration curve.

The slope b_0 can be derived from eq 2 by differentiation:

$$b_0 = (kt)^{-1} \tag{6}$$

Substitution of eq 6 into eq 5b yields

$$TEAC_0 = b_{std}kt \tag{7}$$

After the development of eq 2 into the function series, eq 8 is obtained

$$\exp(x) = 1 + x + x^2/2 + x^3/3 + \ldots \tag{8}$$

where $x = -ktC$.

Including the three lowest function series components and combining them with eqs 4a, 5a, and 7, a linear equation for experimental variables C and S/C is obtained

$$b_{std}/(C/S) = TEAC_0 - aC \tag{9}$$

where a is an empirical constant or

$$TEAC_1 = TEAC_0 - aC \tag{10}$$

where $TEAC_1 = b_{std}/(C/S)$.

Linear regression (LR_a) according to eq 10 or graphical plot of experimental points $TEAC_1$ versus C allows for direct estimation of the constant $TEAC_0$. An example of the regression line is shown as the lower plot in **Figure 2**.

For several materials studied, a good fit with the experimental points was obtained by linear regression for the low range of quenching coefficient S. However, at the higher sample concentration the relationship may not be linear, if higher than second-order function components of eq 8 significantly contribute to the $TEAC_1$ value, and the approximation made for eq 9 becomes invalid. For the majority of samples studied, linearity was observed in a wide range of quenching efficacy, up to 80%, and more, due to the very low concentrations used.

Additional partially alternative evaluations of data can be performed by the use of another approximation, obtaining linear eq 12. By transformation of eq 2 via eq 6 and varying it to the expression 11

$$b_1 = -C/\ln(1 - S) \tag{11}$$

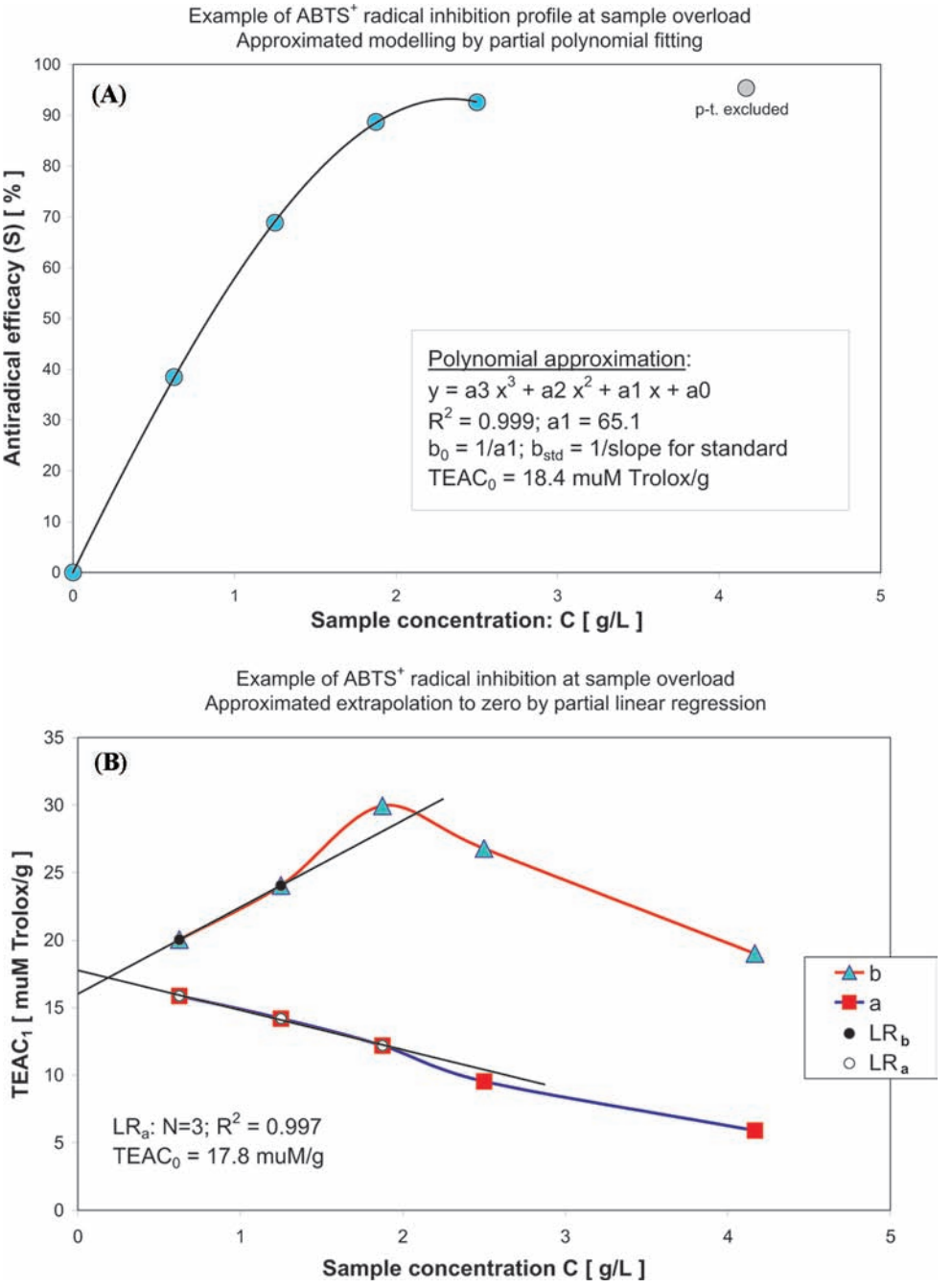

Figure 3. (**A**) Influence of sample concentration overload on dose−response profile. The simplified method of estimation of approximate $TEAC_0$ value via polynomial curve modeling is shown in the inset. (**B**) Spread of experimental points calculated according to the two regression modes (LR_a, LR_b) from the example shown in **A**. Linear regression was applied for selected data points.

then expression 12 was obtained:

$$TEAC_1 = TEAC_0 + a_1 C \qquad (12)$$

where $TEAC_1 = b_{std}/(-C/\ln(1-S))$ and a_1 is an empirical constant.

An example of a regression plot according to the eq 12 (LR_b) is shown as the upper plot in **Figure 2**.

Expressions 12 and 10 converge to the value $TEAC_0$ for the concentration $C = 0$ and are equivalent in the concentration range of applicability of approximation 8 ($-\ln(1 - S)$ approaches S at $C \rightarrow 0$). Because the convergence test is the major reason for introduction of the above equation, an explicit form of driving function for extrapolation is not meaningful. Therefore, both of these expressions allow for alternative estimation of $TEAC_0$, provided that quantity and quality of measurements sufficiently model the range of near zero sample concentration.

For an improper range of sample concentrations or blank correction, these equations did not converge. Equation 10 is a primary method (LR_a, the LR method) suggested for estimation of $TEAC_0$ value; however, eq 12 (LR_b mode) is useful for verification of data quality. It appeared to be very sensitive to deviation of experimental points from linearity, which allows for identification of the points to be rejected from estimation. Moreover, for measurements at not compensated significant spectral background, extrapolation gave higher values of estimates. Examples of the effects of sample overload on the dose−response profile and spread of points estimated for linear regression are shown in **Figure 3**, panels **A** and **B**, respectively.

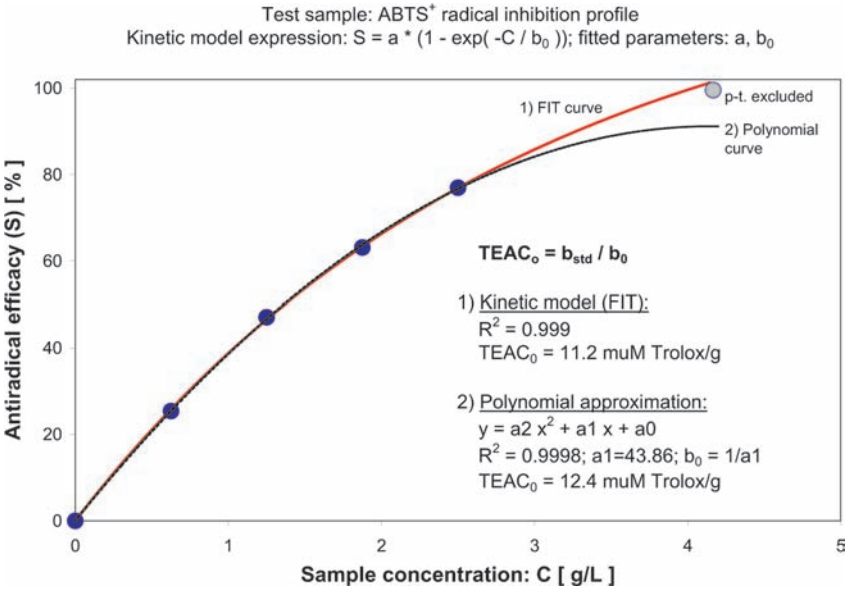

Figure 4. Dose−response profile for the example shown in **Figure 2**: illustration of the method of estimating Trolox equivalent antioxidant capacity at zero sample concentration, $TEAC_0$, by numerical fitting of parameters of eq 13 (FIT method). The approximate method of estimation via polynomial curve modeling is shown in the inset.

The effect of deviation from linearity of the DR profile can be omitted by the alternative method for $TEAC_0$ estimation, that is, numerical fitting of constants in combined kinetic expression (FIT method)

$$S = a(1 - \exp(-C/b_0)) \quad (13)$$

where a is a scaling constant.

Substitution of b_0 into eq 5b allows for alternative estimation of the $TEAC_0$ value.

An example of the use of the FIT method is shown in **Figure 4**.

The FIT method needs to incorporate the scaling constant a in eq 13, which was introduced to achieve fast convergence of numerical fitting. This constant is necessary due to lack of sufficient numbers of experimental points at higher inhibition range (poor modeling of the profile), spectrophotometric background signal deviations, or others.

Due to the nonlinear fitting procedure using one simple function, all subtle details of the DR profile associated with, for example, nonuniform kinetics of a complex mixture, are averaged and represented by the one kinetic mode. This cannot always be accepted; thus, applicability of this method is to some extent limited. Consequently, the profile shape, convergences, and quality of estimations should always be checked. However, this method allows for convenient approximate online fitting with polynomials (e.g., on spreadsheet) capable of modeling of the experimental points profile. By use of a linear coefficient of polynomial expression for approximation of slope b_0 ($b_0 = 1/a_1$, where a_1 is taken from the equation $y = a_i x^i + ... + a_1 x + a_0$ ($y = S$; $x = C$, and $i > 0$), the extrapolated rough activity value can be smoothly obtained, as shown in examples in **Figures 3A** and **4**. The estimated approximate value can then be used as a starting point for numerical fitting according to eq 13.

The extrapolation methods need at least four experimental points in the middle response range (below 80% inhibition). It should be noted that the small number of experimental points and the imprecise shape of the profile in the low sample concentration range result in overestimation of the $TEAC_0$ value by the FIT method and underestimation by the LR method. We used the convergence of $TEAC_0$ values estimated by the two above methods (or difference) as a quality criterion to confirm the precision of measurements and proper range of sample concentration applied for analysis.

The mean $TEAC_0$ value of the estimates by LR and FIT methods averages some of the above deviations (i.e., over- and underestimation) and is suggested as the best value of estimation, if satisfactory concurrence of the LR and FIT methods cannot be achieved. On the basis of the large number of our estimations, the between-method precision was high and was 0.7−1.1% for fruit juices and cereal extracts. Examples of estimates of $TEAC_0$ values by extrapolation to zero sample concentration via linear regression (LR_a mode) and numerical fitting (FIT mode) are shown in **Table 2**.

If extrapolation techniques cannot be performed, then the simpler standardization of AA estimations can be applied by adopting a standard point in the DR profile of inhibition, conventionally chosen at 50%. The sample concentrations providing 50% inhibition (IC_{50}) can be estimated from the DR profile by different methods, for example, by weighted mean, local linear, or log−linear or nonlinear interpolation, via regression or fitting procedures. The respective antioxidant capacity at this point can be defined as

$$TEAC_{50} = IC_{50,standard}/IC_{50,sample} \quad (14)$$

The $TEAC_{50}$ values are lower than $TEAC_0$ values for nonlinear DR profiles of positive shape or equal for linear ones. Both of these parameters cannot be thus generally compared, because different methods are used for their estimation. This can be seen from examples shown in **Table 2**, which also included the TEAC parameters estimated by the method described by Re et al. (*3*), who approximated the DR profiles linearly. When poor linearity is observed, it is suggested to estimate $TEAC_{50}$ parameters for the series studied, instead of linear approximation. The $TEAC_{50}$ and $TEAC_0$ values are not equivalent, as shown by statistical significances in **Table 2**.

The $TEAC_0$ estimate reaches the highest numerical value of all parameters estimated with the same analytical procedure and conditions. However, all of the above parameters (TEAC, $TEAC_{50}$, $TEAC_0$) reach the same value for linear DR profiles.

Table 2. Examples of Estimations of $TEAC_0$, $TEAC_{50}$, and TEAC Values for $ABTS^+$ Radical Scavenging for a Range of Materials[a]

material	$TEAC_0$ (LR)[b] a	$TEAC_0$ (FIT) b	$TEAC_{50}$ c	TEAC d
buckwheat seeds	96.1 ± 4.8	97.5 ± 3.3 cd	82.3 ± 2.2 b	82.5 ± 2.2 b
rye seeds	19.4 ± 0.4 d	20.2 ± 0.4 cd	16.3 ± 0.9 b	16.2 ± 0.8 ab
apple juice	6.3 ± 0.2	6.2 ± 0.2	5.8 ± 0.2	5.8 ± 0.2
grapefruit juice	10.7 ± 0.1 cd	11.2 ± 0.2 cd	7.6 ± 0.4 ab	7.4 ± 0.3 ab
rat plasma	8.5 ± 0.4 cd	8.9 ± 0.5 c	5.8 ± 0.1 ab	6.2 ± 0.1 a

[a] As Trolox equivalents, mean ± standard deviation in millimoles of Trolox per kilogram of dry weight or liter; $TEAC_{50}$ estimated from dose−response profiles by interpolation with bis-log−linear equations; TEAC represents Trolox equivalent antioxidant capacity estimated from dose−response profile by the linear dependence approximation according to Re et al. (*3*). Letters following entries indicate a significant difference (p level of at least 0.05) between parameter mean in column designated by the same letter. [b] Linear regression mode LR_a.

To emphasize the difference and avoid improper comparisons of the parameters TEAC and $TEAC_0$ obtained by extrapolation to zero sample concentration, the above new parameter estimated by extrapolation has been designated by a different abbreviation, that is, $SEAC_0$ (standard equivalent antioxidant capacity at zero sample concentration), in general, or $TEAC_0$ (for Trolox standard). All of the above indices were estimated for the variety of food products and rat plasma and are exemplified in **Table 2**.

DISCUSSION

Fast societal changes in dietary and nutritional habits, extensive food production, modified food quality, and changes in the occupational, physical, and emotional activity of the human population significantly influence pro-/antioxidant homeostasis of the body and can affect health (see, e.g., ref *26*). Thus, the analytical methods for measurement of "antioxidant power" of biological materials should fit a potential "biological window" and the needs of the antioxidant−oxidant system to be useful in diagnostics of pro-/antioxidant status balance and its deviation from normal state in living organisms and in the evaluation of the biological adequacy of the human diet.

Deficient antioxidant capacity of the body has been recognized and is known as "oxidative stress", which leads to disturbance of many biological functions. On the other hand, however, recent studies have suggested that both lower and upper biological antioxidant limits are critical for human health. In humans, an increase in mortality was observed at excessive vitamin E supplementation (*27*). Excess of antioxidants has been found to exhibit pro-oxidant activity (*28*). Recent findings of Ristov et al. (*29*) have concluded that dietary antioxidants prevented health-promoting effects of physical exercise in humans. Because the living organism is functioning via the system of equilibria, there may be potentially many other disadvantageous effects of an excess of antioxidants in the body. In addition, it might hypothetically be expected that the human body is biologically better equipped to function with and recover from antioxidant deficiency than to function under an overload of antioxidants.

There is nonrational public expectation that "more" is better than "less". This may and does stimulate excessive antioxidant consumption from diet or supplementation, and it is known from supplementation of nutrients, microelements, vitamins, etc. Thus, the overdosing tendency by humans in association with underevaluation of antioxidant potential of foods may overlap and act in the same direction with respect to potential disadvantageous effects on health.

With the aim to attain better knowledge on antioxidant properties of a variety of foods, there are many research efforts to estimate and collect databases of antioxidant properties for most food products on local and worldwide scales (*30*−*32*).

There is a strong literature call for reliable, precise, and convenient methods of evaluation of antioxidant activity of biological materials, including foods and animal/human tissues (*15*). To date, a few methods have been established as commonly applicable (*3*, *5*, *6*), and there are increasing efforts for development in this field (*17*, *33*−*37*).

All organic compounds can behave as antioxidants; thus, the antioxidant activities form a quasi-continuum. The antioxidant capacity of a particular sample depends roughly on the oxidative potential of the oxidizer used. For the free radicals of the highest oxidative potential and thus most destructive in vivo, the antioxidant properties of the target molecule are thus less meaningful (*38*), and then spatial features (e.g. availability) and the other factors of the microenvironment may play major roles in the reaction rate and products.

However, under in vitro conditions of antioxidant potential measurement, such as the use of stable radical reagents, which are not present in biological systems, the most important factor is the reaction kinetics between free radical and antioxidant. Potentially fast kinetics of low molecular weight antioxidants may not apply to high molecular weight structures, which can be present in complex animal samples, in which slow kinetics conformational transformations of biomolecules may contribute to the reaction kinetics in a time-dependent manner. Our rat (and human) plasma AA measurements have shown that inhibition of the ABTS radical did not stop even after 3 h. At a shorter measurement time, clearly a nonlinear dose−response profile was observed, which made estimation difficult. Particularly, plasma seems to be one of the most difficult materials for reliable antioxidant capacity measurement. This is probably due to a variety of kinetics of plasma constituents and contribution from secondary processes.

Nonlinearity in the reagent inhibition dose−response profiles appears to be common for natural complex samples. The nonlinearity for a mixture of different antioxidant constituents potentially could result from several factors: (1) spectral background contribution to the measured signal (e.g., in spectrophotometric techniques) due to changes of background absorbance due to reaction of the sample constituents (decrease) or formation of products (increase); (2) different kinetics rates for different compound groups in the sample; (3) multiple or sequential reaction pathways for complex molecules (*21*); (4) different kinetics order for different sample constituents; (5) interactions between intermediate radicals formed during the first step of inhibition (reactions of homo- or heteromolecular species); (6) interactions between intermediate radicals and other antioxidant compounds; (7) possible specific reactivity of ABTS cation (instability) induced by addition of complex sample and not associated directly with radical reactions of tested antioxidants (e.g., traces of transient metals present in the sample).

Some of the above factors should depend on sample concentration. For fast kinetics in comparison to reaction time, the inhibition measurements should not be sample concentration-dependent, resulting in a linear dose−response profile and a flat plot of $TEAC_1$ versus sample concentration. For the second factor, interactions between intermediate radicals, second-order kinetics, and thus strong concentration dependence should be expected. Thus, both of the mentioned factors are expected to be dependent on the reagent-to-sample ratio. The quenching kinetics order may thus be simplified at the significant excess of the reagent, where pseudo-first-order can be observed.

The effect mentioned above was proved in hundreds of our estimations. However, in the range of sample concentration very close to zero, a short flat plateau was sometimes observed (*Z*-shape),

suggesting that the reaction was completed and reached saturation and that no further secondary processes proceeded. These conditions, that is, measurements at a stationary state, would give an accurate value for total inhibition capacity of sample and were sometimes applied for the DPPH radical-based method. This approach not always can be applied to the ABTS cation radical due to its instability. Under such conditions, that is, saturation, the linear extrapolation to zero can slightly overestimate the $TEAC_0$ value. For most measured series the concentration range close to zero was not achieved because of significant imprecision of measurement for small absorbance changes at high optical density. Thus, the experimental points with deviation from linearity in the concentration range close to zero, if observed, were excluded here from extrapolation rather than considered to be valid.

The method described in this paper allows for omitting some problems associated with nonlinearity of dose−response relationships and for estimating standardized antioxidant activities. The estimated parameter ($TEAC_0$) was not sample concentration-dependent and can be estimated with high precision. However, because the incubation time applied was not excluded from kinetic equations and due to possible secondary reactions during the incubation, $TEAC_0$ values are still dependent on the incubation time. The first dependence can be numerically removed and expressed by rate constants (eq 7). The second one can be studied by the use of automatic, high-speed operation or stopped-flow technique, where a shorter reaction time and extrapolation to zero reaction time can be applied. Surprisingly, good linearity was achieved for many materials tested with LR_a regression modes as compared with LR_b (**Figure 3B**), hypothetically suggesting that extrapolations made with mode LR_a could just exclude some contribution of secondary processes. In such a case, an extrapolated activity value may be associated to a higher extent with primary antiradical properties of tested molecules. The complexity of TAA estimates is the major limitation in the studies of structure−activity relationships (*16*, *21*); thus, the above suggestion is worth further studies.

The extrapolation of the measured antioxidant activity to zero sample concentration can be applicable especially to complex materials for which maximal standardized values can be obtained. This approach can minimize the effects of interactions between free radicals generated at the same time from sample constituents and their reactions between themselves and with the other reactive nonradical species present in the reaction medium. The latter effects can be hypothetically associated with lower antioxidant capacities for higher sample concentrations and may also reduce the effectiveness of antioxidants in the organism.

The conditions of low sample concentration also can make secondary radical reactions more uniform, limiting them to the sample associated intermediate species inhibition mainly by the reagent radicals.

It should be emphasized that estimation of antioxidant capacity at zero sample concentration is an approach directed to the antioxidant properties of the sample, that is, antioxidant, not to the reagent, a free radical. Thus, the antioxidant activities should also depend less on the kind of radical reagent used, and estimates for other reagents should be more comparable or correlated, which will be further studied. The estimated maximal ability of the tested substance to react with free radicals can be further interpreted in terms of its importance and biological needs.

Because the $TEAC_0$ values are not sample concentration dependent, they can be compared between different kinds of samples independent of the type of their DR profile. This method standardizes TAA estimates and, in our experience, has given excellent between-method linear correlations (*39*). It can be useful for the evaluation of food quality, for its aging control during storage, and for collection of standard characteristics of foods and evaluation of the nutritional value of the human diet. A simple $SEAC_0$ (or $TEAC_0$) abbreviation is proposed for the index of antioxidant capacity estimated by extrapolation to zero sample concentration, a "zero sample concentration approach".

The method described in this paper is at present under evaluation and further development. It was also employed for different estimations (*22*, *39*, *40*) applied to the DPPH radical inhibition method (*41*) and others, which is being currently evaluated.

ABBREVIATIONS USED

AA, antioxidant activity; ABTS, 2,2′-azobis(3-ethylbenzothiazoline-6-sulfonic acid) and related method based on this reagent; $ABTS^{\bullet+}$, cationic radical obtained by oxidation of ABTS sodium salt; ABTS method, method based on antioxidant activity estimation with the use ABTS cation radical as a reagent; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl, an alternative stable radical used as a reagent for AA estimations; DR profile, dose−response profile, a plot of antiradical efficacy (*S*) versus sample concentration or reverse; IC_{50}, inhibition concentration at half-conversion level (50% reagent quantity inhibition); IC_1, inhibition concentration at 1% reagent inhibition on average, calculated from the ratio of sample concentration to scavenging coefficient in percent, equal to the slope b_1 in the text; *S*, antiradical efficacy, scavenging coefficient, quenching or inhibition efficacy given usually in percentage; $SECA_0$, standard equivalent antioxidant capacity at zero sample concentration expressed by equivalent amount of the chosen standard substance; $TEAC_0$, Trolox equivalent antioxidant capacity at zero sample concentration estimated by extrapolation and expressed by equivalent content of Trolox as standard; TAA (TAC), total antioxidant activity (capacity); $TEAC_1$, Trolox equivalent antioxidant capacity calculated from one-point measurement and equal to the ratio $b_{1,standard}/b_{1,sample}$ or $IC_{1,standard}/IC_{1,\ sample}$; $TEAC_{50}$, Trolox equivalent antioxidant capacity calculated from the sample concentration at 50% inhibition or estimated by interpolation method; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analogue of α-tocopherol.

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There was an error in the first paragraph of the Results section in the version of this paper published ASAP July 29, 2010. The correct version published on August 3, 2010.

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