Antioxidant properties of BHT estimated by ABTS assay in systems differing in pH or metal ion or water concentration

Andrzej L. Dawidowicz · Małgorzata Olszowy

Abstract Antioxidant activity (capacity or potential) is widely used as parameter to characterize different substances and mixtures, which is able to scavenge or neutralize free radicals. Recently, ABTS assay has been the most widely employed method for estimating antioxidant activity. The method is based on the spectrophotometric measurement of ABTS cation radical (ABTS•+) concentration changes resulting from the ABTS•+ reaction with antioxidants. Yet little is known about factors influencing the kinetics that reaction i.e., about factors affecting the estimation of antioxidant activity of examined compounds. The paper shows that metal ion type and concentration, water content and pH of the measuring system all significantly influence the estimation of antioxidant activity in ABTS assay and thus make the estimation of correct antioxidant properties of plant and food extracts difficult. Butylhydroxytoluene (BHT) was used as standard antioxidant in the performed experiments. The relationships discussed in this paper indicate the necessity of standardizing the ABTS method and reveal the complexity of estimating adequate antioxidant activity of examined substances.

Keywords Antioxidant properties · ABTS assay · Butylhydroxytoluene (BHT) · Metal ions impact · pH impact · Water impact

Introduction

Continuously generated in living systems and in exogenous environment, the reactive oxygen and nitrogen species (frequently called free radicals) can cause oxidative damage to unsaturated fatty acids, to the thiol group in proteins, and to the nucleic acid bases in DNA and RNA [1, 2]. These changes in cell components may accelerate the aging process and cause various kinds of diseases as follows: coronary heart diseases, cancer, inflammation, immune system decline, neurological diseases, and atherosclerosis [3–6]. In the struggle with free radicals, the living organisms are supported by substances known as antioxidants that are substances significantly decreasing or preventing the effect of harmful free radicals action on oxidizable substrates and, hence, on normal physiological functions in humans [7].

Antioxidant activity (capacity or potential) is widely used as parameter to characterize different substances and mixtures with the ability of scavenging or neutralizing free radicals. This activity is related to the presence of compounds capable to protecting a biological system against harmful oxidation. A lot of substances occurring in the natural world exhibit antioxidant properties, the best example being plant components responsible for the antioxidant capacity of plant foods.

There are a number of methods for measuring the efficiency of antioxidants. They differ in the applied reactive oxidant, in reaction mechanisms, reaction conditions in which the antioxidant assay is performed, and in the way of presenting result [8, 9]. Irrespective of the applied method, a lack of correlation between antioxidant activities determined on the same material using different assays is very often observed in literature [10–12]. Moreover, antioxidant activities of the same material estimated by the same assay in various laboratories are also frequently different [13–16].
All those differences may be caused by the presence of components in the examined material, e.g., in plant or food extract, which do not exhibit antioxidant activity but can affect the result of the measurement. As it was shown in [17], BHT antioxidant properties determined by β-carotene bleaching assay strongly depend on metal and hydrogen ion concentration. Yet it has not been established whether the presence of these ions in the measuring system influences also the estimation of antioxidant activity evaluated using ABTS assay, an increasingly popular research method these days.

The extraction process from plant and food materials is frequently performed using non-aqueous extractants such as methanol, ethyl acetate, and hexane. Although these solvents exhibit different ability to dissolve water, its concentration in the final extract can be different, depending on both solvent polarity and water concentration in the extracted material. Hence, the influence of water content on the estimation of antioxidant activity using ABTS assay is also worth considering. The raised questions are justified as metal ions, hydrogen ions, and water are natural constituents of plant and foods and do not belong to radical scavengers.

The paper shows and discusses the differences in the antioxidant activity of BHT estimated by the ABTS method applied to systems differing in 1. hydrogen ion concentration, 2. metal ion concentration, and 3. water content.

**Experimental section**

Reagents

Methanol, CuCl₂ × 9 H₂O, Fe₂(SO₄)₃ × 5 H₂O, Zn(NO₃)₂ × 2 H₂O, AlCl₃ × 6 H₂O phosphoric acid, monobasic sodium monophosphate (all of analytical-reagent grade) were purchased from the Polish Chemical Plant-POCh (Gliwice, Poland). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate (dipotassium peroxdisulfate) and butylhydroxytoluene (BHT) were purchased from Sigma Aldrich (Poznań, Poland). Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA).

Methods

**ABTS** [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] radical cation was generated according to Re [14, 18]. The ABTS⁺⁺ solution was prepared by the reaction of 5 mL of a 7 mM aqueous ABTS solution and 88 μL of 140 mM (2.45 mM final concentration) potassium persulfate (K₂S₂O₈) solution. The mixture was incubated in the dark for 16 h. The formed radical cation was then diluted in methanol until the initial absorbance value of 0.7 at 744 nm was reached.

**The influence of the type and concentration of metal on the amount of unreacted ABTS⁺⁺ in BHT/ABTS⁺⁺ system**

Two milliliters of prepared ABTS⁺⁺ solution was mixed in a 4-mL test tube with 20 μL of methanolic BHT solution (0.5 mg/mL), 50 μL of metal ion solution in water, and 30 μL of methanol to reach the total mixture volume equal 2,100 μL. The mixture was stirred vigorously for 30 s and poured into quartz cuvettes (1 cm × 1 cm × 3.5 cm). The decrease in absorbance was monitored for 60 min at 744 nm. To zero the spectrophotometer, the mixtures of appropriate solvents’ volumes without ABTS⁺⁺ and the antioxidant were used. The mixtures of appropriate solvents’ volumes with ABTS⁺⁺ solution without antioxidant were applied as controls. System No. 6 from Table 1 was adopted as a reference system in the experiments. The following metal ion solutions were used: Cu²⁺ at concentration 1.0 × 10⁻⁴, 5.0 × 10⁻⁴, 10 × 10⁻⁴ mg/mL (mg of metal ion per 1 mL); Fe³⁺ at concentration 8.0 × 10⁻³, 14.0 × 10⁻³, 22.0 × 10⁻³; Zn²⁺ at concentration 2.0 × 10⁻⁴, 8.0 × 10⁻⁴, 14.0 × 10⁻⁴, and Al³⁺ at concentration 2.0 × 10⁻³, 8.0 × 10⁻³, 16.0 × 10⁻³.

The percent of unreacted ABTS⁺⁺ was calculated from the following equation:

\[
\% \text{ of unreacted ABTS}^{++} = \left(\frac{A_0}{A_t}\right)100\%,
\]

where A₀ and Aₜ are the values of ABTS⁺⁺ absorbance at 0 min and at time equal to (t) min.

**The influence of water concentration on the amount of unreacted ABTS⁺⁺ in BHT/ABTS⁺⁺ system**

Two milliliters of ABTS⁺⁺ solution was mixed in a 4-mL test tube with 20 μL of methanolic BHT solution (0.5 mg/mL), 50 μL of metal ion solution in water, and 30 μL of methanol to reach the total mixture volume equal 2,100 μL. The mixture was stirred vigorously for 30 s and poured into quartz cuvettes (1 cm × 1 cm × 3.5 cm). The decrease in absorbance was monitored for 60 min at 744 nm. To zero the spectrophotometer, the mixtures of appropriate solvents’ volumes without ABTS⁺⁺ and the antioxidant were used. The mixtures of appropriate solvents’ volumes with ABTS⁺⁺ solution without antioxidant were applied as controls. System No. 6 from Table 1 was adopted as a reference system in the experiments. The following metal ion solutions were used: Cu²⁺ at concentration 1.0 × 10⁻⁴, 5.0 × 10⁻⁴, 10 × 10⁻⁴ mg/mL (mg of metal ion per 1 mL); Fe³⁺ at concentration 8.0 × 10⁻³, 14.0 × 10⁻³, 22.0 × 10⁻³; Zn²⁺ at concentration 2.0 × 10⁻⁴, 8.0 × 10⁻⁴, 14.0 × 10⁻⁴, and Al³⁺ at concentration 2.0 × 10⁻³, 8.0 × 10⁻³, 16.0 × 10⁻³.

The percent of unreacted ABTS⁺⁺ was calculated from the following equation:

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\% \text{ of unreacted ABTS}^{++} = \left(\frac{A_0}{A_t}\right)100\%,
\]

where A₀ and Aₜ are the values of ABTS⁺⁺ absorbance at 0 min and at time equal to (t) min.

**Table 1** Volume (in μL) of the individual components used for formation of the examined systems

| System component | System number |
|------------------|---------------|
| ABTS⁺⁺ in MeOH   | 2,000 2,000 2,000 2,000 2,000 2,000 2,000 2,000 |
| BHT in MeOH      | 20 20 20 20 20 20 20 20 |
| MeOH             | 80 70 60 50 40 30 20 0 |
| Water            | 0 10 20 30 40 50 60 80 |
| Total volume     | 2,100 |

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mg/mL) and supplemented with a proper amount of methanol and water up to the volume of 2,100 μL. Table 1 contains the exact compositions of the examined systems. This type of system formation saves chemical reagents and guarantees the same amount of ABTS•+ and antioxidant in the measuring systems. The water-free system was taken assumed as a reference system. To zero the spectrophotometer, the mixtures of appropriate solvents without ABTS•+ and the antioxidant were used. The percent of unreacted ABTS•+ was estimated as in the previous assay.

The influence of hydrogen ions concentration on the amount of unreacted ABTS•+
in BHT/ABTS•+ system

Two milliliters of ABTS•+ solution was mixed in a 4-mL test tube with 20 μL of methanolic BHT solution (0.5 mg/mL) and 40 μL of phosphoric buffer and supplemented with a 40 μL of methanol up to the volume of 2,100 μL. Phosphoric buffers of the following pH were used 1.5, 1.8, 3.5, 6.0, and 10.1. System No. 5 from Table 1 was adopted as a reference system in the experiments. The percent of unreacted ABTS•+ was estimated as in the assay described earlier.

Statistical analysis

Results are presented as mean values. Each antioxidant activity assay was performed three times in order to determine the measurement reproducibility. RSD of all measured values were lower than 8%. p < 0.01 was assumed as statistical difference between experimental points.

Results and discussion

Fig. 1 presents the influence of the iron ions concentration on the difference between BHT/ABTS•+ reaction rates in the systems with and without the metal ions (Δ). Fig. 2 shows the method of Δ calculation in this experiment. As results from Fig. 1, the presence of iron ions strongly affects the assessment of BHT antioxidant properties. In the studied concentration range, the increase in metal ion concentration decelerates the radical/antioxidant reaction kinetics in relation to the kinetics in a metal ion–free system (negative Δ). The difference in the chemical reaction kinetics in the system with and without iron ions is slower of about 50% at the metal ions concentration equal to 0.022 mg/mL. Generally, the greater the iron ions concentration the smaller are the antioxidant properties of BHT. The influence of copper ion concentration on Δ between BHT/ABTS•+ reaction rates in the systems with and without the metal ions is illustrated by Fig. 3. The presented plot indicates that copper, like iron, diminishes the reaction kinetics between the radical and antioxidant(negative Δ), thus affecting the assessment of its antioxidant properties. However, the shape of the kinetic change for the both metal ions is different. The results presented in Figs. 1 and 3 agree with [17], reporting the decline of BHT antioxidant properties estimated by β-carotene assay in systems containing metal ions.

As appears from the literature [19], ABTS•+ forms complexes with transition metal ions. It is reflected visually by the intensity of ABTS•+/metal green color at the presence of metal ions. The observed decrease in reaction kinetics can be connected with the ABTS•+/metal complex formation in the measuring system, which restrains the BHT/ABTS•+ reaction rate. It is possible that the electron and hydrogen derived from the antioxidant have limited access to the unpaired electron in the newly established structure of the ABTS radical cation/metal complex.
It should be noticed, however, that the ions of both metals can also undergo redox reactions and the reduction in Cu\(^{2+}\) to Cu\(^+\) and/or Fe\(^{3+}\) to Fe\(^{2+}\) in measuring system, resulting from the electron interception from the radical cation or BHT, cannot be excluded. The ABTS assay is classified as an electron transfer (ET) method [20, 21]. ET-based methods detect the ability of a potential antioxidant to transfer one electron to a radical. It is probable that Cu\(^{2+}\) and/or Fe\(^{3+}\) intercept this electron, thus causing the inhibition of the BHT/ABTS\(^{**}\) reaction rate.

The dependencies shown in Fig. 4 indicate a higher probability of the second explanation. The presence of zinc or aluminum ions in the measuring system does not affect the BHT/ABTS\(^{**}\) reaction rate (\(\Delta = 0\)). These ions do not undergo redox reactions. Although ABTS\(^{**}\) does forms a complex with the transition zinc ion, which does not influence the BHT/ABTS\(^{**}\) reaction kinetics.

The presence of metal ions in extracts is a consequence of their natural existence in different samples (plants, foods etc). The concentration in extracts is different and depends many on natural and experimental factors such as plant type, plant growing conditions (including soil pollution by metals), ion type, extrahent type, and extracting conditions. These results indicate that the influence of the concentration of some metal ions on the difference (\(\Delta\)) between the inhibition percent of the sample polluted with metal ion in relation to the sample free of metal ion can be the source of erroneous conclusions in comparing different samples. In this experiment, the dependences presented in Figs. 1, 3, and 4 are limited to metal concentration probable in plant extracts, assuming that the whole amount of a given metal ion is extracted from 1 g of plant sample to 50 mL of extrahent.

Extracts can differ not only in qualitative and quantitative composition of the contained antioxidants but also in the presence and concentration of natural acids, which makes their pH different. The presence of natural acids in extracts is rarely taken into consideration when comparing their antioxidant properties. Moreover, to prepare extracts from matrices, investigators frequently apply extrahents of different pH. In this context, the question appears whether the concentration of hydrogen ions in the measuring systems influences the estimation of antioxidant activity. The plots in Fig. 5 shows that pH influences the difference between the BHT/ABTS\(^{**}\) reaction kinetics in buffered and non-buffered measuring systems, i.e., they illustrate the influence of pH on estimated antioxidant BHT activity. As results from the figure, higher hydrogen ion concentration (below 2.5 pH unit) decelerates the BHT/ABTS\(^{**}\) reaction rate (negative \(\Delta\)), whereas lower concentration (above 2.5 pH) accelerates this reaction (positive \(\Delta\)). These results agree with Ozgen et al. [22], who reported greater antioxidant capacity of phenolic compounds estimated by ABTS assay at higher pH than that determined at lower pH. As already mentioned, ABTS is a method in which electron or hydrogen transfer occurs. In methods of this type, the antioxidant activity can be explained by the deprotonation and ionization potential of the reactive functional group of antioxidant (BHT in the presented study). Hence, the reaction occurring between the examined antioxidant and the cation radical is pH dependent [9, 23]. In general, the ionization potential values of phenolic compounds (including BHT) decrease with the increase in pH, reflecting enhanced electron donating capacity with deprotonation [24]. At acidic conditions, the reduced capacity may be restrained due to protonation on antioxidant.

The influence of pH on the BHT antioxidant properties estimated by \(\beta\)-carotene bleaching assay is opposite to that shown in Fig. 5, i.e., the pH increase causes the decrease in BHT antioxidant properties [17]. It results from different radical scavenging mechanisms operating in both methods. The opposite pH influence on antioxidant activity is a good
explanation of the contradictory conclusions found in many reports concerning a comparison of antioxidant properties of examined materials estimated by β-carotene and ABTS (or DPPH) methods [e.g., 11, 25].

Water is one of the fundamental components in all organisms and food products in which it occurs in different amount. Even seemingly, dry plant and food materials contain water. Different water content in the examined samples is responsible for different water concentration in their extracts. The water content in an examined extract depends also on the polarity of the applied extractant. Figure 6 presents the influence of water content on the BHT/ABTS•+ reaction kinetics. The water amounts applied in these experiments approximately correspond to the water amount present in methanolic extracts from differently dried and stored plants at various ratios of plant mass to extractant volume. As appears from the figure, an acceleration of the reaction kinetics takes place with the increase in water concentration in the measuring system. In the examined water concentration range, the relationship is linear. The obtained results indicate that the presence of water in the measuring system facilitates the donation of an electron or hydrogen atom from the BHT to the ABTS radical cation. As appears from [21], the radical cation in the ABTS method is scavenged by electron transfer from antioxidant. This process is accelerated in a medium supporting the ionization of antioxidant. Since water strongly supports BHT ionization [26], an increase in the reaction rate with the increase in water amount in the measuring system is observed. Therefore, studying the effects of water content in the antioxidant/ABTS•+ system on the amount of unreacted ABTS radical is necessary, particularly in solvents miscible with water.

Conclusions

The antioxidative potential of an examined material is usually described by qualitative and quantitative specification of its antioxidative components and/or by the estimation of their antioxidative properties. Recently, ABTS assay has become one of the most widely employed methods for estimating antioxidant activity. The presented results show that such factors in the measuring system as metal ion type and their concentration, water content and pH influence the estimation of antioxidant activity in ABTS assay and create the difficulty in the estimation of exact and correct antioxidant properties of plant and food extracts. The demonstrated relationships confirm the necessity of standardizing the ABTS method and show the complexity of estimating antioxidant activity, even in the case of very simple antioxidant/ABTS•+ systems. In fact, the antioxidant activity of antioxidants depends also on the presence of other components occurring in the examined material, which do not exhibit antioxidant activity.

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