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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].

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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, $CuCl_2 \times 9\ H_2O$, $Fe_2(SO_4)_3 \times 5\ H_2O$, $Zn(NO_3)_2 \times 2\ H_2O$, $AlCl_3 \times 6\ H_2O$ 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 (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 $ABTS^{\bullet+}$ 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 ($K_2S_2O_8$) 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 $ABTS^{\bullet+}$ in BHT/$ABTS^{\bullet+}$ system

Two milliliters of prepared $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ and the antioxidant were used. The mixtures of appropriate solvents' volumes with $ABTS^{\bullet+}$ 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: Cu^{2+} at concentration 1.0×10^{-4}, 5.0×10^{-4}, 10×10^{-4} mg/mL (mg of metal ion per 1 mL); Fe^{3+} at concentration 8.0×10^{-3}, 14.0×10^{-3}, 22.0×10^{-3}; Zn^{2+} at concentration 2.0×10^{-4}, 8.0×10^{-4}, 14.0×10^{-4}; and Al^{3+} at concentration 2.0×10^{-3}, 8.0×10^{-3}, 16.0×10^{-3}.

The percent of unreacted $ABTS^{\bullet+}$ was calculated from the following equation:

$$\% \ of \ unreacted \ ABTS^{*+} = \left(\frac{A_t}{A_{t_0}}\right)100\%,$$

where A_{t0} and A_t are the values of $ABTS^{\bullet+}$ absorbance at 0 min and at time equal to (t) min.

The influence of water concentration on the amount of unreacted $ABTS^{\bullet+}$ in BHT/$ABTS^{\bullet+}$ system

Two milliliters of $ABTS^{\bullet+}$ solution was mixed in a 4-mL test tube with 20 μL of methanolic BHT solution (0.5

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

System component	System number							
	1	2	3	4	5	6	7	8
$ABTS^{*+}$ 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							

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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ and the antioxidant were used. The percent of unreacted $ABTS^{\bullet+}$ was estimated as in the previous assay.

The influence of hydrogen ions concentration on the amount of unreacted $ABTS^{\bullet+}$ in BHT/$ABTS^{\bullet+}$ system

Two milliliters of $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ was estimated as in the assay described earlier.
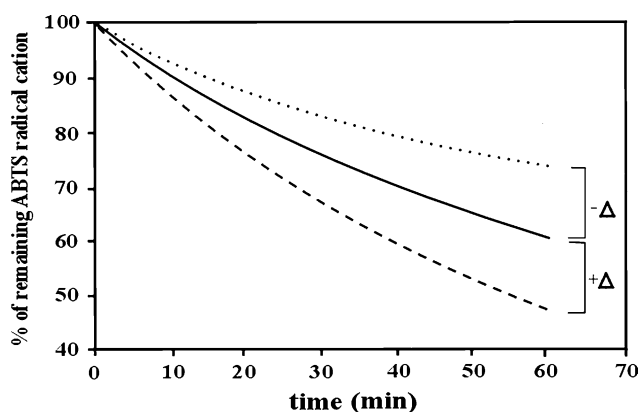
Statistical analysis

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/$ABTS^{\bullet+}$ 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/$ABTS^{\bullet+}$ reaction rates in the systems with
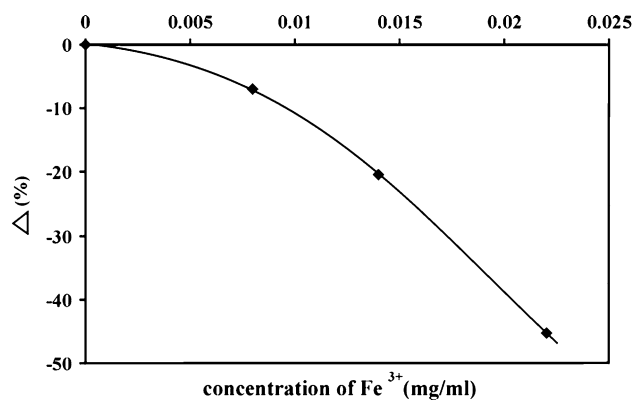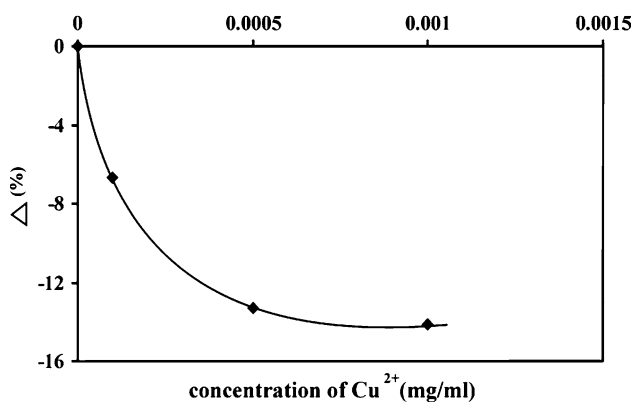

Fig. 1 The influence of the iron ions concentration on the difference (Δ) between BHT/$ABTS^{\bullet+}$ reaction rates in the systems with and without the metal ions


Fig. 2 The way of (Δ) calculation. *Solid line*—kinetic curve in reference system; *dashed and dotted lines*—kinetic curves in systems containing components accelerating and decelerating antioxidant/$ABTS^{\bullet+}$ reaction rate, respectively

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], $ABTS^{\bullet+}$ forms complexes with transition metal ions. It is reflected visually by the intensity of $ABTS^{\bullet+}$ green color at the presence of metal ions. The observed decrease in reaction kinetics can be connected with the $ABTS^{\bullet+}$/metal complex formation in the measuring system, which restrains the BHT/$ABTS^{\bullet+}$ 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.

Fig. 3 The influence of the copper ions concentration on the difference (Δ) between BHT/ABTS$^{\bullet+}$ reaction rates in the systems with and without the metal ions
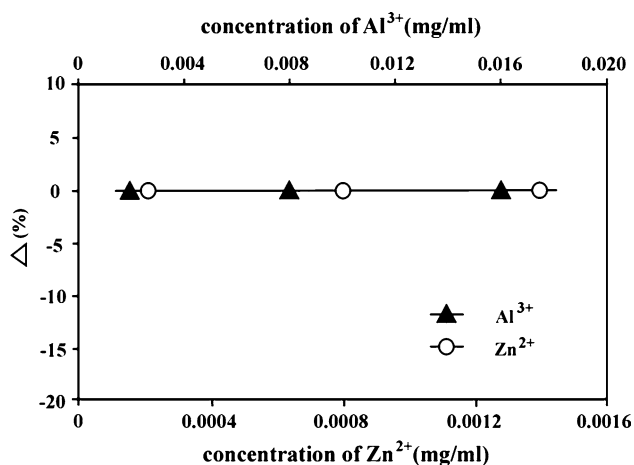
Fig. 4 The influence of the zinc and aluminum ions concentration on the difference (Δ) between BHT/ABTS$^{\bullet+}$ reaction rates in the systems with and without the metal ions

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$^{\bullet+}$ 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$^{\bullet+}$ reaction rate (Δ = 0). These ions do not undergo redox reactions. Although ABTS$^{\bullet+}$ does forms a complex with the transition zinc ion, which does not influence the BHT/ABTS$^{\bullet+}$ reaction kinetics.

The presence of metal ions in extracts is a consequence of their natural existence in different samples (plants, foods etc).
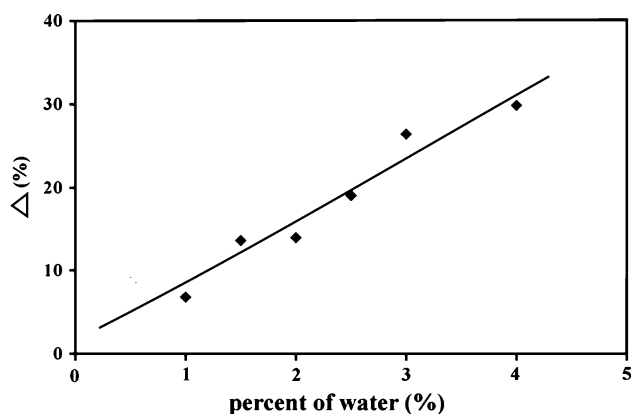
The concentration in extracts is different and depends many on natural and experimental factors such as plant type, plant growing conditions (including soil pollution by metals), ion type, extrahent type, and extracting conditions. These results indicate that the influence of the concentration of some metal ions on the difference (Δ) 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$^{\bullet+}$ reaction kinetics in buffered and non-buffered measuring systems, ie., 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$^{\bullet+}$ reaction rate (negative Δ), whereas lower concentration (above 2.5 pH) accelerates this reaction (positive Δ). 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 occuring 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 β-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
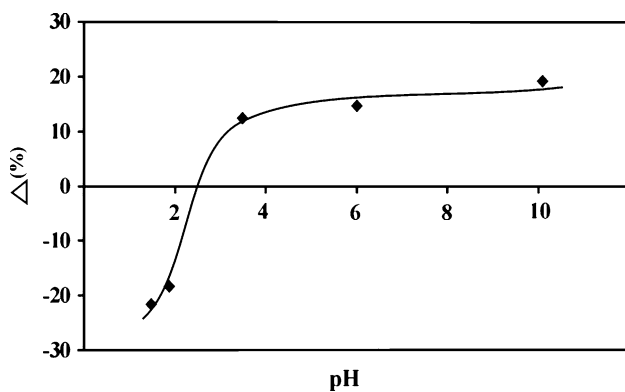
Fig. 5 The influence of pH on the difference between the BHT/ABTS$^{\bullet+}$ reaction kinetics in buffered and non-buffered measuring systems

Fig. 6 The influence of water volume in the measuring systems on antioxidant activity BHT

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$^{\bullet+}$ 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$^{\bullet+}$ 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.

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$^{\bullet+}$ 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 extrahent 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

References

1. Ceconi C, Boraso A, Cargnoni A, Ferrari R (2003) Oxidative stress in cardiovascular disease: myth or fact? Arch Biochem Biophys 420:217–221
2. Ak T, Gülcin I (2008) Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact 174:27–37
3. Kim DO, Chun OK, Kim YJ, Moon HY, Lee CHY (2003) Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric Food Chem 51:6509–6515
4. Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants and the degenerative diseases of aging. Proc Natl Acad Sci United States Am 90:7915–7922
5. Halliwell B, Gutteridge JMC (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1–85

6. Halliwell B (1997) Antioxidants in human health and disease. Annu Rev Nutr 16:33–50
7. Prior RL, Cao G (1999) In vivo total antioxidant capacity: comparison of different analytical methods. Free Radic Biol Med 27:1173–1181
8. Niki E (2010) Assessment of antioxidant capacity in vitro and in vivo. Free Radic Biol Med 49:503–515
9. Karadag A, Ozcelik B, Saner S (2009) Review of methods to determine antioxidant capacities. Food Anal Methods 2:41–60
10. Yang J, Guo J, Yuan J (2008) In vitro antioxidant properties of rutin. LWT Food Sci Technol 41:1060–1066
11. Juntachote T, Berghofer E (2005) Antioxidative properties and stability of ethanolic extracts of Holy basil and Galangal. Food Chem 92:193–202
12. Mata AT, Proença C, Ferreira AR, Serralheiro MLM, Nogueira JMF, Araújo MEM (2007) Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. Food Chem 103:778–786
13. Arts MJTJ, Dallinga JS, Voss HP, Haenen GRMM, Bast A (2004) A new approach to assess the total antioxidant capacity using the TEAC assay. Food Chem 88:567–570
14. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237
15. Williamson G, Plumb GW, Garcia-Conesa MT (1999) Glycosylation, esterfication and polymerization of flavonoids and hydroxycinnamates: effect on antioxidant properties. Basic Life Sci 66:483–494
16. Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956
17. Dawidowicz AL, Olszowy M (2010) Influence of some experimental variables and matrix components in the determination of antioxidant properties by β-carotene bleaching assay: experiments with BHT used as standard antioxidant. Eur Food Res Technol 231:835–840
18. Nenadis N, Wang LF, Tsimidou M, Zhang HY (2004) Estimation of scavenging activity of phenolic compounds using the ABTS assay. J Agric Food Chem 52:4669–4674
19. Deng W, Tan Y, Fang Z, Xie Q, Li Y, Liang X, Yao S (2009) ABTS-multiwalled carbon nanotubes nanocomposite/Bi film electrode for sensitive determination of Cd and Pb by differential pulse stripping voltammetry. Electroanalysis 21:2477–2485
20. Sun T, Tanumihardjo SA (2007) An integrated approach to evaluate food antioxidant capacity. J Sci 72:159–165
21. Huang DJ, Ou BX, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem 53:1841–1856
22. Ozgen M, Reese RN, Tulio AZ, Scheerens JC, Miller AR (2006) Modified 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2, 2′-diphenyl-1-ppicrylhydrazyl (DPPH) methods. J Agric Food Chem 54:1151–1157
23. Lemańska K, Szymusiak H, Tyrakowska ZielińskiR, Soffers EMF, Rietjens IMCM (2001) The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. Free Radic Biol Med 31:869–881
24. Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:3101–3113
25. Valgimigli L, Amorati R, Petrucci S, Peduli GF, Hu D, Hanthorn JJ, Pratt DA (2009) Unexpected acid catalysis in reaction of peroxyl radicals with phenols. Angewandte Chemie International Edition 48: 8348–8351
26. Musialik M, Litwinienko G (2008) Scavenging of DPPH radicals by vitamin E is accelerated by its partial ionization: the role of sequential proton loss electron transfer. Org Lett 7:4951–4954