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Mechanism change in estimating of antioxidant activity of phenolic compounds

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ABSTRACT

The 2,2′-diphenyl-1-picrylhydrazyl (DPPH•) method is commonly applied for the estimation of antioxidant activity of single compounds and plant extracts. In this method, the amount of disappeared DPPH• in the examined system, determined spectrophotometrically, is a measure of the antioxidative (hydrogen-donating) activity of compounds. The present paper discusses the influence of buffer components on the estimation of antioxidant activity of phenolic compounds by this method. According to the obtained results, the change of hydrogen ion concentration changes the mechanism of scavenging process of DPPH radicals by phenolic antioxidants, and the introduction of metal ions into measuring system blocks the scavenging process of DPPH radicals. Both factors depend on the anion type used in the measuring system. The presented results may be especially important for the researches examining plant extract which differ in the content and composition of natural acids and metal ions, and for those who investigate the mechanisms of the reaction applied for the estimation of antioxidant properties.

1. Introduction

Negative influence of reactive oxygen species (ROS) on living organisms [1–3] and on the stability of food products [4–6] is the reason of a significant interest, especially over the last 20 years, in substances exhibiting antioxidant properties and in methods used for the estimation of such properties. Although the human organism has developed defense systems highly efficient in ROS detoxification, the numerous studies have shown that exogenic antioxidants, especially those supplied in foods, are also very important in counteracting oxidative stress. Antioxidants counteract oxidation process in different ways [7–10], for instance by protecting target molecules from oxidation initiators or by stalling the propagation phase. In the first case, the so-called preventive antioxidants hinder ROS formation or scavenge species responsible for oxidation initiation. In the second case, the so-called 'chain breaking' antioxidants intercept radical oxidation propagators or indirectly participate in stopping radical chain propagation.

Phenolic compounds, which mainly come from plants (flavonoids, phenolic acids, stilbenes, tocopherols, tocotrienols etc.), are the biggest group of exogenic antioxidants. Classified as 'chain breaking antioxidants, phenolic compounds are reported to quench free radicals by donating a hydrogen atom and/or an electron to free radicals [11]. Chemical reactions involving the transfer of an electron and a proton can occur by means of concerted or stepwise mechanisms. The position and degree of hydroxylation, polarity, solubility and reducing potential are the main factors influencing the antioxidant activity of phenolic compounds [12,13].

In most methods used to measure the antioxidant properties, the ability of antioxidants (for example phenolic compounds) to trap free radicals is measured by the reaction kinetics between the examined antioxidant and the radical. The methods applying chromogen compounds are commonly used due to their ease, speed and sensitivity [14,15] most popular being those employing the stable 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical ($ABTS^{\bullet+}$).

As results from the literature [16–18], the reaction of radical neutralization depends on many factors among them the hydrogen ion concentration in measuring system. In the case of the ABTS and DPPH methods, the increase of hydrogen ion concentration leads to the decrease of the reaction rate of chromogen radical scavenging. The reported investigations have been limited to phosphoric buffer only. The present paper shows the influence of pH and buffer type on the kinetics of scavenging DPPH radicals; in other words, it shows the influence of pH and buffer type on the estimation of antioxidant properties of the examined antioxidant by the DPPH method. Butylhydroxytoluene (BHT) was used in the reported experiments as standard antioxidant.

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2. Experimental

2.1. Reagents

Methanol, phosphoric acid, acetic acid, monobasic sodium monophosphate, monobasic potassium monophosphate, citric acid, sodium citrate, oxalic acid, potassium oxalate, boric acid, sodium borate, sulphuric acid, hydrochloric acid, nitric acid, silver, cobalt, aluminum, mercury, nickel, copper, iron, zinc, cadmium, potassium and calcium nitrates, potassium, copper, aluminum sulphates and chlorides (all of analytical-reagent grade) were all purchased from the Polish Chemical Plant - POCh (Gliwice, Poland). 2,2′-diphenylpicrylhydrazyl (DPPH) and butylhydroxytoluene (BHT) were purchased from Sigma Aldrich (Poznań, Poland). Water was purified on a Milli-Q system from Millipore (Millipore, Bedford, MA, USA).

3. Methods

The estimation of the unreacted $DPPH^{\bullet}$ concentration in $DPPH^{\bullet}$/BHT systems was performed by the slightly modified Brand-Williams method [19]. To zero the spectrophotometer, the mixtures of appropriate solvents volumes without $DPPH^{\bullet}$ and the antioxidant were used. The mixtures of appropriate solvents' volumes with methanolic $DPPH^{\bullet}$ solution without antioxidant were applied as controls. The assays were carried out in triplicate and the data points are expressed as average values.

Aliquot (2.91 ml) of methanolic $DPPH^{\bullet}$ solution placed in a glass optical cuvette (1 cm × 1 cm × 3.5 cm) containing 60 μl of BHT solution in methanol was examined in several combinations.

- With 30 μl of buffer solution, to estimate the influence of buffer type and its pH on $DPPH^{\bullet}$/BHT reaction kinetics. Acetic, oxalic, phosphoric, boric and citric buffer of different pH in the range to 9 were used in these experiments. $DPPH^{\bullet}$ and BHT concentration in these experiments equal 0.024 mg/ml ($n=2.31\times10^{-7}$ mol) and 0.5 mg/ml ($n=1.36\times10^{-7}$ mol), respectively. In all these experiments 500 mM buffer solution were applied. The precipitation of buffer components was not observed.
- With 30 μl of phosphoric buffers solutions (pH values in the range1–4), to estimate the influence of pH on the inhibition percent in the systems:
  a) containing the same amount of free radicals ($c=0.024$ mg/ml; $n=2.31\times10^{-7}$ mol) and the increasing amount of BHT at the concentrations of BHT methanolic solutions used in these experiments of 0.25; 0.50; 1.00; 1.50; 2.00 mg/ml ($n=0.68\times10^{-7}$ mol; $n=1.36\times10^{-7}$ mol; $n=2.72\times10^{-7}$ mol; $n=4.08\times10^{-7}$ mol; $n=5.44\times10^{-7}$ mol; respectively)
  b) containing the same amount of BHT ($c=0.5$ mg/ml; $n=1{,}36\times10^{-7}$ mol) and the increasing amount of free radicals, the number of $DPPH^{\bullet}$ moles used in these experiments being -1.36×10^{-7}; 3.3×10^{-7}; 8.2×10^{-7} mol.
- With 30 μl of acid solutions to estimate the influence of anion type at the presence of hydrogen ion on the antioxidant activity of BHT. Sulphuric, phosphoric, hydrochloric, acetic and nitric acid were applied in these experiments. pH range from 2 to 8 was used whereas $DPPH^{\bullet}$ and BHT concentrations in these experiments equal 0.024 mg/ml ($n=2.31\times10^{-7}$ mol) and 0.5 mg/ml ($n=1.36\times10^{-7}$ mol), respectively.
- With 30 μl of salt solutions of silver, cobalt, aluminum, mercury, nickel, copper, iron, zinc, cadmium, potassium and calcium nitrates; potassium, copper, aluminum sulphates and chlorides, to estimate the influence of cation and anion type on $DPPH^{\bullet}$/BHT reaction kinetics. The concentration of metal ions was 10^{-2} mol/dm in the applied salt solutions. $DPPH^{\bullet}$ and BHT concentration in these experiments equaled as above, i.e., 0.024 mg/ml ($n=2.31\times10^{-7}$ mol) and 0.5 mg/ml ($n=1{,}36\times10^{-7}$ mol), respectively.
- With 30 μl of salt solutions to estimate the influence of metal ion concentration (potassium and zinc) on the inhibition percent (I) in two systems:
  a) containing the same amount of free radicals ($c=0.024$ mg/ml; $n=2.31\times10^{-7}$ mol) and the increasing amount of BHT. The concentrations of BHT methanolic solutions used in these experiments were 0.25; 0.50; 1.00; 1.50; 2.00 mg/ml ($n=0.68\times10^{-7}$ mol; $n=1.36\times10^{-7}$ mol; $n=2.72\times10^{-7}$ mol; $n=4.08\times10^{-7}$ mol; $n=5.44\times10^{-7}$ mol; respectively)
  b) containing the same amount of BHT ($c=0.5$ mg/ml; $n=1.36\times10^{-7}$ mol) and the increasing amount of free radicals. The number of $DPPH^{\bullet}$ moles used in these experiments were 2.31×10^{-7}; 4.00×10^{-7}; 5.6×10^{-7} mol; potassium and zinc nitrates were applied. The concentration of metal ions salt solutions in (a) and (b) were 10^{-1}; 10^{-3}; 10^{-5}; 10^{-7} mol/l

Each mixture was vigorously shaken for 30 s and immediately transferred into a quartz cuvette (1 cm × 1 cm × 3.5 cm). The decrease in the absorbance at 516 nm was registered in a continuous manner during 60 min employing a UV Probe-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Subsequent readings were taken at regular intervals (60 s).

The percent of remaining $DPPH^{\bullet}$ (% $DPPH^{\bullet}_{rem}$) was calculated from the following equation:

$$\%DPPH^{\bullet}_{rem}=\frac{A_t}{A_{t=0}}\cdot100\%$$

where $A_{t=0}$ and A_t are the values of absorbance at 0 min and at time equal to (t) min, respectively.

The antioxidant activity expressed as inhibition percent (I) was established using the following equation:

$$I(\%)=100\%-\%DPPH^{\bullet}_{rem}$$

3.1. Statistical analysis

Results are presented as mean values ± SD. In order to determine the measurements reproducibility, each antioxidant activity assay was done three times. RSD of all measurements were lower than 10%. $p<0.01$ was assumed as statistical difference between experimental points.

4. Results and discussion

Fig. 1 illustrates the influence of pH on the difference (ΔI) between the percent of remaining DPPH radical in the buffered and the non-buffered samples, after 60 min of the BHT/$DPPH^{\bullet}$ reaction. For clarity, Fig. 2 shows the method of ΔI calculation in these experiments. This figure presents three exemplary kinetic curves: kinetic curve for reference system (solid line), kinetic curve for system with acetic buffer of pH=4.29 (dotted line) and kinetic curve for system with acetic buffer of pH=5.17 (dashed line). As results from the figure, the greater hydrogen ions concentration causes the deceleration of $DPPH^{\bullet}$/BHT kinetic in relation to the kinetic in reference system (negative values of ΔI), whereas the acceleration this reaction is observed when the hydrogen ions concentration is smaller (positive values of ΔI). The presented way of ΔI calculation was applied to obtain the dependences shown in Fig. 1. These relationships were established for different buffers.

Considering the results presented in Fig. 1 and in other figures of this paper it worth noticing that the measurement of

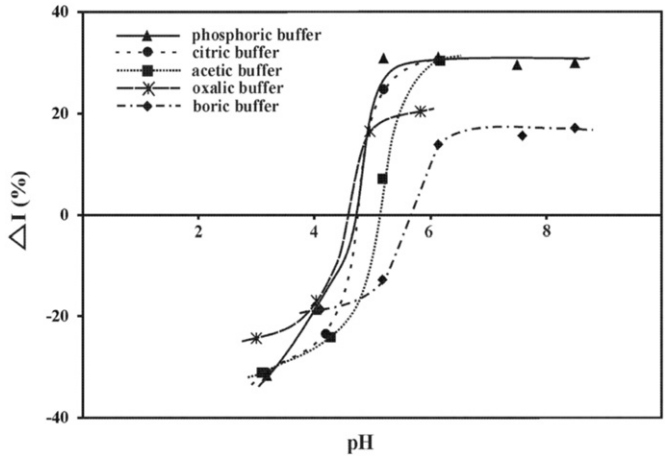

Fig. 1. The influence of pH and the buffer type on the difference (ΔI) between antioxidant activity percent of BHT in buffered and non–buffered samples after 60 min of reaction.

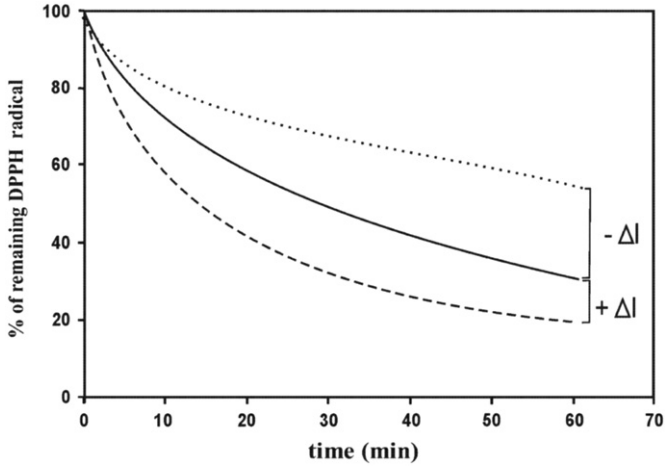

Fig. 2. The method of ΔI calculation. Solid line corresponds to kinetic curve for reference system; dotted line to kinetic curve for system with acetic buffer of pH=4.29 and dashed line to kinetic curve for system with acetic buffer of pH=5.17.

pH (by a pH meter) for solvent mixture that contains organic solvent is imprecise because electrode response tends to drift — the addition of organic solvent changes the pH. In liquid chromatography, for example, it is recommended to relate retention parameters to pH of the buffer being the component of buffered organic mobile phase. This way was assumed in this paper.

The plot for each applied buffer shows that the increase in hydrogen ion concentration results in the decrease in ΔI toward negative values. Moreover, for each applied buffer the s-shape dependences are observed. The results suggest that BHT exhibits higher or lower antioxidant activity in relation to that measured for system containing only water. Higher BHT antioxidant activity is observed in less acidic media and lower in more acidic ones.

According to the literature [20,21], the redox reactivity of phenolic compounds to scavenge free radicals can follow four chemical pathways: Proton Coupled-Electron Transfer (PC-ET), Electron Transfer–Proton Transfer (ET–PT), Sequential Proton Loss Electron Transfer (SPLET) and Adduct Formation (AF). The balance between these mechanism depends on the reaction environment. Following [22], the reaction between phenolic antioxidants and DPPH radical occurs by a combination of the PC-ET and SPLET mechanism. The first one is slower and dominates in non-polar solvents of low dielectric constant and of low basicity, whereas the second one is faster and is characteristic for solvents of high dielectric constant and of high basicity supporting antioxidant ionization [20,23]. The degree of ionization of phenolic antioxidant (ArOH) depends on both a bulk property of the solvent, (its relative permittivity, ε_r), and a molecular property, [its relative ability to solvate, and hence stabilize, anions (ArO^-)] [20]. Taking the last into account, SPLET mechanism is favored in methanol

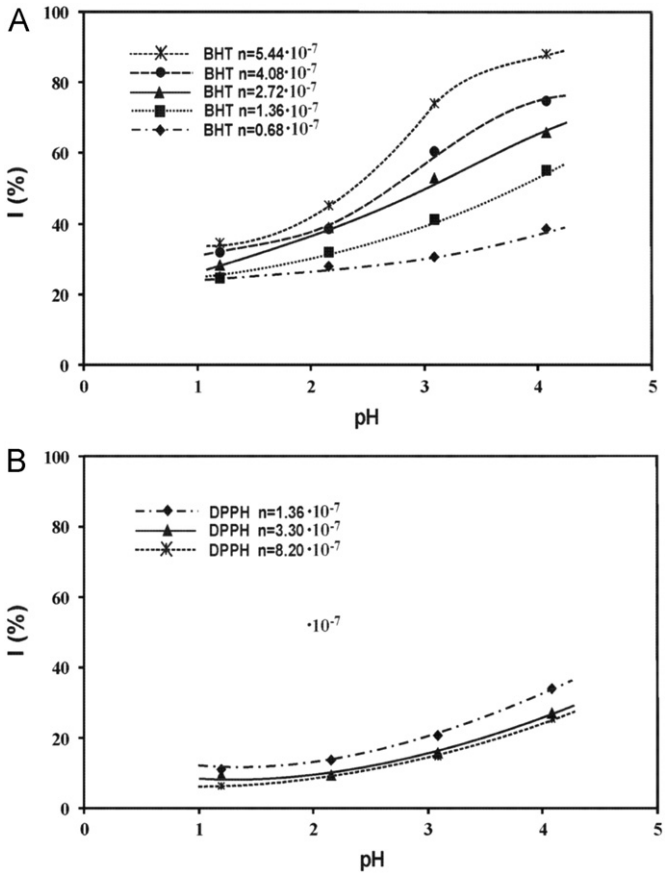

Fig. 3. The influence of pH on the inhibition percent (I) for systems: (A) Containing the same amount of free radicals and the increasing amount of antioxidant; (B) Containing the same amount of antioxidant and the increasing amount of free radical.

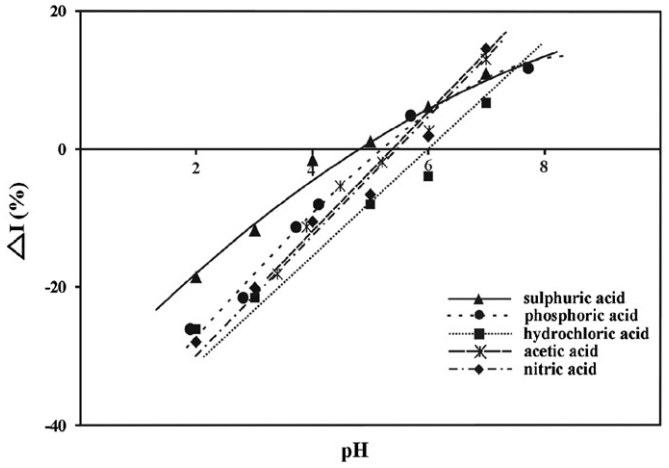

Fig. 4. The influence of hydrogen ion concentration on the difference (ΔI) between the percent of the remaining DPPH radical in the acidified sample and the percent of the remaining DPPH radical in the non-acidified sample after 60 min of the BHT/DPPH• reaction.

which possess high dielectric constant ($\varepsilon = 33$) and high ability to solvate phenolic anions [20]. In non-ionizing solvents and in solvents causing ionization but containing small amounts of hydrogen ions, the SPLET mechanism can even be eliminated [24]. Hence, the change of ΔI from negative to positive value with the increase of pH (see Fig. 1) is connected with the domination change of the mechanism of BHT/DPPH$^{\bullet}$ reaction kinetics from PC-ET into SPLET.

The relationships presented in Fig. 1 were obtained applying systems containing the same amount of the antioxidant (BHT) and the free radical (DPPH$^{\bullet}$). Fig. 3 illustrates the influence of pH on the inhibition percent (I) for systems containing the same amount of the free radical and the increasing amount of the antioxidant (Fig. 3(A) and for systems containing the same amount of the antioxidant and the increasing amount of the free radical (Fig. 3(B). In these experiments phosphoric buffer as pH regulator was applied. According to the SPLET mechanism, the reaction between antioxidants and free radicals begins from the antioxidant dissociation (the first step of the reaction). pK_a of BHT equals 12.2 [25]. In this experiment the dissociation degree of BHT at pH = 7.0 is very low and equals 6.37×10^{-6}. The increase of hydrogen ion concentration leads to further decrease of BHT dissociation, which results in the decrease of SPLET mechanism domination in the examined system. The dissociation decrease of BHT with pH decrease is slower for higher BHT concentrations. Hence, the observed relation between the curves in Fig. 3(A) results from the influence of BHT concentration on the domination of SPLET mechanism: the greater BHT concentration the smaller suppression of the domination of the SPLET. Fig. 3(B) presents the influence of pH on inhibition percent (I) for systems containing the same amount of BHT and the increasing amount of the free radicals. From the comparison of Fig. 3(A) and (B) appears that, at each examined pH, the impact of DPPH$^{\bullet}$ concentration on the inhibition percent is much smaller than of BHT, which additionally indicates the pH influence on the change of the domination mechanism in BHT/DPPH$^{\bullet}$ reaction.

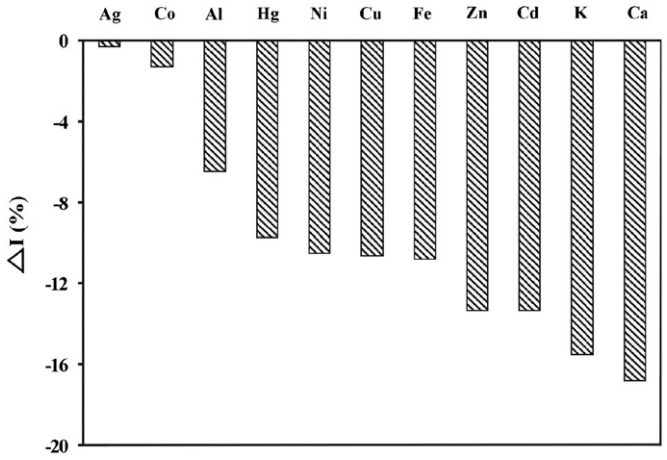

Fig. 5. The difference (ΔI) between the percent of remaining DPPH radical in measuring system with and without metal ions after 60 min of the BHT/DPPH$^{\bullet}$ reaction.

A more detailed consideration of the plots in Fig. 1 shows that their position in relation to the pH scale is different. It is thus probable that there are other factors, not only hydrogen ion concentration, affecting the estimation of BHT antioxidant activity. The components of the applied buffers, cations and anions can be such factors. The application of pure acids instead of buffers is the simplest way of examining the influence of the anion type at the presence of hydrogen ion on the estimation of the antioxidant activity of BHT. The influence of hydrogen ion concentration on

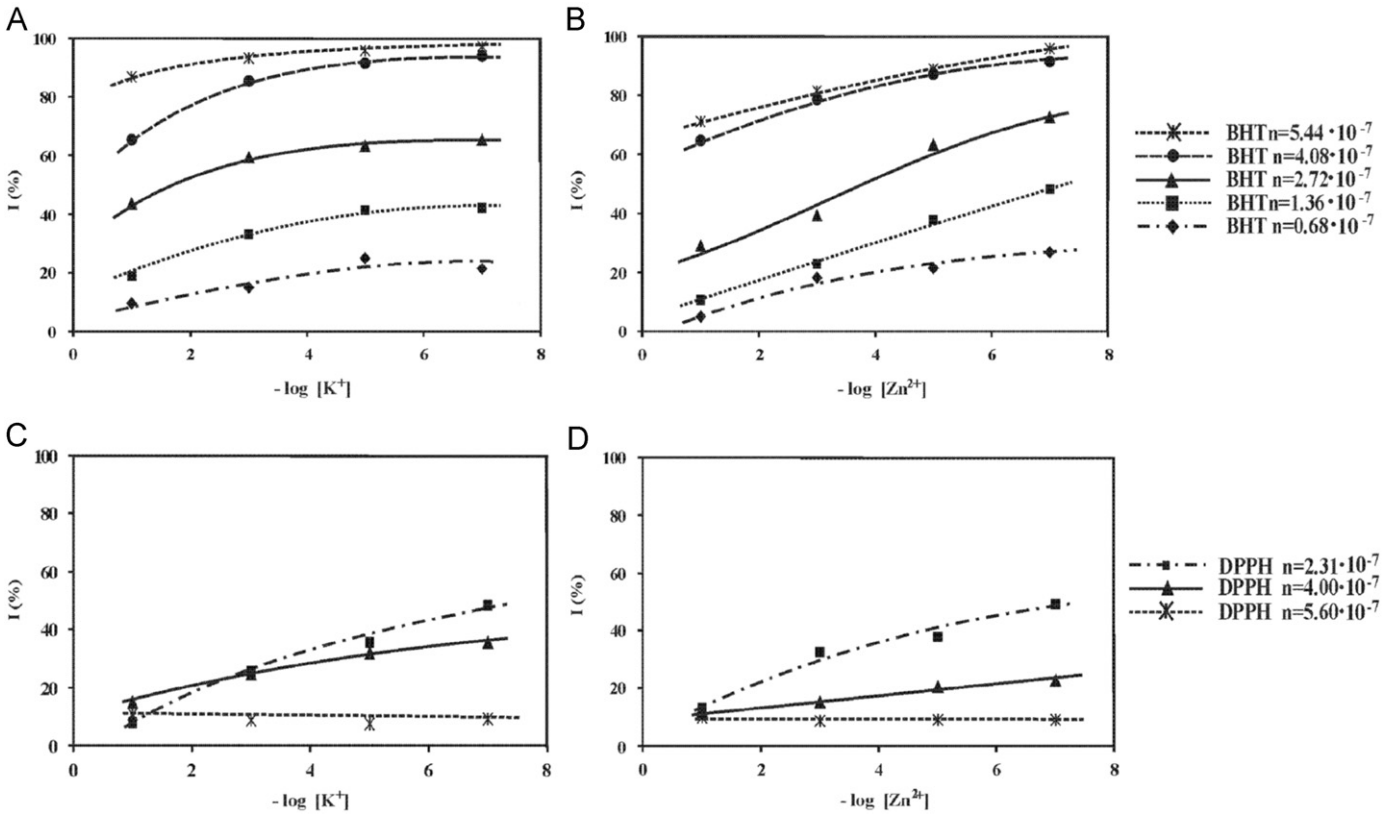

Fig. 6. The influence of metal ion concentration (potassium (A, C) and zinc(B, D)) on the inhibition percent (I) for systems. Containing the same amount of free radicals and the increasing amount of antioxidant – see (A) and (B). Containing the same amount of antioxidant and the increasing amount of free radical – see (C) and (D).

the difference (ΔI) between the percent of the remaining DPPH radical in the acidified sample and the percent of the remaining DPPH radical in the non-acidified sample after 60 min of the BHT/DPPH$^{\bullet}$ reaction is presented in Fig. 4. The figure shows that the decrease in hydrogen ion concentration results in the increase in ΔI from negative to positive values. The run of these dependences can be explained in the same way as in the former case: the change of the domination mechanism of BHT/DPPH$^{\bullet}$ reaction kinetics from PC-ET into SPLET occurs with the decrease of hydrogen ion concentration. These results confirm that the anion type affects the velocity of BHT/DPPH$^{\bullet}$ reaction kinetics.

Fig. 5 illustrates the influence of the metal ion type on the difference (ΔI) between the percent of remaining DPPH radical in the measuring system and the percent of the remaining DPPH radical in the system without metal ions after 60 min of the BHT/DPPH$^{\bullet}$ reaction. All ΔI values were obtained for systems containing the same concentration of metal ions applied in the form of nitrate salts. As results from the diagram, the deceleration of the BHT/DPPH$^{\bullet}$ reaction kinetics takes place for each used cation. However, its suppressive influence on the reaction rate is different: the greatest for alkaline metal ions and the slightest for cobalt and silver ions. The straightforward impact of metal ion and its type on the reaction kinetic changes is observed only in the case of calcium and potassium ions. The acidic character of water solutions of other salts does not allow for the reliable estimation of the effect of the metal ion type on the BHT/DPPH$^{\bullet}$ reaction kinetics. Nevertheless, the decelerating influence of metal ions on the change of the BHT/DPPH$^{\bullet}$ reaction kinetics is obvious and can results from their interaction with BHT molecules and/or DPPH radicals. Fig. 6 illustrates the influence of metal ion concentration (potassium and zinc) on the inhibition percent (I) for systems containing the same amount of the free radical and the increasing amount of the antioxidant (Fig. 6(A) and (B)) and for systems containing the same amount of the antioxidant and the increasing amount of the free radical (Fig. 6(C) and (D)). In these experiments potassium and zinc nitrates were applied. As results from Fig. 6(A) and (B), the increase of metal ion concentration causes the decrease of BHT/DPPH$^{\bullet}$ reaction rate; however, this trend is less pronounced at higher BHT concentration. Fig. 6(C) and (D) leads to the same conclusion. The relative position of dependence curves is the only difference between Fig. 6(A) and (B) and between Fig. 6(C) and (D). The increase of BHT concentration in the measuring system leads to the increase of inhibition percent whereas the increase of DPPH radical concentration in the measuring system has the opposite effect. All these facts indicate a stronger impact of metal ions than of the antioxidant than on DPPH radical. Yet as phenolic compounds with metal ions form phenolates (phenoxides) [26], electron transfer from phenolate ions is impossible and the scavenging process of the free radicals is blocked.

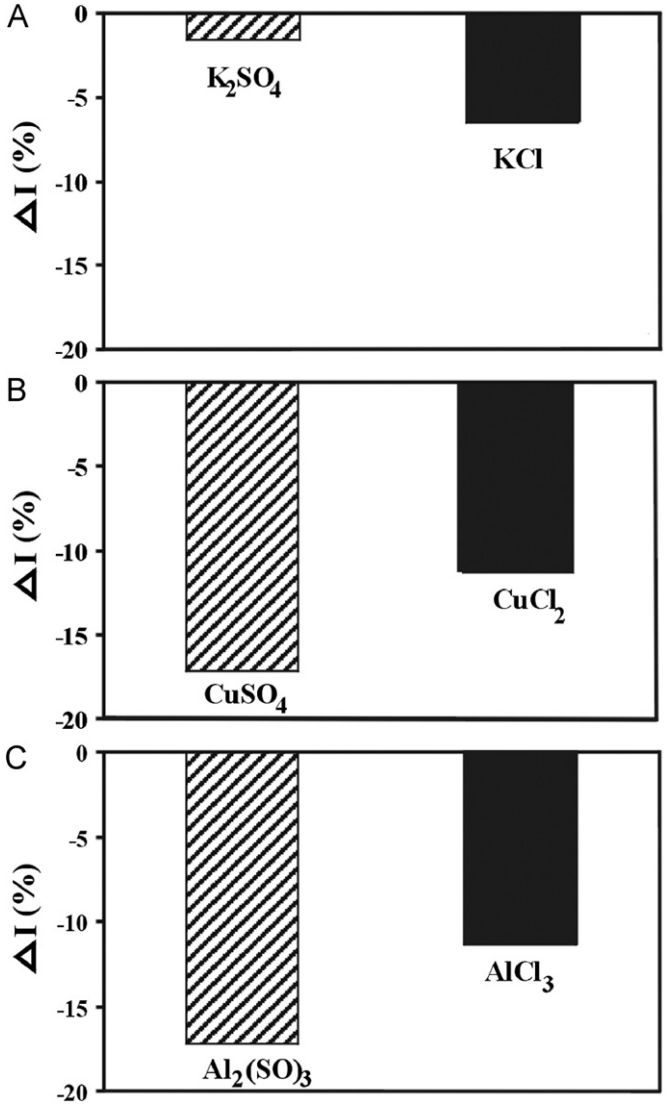

Fig. 7. The difference (ΔI) between the percent of the remaining DPPH radical in the measuring system containing various potassium, copper and aluminum salts and in the system without the salts after 60 min of the BHT/DPPH$^{\bullet}$ reaction.

As results from Fig. 4, the anion type influences the depletion degree of BHT/DPPH$^{\bullet}$ reaction rate caused by the presence of hydrogen ions in the measuring system. Fig. 7 shows that the analogous effect in the BHT/DPPH$^{\bullet}$ reaction rate resulting from the presence of metal ions. Yet the straightforward impact of the anion type on the reaction kinetic changes is possible only in the case of potassium salts. In the case of aluminum and copper salts, hydrogen ions present in the water solutions of these salts disturb the real influence of the anion type on the degree BHT/DPPH$^{\bullet}$ reaction rate depletion.

5. Conclusions

The presented results and discussion show that:

- the change of hydrogen ion concentration causes the change of the mechanism of scavenging process of DPPH radicals by phenolic antioxidants. The increase of hydrogen ion concentration leads to the domination of the PC-ET mechanism;
- the presence of metal ions in the measuring system blocks the scavenging process of DPPH radicals. In consequence electron transfer from phenolate ions into DPPH radicals is impossible;
- the change of the scavenging process mechanism resulting from the change of hydrogen ion concentration and the blocking degree of scavenging process caused by the presence of metal ions depends on the anion type occurring in the measuring system.

The presented results may be especially important for the researches examining plant extract which differ in the content and composition of natural acids and metal ions, and for those who investigate the mechanisms of the reaction applied for the estimation of antioxidant properties. This study show to what extent the mentioned factors have the influence on the concentration of unreacted DPPH$^{\bullet}$, leading to differences in the estimation of antioxidant activity. Moreover, it proves that the

estimation of exact and correct antioxidant properties of plant and food extracts seems to be impossible.

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