Biophysical Chemistry 212 (2016) 9–16

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Kinetics of ABTS derived radical cation scavenging by bucillamine, cysteine, and glutathione. Catalytic effect of Cu^{2+} ions

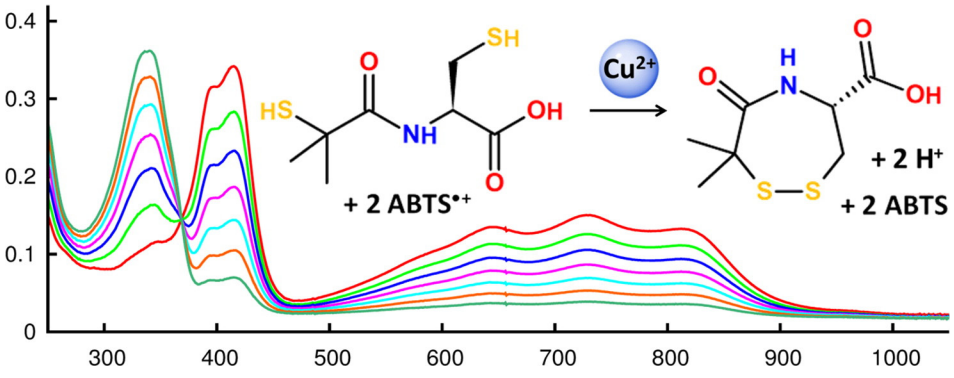
Ivan Valent [a,*], Dominika Topoľská [b], Katarína Valachová [b], Juraj Bujdák [a], Ladislav Šoltés [b]

[a] Department of Physical and Theoretical Chemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina, Ilkovičova 6, 842 15 Bratislava, Slovak Republic
[b] Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dúbravská cesta 9, 841 04 Bratislava, Slovak Republic

HIGHLIGHTS

- Decay of $ABTS^{\bullet+}$ is governed by pseudo-first order kinetics
- H^+ ions display second order inhibition with all the studied thiols
- BUC exhibits zero order kinetics to $ABTS^{\bullet+}$ with H^+-catalysis at pH > 2.7
- Cu^{2+} ions show strong catalysis for all the species in the order BUC > Cys > GSH
- Significant effects of EDTA and bathocuproine disulfonate were observed

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 December 2015
Received in revised form 18 February 2016
Accepted 18 February 2016
Available online 3 March 2016

Keywords:
Antioxidant
Electron transfer
Thiol
Copper catalysis
TEAC
SA96

ABSTRACT

Kinetics of reduction of the stable radical cation derived from 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in reaction with the anti-rheumatic drug bucillamine (BUC) and two reference thiols – cysteine (Cys) and glutathione (GSH) was followed spectrophotometrically in acidic medium with 10-fold molar excess of the reductant. Decay of the radical is governed by pseudo-first order kinetics with small deviation in the case of GSH. H^+ ions displayed second order inhibition of the reaction with all the studied compounds. The reaction of BUC exhibits zero order kinetics to the radical at lower acidities with a moderate acceleration of the reaction rate by H^+ ions. A significant catalytic effect of Cu^{2+} ions on the reactions with all the reductants was observed. The most sensitive to Cu^{2+}-catalysis was the reaction of BUC with the radical cation, while Cu^{2+} ions showed much lower effect on the reaction with GSH. The presence of EDTA strongly inhibited the reactions and equalized the reaction rates for all the reductants. A Cu(I) selective chelator bathocuproine disulfonate reduced the reaction rate with Cys, but accelerated the reaction with BUC at the lower acidities. The experimental results were rationalized in the framework of the mechanism of reductive chelation. The conclusions may have important consequences for interpretation of antioxidant capacity assays, such as TEAC, utilizing the ABTS derived radical cation.

1. Introduction

Thiol-type antioxidants, constituting a class of organic sulfur derivatives having sulfhydryl functional groups, play a crucial role in protecting cells from oxidative damage by interacting with the

* Corresponding author.
E-mail address: valent@fns.uniba.sk (I. Valent).

http://dx.doi.org/10.1016/j.bpc.2016.02.006
0301-4622/

electrophilic groups of reactive oxygen species (ROS) as a first and major member of physiological antioxidative defense system [1]. Decreased levels of thiol compounds in the organism have been shown to cause various disorders. Biologically derived thiols such as glutathione (GSH), cysteine (Cys), and homocysteine are often called biothiols. The side chain functional group —CH_2—SH of cysteinyl residues serves as an active site for most biologically important thiols. The integrity of the —SH groups of intracellular and plasma membrane proteins and soluble thiols are essential to a large number of biological functions. Low molecular weight nonprotein thiols have been shown to have a protective effect against free radical-, radiation- and chemically reactive metabolite-induced toxicity [2].

Bucillamine, *N*-(2-mercapto-2-methylpropionyl)-L-cysteine (Fig. 1a), is a cysteine derivative with two thiol (sulfhydryl) groups per molecule and is known as an efficient anti-rheumatic drug [3] with immunological effects [4]. The exact mechanism of bucillamine action is unknown, but many studies to date suggest that it is an immunomodulator [2,5]. Bucillamine (BUC), also referred to as SA96, is rapidly metabolized to SA981 which is a disulfide compound formed by intramolecular binding of two sulfhydryl groups (Fig. 1b). The disulfide structure of bucillamine metabolites plays a critical role in the pharmacological action of the drug [5]. Most likely, the two sulfhydryl groups which are predisposed to close a stable ring underlie to a different pharmacological action of bucillamine in comparison with D-penicillamine, another substituted cysteinyl anti-rheumatic drug [5].

Bucillamine is capable of replenishing the thiol group in glutathione, thereby assisting defense against oxidant injury [6]. BUC as the effective thiol donor has potential to attenuate or prevent damage during myocardial infarction, cardiac surgery and organ transplantation [6,7]. On the other hand, the drug has been reported to act as an inducer of apoptosis via generation of ROS in the presence of copper ions [8]. Kładna et al. [9] showed that BUC can directly scavenge ROS or inhibit reactions generating them. However, the drug may have pro-oxidant activity under some conditions [9].

In studies of antioxidant properties of various compounds with respect to their radical scavenging capacity, a radical cation derived from 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS (Fig. 2), is frequently used [10]. $ABTS^{\bullet+}$ has been suggested [11] as a useful reference free radical for studies of reactions of organic radicals with sulfhydryl compounds. The aim of this contribution was to examine the reaction of BUC and two reference sulfhydryl compounds (Cys and GSH) with $ABTS^{\bullet+}$ as potential models for more precise kinetic and mechanistic studies of reactions of cysteine-derived drugs with free radicals. Particular attention was given to effect of cupric ions as the catalytic action of copper on oxidation reactions of Cys [12] and its derivatives including BUC [8] is well-known. Despite the amount of experimental data that have been collected so far, detailed mechanisms of these reactions are not fully understood.

2. Materials and methods

Bucillamine was provided by Santen Pharmaceutical Co., Osaka, Japan and was used without purification as well as L-cysteine (97%, Aldrich), glutathione (puriss, Fluka), ABTS diammonium salt (purum, >99%, Fluka), $K_2S_2O_8$ (p.a., 99%, Merck), perchloric acid (p.a., 70–72%, Merck), disodium ethylenediaminetetraacetate dihydrate (complexon III, p.a., Lachema), bathocuproine disulfonate ($Na_2BCS \cdot xH_2O$, Aldrich), and $CuSO_4 \cdot 5H_2O$ (p.a., Lachema).

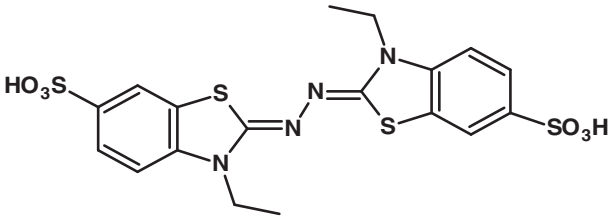


Fig. 2. Structure of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS.

$ABTS^{\bullet+}$ was pre-formed by addition of 18.3 mg of $ABTS(NH_4)_2$ into an aqueous solution of $K_2S_2O_8$ (3.3 mg in 5 ml H_2O). The resulting mixture was stored overnight in the dark below 0 °C. This way a primary stock solution of the radical (approximately of 4.7 mmol dm^{-3}) was prepared which was defrosted before an experiment and used to prepare a working stock solution of the required concentration. Remaining primary stock solution may be frozen back and stored for a repeat use. The primary stock solution of $ABTS^{\bullet+}$ prepared by this procedure contains about 25 molar% of unreacted ABTS which supports stability of the radical [13].

UV–vis spectra were recorded on a Lambda 25 Perkin-Elmer UV/VIS spectrophotometer and an Agilent 8453 diode-array spectrophotometer. Both spectrophotometers were used also for kinetic measurements performed at the temperature of 20 °C. The temperature was regulated by water circulation using a Julabo F12 thermostat when the Lambda 25 spectrophotometer was used, while the diode-array spectrophotometer was equipped with an Agilent 89090A temperature controller based on the Peltier effect. Measurements were carried out in a 10 mm QS stoppered cuvette. Kinetic runs were recorded on the Lambda 25 spectrophotometer immediately after rapid mixing of the reactants in the cuvette by following the absorbance at 734 nm without a stirring of the reaction solution during the measurement. Alternatively, or in parallel, kinetics were followed using the diode-array spectrophotometer and recording full spectra 190–1100 nm, usually with 0.5–2 s cycle time. In this case, the reaction solution was continuously stirred at 600 rpm by 7 mm teflon-coated stirring bar. The time dependences of absorbance at 734 and 340 nm were selected from the acquired data for kinetic analysis. These wavelengths correspond to the absorption maxima of $ABTS^{\bullet+}$ and ABTS, respectively. Although, the reported values for the absorption maximum of the radical cation in this region slightly vary from 725 nm [14] to 734 nm [10], the plateau is relatively wide and a value for the molar absorptivity of the radical $\varepsilon_{728} = 1.50 \times 10^4$ mol^{-1} dm^3 cm^{-1} [15] can be accurately used for estimation of concentration of $ABTS^{\bullet+}$ in aqueous solutions.

Stock solutions of BUC, Cys, and GSH were prepared by weight. A stock solution of perchloric acid was standardized by titration with a solution of sodium hydroxide (p.a., Centralchem, Slovakia) using a Titrino automatic titrator (type 785 DMP, Metrohm). The volumetric NaOH solution was standardized by titration of a weighted amount of potassium hydrogen phthalate (p.a., Merck).

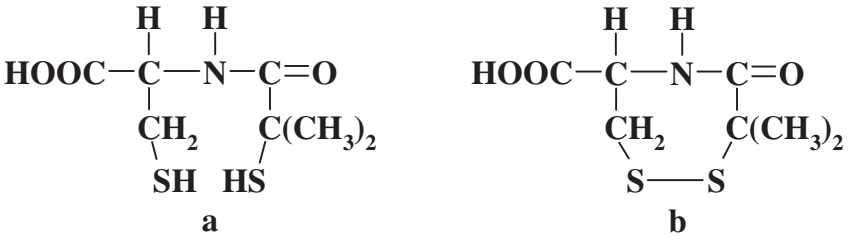


Fig. 1. Structure of bucillamine (a) and SA981 (b).

The recorded absorbance vs. time data were fitted to an exponential function $y = ae^{-bx} + c$ via the parameters a, b, c, where the parameter b corresponds to the pseudo-first order rate constant k_{obs}. Fitting was performed by the nonlinear least squares regression using an interactive plotting program Gnuplot — version 4.6 (T. Williams & C. Kelley) or Matlab — version R2008a (Mathworks).

The data sets of spectra changing during progress of the reaction obtained by the diode-array spectrophotometer were subjected to the chemometric analysis [16]. The original data was adapted by adjusting the baseline offset and the principal component analysis (PCA) and multivariate curve resolution (MCR) were performed using a software Unscrambler X — version 10.3 (Camo).

For needs to study reaction progress in the absence of oxygen, a reaction solution without a final reactant (usually the thiol-compound) was bubbled by N_2 for 4–5 min in the cuvette. After that, the reaction was started by addition of the final reactant from its stock solution, that was bubbled separately before by N_2 for 5–6 min, to the cuvette.

The total volume of the reaction solution was usually 1.8 ml.

3. Results

3.1. Stoichiometry

Stoichiometric ratios $ABTS^{\bullet+}$/BUC and $ABTS^{\bullet+}$/Cys were estimated in excess of the radical by measuring a decrease in the absorbance at 734 nm (A_{734}) after 3–4 additions of BUC or Cys. The reactions proceeded in 9.0×10^{-4} mol dm^{-3} $HClO_4$ in the presence of 10^{-5} mol dm^{-3} Cu^{2+}. The initial concentration of $ABTS^{\bullet+}$ was approximately 62 μmol dm^{-3} and 8 μmol dm^{-3} of BUC was repeatedly added. Completion of the reaction was followed by monitoring A_{734}. To assess the $ABTS^{\bullet+}$/Cys stoichiometric ratio, ~55 μmol dm^{-3} of $ABTS^{\bullet+}$ was allowed to react with several additions of Cys in concentration of 20 μmol dm^{-3}. The stoichiometric ratios were estimated from molar ratios of the consumed radical, calculated from ΔA_{734}, to the amount of added BUC or Cys. The results suggest that 2 moles of $ABTS^{\bullet+}$ react with 1 mole of BUC and 1 mole of the $ABTS^{\bullet+}$ reacts with 1 mole of Cys. These ratios are consistent with the presumed equations:

$$2\ ABTS^{\bullet+} + BUC \rightarrow 2\ ABTS + SA981 + 2\ H^{+} \quad (1)$$

$$2\ ABTS^{\bullet+} + 2\ Cys \rightarrow 2\ ABTS + CySS + 2\ H^{+} \quad (2)$$

where CySS stands for cystine.

3.2. Chemometrics

To justify validity and accuracy of the UV/vis spectrophotometry to monitor kinetics of $ABTS^{\bullet+}$ to ABTS conversion, the measured time series of spectra (an example is shown in Fig. 3a) were processed using the multivariate curve resolution. The analysis proved that $ABTS^{\bullet+}$ and ABTS can be considered as the only two components of the reaction system which are detectable in UV/vis spectra. Providing that the sum of the component concentrations may be estimated from the weighted amount of ABTS used for the radical cation preparation, spectra of the components can be converted to the molar absorptivity coefficient (ε) vs. wavelength dependence (Fig. 3b). Wavelengths of absorption maxima (subscripts in nm) with the corresponding values of ε (in mol^{-1} dm^3 cm^{-1}) assessed from the component spectra: $\varepsilon_{729}(ABTS^{\bullet+}) = 1.6 \times 10^4$, $\varepsilon_{415}(ABTS^{\bullet+}) = 4.0 \times 10^4$, $\varepsilon_{338}(ABTS) = 3.6 \times 10^4$ agree with the published data: [15] $\varepsilon_{728}(ABTS^{\bullet+}) = 1.50 \times 10^4$, $\varepsilon_{417}(ABTS^{\bullet+}) = 3.47 \times 10^4$, $\varepsilon_{340}(ABTS) = 3.66 \times 10^4$. Also, a measured spectrum of pure ABTS (not shown) compares very well with the spectrum of the second component.

The time courses of the component concentrations (Fig. 3c) can be perfectly fitted to the exponential function, suggesting pseudo-first order kinetics under the used reaction conditions. The values of the pseudo-first order rate constants for both components match very well (1.72×10^{-2} s^{-1} for $ABTS^{\bullet+}$ and 1.74×10^{-2} s^{-1} for ABTS) and they agree with values obtained from the $A_{734}(t)$ and $A_{340}(t)$ recordings. The sum of the component concentrations preserves a constant value during the reaction, confirming that no

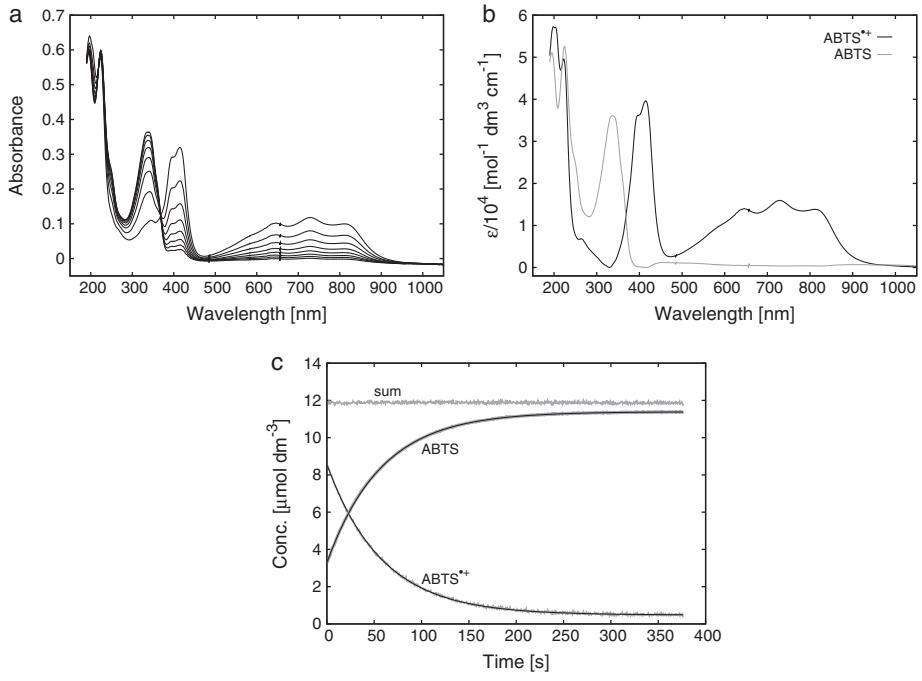


Fig. 3. UV/vis spectrophotometric monitoring of the reaction of $ABTS^{\bullet+}$ (~9 μmol dm^{-3}) with cysteine (100 μmol dm^{-3}) in 900 μmol dm^{-3} perchloric acid at 20 °C. (a) Selected spectra with time interval of 20 s. (b) Spectra of two components obtained by MCR analysis of all spectra (measured with the cycle of 0.5 s). (c) Time courses of the concentrations of components during progress of the reaction. The gray lines illustrate experimental data and the black lines represent the exponential fits. The top line corresponds to the sum of the component concentrations.

ABTS derived intermediates can accumulate in a significant amount during the reaction. In addition, the constant [$ABTS^{\bullet+}$] + [ABTS] level indicates good stability of the radical cation for the followed period. Similar results were obtained by the MCR analysis of the reaction of $ABTS^{\bullet+}$ with BUC, but the resulting molar absorptivities are slightly underestimated in this case (supplementary information, Figure S1).

3.3. Kinetics

3.3.1. Effect of H^+ ions

Hydrogen ions show inhibitory effect on the reaction of $ABTS^{\bullet+}$ with all of the studied thiol compounds. However, more complex effect of H^+ ions was observed in the case of bucillamine. For examined concentrations of $[H^+] < 2 \times 10^{-3}$ mol dm^{-3} decay of $ABTS^{\bullet+}$ in reaction with BUC is linear with time for most of the reaction progress, indicating zero order kinetics to $ABTS^{\bullet+}$ (Fig. 4a). Interestingly, hydrogen ions moderately increase rate of the reaction under those conditions as it can be detected from the slopes of A_{734} vs. time traces. On the contrary, at examined concentrations $[H^+] > 10^{-2}$ mol dm^{-3} the reaction exhibits pseudo-first order kinetics with strong inhibition by hydrogen ions (Fig. 4b). The values of the pseudo-first order rate constants k_{obs} suggest second order for H^+ inhibition.

Effect of H^+ ions on kinetics of the reaction of $ABTS^{\bullet+}$ with Cys was examined for H^+ concentrations ranging from 9.0×10^{-4} mol dm^{-3} to 2.25×10^{-2} mol dm^{-3} under similar conditions as described above for BUC. The reaction exhibits perfect pseudo-first order kinetics under these conditions with second order inhibition by H^+ ions. Similar behavior was observed also for the reaction of $ABTS^{\bullet+}$ with GSH, however, the recorded absorbance vs. time traces showed small deviations from pseudo-first order kinetics.

3.3.2. Effect of O_2

Effects of dissolved atmospheric oxygen, normally present in reaction solutions, on kinetics of the studied reactions was tested by comparing time courses measured in the presence and absence of oxygen (see Materials and methods). Oxygen shows a moderate retardation effect on the reaction of $ABTS^{\bullet+}$ (~10 μmol dm^{-3}) with bucillamine (100 μmol dm^{-3}) measured in 0.011 mol dm^{-3} perchloric acid as well as with cysteine (100 μmol dm^{-3}, in perchloric acid of 900 μmol dm^{-3}). The observed pseudo-first order rate constant increased by a factor of ~1.5 upon removing O_2 from the reaction solution. No significant effect of oxygen on the reaction of $ABTS^{\bullet+}$ with glutathione was observed under the same conditions.

3.3.3. Effect of a nonspecific chelator

In order to inspect whether kinetics of the studied reactions is influenced by traces of metal ions present as impurities in the used chemicals and water, we performed a kinetic measurement with an addition of complexon III (disodium salt of EDTA). The obtained results are summarized in Fig. 5. Even a low concentration of the chelator (50 μmol dm^{-3}) showed a significant effect on the reactions of $ABTS^{\bullet+}$ with BUC and Cys in 900 μmol dm^{-3} $HClO_4$. In the case of BUC the linear zero order course (Fig. 4a, line 2) was changed to an exponential one with a substantial decrease of the reaction rate (Fig. 5). Similarly, a significant retardation of the reaction kinetics by complexon III can be observed in the case of cysteine, whereas it showed a negligible effect on the reaction of $ABTS^{\bullet+}$ with GSH. Interestingly, the kinetics of all the three thiol compounds is closely comparable in the presence of the chelator. Of note are also moderate deviations from exponential course that are obvious in Fig. 5. In contrast with GSH, cysteine exhibits perfect pseudo-first order kinetics in the absence of complexon III at the given acidity as well as BUC does at higher acidities (see Fig. 4b).

3.3.4. Effect of Cu^{2+} ions

Significant effect of the chelator on kinetics of the studied reactions suggests catalysis by metal ions. We examined effect of Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, and Fe^{3+} on the reactions of $ABTS^{\bullet+}$ with all the three thiols. Only Cu^{2+} and Fe^{3+} showed marked influence on the reaction rate. The effect of cupric ions on the reaction of $ABTS^{\bullet+}$ with BUC is documented in Fig. 6. The reaction was retarded by the use of higher concentration of H^+ ions and additions of Cu^{2+} at micromolar level induced a dramatic acceleration of the reaction. The Cu-catalyzed reaction exhibits pure pseudo-first order kinetics with values of the rate constants proportional to the amount of added Cu^{2+} (see the legend of Fig. 6). In the absence of the second reactant (thiol) no reaction between $ABTS^{\bullet+}$ and Cu^{2+} was observed.

A significant effect of Cu^{2+} ions was observed also in the reaction of $ABTS^{\bullet+}$ with cysteine. We compared kinetics of the reaction under study with cysteine from various sources and from stock solutions of a different storage time with and without addition of Cu^{2+}. Cys or Cys. HCl from several different suppliers displayed the pseudo-first order rate constants in the range of 0.55–2.7 min^{-1} under conditions of ~10 μmol dm^{-3} $ABTS^{\bullet+}$ with 100 μmol dm^{-3} Cys in 900 μmol dm^{-3} $HClO_4$ without additional Cu^{2+}. Such a wide range of the k_{obs} values is most likely due to a various content of copper impurities in different chemical products. If the level of Cu^{2+} is controlled by addition of a defined amount of Cu^{2+}, the kinetics becomes better reproducible and independent of the origin of cysteine as it is illustrated in Fig. 7 which shows data measured in 0.011 mol dm^{-3} perchloric acid. At lower acidities, such as 900 μmol dm^{-3} $HClO_4$, the Cu-catalyzed reaction between $ABTS^{\bullet+}$ and Cys is very fast, proceeding to completion immediately after the addition of 10 μmol dm^{-3} Cu^{2+} even in the presence of 100 μmol dm^{-3} of complexon III.

Glutathione is substantially less susceptible to Cu^{2+}-catalysis in the reaction with $ABTS^{\bullet+}$ than bucillamine and cysteine. Addition of Cu^{2+}

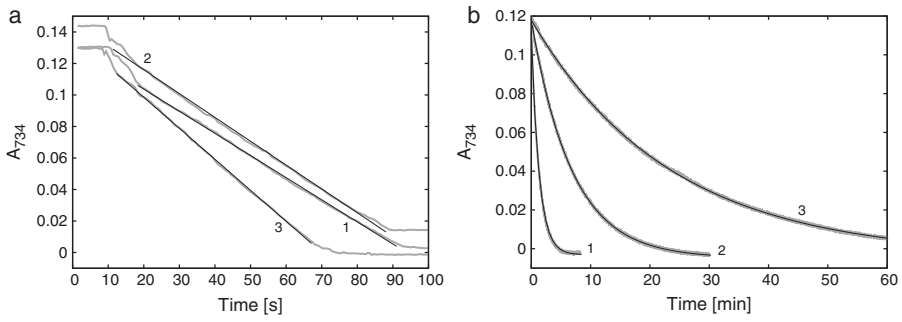


Fig. 4. Effect of hydrogen ions on kinetics of reaction of $ABTS^{\bullet+}$ (~10 μmol dm^{-3}) with bucillamine (100 μmol dm^{-3}) in perchloric acid at 20 °C. (a) Reaction progress at lower H^+ concentrations: $[H^+] = 4.5 \times 10^{-4}$ mol dm^{-3}, line 1, slope 1.40×10^{-3} s^{-1}; $[H^+] = 9.0 \times 10^{-4}$ mol dm^{-3}, line 2, slope 1.51×10^{-3} s^{-1}; $[H^+] = 1.8 \times 10^{-3}$ mol dm^{-3}, line 3, slope 1.97×10^{-3} s^{-1}. (b) Reaction progress at higher H^+ concentrations: $[H^+] = 1.1 \times 10^{-2}$ mol dm^{-3}, line 1, $k_{obs} = 7.63 \times 10^{-1}$ min^{-1}; $[H^+] = 2.25 \times 10^{-2}$ mol dm^{-3}, line 2, $k_{obs} = 1.46 \times 10^{-1}$ min^{-1}; $[H^+] = 4.5 \times 10^{-2}$ mol dm^{-3}, line 3, $k_{obs} = 4.29 \times 10^{-2}$ min^{-1}. The gray lines are measured records and the black lines correspond to the linear and exponential fits.

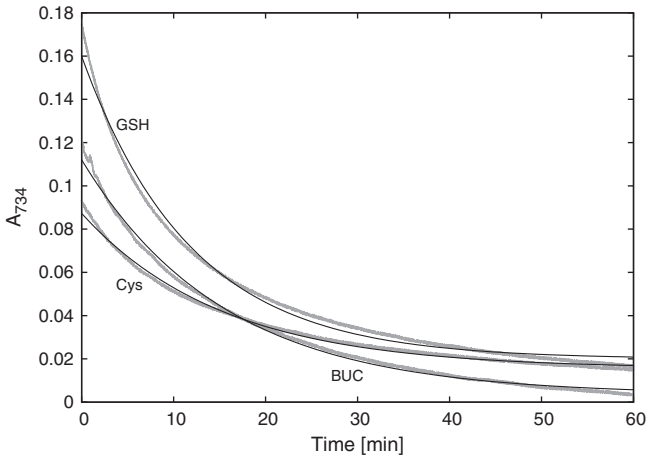


Fig. 5. Absorbance at 734 nm vs. time records of reactions of $ABTS^{\bullet+}$ with bucillamine (label 'BUC'), cysteine ('Cys'), and glutathione ('GSH'). Initial conditions: ~10 μmol dm^{-3} $ABTS^{\bullet+}$, 100 μmol dm^{-3} thiol, 900 μmol dm^{-3} $HClO_4$, 50 μmol dm^{-3} complexon III; 20 °C. The black lines represent exponential fits of the measured time traces (grey lines) with the following pseudo-first order rate constants (in min^{-1}): $k_{BUC} = 6.5 \times 10^{-2}$, $k_{Cys} = 6.5 \times 10^{-2}$, $k_{GSH} = 8.4 \times 10^{-2}$.

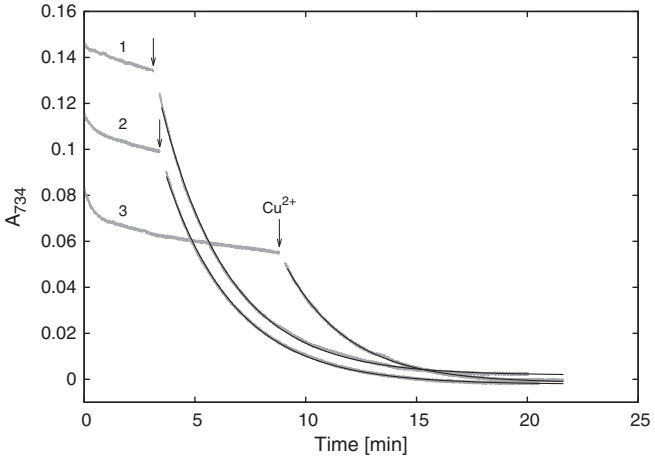


Fig. 7. Effect of additions of Cu^{2+} ions to the reaction of $ABTS^{\bullet+}$ (~10 μmol dm^{-3}) with cysteine (100 μmol dm^{-3}) in perchloric acid (0.011 mol dm^{-3}) at 20 °C. At the indicated moments (arrows) 10 μmol dm^{-3} of Cu^{2+} was added to the reaction solution. Labels are assigned to various cysteine products with the corresponding pseudo-first order rate constants (in min^{-1}) as follows: (1 – Cys-Aldrich) 0.326, (2 – Cys·HCl-Lachema) 0.318, (3 – Cys·HCl-Sigma) 0.350.

ions in the final concentration of 20 μmol dm^{-3} to a reaction solution of $ABTS^{\bullet+}$ (~10 μmol dm^{-3}) and GSH (100 μmol dm^{-3}) in 0.045 mol dm^{-3} $HClO_4$ exhibits no change of the reaction rate. Small acceleration of the reaction was observed in 3.6 mmol dm^{-3} $HClO_4$ upon addition of 10 μmol dm^{-3} Cu^{2+} yielding $k_{obs} = 6.5 \times 10^{-2}$ min^{-1} (20 °C). At lower acidity of 900 μmol dm^{-3} $HClO_4$ in the presence of 500 μmol dm^{-3} complexon III and 40 μmol dm^{-3} Cu^{2+}, $ABTS^{\bullet+}$ and GSH in the above-mentioned concentrations reacted showing pseudo-first order kinetics with the rate constant of approximately 0.14 min^{-1} at 20 °C. This value is less than two times higher than the corresponding rate constant, 0.08 min^{-1}, obtained in the absence of Cu^{2+}. It should be noted that, in contrast with BUC and Cys, kinetics of $ABTS^{\bullet+}$ and GSH reaction subtly deviate from a perfect exponential course under the used conditions in the presence as well as in the absence of Cu^{2+} ions.

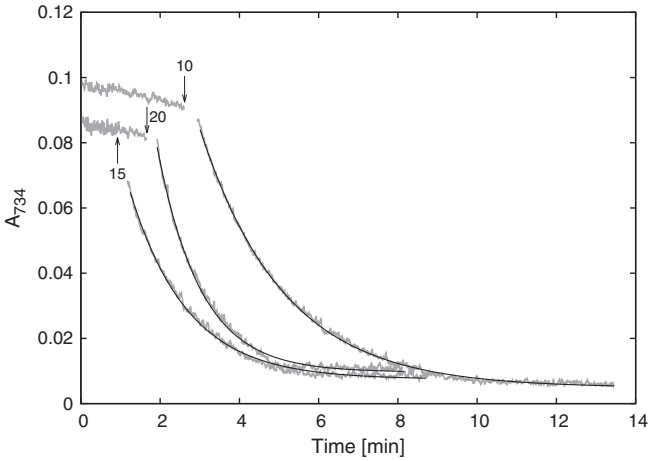


Fig. 6. Effect of additions of Cu^{2+} ions to the reaction of $ABTS^{\bullet+}$ (~10 μmol dm^{-3}) with bucillamine (100 μmol dm^{-3}) in perchloric acid (0.12 mol dm^{-3}) at 20 °C. At the indicated moments (arrows) various amounts of Cu^{2+} (labels in μmol dm^{-3}) were added to the reaction solution. The black lines represent exponential fits of the experimental records (grey lines) with the following pseudo-first order rate constants (in min^{-1}): (10) 0.474, (15) 0.691, (20) 0.914. Stirring rate was 600 rpm.

3.3.5. Effect of Fe^{3+} ions

Effect of ferric ions was examined only on the reaction of $ABTS^{\bullet+}$ (10 μmol dm^{-3}) with cysteine (100 μmol dm^{-3}) in $HClO_4$ (0.011 mol dm^{-3}). An addition of Fe^{3+} ions in concentration of 10 μmol dm^{-3} was able to reverse temporarily consumption of $ABTS^{\bullet+}$ in the reaction system. Production of $ABTS^{\bullet+}$ radical was proved by the measured UV–vis spectra. The transient rise of $ABTS^{\bullet+}$ concentration evoked by the Fe^{3+} addition was then followed by slow, linear in time, decay of $ABTS^{\bullet+}$. Next addition of the same amount of Fe^{3+} brought about a similar effect repeatedly. When Cu^{2+} ions (10 μmol dm^{-3}) were finally added into the reaction mixture, the reaction rate was significantly accelerated and the course of the reaction was turned to the pseudo-first order kinetics as reported above for the Cu-catalysis. However, the observed rate constant was lower in dependence on the amount of Fe^{3+} added than that measured for the Cu-catalysis without addition of Fe. A record of the experiment can be found in the Supplementary data (Figure S2).

3.3.6. Effect of a Cu^{+}-specific chelator

In order to confirm catalytic action of copper ions and to get an additional experimental evidence useful for mechanistic analysis, for example, with respect to the oxidation state of copper, we examined effect of Cu-specific chelators on the reactions of $ABTS^{\bullet+}$ with cysteine and bucillamine. We used a Cu^{2+}-chelator cuprizone and Cu^{+}-chelators neocuproine and bathocuproine disulfonate (BCS) which are commonly used in trace analysis and biological research [17,18]. Cuprizone has appeared to be inappropriate for usage in the studied systems due to its reactivity with $ABTS^{\bullet+}$ and the pH necessary for the complexation with Cu^{2+}. More information on this issue is given in the Supplementary data. However, the presence of BCS in the reaction solution with the concentration of 100–150 μmol dm^{-3} showed significant effects on both the $ABTS^{\bullet+}$-Cys and $ABTS^{\bullet+}$-BUC systems as it can be seen in Fig. 8. Interestingly, BCS inhibits the reaction with cysteine (Fig. 8a), while the reaction with BUC in the zero order kinetic mode is substantially accelerated by the Cu^{+}-chelator (Fig. 8b). The effect of BCS was less profound at higher acidities probably due to protonization of BCS. Negligible effect of BCS on the $ABTS^{\bullet+}$-Cys reaction was observed in 800 μmol dm^{-3} $HClO_4$. On the other hand, the effect of BCS on the $ABTS^{\bullet+}$-BUC reaction in 700 μmol dm^{-3} $HClO_4$ (supplementary information, Figure S5) was comparable (or even higher) to that observed in 400 μmol dm^{-3} $HClO_4$.

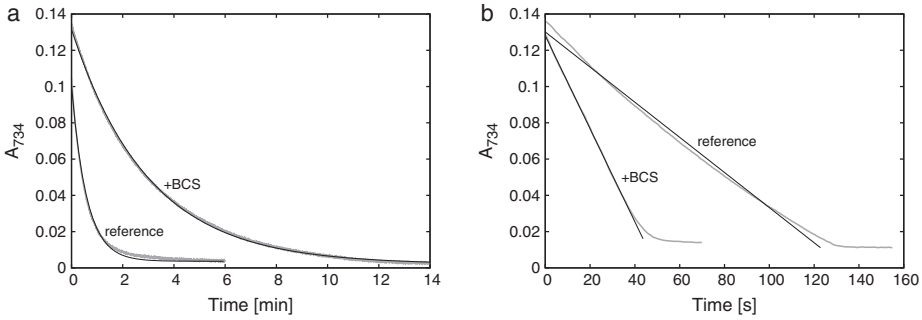


Fig. 8. Effect of bathocuproine disulfonate (BCS) on kinetics of reaction of $ABTS^{\bullet+}$ with cysteine (a) and bucillamine (b) in perchloric acid at 20 °C. Initial concentrations (in μmol dm^{-3}): $ABTS^{\bullet+}$ ~10, thiol 100, $HClO_4$ 700 (a) and 400 (b), BCS 100 (a) and 150 (b). The gray lines are measured records and the black lines correspond to the exponential (a) and linear (b) fits. The obtained rate constants and slopes (in s^{-1}): 2.82×10^{-2} - reference, 5.57×10^{-3} - with BCS (a) and 9.69×10^{-4} - reference, 2.57×10^{-3} - with BCS (b).

4. Discussion

4.1. Stoichiometry

Measured molar ratios of consumed $ABTS^{\bullet+}$ radical cation to reacted cysteine and bucillamine agree with the stoichiometric Eqs. (1), (2). The proposed equations assume one-electron reduction of $ABTS^{\bullet+}$ to ABTS and formation of a disulfide bridge, either intermolecular, producing cystine, or intramolecular that leads to the oxidized form of BUC (SA981). It can be deduced that $ABTS^{\bullet+}$ is a mild oxidizing agent avoiding oxidation of thiols to higher oxidation products under the used conditions even at excess of $ABTS^{\bullet+}$. However, it may not be the case for media of a lower acidity. Aliaga and Lissi [19] reported $ABTS^{\bullet+}$/Cys stoichiometric ratio 1.8:1 with an excess of the radical cation for pH = 5 or higher. Formation of disulfides and equimolar oxidant/thiol stoichiometric ratio in excess of a thiol was reported by Kapoor et al. [20] for reaction of ferricyanide with thioglycolic acid as well as by Mishra et al. [21] for Cu^{2+} catalyzed oxidation of cysteine, 2-mercaptoethanol, and thioglycolic acid by a superoxide-cobalt complex.

4.2. Kinetics and mechanism

4.2.1. Copper catalysis

4.2.1.1. Cysteine. The observed significant effect of Cu^{2+} ions on the rate of $ABTS^{\bullet+}$ decay in reaction with all the three substrates studied here is a main feature outlining mechanistic considerations. Strong catalytic effect of copper and iron on "autoxidation" of cysteine by atmospheric oxygen is known for a long time [12,22,23] suggesting an intermediate complex of cysteine with the metal ions or a metal oxide. More recent studies [24,25] offer a deeper insight to the mechanism of cysteine oxidation associated with the copper complexation by the thiol compound. Pecci et al. [25] suggested the reductive chelation [26] of Cu^{II} as a first step of the oxidation process with formation of a Cu^{I} complex with two cysteine molecules. The complex is oxidized by O_2 or other electron acceptor in a subsequent step producing a transiently bound thiyl radical to the metal which remains in the reduced form. The catalytic complex is recovered by rapid substitution of the thiyl radical by cysteine. The thiyl radicals may directly combine to form the final product cystine or, perhaps more probably, be scavenged by thiolate anion producing cystine in a following reaction with the electron acceptor.

We suppose that a similar mechanism as proposed by Pecci et al. [25] for copper catalyzed oxidation of cysteine by O_2, ferricytochrome *c*, and nitro blue tetrazolium accounts also for the presented reaction of cysteine with $ABTS^{\bullet+}$ radical cation as the electron acceptor. This is supported by the observed reciprocal second order kinetics with respect to hydrogen ions and the observed inhibitory effect of BCS on the $ABTS^{\bullet+}$-Cys reaction. The following scheme can be suggested:

$$RSH \rightleftarrows RS^- + H^+ \quad (3)$$

$$Cu^{2+} + RS^- \rightleftarrows Cu^{II}-SR^+ \quad (4)$$

$$Cu^{II} - SR^+ + RS^- \rightarrow RS - Cu^{I\bullet}SR \quad (5)$$

$$RS - Cu^{I\bullet}SR + RS^- \rightarrow RS - Cu^{I} - SR^- + RS^{\bullet} \quad (6)$$

$$RS - Cu^{I} - SR^- + ABTS^{\bullet+} \rightarrow RS - Cu^{I\bullet}SR + ABTS \quad (7)$$

$$RS^{\bullet} + RS^- \rightarrow RSSR^{\bullet-} \quad (8)$$

$$RSSR^{\bullet-} + ABTS^{\bullet+} \rightarrow RSSR + ABTS \quad (9)$$

where RSH denotes cysteine.[1] Decay of $ABTS^{\bullet+}$ according to first order kinetics and the effect of the Cu(I)-selective chelator suggest the reaction (7) to be the rate-determining step.

4.2.1.2. Bucillamine. It can be assumed that an analogous mechanism as that described above for Cys may be applicable also for the reaction of bucillamine with $ABTS^{\bullet+}$. The reciprocal second order kinetics with respect to hydrogen ions suggests that either one molecule of BUC with both SH-groups ionized binds to cupric ion, or two BUC molecules form a complex with copper with one of the two SH-groups in each BUC molecule remaining protonized. Our preliminary kinetic measurements revealed a second order dependence of the reaction rate on the concentration of bucillamine in the presence of Cu^{2+} which favors 1:2 complexation of Cu^{2+} with BUC. However, additional comprehensive kinetic studies resulting in a complete rate law valid for wide ranges of concentrations of the reactants, hydrogen and cupric/cuprous ions, and possibly of other effectors are necessary to resolve the mechanistic details. Also, a direct characterization of the structure and stability of the $Cu^{2+/1+}$–BUC complex could bring another valuable knowledge for deeper understanding of the properties and therapeutic effects of BUC.

The observed switch of $ABTS^{\bullet+}$ decay kinetics from zero to first order in dependence on the hydrogen ion concentration deserves further investigation. Although coexistence of zero and first order kinetics is rare, shifting between linear and exponential kinetic profiles under various conditions has been reported for copper catalyzed oxidation of thiols [24,27,28]. This phenomenon points to variation in rate-limiting

[1] We used the common abbreviation $ABTS^{\bullet+}$ for the ABTS derived radical cation in the scheme and throughout all the text, although the two sulfonic groups are usually ionized in aqueous solutions. Therefore a more correct denotation would be $ABTS^{\bullet-}$. Similarly, ABTS, or rather $ABTS^{2-}$, is protonated at one of the nitrogen atoms below pH 3 [15], hence $HABTS^-$ should stand for ABTS reflecting better the conditions used in our experiments.

step upon changes in reaction conditions. The observed linear decay of $ABTS^{\bullet+}$ at lower acidities (Fig. 4a) suggests that the rate-limiting process occurs in earlier stages of the reaction sequence than (7), perhaps in the reductive chelation step (5). However, moderate catalytic effect of hydrogen ions on this reaction pattern, which is in dramatic contrast with the second order H^+-inhibition observed for the exponential $ABTS^{\bullet+}$ decay, together with the surprising acceleration of the reaction by BCS (Fig. 8b) indicate a higher complexity of the whole process than that rendered by the simplified scheme (3–9). Therefore, finding precisely defined reaction conditions for zero order kinetics with in-depth mechanistic analysis of this phenomenon is challenging.

4.2.1.3. Glutathione. The reaction of glutathione with $ABTS^{\bullet+}$ radical cation exhibits the lowest catalytic effect of cupric ions in comparison with the reactions of the other two thiols investigated in this study. For example, the pseudo-first order rate constant measured for 100 μmol dm^{-3} concentration of GSH and 10 μmol dm^{-3} Cu^{2+} has the value of ~6.5×10^{-2} min^{-1} in 3.6 mmol dm^{-3} $HClO_4$, while the same concentrations of BUC and Cu^{2+} yield the k_{obs} value approximately eight times higher, but in $HClO_4$ more than 35-times concentrated (0.13 mol dm^{-3}). Observed differences in the rates of copper catalyzed reaction of the studied thiols may arise from several factors. First, it is the pK_a value of the -SH-group and affinity of the thiol/thiolate ligand to Cu^{2+} ion. The second important factor is stability of the Cu^{I}-SR complex formed by the reductive chelation. It is known that a stable complex is formed between Cu^{+} and GSH, whereas the complex between Cu^{+} and cysteine is much less stable [29]. On the other hand, high stability of the Cu^{I}-SR complex may correlate with lower readiness for electron transfer to the oxidant. The process of reoxidation of the complexed Cu^{I} is often the rate limiting step of the reaction with the rate dependent on the R-group of the thiol [30]. Thus the relatively lower rate of the copper catalyzed reaction of GSH with $ABTS^{\bullet+}$ could be explained in terms of hindered rate-limiting electron transfer due to higher stabilization of the Cu^{I}-GSH complex. However, as we mentioned above for Cys, some authors [25] mean that copper remains in the reduced form during the oxidation suggesting sulfur as the electron donor. In that case, stability of the intermediate thiyl radical, alone or in a Cu^{I}-complex, could be the most important factor.

4.2.2. Effect of Fe^{3+} and O_2

Catalytic effect of iron on oxidation of cysteine has been previously described [12,24,31–33]. Under certain conditions, iron ions can cause inhibition of the copper-catalyzed cysteine oxidation [24,33], even at a very low concentration of Fe ions. However, the rise of $ABTS^{\bullet+}$ concentration after the Fe^{3+} addition observed in this study can be most probably explained simply by a direct reaction of reduced ABTS with Fe^{3+} producing $ABTS^{\bullet+}$. According to Scott et al. [15], ferric ions are capable of reversible oxidation of ABTS to $ABTS^{\bullet+}$ with the rate constant for the forward reaction of 1.3×10^{2} mol^{-1} dm^{3} s^{-1} at 25 °C in 1 mol dm^{-3} perchloric acid. Also, the observed retardation of the Cu-catalyzed $ABTS^{\bullet+}$-Cys reaction can be ascribed to continual regeneration of $ABTS^{\bullet+}$ from producing ABTS by the reaction with Fe^{3+}. The investigated $ABTS^{\bullet+}$-Cys system could be useful as a model for study of the interesting issue of combined effect of Cu and Fe ions on cysteine oxidation, providing that appropriate conditions are found for elimination of the direct ABTS-Fe^{3+} reaction.

The observed inhibition of the reactions $ABTS^{\bullet+}$-Cys and $ABTS^{\bullet+}$-BUC by oxygen (the rates increase approximately 1.5-times upon removing atmospheric O_2 from the reaction solution) can be attributed to competition of O_2 as another electron acceptor with $ABTS^{\bullet+}$ in the reaction with the complex RS-Cu^{I}-SR^{-} (step 7). Produced superoxide anion radical is most probably scavenged by a direct reaction with the thiol present in excess.

4.2.3. Uncatalyzed reactions

Addition of a chelating agent such as EDTA which forms a stable complex with a variety of metal ions enables to study uncatalyzed reactions of the selected thiols with $ABTS^{\bullet+}$ radical cation. Interestingly, BUC, Cys, and GSH show very similar reaction rates in the presence of EDTA implying that the structure of the cysteine surrounding plays a minor role in reactivity of the cysteine -SH-group of the examined thiols. In addition, similar reaction rate of BUC possessing two sulfhydryl groups with that of Cys and GSH points to preference reactivity of one of the -SH-groups in the rate-limiting step. The observed comparability of the reactivity of all the three Cys-derived thiols implies that the cysteine thiol group is the key target for the $ABTS^{\bullet+}$ radical also in the case of bucillamine.

The observed deviations of the uncatalyzed reactions from pure pseudo-first order kinetics may originate from several causes. One explanation offers a possible autocatalysis of the studied reactions by the producing disulfide. Dixon and Tunnicliffe [34] noticed the autocatalytic nature of the aerobic and anaerobic oxidation of glutathione, cysteine, and thioglycollic acid and showed that the disulfide group accelerates the rate of oxidation.

4.3. Implications and perspectives

The presented results demonstrate by means of kinetic data important effect of copper ions on reactions of some thiol antioxidants with $ABTS^{\bullet+}$ radical cation. Although the catalytic effect of copper on oxidation of various thiols is generally known, such effect has not, to our knowledge, been noticed with respect to $ABTS^{\bullet+}$ radical cation scavenging. However, this phenomenon requires a special attention particularly in this case, since $ABTS^{\bullet+}$ is utilized for quantification of antioxidant capacity of pure compounds or mixtures in various matrices. For example, the Trolox equivalent antioxidant capacity (TEAC) assay [10,35] represents a standard procedure broadly applied in assaying food samples [36]. Forasmuch as the operational protocol of the TEAC assay usually does not include an assay or a control of trace metals such as copper or iron, the obtained results may be seriously affected by the adventitious presence of such ions in the sample. Additionally, the metal ion catalysis of $ABTS^{\bullet+}$ scavenging may not be restricted to reactions with thiols, since, for example, oxidation of a wide-spread antioxidant ascorbic acid shows copper catalysis as well [37,38]. Thus the TEAC results may be misinterpreted and may lead to erroneous conclusions. This way a surprisingly weak correlation between the measured TEAC values of various antioxidants and their chemical structures [36] could be explained. Moreover, TEAC assay results for pure antioxidant compounds should be interpreted with respect to mechanisms of their reactions with $ABTS^{\bullet+}$ that are not generally known, however. For illustration, Walker and Everette [39] in their comparative study of twenty antioxidants including several thiols (with Cys and GSH among them) pointed to different kinetic patterns in reactions with $ABTS^{\bullet+}$, where most of the studied species showed biphasic kinetics in contrast with simple first order kinetics displayed by merely three compounds. So it can be concluded that more information on kinetics and mechanism of reactions of a wide ensemble of antioxidants with $ABTS^{\bullet+}$ radical cation is needed to better understand the relation between the structure and antioxidant capacity, in particular with respect to action of metal ions.

Formation of complexes of antioxidants with metal ions and their effect on biological systems deserves a special attention. It is known that copper(II) complexes of many anti-inflammatory compounds exhibit enhanced therapeutic activity, relative to the parent ligands, in selected model systems [40]. It may come out that biological activity of many antioxidants relays on a specific complexation with metal ions, though present in very low concentrations, rather than on their direct reactions with the target species such as ROS.

We believe that the reaction system of $ABTS^{\bullet+}$ radical cation with some thiols as described above, and possibly other structurally diverse antioxidants, can serve as an appropriate model for precise studies of

kinetics and mechanism of biologically relevant reactions with the aim to reveal or clarify mechanisms of biological activity of the surveyed compounds.

Acknowledgement

This work was supported by the Scientific Grant Agency of the Ministry of Education of Slovak Republic and of Slovak Academy of Sciences VEGA 2/0065/15 and Slovak Research and Development Agency under the contract No. APVV-0291-11. The authors thank Miss Táňa Sebechlebská for assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2016.02.006.

References

[1] M. Valko, C. Rhodes, J. Moncol, M. Izakovic, M. Mazur, Chem. Biol. Interact. 160 (2006) 1–40.
[2] J.H. Yeung, Biochem. Pharmacol. 42 (1991) 847–852.
[3] Y. Ichikawa, et al., Mod. Rheumatol. 15 (2005) 323–328.
[4] K. Hashimoto, P. Lipsky, J. Rheumatol. 20 (1993) 953–957.
[5] H. Matsuno, E. Sugiyama, A. Muraguchi, T. Nezuka, T. Kubo, K. Matsuura, H. Tsuji, Int. J. Immunopharmacol. 20 (1998) 295–304.
[6] L.D. Horwitz, Cardiovasc. Drug Rev. 21 (2003) 77–90.
[7] F. Amersi, S.K. Nelson, X.D. Shen, H. Kato, J. Melinek, J.W. Kupiec-Weglinski, L.D. Horwitz, R.W. Busuttil, M.A. Horwitz, Proc. Natl. Acad. Sci. 99 (2002) 8915–8920.
[8] T. Sawada, S. Hashimoto, H. Furukawa, S. Tohma, T. Inoue, K. Ito, Immunopharmacology 35 (1997) 195–202.
[9] A. Kładna, H.Y. Aboul-Enein, I. Kruk, T. Michalska, K. Lichszteld, Luminescence 21 (2006) 90–97.
[10] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Radic. Biol. Med. 26 (1999) 1231–1237.
[11] B.S. Wolfenden, R.L. Willson, *J. Chem. Soc., Perkin Trans. 2* (1982) 805–812.
[12] A.P. Mathews, S. Walker, J. Biol. Chem. 6 (1909) 299–312.
[13] C. Henriquez, C. Aliaga, E. Lissi, In. J. Chem. Kinet. 34 (2002) 659–665.
[14] R.E. Childs, W.G. Bardsley, Biochem. J. 145 (1975) 93–103.
[15] S.L. Scott, W.J. Chen, A. Bakac, J.H. Espenson, J. Phys. Chem. 97 (1993) 6710–6714.
[16] K. Mardia, J. Kent, J. Bibby, Multivariate Analysis; Probability and Mathematical Statistics, Academic Press, 1979.
[17] R. Halko, S. Procházková, L. Okenicová, M. Hutta, P. Božek, Chromatographia 77 (2014) 1019–1025.
[18] M. Gordge, D. Meyer, J. Hothersall, G. Neild, N. Payne, A. Noronha-Dutra, Br. J. Pharmacol. 114 (1995) 1083–1089.
[19] C. Aliaga, E.A. Lissi, Can. J. Chem. 78 (2000) 1052–1059.
[20] R.C. Kapoor, O.P. Kachhwaha, B.P. Sinha, J. Phys. Chem. 73 (1969) 1627–1631.
[21] R. Mishra, R. Banerjee, S. Mukhopadhyay, J. Phys. Org. Chem. 25 (2012) 1193–1197.
[22] O. Warburg, Sakuma, S, Pflugers Arch. Gesamte Physiol. Menschen Tiere 200 (1923) 203–206.
[23] K.A.C. Elliott, Biochem. J. 24 (1930) 310–326.
[24] L. Ehrenberg, M. Harms-Ringdahl, I. Fedorcsák, F. Granath, Acta Chem. Scand. 43 (1989) 177–187.
[25] L. Pecci, G. Montefoschi, G. Musci, D. Cavallini, Amino Acids 13 (1997) 355–367.
[26] V. Vortisch, P. Kroneck, P. Hemmerich, J. Am. Chem. Soc. 98 (1976) 2821–2826.
[27] S.K. Ghosh, S.K. Saha, M.C. Ghosh, R.N. Bose, J.W. Reed, E.S. Gould, Inorg. Chem. 31 (1992) 3358–3362.
[28] S. Mandal, R.N. Bose, J.W. Reed, E.S. Gould, Inorg. Chem. 35 (1996) 3159–3162.
[29] M.R. Ciriolo, A. Desideri, M. Paci, G. Rotilio, J. Biol. Chem. 265 (1990) 11030–11034.
[30] R.C. Smith, V.D. Reed, W.E. Hill, Phosphorus Sulfur Silicon Relat. Elem. 90 (1994) 147–154.
[31] L. Michaelis, J. Biol. Chem. 84 (1929) 777–787.
[32] R.G. Neville, J. Am. Chem. Soc. 79 (1957) 2456–2457.
[33] R. Munday, C.M. Munday, C.C. Winterbourn, Free Radic. Biol. Med. 36 (2004) 757–764.
[34] M. Dixon, H.E. Tunnicliffe, Proc. R. Soc. Lond. B Biol. Sci. 94 (1923) 266–297.
[35] N.J. Miller, C. Rice-Evans, M.J. Davies, V. Gopinathan, A. Milner, Clin. Sci. 84 (1993) 407–412.
[36] D. Huang, B. Ou, R.L. Prior, J. Agric. Food Chem. 53 (2005) 1841–1856.
[37] E.S.G. Barron, R.H. DeMeio, F. Klemperer, J. Biol. Chem. 112 (1936) 625–640.
[38] F. Sahbaz, G. Somer, Food Chem. 47 (1993) 345–349.
[39] R.B. Walker, J.D. Everette, J. Agric. Food Chem. 57 (2009) 1156–1161.
[40] D.P. Naughton, J. Knappitt, K. Fairburn, K. Gaffney, D.R. Blake, M. Grootveld, FEBS Lett. 361 (1995) 167–172.