Scientific Paper

The Influence of Halide and Pseudo-Halide Antioxidants in Fenton-Like Reaction Systems

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Abstract

An application of the *N,N'*-(5-nitro-1,3-phenylene)bisglutaramide (NPG) hydroxylation assay for spectrophotometric determination of the rate of oxidising species generation in Fenton-like systems in the presence of halide and pseudo-halide antioxidants was evaluated. Using ion chromatography it was demonstrated that the concentration of antioxidants did not decrease during the course of the experiment. Although the stoichiometry was not constant, determination of the rate constant of NPG hydroxylation was not significantly affected. Consequently, a reasonably good correlation was obtained between the kinetic data obtained spectrophotometrically by studying the reaction mixtures at 432 nm and the sum of concentrations of *p*- and *o*- hydroxylated NPG derivatives determined chromatographically. This indicates that the less time consuming spectrophotometric *N, N'*-(5-nitro-1,3-phenylene)bisglutaramide hydroxylation assay may be used for facile determination of the rate of oxidising species generation in Fenton-like systems containing various halide and pseudo-halide antioxidants.
Employing the assay, it was demonstrated that the efficiency of the antioxidants is affected by their concentration and decreases in the order $I^- > SCN^- \geq Br^-$. In addition, the effect of the corresponding cations was also studied. It was shown that there is a pronounced effect on the rate of hydroxyl radical production, with quaternary ammonium bromides being more effective than potassium bromide or methyltriphenylphosphonium bromide.

Keywords: fenton reaction, hydroxylation, antioxidant, halide, pseudo-halide

1. Introduction

Fenton chemistry[1-6] is of immense importance in biological systems[7-10], ecology[11,12], food chemistry[13,14] and also material ageing.[15,16] The production of oxidising species, often assumed to be hydroxyl radicals, in Fenton-like systems may lead to extensive unwanted oxidative damage in a variety of systems and antioxidants are often employed to minimize it. Their effectiveness may be studied using aromatic hydroxylation assays. Hydroxyl radicals, produced in Fenton-like reaction systems composed of iron ions and hydrogen peroxide, react with an aromatic radical scavenger and the yield of hydroxylated product is used to assess the kinetics of oxidizing species production. Depending on the substrate (benzoate, salicylate, 4-nitrophenol, phenol) hydroxylation products may be numerous[17] or even remove iron from the reaction mixture by formation of stable coordination compounds (*e.g.* with catechol species). Therefore, a substituted nitrobenzene substrate *N, N'*-(5-nitro-1,3-phenylene)bisglutaramide (NPG, Figure 1), was designed,[18] the two predominant products being *o*- and *p*-hydroxylated derivates (*i.e.* 4- or 6-hydroxy and 2-hydroxy, respectively designated as *o*- and *p*-HNPG). In our previous work, hydroxylated products were characterized regarding respective spectroscopic properties and yields at various pH values of reaction media and temperature.[19] The methodology had been utilized to perform a comparative study of several transition metals in Fenton-like reaction systems at circum-neutral pH[20], for evaluation of antioxidant efficiency of various iron chelators.[21] The assay was also used to evaluate the antioxidant efficiency of various halides and a pseudo-halide in an aqueous reaction system at 40 °C containing copper(II) ions and hydrogen peroxide in phosphate buffer of pH 9.[22] In the present paper, the assay is used to evaluate antioxidant efficiency of various halides and a pseudo-halide in an aqueous reaction system at 70 °C containing Fe(III) ions and hydrogen peroxide in phosphate buffer. Higher temperature was used in comparison to our previous experiments[22], as the reaction between Fe(III) ions and hydrogen peroxide is significantly slower than in the case of Cu(II) ions. Raising the reaction temperature to 70 °C enabled the experiments to be performed within one day.

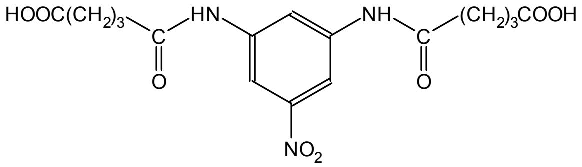

Figure 1. *N, N'*-(5-nitro-1,3-phenylene)bisglutaramide (NPG).

Iodide is an excellent scavenger of hydroxyl radicals.[23] It was demonstrated[24] that $^\bullet I_2^-$ is the product of reaction of hydroxyl radicals with iodide ions in neutral solution (Eqs.1 & 2).

$$I^- + HO^\bullet \rightarrow I^\bullet + HO^- \qquad (1)$$

$$I^\bullet + I^- \rightarrow {}^\bullet I_2^- \qquad (2)$$

Iodide and bromide were shown to scavenge radicals in a wide pH and temperature range, also in alkaline solutions of pH 9 and 10.5.[25-27] The rate constants for reaction of hydroxyl radicals with iodide and thiocyanate ions are nearly diffusion controlled.[27] While bromide ions react with hydroxyl radicals more slowly, reactions between hypobromite or hypobromous acid with hydroxyl radicals proceed at a very fast rate.[28]

Iodide and bromide ions are also known to act as catalytic decomposers of hydrogen peroxide in acidic solutions.[29,30] A cyclic oxidation-reduction is possible at low acidity (10^{-5} mol L^{-1} H_3O^+; Eqs. 3 & 4). Under alkaline conditions (10^{-13} mol L^{-1} H_3O^+), the reaction between hydrogen peroxide and iodine (Eq.5) is very rapid while the reaction between hydrogen peroxide and iodide does not take place.[29]

$$2I^- + 2H^+ + H_2O_2 \rightarrow I_2 + 2H_2O \qquad (3)$$

$$I_2 + 2H_2O_2 \rightarrow 2I^- + 2H^+ + O_2 \qquad (4)$$

$$2HO^- + I_2 + H_2O_2 \rightarrow 2I^- + 2H_2O + O_2 \qquad (5)$$

2. Experimental

The reaction mixture was composed of 0.1 mmol L^{-1} $FeCl_3 \cdot 6H_2O$ (Fluka, Buchs, p.a. quality), 1 mmol L^{-1} NPG, 20 mmol L^{-1} phosphate buffer, 20 mmol L^{-1} H_2O_2 (Fluka, Buchs, non-stabilised). Various halides and a pseudo-halide were added in four different concentrations (0.1, 0.5, 2.5 or 12.5 mmol L^{-1}).

The substrate, *N, N´*-(5-nitro,1,3-phenylene) bisglutaramide (NPG) was synthesized according to the literature.[18] The solution of NPG with pH 9 was prepared in phosphate buffer (composed of Na_3PO_4 and Na_2HPO_4, microselect quality, Fluka, Buchs).

The halides and pseudo-halides studied in this work were potassium iodide (p.a. quality, Carlo Erba, Milano), potassium bromide (p.a. quality, Riedel de Haen, Seelze), potassium thiocyanate (p.a. quality, Merck, Darmstadt), tetrabutylammonium bromide (purum, Fluka, Buchs), tetrabutylammonium iodide (puriss, Fluka, Buchs). Tetrabutylammonium chloride (purum, Fluka, Buchs), benzyltributylammonium bromide, dodecyltrimethylammonium bromide, tetramethylammonium bromide, tetrapropylammonium bromide, tetraethylammonium bromide and methyltriphenylphosphonium bromide were all from Dishman, Ahmedabad, India. The pH of quaternary ammonium and phosphonium bromide solutions was adjusted with NaOH to pH of the reaction mixtures, i.e. 8.8.

The actual pH (8.8±0.1) of the reaction mixtures was determined separately with a combined glass electrode and a pH meter. All solutions were prepared with additionally purified deionised water (MilliQ water purification system, Millipore, Molsheim).

The photometric experiments were conducted in a 3-mL Peltier-thermostated cuvette equipped with a magnetic stirrer and a Pt 1000 resistance thermometer in a Cary 50 Probe spectrophotometer.

On hydroxylation, the substrate yields derivates, which can be determined spectrophotometrically, the mixture exhibiting an absorption maximum at 431 nm. Separation, isolation and UV-VIS absorption properties of the hydroxylated derivatives were already described.[19] The extinction coefficients presented were used for calculation of rate constants from the slopes of absorbance vs. reaction time curves. The concentration ratio of *o*- and *p*-hydroxylated derivatives was determined by HPLC.

For liquid chromatographic analyses, reaction mixtures (5 mL) were prepared as described above and maintained at 70 °C. The reaction was terminated by an addition of 0.5 mL of catalase solution to 0.5 mL of reaction mixture. The catalase solution was prepared by dissolving 4 uL catalase suspension (1300000 umg^{-1}, Merck, Darmstadt) in 100 mL phosphate buffer pH 7.

High-pressure liquid chromatography was performed with a Hewlett-Packard 1100 Series chromatographic system equipped with a diode-array detector. A Hypersil ODS column was used (250x4 mm, 5 um particle size) and 100 uL of sample was injected. Gradient elution of 1.5 mL min^{-1} was used and consisted of 3-8% acetonitrile and 97-92% of phosphate buffer (corresponding mixture of KH_2PO_4/Na_2HPO_4, pH 7, 20 mmol L^{-1}; K_2HPO_4, p.a.; KH_2PO_4, for chromatography, Kemika, Zagreb) in 0-5 min, after which the unreacted NPG was flushed out of the column with a 30% acetonitrile/70% phosphate buffer eluent.

For ion chromatographic determinations, the Fenton-like reaction mixture was prepared as described above. Potassium iodide or potassium bromide was added to the reaction mixture in various concentrations (0.02, 0.05, 0.1 and 0.2 mmol L^{-1}). In some cases the mixtures were prepared without NPG. The reaction mixtures were maintained at 70 °C. The reaction (495 uL of the mixture) was terminated by an addition of 5 uL catalase solution, prepared by dissolving 10 uL of catalase suspension (1300000 umg^{-1}, Merck, Darmstadt) in 5 mL of phosphate buffer pH 7. Ion chromatography was performed with a Merck Hitachi chromatographic system (D-6000 Interface, AS-2000A Autosampler, L-6200A pump) equipped with a UV-VIS detector (L-4250). An Ion Pac AS4A-SC analytical column with an IonPac AG4A-SC guard column were used (Dionex) and 100 uL of sample was injected. Isocratic elution at 2 mL min^{-1} of carbonate buffer (1.8 mmol L^{-1} Na_2CO_3 and 1.7 mmol L^{-1} $NaHCO_3$ in deionised water) was used. Iodide and bromide were detected at 200 nm.

3. Results and Discussion

It may be assumed that NPG reacts with hydroxyl radicals at a virtually diffusion-controlled rate, so that in a system with an excess of NPG, hydroxyl radical production rate can be approximated by the rate of NPG hydroxylation.[18] The yield of hydroxylated product may be determined chromatographically, which is a rather time-consuming approach, or spectrophotometrically. In the latter case, the product concentration ratio (*p*-HNPG/*o*-HNPG*)* and the ratio of molar absorptivites of both derivates have to be determined and taken into account.[19] At experimental conditions used in the present study, the rate constant is obtained in approximately 60-140 min, after which curvature of the otherwise linear plot of absorbance vs. time is observed.

In order to enable rapid and undemanding spectrophotometric determination of relative antioxidant efficiency, the following parameters, which may influence the quality of the obtained results, had to be evaluated:

-change in concentration of the antioxidant during an experiment;

-change in *p*-HNPG/*o*-HNPG product ratio;

-correlation between determination of hydroxylated products using chromatography and VIS spectrometry.

4. Evaluation of the NPG Hydroxylation Assay for Various Halides

4.1. Change in concentration of antioxidant during experiment

During the course of a reaction, concentration of the antioxidant in the reaction system may change, thus affecting interpretation of results.

The concentration of iodide and bromide ions could decrease due to reactions with different components of the reaction mixture. For example, iron(III) ions are known to bind to halide and pseudo-halide ions.[31,32] A series of reactions can take place between iodide and hydrogen peroxide forming various products, such as iodine, hypoiodite, triiodide anion etc.[32]

To assess the error arising from e.g. depletion of antioxidant in the system, the concentration of iodide and bromide anions was followed using ion chromatography. Results indicate that the concentration of iodide does not change significantly during the experimental time used in our study. Furthermore, we may conclude that it is not affected by the presence of NPG in the reaction system (Figure 2). Similar results were obtained with bromide added to the reaction mixture.

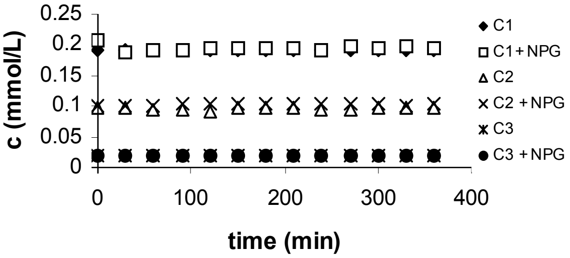

Figure 2. Concentration of iodide during 360 minutes of reaction in Fenton-like reaction system containing potassium iodide, Fe(III) ions, and hydrogen peroxide at pH 9 and 70 ºC (C1-C3) and the same system with addition of NPG (C1-C3 + NPG) determined by ion chromatography. Typical RSD was below 3% (n=5). Potassium iodide was added to the reaction mixtures in following concentrations: $C_1 = 0.02$ mmol L^{-1}, $C_2 = 0.1$ mmol L^{-1} and $C_3 = 0.2$ mmol L^{-1}.

4.2. *p*-HNPG to *o*-HNPG derivate ratio

As demonstrated earlier,[19] extinction coefficients at pH 7 of *o*-HNPG and *p*-HNPG products are different (*o*-HNPG: $\varepsilon_{222}=8710$ L cm^{-1} mol^{-1}, $\varepsilon_{431}=5952$ L cm^{-1} mol^{-1}; and *p*-HNPG $\varepsilon_{222}=9950$ L cm^{-1} mol^{-1}, $\varepsilon_{431}=2296$ L cm^{-1} mol^{-1}). In order to enable the determination of reaction rates using spectrophotometry, it is preferable if the molar ratio of *p*-HNPG to *o*-HNPG derivative is constant during an experiment. In addition, the ratio of the two products should not vary significantly if various antioxidants are studied, in order to enable a direct comparison of their antioxidant efficiency.

To evaluate the possible error arising from the difference in the ratio of hydroxylated products, liquid chromatography was used. A steady decrease of *p*-HNPG to *o*-HNPG derivative ratio was observed during experiments, however, this change is small and has no significant effect on the rate constant. E.g., a decrease of *p*-HNPG/*o*-HNPG product ratio (Figure 3) from 1.79 to 1.73 accounts for a relative systematic error of +0.01% (R.S.D.) in the rate constant.

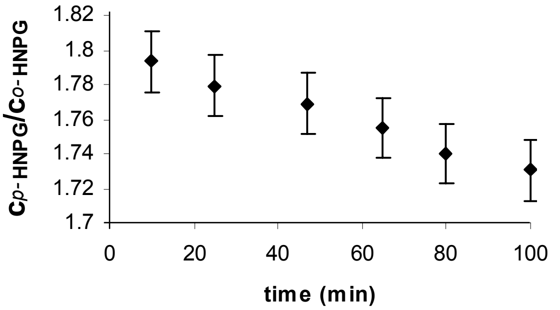

Figure 3. *p*-HNPG/*o*-HNPG product ratio for pseudo-Fenton reaction system containing iron(III) during 100 min of reaction at 70 °C.

Nevertheless, during 100 min of experiment in Fenton-like reaction mixtures containing various halides and a pseudo-halide, six determinations of *p*-HNPG/*o*-HNPG product ratio were performed and average values (Figure 4) were used for calculations of the rate constants from spectrophotometric data.

As evident from the experiment shown in Figure 4, where the concentration of KBr was varied between 0.1 and 12.5 mmol L^{-1}, *p*-HNPG/*o*-HNPG product ratio is not dependent on the antioxidant concentration. However, the average values for the *p*-HNPG/*o*-HNPG product ratio depend on the type of antioxidant used. The effect of anion used is demonstrated by the various ratios obtained with KI, KBr or KSCN. The influence of cation is evident from the *p*-HNPG/*o*-HNPG product ratios obtained when different bromides are added to the system.

However, the observed differences are small and do not prevent the use of spectrophotometry to obtain the kinetic data. The values of *p*-HNPG/*o*-HNPG product ratio are between 1.5-1.9 (16%), which represents a systematic error in determination of the rate constant of +0.5% R.S.D., which is not significant if compared to the experimentally determined R.S.D. for determination of the rate of NPG hydroxylation, which is typically 17%.

4.3. Correlation between determinations of hydroxylated products using chromatography and VIS spectrometry

A comparison between spectrophotometric and chromatographic determination of NPG derivates was necessary in order to establish whether halides or the pseudo-halide interfere with colour formation. E.g., an addition of thiocyanate ion to a solution of iron(III) produces red coloured $[Fe(H_2O)_5(CNS)_2]^{2+}$.[31]

Table 1. The calibration curves (absorbance at 432 nm (AU) vs. summary concentration of hydroxylated NPG derivates (mmol L^{-1})) during 360 min of reaction containing Fe(III) and 0.5 mmol L^{-1} of KBr, KSCN or KI.

	Absorbance (AU) *vs.* concentration (mmol L^{-1})	R^2	*N*
Fe	Y = 0.145x - 0.0076	0.995	7
Fe, KBr	Y = 0.1509x – 0.0093	0.987	7
Fe, KSCN	Y = 0.1459x – 0.0083	0.991	7
Fe, KI	Y = 0.1191x – 0.0014	0.996	6

A reasonably good correlation is obtained between the spectrophotometric measurements of reaction

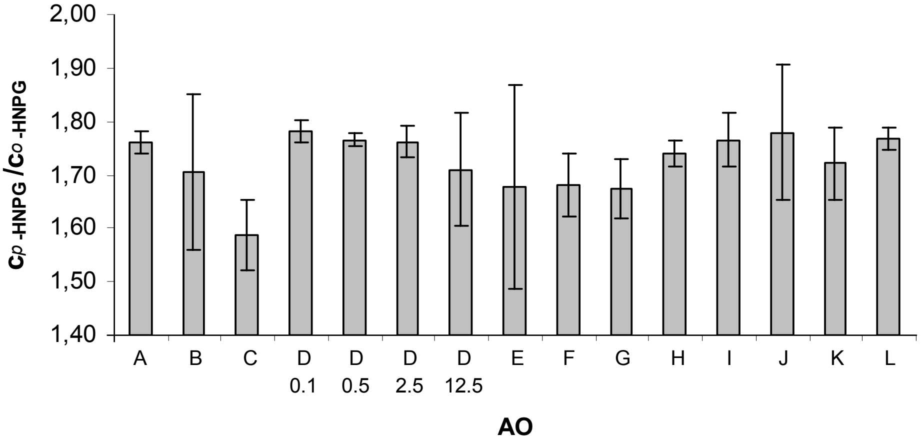

Figure 4. *p*-HNPG/*o*-HNPG product ratio for pseudo-Fenton reaction system containing iron (III) and different antioxidants (AO). Concentration of antioxidant was 0.5 mmol L^{-1}, unless specified differently (0.1; 0.5; 2.5 and 12.5 mmol L^{-1} addition of potassium bromide). A represents sample without addition of antioxidant potassium iodide (B), potassium thiocyanate (C), or potassium bromide (D), tetrabutylammonium iodide (E), tetrabutylammonium chloride (F), tetrabutylammonium bromide (G), tetraethylammonium bromide (H), tetrapropylammonium bromide (I), dodecyltrimethylammonium bromide (J), benzyltributylammonium bromide (K), methyltriphenylphosphonium bromide (L).

mixtures at 432 nm and the sum of concentrations of hydroxylated derivatives as determined by HPLC (Table 1) indicating that the non-demanding spectrophotometric approach may be used for determination of the rate of oxidising species generation in reaction systems containing various halide and pseudo-halide antioxidants.

5. Comparative Study of the Antioxidant Effects

The relative antioxidant effect of various anions is presented in Figure 5, where KI, KBr and KSCN are compared. It is demonstrated that their efficiency decreases in the order $I^- > SCN^- \geq Br^-$. Also, the effect of concentration of antioxidant is shown, higher concentrations resulting in a lower rate constant of NPG hydroxylation.

These results are consistent with the literature. The relative rate constants for reaction of hydroxyl radicals with iodide ions are more than ten times higher when compared to relative rate constants for reaction of hydroxyl radicals with bromide anions.[24-26] For example, relative rate constant for reaction of hydroxyl radicals at pH 9 and at room temperature with iodide anion was established to be $114 \cdot 10^{10}$ mol L^{-1} s^{-1} and for bromide $8.9 \cdot 10^{10}$ mol L^{-1} s^{-1}.[25] Chloride anion, which was also a subject of the same study, had a relative rate constant for reaction of hydroxyl radicals lower than $0.01 \cdot 10^{10}$ mol L^{-1} s^{-1}.

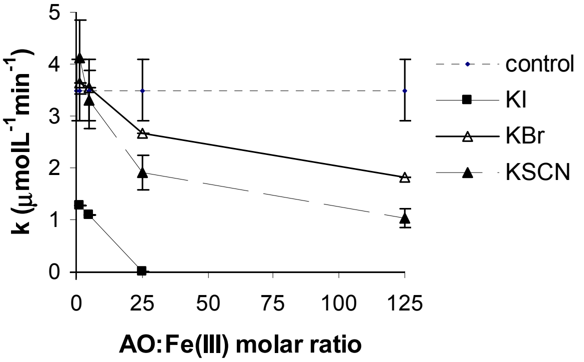

Figure 5. The effect of concentration of halides and a pseudo-halide in Fenton-like reaction mixtures containing Fe(III) ions as catalysts on the rate of NPG hydroxylation. The control sample contained no antioxidant (AO), KI represents addition of potassium iodide, KBr addition of potassium bromide and KSCN addition of potassium thiocyanate. Error bars represent standard deviation for determination of rate constants.

Interestingly, cations have a strong effect on the rate of hydroxyl radical production (Figure 6, Figure 7). The results indicate that the addition of tetrabutylammonium bromide to the reaction mixture decreases the rate of hydroxyl radical production more effectively than potassium bromide (Figure 6).

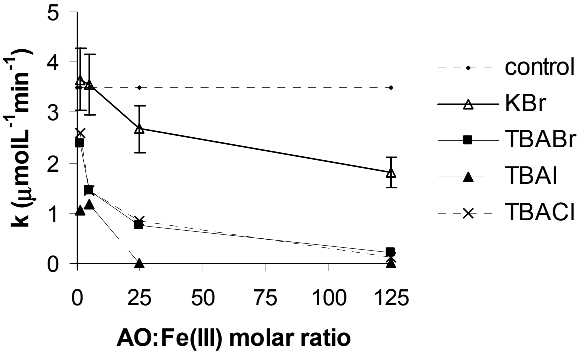

Figure 6. Effect of concentration of halide (AO) in Fenton-like reaction mixture containing Fe(III) ions as catalyst on the rate of NPG hydroxylation. The control sample contained no antioxidant, other samples contained potassium bromide (KBr), tetrabutylammonium bromide (TBABr), tetrabutylammonium iodide (TBAI) and tetrabutylammonium chloride (TBACl). Error bars represent standard deviation for determination of rate constants.

Tetrabutylammonium chloride has a similar effect to tetrabutylammonium bromide, indicating a prevailing influence of the cation on the rate of NPG hydroxylation. The most effective water soluble bromides are the ones containing longer aliphatic chains bound to the quaternary ammonium ion (Figure 7). The results are consistent with studies in pseudo-Fenton reaction systems containing copper ions.[22]

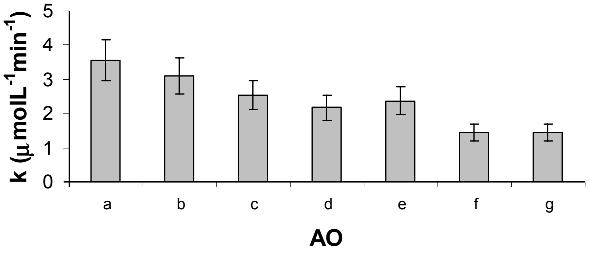

Figure 7. Effect of various bromides (AO) with bromide: Fe(III) molar ratio 5:1 on the rate of hydroxyl radical production in the pseudo-Fenton reaction mixture containing NPG. Sample a contained potassium bromide, b-methyltriphenylphosphonium bromide, c-benzyltributylammonium bromide, d-tetraethylammonium bromide, e-tetrapropylammonium bromide, f-tetrabutylammonium bromide and g-dodecyltrimethylammonium bromide. Error bars represent standard deviation for determination of rate constants.

This observation appears to be contrary to the earlier findings, where it was demonstrated that the rate of reaction of benzoyl peroxide with various iodides in 95% ethanol solution decreases with increasing size of the cations.[33] The difference may be ascribed to the fact that although the rate of decomposition of peroxides may be slower in case of bigger cations, they are more likely to scavenge hydroxyl radicals formed. This may result in lower rates of NPG hydroxylation, as observed in our work.

6. Conclusions

An aromatic substrate, *N, N'*-(5-nitro,1,3-phenylene)bisglutaramide, was used to obtain the rate constants of $HO^\bullet$ radical production in buffered aqueous solutions of H_2O_2 and iron ions in presence of iodide, thiocyanate and bromide antioxidants. Results demonstrate that when the same cation is used, iodide, thiocyanate and bromide exhibit antioxidative behaviour in the order of decreasing efficiency. A strong effect of cation is demonstrated by antioxidative properties of some water-soluble bromides, with the highest antioxidant effectiveness exhibited by bromide with the longest aliphatic chain connected to the quaternary ammonium bromide.

7. Acknowledgements

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Povzetek

Ovrednotena je bila uporaba hidroksilacije z *N, N'*-(5-nitro-1,3-fenilen) bis glutaramidom (NPG) za spektrofotometrično določevanje hitrosti nastanka oksidirajočih zvrsti v Fentonskih oz. njim podobnih sistemih ob prisotnosti halidnih in psevdohalidnih antioksidantov. Z ionsko kromatografijo je bilo dokazano, da se koncentracija antioksidantov med poskusom ne zmanjšuje. Čeprav stehiometrija reakcij ni bila konstantna, to ni vplivalo na določitev konstante reakcijske hitrosti hidroksilacije NPG. Ugotovljena je bila dobra korelacija med kinetičnimi podatki na osnovi spektrofotometričnih meritev reakcijske zmesi pri 432 nm in vsoto kromatografsko določenih koncentracij *p*- in *o*-hidroksiliranih derivatov NPG.

To potrjuje, da lahko za enostavno določevanje hitrosti nastanka oksidirajočih zvrsti v Fentonskih in podobnih sistemih, ki vsebujejo različne halidne in psevdohalidne antioksidante, uporabimo časovno manj zamudno spektrofotometrično metodo na osnovi hidroksilacije *N,N'*-(5-nitro-1,3-fenilen) bis glutaramida.

Z uporabo te metode smo pokazali, da učinkovitost antioksidantov upada v zaporedju $J^- > SCN^- \geq Br^-$ nanjo pa vpliva tudi njihova koncentracija. Poleg tega smo ugotovili očiten vpliv kationov na nastanek hidroksilnih radikalov, pri čemer je kvarterni amonijev bromid učinkovitejši od kalijevega ali metil-trifenilfosfonijevega bromida.