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THE EFFECT OF METAL ION, pH AND TEMPERATURE ON THE YIELD OF OXIDISING SPECIES IN A FENTON-LIKE SYSTEM DETERMINED BY AROMATIC HYDROXYLATION *

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Abstract

The *N,N'*-(5-nitro,1,3-phenylene)bisglutaramide hydroxylation assay for rapid spectrophotometric determination of the rate of oxidising species production in Fenton-like systems has been evaluated and modified for use in different reaction conditions, *i.e.* at different temperatures, pH and transition metal used. The two predominant hydroxylated derivates were isolated and their spectral properties were demonstrated to depend significantly on pH. The ratio of products was found to be constant at different reaction temperatures, but dependent on pH, therefore it has to be determined by high-pressure liquid chromatography to enable us to calculate the apparent activation energies of the oxidising species production, as used for the systems Fe(III)/phosphate buffer/H_2O_2 (~70 kJ/mol) or Cu(II)/phosphate buffer/H_2O_2 (~95 kJ/mol) at pH 6.5-8. The rate of hydroxylation increases with pH in both systems and is ~$5 \cdot 10^3$-$10^4\times$ higher in the system containing Cu(II), probably reflecting its higher rate of reaction with superoxide anion and the higher rate of reaction of Cu(I) with H_2O_2.

* Dedicated to Prof. Dr. Drago Leskovšek at his 80th anniversary.

INTRODUCTION

The importance of Fenton chemistry [1-3] has long been recognised in biological systems [4-7], ecology [8-11], food chemistry [12,13] and material ageing [14-16]. Broadly regarded, a Fenton type reagent can be a mixture composed of a lower valence transition metal [*e.g.* Fe(II), Cu(I), Co(II), Mn(II)] and H_2O_2, an organic hydroperoxide (*e.g.* alkyl hydroperoxide, ROOH) or hypohalous acid (*e.g.* HOCl). Numerous methods have been devised to asses the kinetics of oxidising species production, although its exact identity is elusive and the subject of an ongoing debate on whether a *free* hydroxyl radical or rather a reactive complex iron intermediate, *e.g.* ferryl ion, FeO^{2+} [17-23] is present. While the vast majority of *in vitro* work was done at the physiological pH and at 37 ^{o}C, for purposes of other than biological, other pH values and reaction temperatures are also of interest, although changes in buffer systems may induce changes in kinetics [24,25].

Oxidising species detection methods are versatile and include discoloration of *p*-nitroso-*N*,*N*-dimethylaniline [26], reaction with tryptophan [27], spin trapping [28-32], conversion of dimethylsulfoxide to methanesulfinic acid [33-35], hydroxylation of deoxyguanosine [36], and aromatic hydroxylation [37-42].

While instrumentally relatively non-demanding and rapid, aromatic hydroxylation may provide comparative data on the kinetics of production of the oxidising species. It is usually assumed that the hydroxylation step proceeds at a diffusion controlled rate, therefore the yield of hydroxylated product is used to asses the kinetics of oxidising species production in comparable systems. Besides possible hydroxylation products being numerous [43], the usual aromatic compounds used for the purpose (benzoate, salicylate, 4-nitrophenol, phenol) have another undesirable feature: certain products remove iron from the reaction mixture by formation of stable co-ordination compounds (*e.g.* with catechol species), which may have a pronounced effect on the overall kinetics. Therefore, Singh & Hider [40] designed a substituted nitrobenzene, which through the combined electron-releasing and withdrawing properties of ring substituents provides a rapidly oxidisable substrate *N*,*N*'-(5-nitro,1,3-phenylene)bisglutaramide (NPG), the two

predominant products being *o*- and *p*- hydroxylated derivates (*i.e.* 4- or 6-hydroxy and 2-hydroxy, respectively).

However, the analytical data that the authors provided on the products are not sufficient to justify the use of the method under various reaction conditions without objections. Therefore, our aim was to evaluate the method if it should be used at different reaction temperatures and pH. Finally, the hydroxylation assay was successfully applied to the study of the effect of pH, temperature and transition metal (Fe^{3+}, Cu^{2+}) on the kinetics of oxidising species production.

EXPERIMENTAL

N,*N*'-(5-nitro,1,3-phenylene)bisglutaramide (NPG) was synthesised according to the literature [40]. Its hydroxylation proceeded according to the following procedure: The reaction mixture (5 mL), containing 20 mmol L^{-1} phosphate buffer (KH_2PO_4/Na_2HPO_4; composition according to the desired pH), NPG (1 mmol L^{-1}), metal chloride ($FeCl_3 \times 6H_2O$, Fluka, Buchs; $CuCl_2 \times 2H_2O$, Merck, Darmstadt; 0.1 mmol L^{-1}) and non-stabilised H_2O_2 (20 mmol L^{-1}, Fluka, Buchs) was thermostatted in a 10 mL volumetric flask at the desired temperature in a water-bath. The NPG solution was prepared in phosphate buffer. The actual pH was measured initially and did not change during reaction. The reaction was stopped by an addition of 0.5 mL of catalase solution, prepared by dissolving 0.1 mL catalase suspension (1300000 u mg^{-1}, Merck, Darmstadt) in 25 mL phosphate buffer (0.1 mol L^{-1}, pH 7). The flask was subsequently filled up with the same buffer to the 10 mL mark. All reagents were prepared on a daily basis in MilliQ water, except the peroxide and metal chloride solutions, which were prepared immediately prior to use.

Spectrophotometric measurements were done with a Milton Roy Spectronic 1201 spectrophotometer. 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 (250×4 mm, 5 μm particle size) and 100 μL of sample was injected. Gradient elution of 1.5 mL min^{-1} was used and consisted of 3-7% acetonitrile and 97-93% of phosphate buffer (20 mmol L^{-1}, pH 7) in 7 min, after

which the unreacted NPG was flushed out of the column with a 30% acetonitrile/70% phosphate buffer eluent.

NPG hydroxylation products were isolated by preparative HPLC (ODS Hyperprep, 8 μm). The resulting eluates were acidified with sulphuric acid and extracted with ethylacetate (for HPLC, Carlo Erba, Milano). The solvent was removed and the derivates were dried over P_2O_5. The remaining inorganic impurities (PO_4^{3-}, SO_4^{2-}) were determined using a Merck-Hitachi HPLC ion chromatograph with a Dionex IonPac AS4A-SC 4 mm column, carbonate buffer eluent, and conductometric detection with anion suppressor and H_2SO_4 as regenerant. The unreacted NPG and inorganic impurities amounted to 1% (w/w) of the isolated products.

RESULTS AND DISCUSSION

Evaluation of the NPG hydroxylation assay

The *o*- and *p*-hydroxylated derivates (*o*-HNPG and *p*-HNPG, respectively) exhibit strongly pH-dependent absorption properties in the region between 200 and 600 nm (Figs. 1 & 2). In an assay where various pH values of the reaction mixture are to be used, the final pH prior to spectrophotometric measurements should be adjusted to pH 7, so that comparable results can be obtained. The absorption maxima at 422 nm and 444 nm for *o*-HNPG and *p*-HNPG respectively, achieve the maximum values at pH 7 and do not increase with an even higher pH, therefore the reaction mixture, irrespective of the initial pH, was adjusted to pH 7 with an addition of a more concentrated phosphate buffer (0.1 mol L^{-1}). This pH value coincides with both catalase maximum activity and with chromatographic requirements. With the pure derivates at hand, the molar extinction coefficients of absorbance maxima in this solvent can be determined (*o*-HNPG: ε_{222} = 8710 L cm^{-1} mol^{-1}, ε_{422} = 6020 L cm^{-1} mol^{-1}; and *p*-HNPG: ε_{232} = 10000 L cm^{-1} mol^{-1}; ε_{444} = 2380 L cm^{-1} mol^{-1}). The calibration curves (absorbance [AU] *vs.* concentration [mmol L^{-1}]) are linear in the absorbance region of interest, *i.e.* 0.05-1 AU, for *o*-HNPG ($A = 5.8 \cdot c + 0.0023$, $R^2 = 0.9999$, $N = 10$) and *p*-HNPG ($A = 2.3 \cdot c + 0.0013$, $R^2 = 0.9996$, $N = 10$).

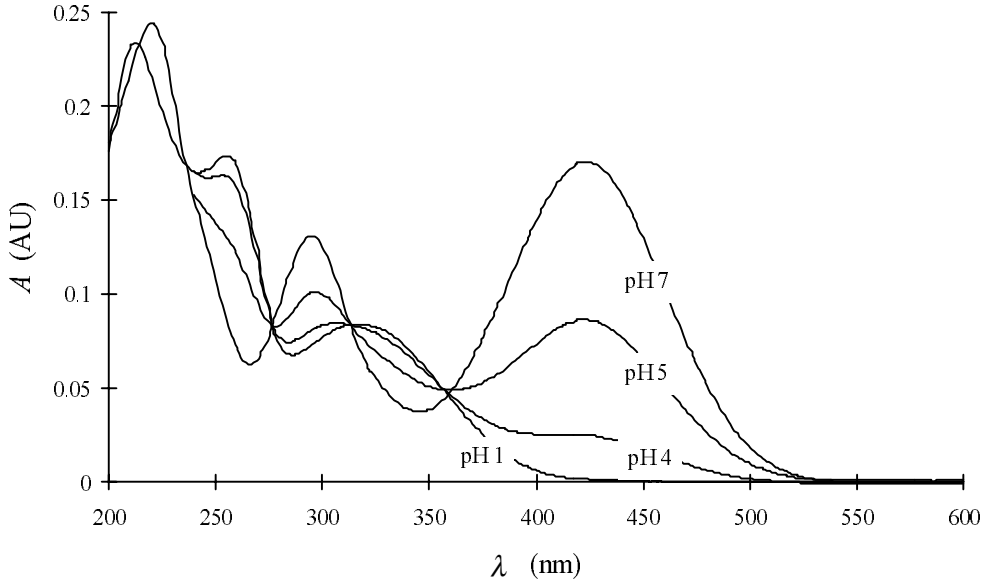

Fig. 1: Absorbance spectra of *o*-HNPG in buffer solutions with pH as indicated, c = 0.014 mmol L^{-1}. The solution exhibits a colour change in the pH region 4-5 from colourless to yellow.

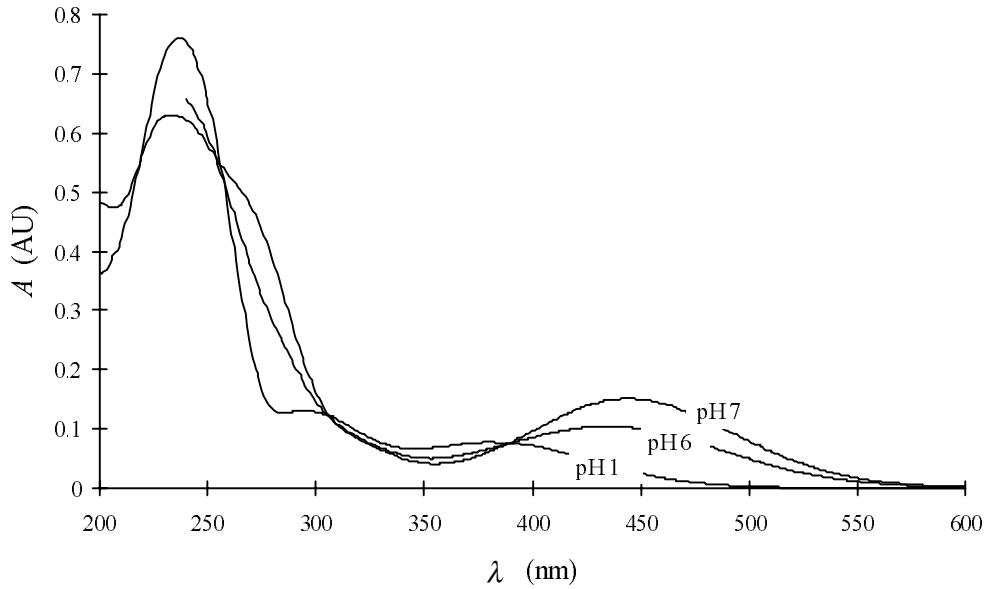

Fig. 2: Absorbance spectra of *p*-HNPG in buffer solutions with pH as indicated, c = 0.031 mmol L^{-1}. The solution exhibits a colour change at pH 6 from yellow to brown.

Detection limits, calculated as threefold standard deviation (N = 20) of the blank sample absorbance at 431 nm, is 1.6 μmol L^{-1} for *o*-HNPG and 4.7 μmol L^{-1} for *p*-HNPG. The absorbance spectrum of the reaction mixture, being a sum of absorbance

spectra of all components, exhibits an absorbance maximum at 431 nm, at which wavelength spectrophotometric measurements can be performed. Although both derivates show maximum absorption in UV region, the addition of catalase does not permit measurements below 300 nm.

Both derivates in phosphate buffer solution (pH 7) show a limited stability at room conditions indicated by a decreasing absorbance amounting to 1% per day. In the temperature interval of interest (20-80 ^{o}C), no difference in absorbance spectra can be observed.

To achieve maximum sensitivity for each derivate, chromatographic detection was performed at 222 nm (*o*-HNPG) and 232 nm (*p*-HNPG). The calibration curves (peak area [mAU·s] *vs.* concentration [mmol L^{-1}]) are linear in the concentration range 0.002-0.1 mmol L^{-1} for both *o*-HNPG ($A_{222} = 30500 \cdot c + 15.8$, $R^2 = 0.9998$, $N = 10$) and *p*-HNPG ($A_{232} = 37450 \cdot c + 12.9$, $R^2 = 0.9997$, $N = 10$). Detection limits, calculated as threefold standard deviation of the background noise, is 0.018 μmol L^{-1} for *o*-HNPG and 0.014 μmol L^{-1} for *p*-HNPG.

A comparison of both analytical methods by linear regression (peak area [mAU·s] *vs.* absorbance [AU]) shows a relatively good agreement for both *o*-HNPG ($Ar = 2950 \cdot A + 27$, $R^2 = 0.999$, $N = 10$) and *p*-HNPG ($Ar = 6800 \cdot A + 29$, $R^2 = 0.999$, $N = 10$) indicating the absence of systematic errors in both methods.

The ratio of products was calculated from the ratio of peak areas taking into account the slopes of chromatographic calibration curves. For the calculation of apparent activation energies, it is advantageous if the product ratio is constant. As can be seen in Fig. 3, this is indeed the case. The ratio, as estimated by Singh and Hider [40] was $c_{p\text{-HNPG}}/c_{o\text{-HNPG}} = 0.17$, while our studies undoubtedly show it to be in the range of 1.6-2.0. This would imply that there is considerable ring substituent effect on the relative yields. On the contrary, the product ratios do show a dependence on the reaction mixture pH (Fig. 4). Besides the acid-base equilibria involved in Fenton chemistry, *e.g.*

$$O_2^{\bullet -} + H^+ \rightleftharpoons HO_2^{\bullet} \ (pK_a = 4.8 \ [44,45]), \qquad (1)$$

the changes in product ratios may also reflect the changes in stability of Fe/phosphate and Fe/NPG complexes, although changes in reaction mechanism cannot be ruled out, *e.g.* changes in electron releasing/withdrawing properties of ring substituents. Phosphate

buffer covers the pH range of interest (pH 6-8) and it was shown to be the least interfering buffer system in a salycilate hydroxylation study [25].

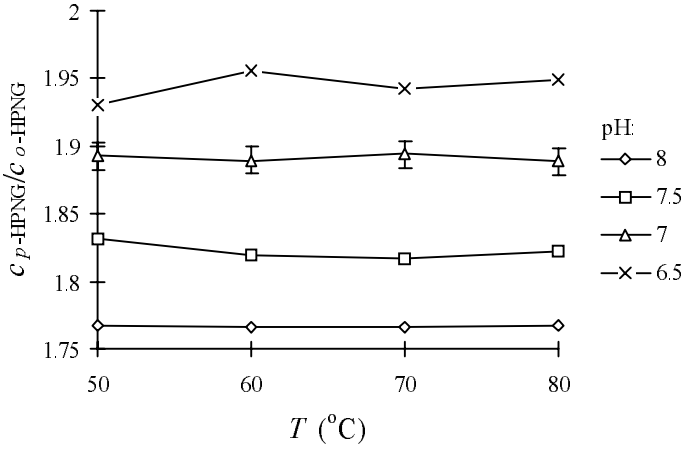

Fig. 3: Dependence of product ratios on reaction temperature at the pH values indicated. The error bars represent standard deviation, SD = 0.01 (N = 3), $FeCl_3$.

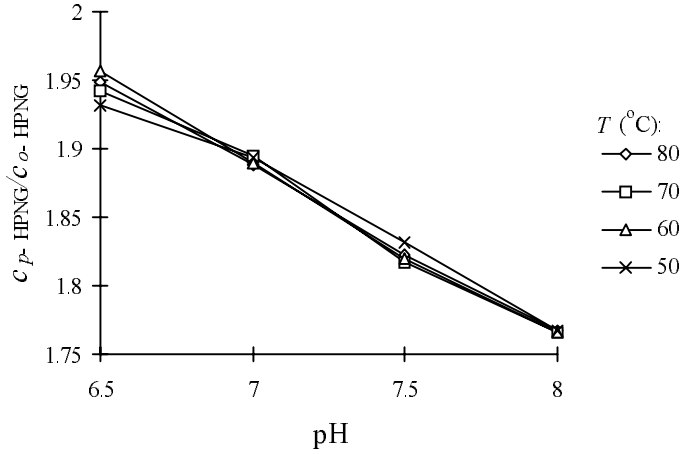

Fig. 4: Dependence of product ratios on reaction mixture pH at the reaction temperatures indicated. $FeCl_3$, SD = 0.01, N = 3.

Study of effects of metal, pH and temperature

Although important in assessing the role of Fenton chemistry in ecological processes and material ageing, its temperature dependence has been studied insufficiently. From the same point of view, it is of considerable interest to study the reaction in aerobic conditions starting with Fe(III) or Cu(II), although for production of $HO^{\bullet}$ in the Fenton reaction, Fe(II) or Cu(I) is needed. A steady-state concentration of Fe(II) is quickly attained and no change in kinetics can be observed in the time-scale in

our study. Furthermore, a system with excess H_2O_2 initially produces its own O_2 atmosphere [19]. It is frequently assumed that reduction of Fe(III) by H_2O_2 proceeds according to the Haber-Weiss scheme later elaborated by Barb *et al.* [46] via the following reaction, promoted by a higher pH:

$$Fe^{3+} + H_2O_2 \rightleftharpoons H^+ + FeOOH^{2+} \longrightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+, \quad (2)$$

The reaction is additionally pH-dependent due to Fe(III) hydrolysis. Besides the reaction:

$$O_2^{\bullet-} + Fe^{3+} \rightleftharpoons O_2 + Fe^{2+} \quad (3)$$

oxidation by Fe(III) of the hydroxycyclohexadienyl radical formed by addition of $HO^\bullet$ to the aromatic ring is another pathway leading to Fe(II) production, also promoted by higher pH:

$$Fe^{3+} + HR^\bullet \longrightarrow Fe^{2+} + R + H^+. \quad (4)$$

If molecular oxygen acts as oxidant in reaction (4), superoxide anion is produced [47]. Fe(II) is oxidised in the classical Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^\bullet + OH^-, \quad (5)$$

a potential source of hydroxyl radicals, though at a higher peroxide concentration, the Haber-Weiss reaction:

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + HO^\bullet + OH^-, \quad (6)$$

may provide the oxidant, as well [3], although it is inhibited by higher pH and its rate is negligible in the absence of metal catalysts [48].

Reduction of Fe(III) (reaction 3) by superoxide anion is a competitive reaction to dismutation of hydroperoxyl radical, the conjugated acid of $O_2^{\bullet-}$ (reaction 1):

$$2HO_2^\bullet \longrightarrow O_2 + H_2O_2, \quad (7)$$

and since it is inhibited by increasing pH (at pH 7.4, only 0.25% of $O_2^{\bullet-}$ exists in the form of $HO_2^\bullet$, and $k_7=10^2$ L mol^{-1} s^{-1} at pH 7; $k_7=5\times10^5$ L mol^{-1} s^{-1} at pH 11 [48]), reaction (3) is promoted. The production of Fe(II) and consequently of hydroxyl radical or ferryl cation thus increases.

Due to Fe(III)-mediated homolytic cleavage of H_2O_2, presence of Fe(II) for hydroxyl radical production may not be strictly necessary [40]. Production of a reactive intermediate Fe(IV) species may be the first step, and by release of another $HO^\bullet$, it converts back to Fe(III), thus playing a catalytic role in decomposition of H_2O_2 and production of $HO^\bullet$:

$$Fe^{3+} + H_2O_2 \longrightarrow FeOH^{3+} + HO^\bullet \longrightarrow Fe^{3+} + 2HO^\bullet. \quad (8)$$

The oxidising species, if not hydroxyl radical, may well be ferryl cation [23]:

$$Fe^{2+} + H_2O_2 \longrightarrow FeO^{2+} + H_2O. \quad (9)$$

The rate of NPG hydroxylation is constant at a given temperature and using the Arrhenius equation, apparent activation energies can be calculated (Fig. 5). Experiments were made at 50-80 °C, and the E_a obtained was used to calculate the reaction rates at 20 °C taking into account the product ratios and molar extinction coefficients for both derivates at 431 nm.

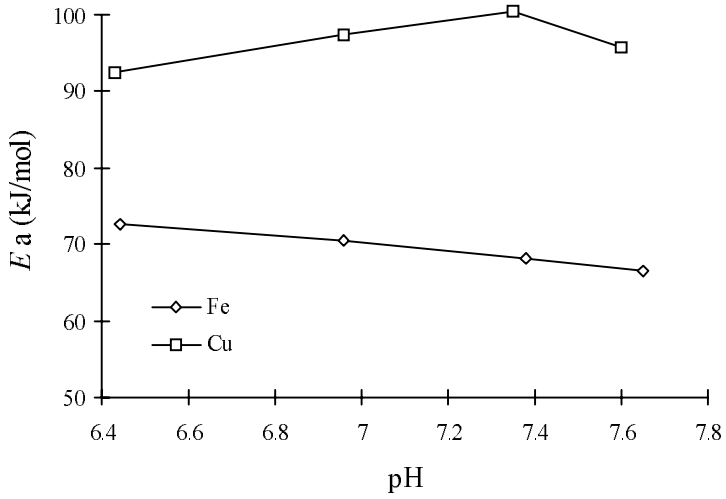

Fig. 5: Effect of pH and metal ion on apparent activation energies of NPG hydroxylation reaction.

The results confirm the estimation by Barb *et al.* [46], that the reactions by Cu(II) ions are more endothermal. On the other hand, complexation with phosphate and/or precipitation of metal ions from the solution, promoted by higher pH, results in a lower activity, since a free metal coordination site is required [49]. Though no precipitates were observed during our experiments, removal of metal from the solution or its inactivation by complexation with phosphate may well result in a lower determined apparent activation energy.

The calculated hydroxylation rates at 20 and 37 °C for the two transition metal cations at pH values 6.4-7.7 are shown in Figs. 6 & 7. The rising trend of oxidising species production with increasing pH mirrors the pH dependence of reactions (2) and (4), though an increased availability of $O_2^{\bullet -}$ for Fe(III) reduction (reaction 3) may play a vital role.

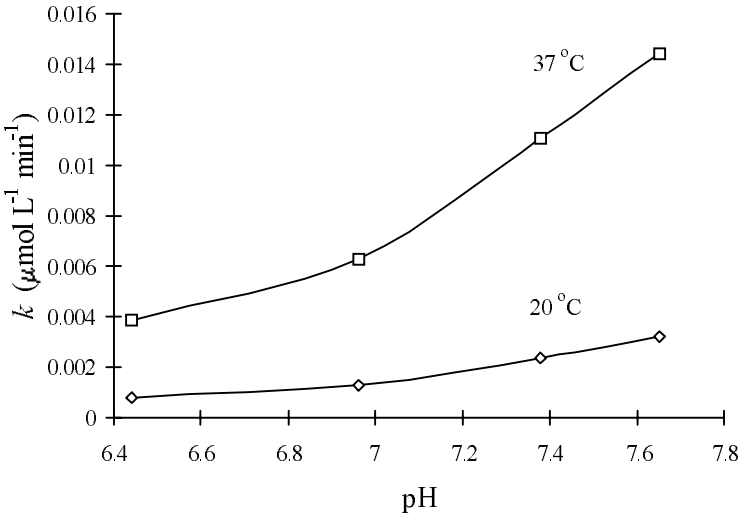

Fig. 6: Rates of NPG hydroxylation with Fe^{3+} as catalyst.

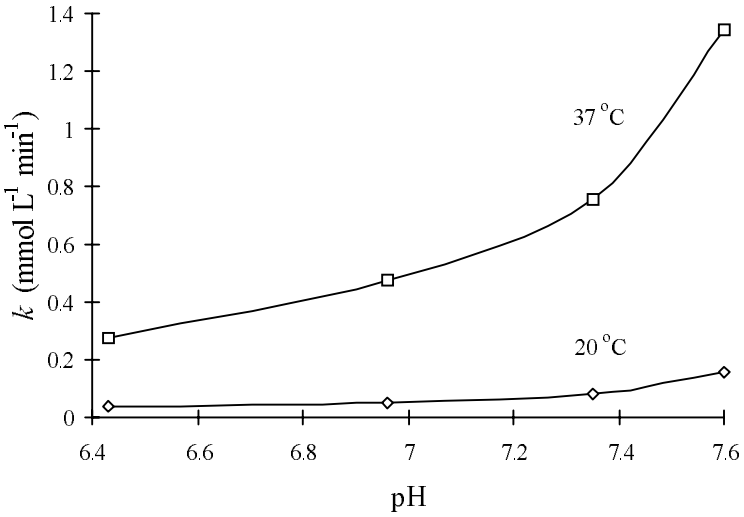

Fig. 7: Rates of NPG hydroxylation with Cu^{2+} as catalyst.

A similar dependence was obtained by other authors [50] during benzoic acid hydroxylation in phosphate buffer of pH 5-9, though at pH > 8 a plateau can be observed. Although suggested by Barb *et al.* [46], the inverse correlation between peroxide decomposition rate and oxonium ion concentration is not valid in this pH region.

As summarised recently [51,52], the reaction (5) proceeds at a ~100× higher rate if instead of Fe(II), Cu(I) is used. Besides, Cu(II) reacts at a ~25× higher rate than Fe(III) in reaction (3) [46]. Both facts may account for the higher rate of NPG hydroxylation when Cu(II) is used.

CONCLUSIONS

N,N'-(5-nitro,1,3-phenylene)bisglutaramide hydroxylation assay was shown to be a rapid spectrophotometric method for detection of oxidising species production in Fenton-like systems. The primary derivates were isolated and their spectroscopic properties evaluated, and the assay procedure changed accordingly. The ratio of derivates was followed and found not to change in the temperature interval 30-80 ^{o}C. On the other hand, an effect of pH was observed. The assay was used for determination of apparent activation energies for the oxidising species production process in the systems Fe(III)/phosphate buffer/H_2O_2 or Cu(II)/phosphate buffer/H_2O_2. While the influence of transition metals on the ageing of materials is well known, and the accelerated ageing experiments are usually run at elevated temperatures (50-90 ^{o}C), the knowledge of temperature dependence of degradation reactions is needed to evaluate the rate of ageing processes at room temperature. Our future work will focus on estimating the correlation between the hydroxylation assay and accelerated ageing experiments in comparable systems to be able to estimate the rates of material degradation at the conditions of use.

REFERENCES

[1] C. Walling, *Acc. Chem. Res.* **1975**, *8*, 125-131.
[2] C. E. Thomas, S. D. Aust, In: J. Miquel, A. T. Quintanilha, H. Weber (Eds.), *Handbook of Free Radicals and Antioxidants in Biomedicine*, Vol. 1, CRC Press, Boca Raton, 1989, pp. 37-48.
[3] P. Wardman, L. P. Candeias, *Radiat. Res.* **1996**, *145*, 523-531.
[4] B. Halliwell, J. M. C. Gutteridge, *Arch. Biochem. Biophys.* **1986**, *246*, 501-514.
[5] A. Singh, In: J. Miquel, A. T. Quintanilha, H. Weber (Eds.), *Handbook of Free Radicals and Antioxidants in Biomedicine*, Vol. 1, CRC Press, Boca Raton, 1989, pp. 17-28.
[6] E. Hideg, C. Spetea, I. Vass, *Biochim. Biophys. Acta* **1994**, *1186* , 143-152.
[7] G. Lubec, *J. Invest. Med.* **1996**, *44*, 324-346.
[8] R. G. Zepp, B. C. Faust, J. Hoigne, *Environ. Sci. Technol.* **1992**, *26*, 313-319.
[9] B. C. Faust, R. G. Zepp, *Environ. Sci. Technol.* **1993**, *27*, 2517-2522.
[10] Y. Sun, J. J. Pignatello, *J. Agric. Food Chem.* **1993**, *41*, 308-312.
[11] Y. Sun, J. J. Pignatello, *J. Agric. Food Chem.* **1992**, *40*, 322-327.
[12] R. Kahl, A. G. Hildebrandt, *Food Chem. Toxic.* **1986**, *24*, 1007-1014.
[13] J. K. Donnely, D. S. Robinson, *Free Rad. Res.* **1994**, *22*, 147-176.
[14] Z. Osawa, *Polym. Degrad. Stab.* **1988**, *20*, 203-236.
[15] A. J. Chirinos-Padrón, P. H. Hernández, F. A. Suárez, *Polym. Degrad. Stab.* **1988** , *20*, 237-255.
[16] J. Kolar, M. Strlič, G. Novak, B. Pihlar, *J. Pulp Pap. Sci.* **1998**, *24*, 89-94.
[17] D. T. Sawyer, C. Kang, A. Llobet, C. Redman, *J. Am. Chem. Soc.* **1993**, *115*, 5817-5818.
[18] J. P. Hage, A. Llobet, D. T. Sawyer, *Bioorg. Med. Chem.* **1995**, *3*, 1383-1388.
[19] D. T. Sawyer, A. Sobkowiak, T. Matsushita, *Acc. Chem. Res.* **1996**, *29*, 409-416.
[20] R. V. Lloyd, P. M. Hanna, R. P. Mason, *Free Rad. Biol. Med.* **1997**, *22*, 885-888.
[21] C. Walling, *Acc. Chem. res.* **1998**, *31*, 155-157.
[22] P. A. MacFaul, D. D. M. Wayner, K. U. Ingold, *Acc. Chem. Res.* **1998**, *31*, 159-162.

[23] M. L. Kremer, *Phys. Chem. Chem. Phys.* **1999**, *1*, 3595-3605.
[24] H. Iwahashi, T. Ishii, R. Sugata, R. Kido, *Arch. Biochem. Biophys.* **1990**, *276*, 242-247.
[25] B. R. V. Dyke, D. A. Clopton, P. Saltman, *Inorg. Chim. Acta* **1996**, *242*, 57-61.
[26] W. Bors, C. Michel, M. Saran, *Eur. J. Biochem.* **1979**, *95*, 621-627.
[27] A. Singh, S. A. Antonsen, G. W. Koroll, W. Kremers, H. Singh, In: W. Bors, M. Saran, D. Tait (Eds.), *Oxygen Radicals in Chemistry and Biology*, Walter de Gruyter, Berlin, 1984, p. 491.
[28] W. Bors, M. Saran, E. Lengfelder, C. Michel, C. Fuchs, *Photochem. Photobiol.* **1978**, *28*, 629.
[29] E. G. Janzen, In: W. A. Pryor (Ed.), *Free Radicals in Biology*, Academic, New York, 1980, p. 116.
[30] G. M. Rosen, E. J. Rauckman, In: L. Packer (Ed.), *Oxygen Radicals in Biological Systems, Methods in Enzymology*, Vol. 105, Academic, New York, 1984, p. 198.
[31] G. Czapski, In: L. Packer (Ed.), *Oxygen Radicals in Biological Systems, Methods in Enzymology*, Vol. 105, Academic, New York, 1984, p. 209.
[32] I. Yamazaki, L. H. Piette, *J. Biol. Chem.* **1990**, *265*, 13589-13594.
[33] C. F. Babbs, M. J. Gale, In: C. Rice-Evans, B. Halliwell (Eds.), *Free Radicals, Methodology and Concepts*, Richelieu Press, London, 1988, p. 91.
[34] R. C. Scaduto Jr., *Free Rad. Biol. Med.* **1997**, *18*, 271-277.
[35] S. Fukui, Y. Hanasaki, S. Ogawa, *J. Chromatogr.* **1993**, *630*, 187-193.
[36] P. Leanderson, C. Tagesson, *Agents Actions* **1992**, *36*, 50-57.
[37] R. A. Floyd, J. J. Watson, P. K. Wong, *J. Biochem. Biophys. Methods* **1984**, *10*, 221.
[38] M. Grootveld, B. Halliwell, *Biochem. J.* **1986**, *237*, 499-504.
[39] B. Halliwell, M. Grootveld, H. Kaur, I. Fagerheim, In: C. Rice-Evans, B. Halliwell (Eds.), *Free Radicals, Methodology and Concepts*, Richelieu Press, London, 1988, pp. 33-59.
[40] S. Singh, R. Hider, In: C. Rice-Evans, B. Halliwell (Eds.), *Free Radicals, Methodology and Concepts*, Richelieu Press, London, 1988, pp. 61-90.
[41] Z. Maskos, J. D. Rush, W. H. Koppenol, *Arch. Biochem. Biophys.* **1992**, *296*, 521-529.
[42] S. Fujimoto, S. Ishimitsu, H. Kanazawa, T. Mizutani, A. Ohara, T. Hayakawa, *Agric. Biol. Chem* **1987**, *51*, 2851-2853.
[43] M. A. Oturan, J. Pinson, *J. Phys. Chem.* **1995**, *99*, 13948-13954.
[44] B. H. J. Bielski, A. O. Allen, *J. Phys. Chem.* **1977**, *81*, 1048-1050.
[45] B. H. J. Bielski, D. E. Cabelli, R. L. Arudi, *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041-1100.
[46] W. G. Barb, J. H. Baxendale, P. George, K. R. Hargrave, *Trans. Faraday Soc.* **1951**, *47*, 591-616.
[47] C. Walling, R. A. Johnson, *J. Am. Chem. Soc.* **1974**, *84*, 363-367.
[48] B. Halliwell, J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 3rd ed., Oxford University Press, Oxford, 1999, p. 62, 131.
[49] E. Graf, J. R. Mahoney, R. G. Bryant, J. W. Eaton, *J. Biol. Chem.* **1984**, *259*, 3620-3624.
[50] J. M. van Zyl, B. J. van der Walt, *Biochem. Pharmac.* **1994**, *48*, 2033-2042.
[51] S. Goldstein, D. Meyerstein, G. Czapski, *Free Rad. Biol. Med.* **1993**, *15*, 435-445.
[52] W. H. Koppenol, In: C. A. Rice-Evans, R. H. Burdon (Eds.), *Free Radical Damage and its Control*, Elsevier, Amsterdam, 1994, pp. 3-24.

Povzetek

Ocenili smo primernost hitre spektrofotometrične metode hidroksilacije *N,N'*-(5-nitro,1,3-fenilen)bisglutaramida za določevanje hitrosti nastajanja oksidirajočih zvrsti v sistemih podobnih Fentonovemu. Postopek smo prilagodili delu v različnih reakcijskih pogojih, t.j. pri različnih temperaturah, pH in z različnimi prehodnimi kovinami. Oba prevladujoča derivata smo izolirali in pokazali, da so njune spektroskopske lastnosti izrazito odvisne od pH. Razmerje produktov je sicer neodvisno od reakcijske temperature, a je odvisno od pH, zato ga je potrebno določiti z visokotlačno tekočinsko kromatografijo, da bi lahko določili navidezne aktivacijske energije procesa nastajanja oksidirajočih zvrsti, kot smo to uporabili za sistema Fe(III)/fosfatni pufer/H_2O_2 (~70 kJ/mol) ali Cu(II)/fosfatni pufer /H_2O_2 (~95 kJ/mol) pri pH 6,5-8. Hitrost hidroksilacije narašča s pH v obeh primerih in je ~5000-10000× višja v sistemu, ki vsebuje Cu(II), kar verjetno odraž večji hitrosti reakcij Cu(II) s superoksidnim anionom in Cu(I) s H_2O_2.