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Sensitive Amperometric Oxalate Biosensor for Food Analysis

Stjepan Milardović[1]*, *Zorana Grabarić*[1] *and Božidar S. Grabarić*[2]

[1] University of Zagreb, Faculty of Chemical Engineering and Technology, Marulićev trg 19, HR-10000 Zagreb, Croatia

[2] University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, HR-10000 Zagreb, Croatia

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Summary

An oxalate biosensor developed by covalent immobilisation of oxalate oxidase enzyme on the top of ruthenium, nickel and iron hexacyanometallate (Ru-FeNiHCM) modified graphite electrode is described. It was noticed that the addition of ruthenium to FeNiHCM layer results in better catalytic efficiency for reduction of hydrogen peroxide at pH=2. To obtain the optimal response of biosensor the influence of several experimental parameters in batch mode, such as working potential, pH and linear concentration range, was examined. Enzymatically produced H_2O_2 was measured at –50 mV *vs.* Hg | Hg_2Cl_2 | 3 M KCl electrode in succinic buffer (pH=3.6). The linear concentration range was up to 100 μM with squared correlation coefficient R^2=0.9985. Sensitivity of biosensor in this range was 43.2 nA/(μM cm^2). Oxalate was determined in real samples of spinach after extraction in water, EDTA and HCl and in samples of different brands of beer. During 11 weeks biosensor sensitivity decreased only 15 % of the initial sensitivity, which means that the biosensor lifetime is much longer than three months.

Key words: amperometric biosensor, graphite electrode modified with nickel, iron, ruthenium, hexacyanometallate, oxalate oxidase, determination of oxalate, food samples

Introduction

Determination of oxalate in different food matrices is of great interest because a high oxalate concentration level in foodstuff may cause formation of insoluble kidney stones as a result of unbalanced nutrition habits. Current analytical methods for determination of oxalate in food industry are time consuming, often require highly trained staff and need relatively expensive instrumentation such as fluorescence spectrometer (*1*), flow injection apparatus with spectrophotometric detection (*2*), ion chromatograph (*3,4*), HPLC (*5*), and system for capillary electrophoresis (*6,7*).

Enzymatic methods are also very suitable for oxalate determination and are used either as two enzymes reactor (*8*) or as enzymatic biosensors (*9–12*). The latter approach is increasingly investigated in the last decade in order to develop accurate enough, stable and reliable biosensor for oxalate determination, possibly without any chemical pre-treatment of samples, especially in the field of clinical chemistry (*e.g.* urine samples), food analysis, *etc*.

Many electrochemical biosensors are designed to detect the concentration of an analyte (*e.g.* oxalate ion) through the electrode reaction of H_2O_2 produced by the enzymatic reaction at the electrode. The main problems to be solved in this type of biosensors are: to lower working potential in order to diminish the possible oxi-

* Corresponding author; Phone: ++ 385 (0)1 4597 286; Fax: ++ 385 (0)1 4597 142; E-mail: Stjepan.Milardovic@pierre.fkit.hr

dation of interferences, to enable easier electron transfer at the electrode and to enable higher sensitivity. The two latter goals are usually achieved through modification of electrode surface with layers having good catalytic properties for H_2O_2 as well as some exchange properties. Efficient modifiers of the electrode surface are different hexacyanometallates such as those of iron(II) and nickel(II) (FeNiHCM), which have been successfully used in biosensors for determination of ethanol in urine (*13*), glucose in blood (*14,15*), catalytic oxidation of thiosulphate (*16*), *etc*.

Incidentally, in most of the papers on biosensors this hexacyanometallate is incorrectly called nickel(II) hexacyanoferrate(II), which will imply the nickel ion as cation and hexacyanoferrate as anion. The crystal structure of this type of hexacyanometallate is known and it was found that in a cubic crystal lattice exists negatively charged ion of the form $[Fe^{3+/2+}M^{z+}(CN)_6]^{(6-z-3/2)-}$, where the electron pair of carbon in CN^- group is bound preferably to iron(III/II) ion, while the electron pair of nitrogen is bound preferably to the other transition metal ion (*17–20*). Such structure of hexacyanometallates enables the movement of counter cations present in solution (*e.g.* K^+, Na^+, *etc.*) through the channel structure of the polymeric hexacyanometallate skeleton in order to neutralise the residual charge, showing an ion exchange property, which can also be used for biosensing (*21*).

In our previous paper the ion-exchange property of FeNiHCM layer, electrocrystalised on nickel electrode by cyclic voltammetry, was used for determination of urea (*21*). Cataldi *et al.* (*22–26*) have recently shown that ruthenium significantly stabilises the hexacyanometallate film grown on a conductive surface (*e.g.* carbon, nickel, *etc*. electrode), giving improved chemical stability (lower solubility), better electrochemical reversibility and longer life time than the same on hexacyanometallate electrode wihout ruthenium. Ruthenium was also used for preparation of screen-printed ruthenium dioxide electrodes for pH measurements (*27*), mixed-valence ruthenium oxide-ruthenium cyanide film electrode for oxidation of aliphatic alcohols (*28*) and ruthenised screen-printed biosensors for measurement of anticholinesterase activity (*29*) and pesticides monitoring (*30*).

In this paper the properties of Ru-FeNiHCM and FeNiHCM modified graphite electrode were investigated and compared to catalytic reduction of hydrogen peroxide with the aim to design sensitive and stable amperometric oxalate biosensor based on oxalate oxidase immobilised on the top of such hexacyanometallate electrode. Some parameters like pH, working potential, linear concentration range, sensitivity and the influence of chemical constituents present in the buffer solutions were also studied to optimise the biosensor performance.

Materials and Methods

Chemicals and solutions

All chemicals used were of analytical reagent grade. Oxalate oxidase was from barley seedlings as lyophilised powder (0.71 U/mg), bovine serum albumin (BSA) and glutaraldehyde (GLA, mass fraction 25 %) were from Sigma (USA). Potassium hexacyanoferrate(III) trihydrate, succinic acid, oxalic acid dihydrate, hydrogen peroxide, hydrochloric acid, potassium chloride, disodium salt dihydrate of ethylenediaminetetraacetic acid (EDTA), nickel(II) chloride and sodium hydroxide were from Kemika (Zagreb, Croatia).

The supporting electrolyte for oxalate measurements contained succinic buffer pH=3.6 or 3.8 and 0.1 M KCl.

The plating solution used for preparation of hexacyanometallate of iron(II) and nickel(II) film (FeNiHCM) contained 0.6 M KCl, 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $NiCl_2$. The pH of this solution was 2, adjusted with 1 M HCl.

Deionised water used throughout all the experiments was purified using a Millipore-MilliQ system.

Apparatus

All electrochemical experiments were performed using a Polarographic Analyser (EG&G PAR, USA) Model 264A interfaced to a personal computer for data acquisition and data handling. Three-electrode electrochemical cell was used having the electrochemical biosensor as a working electrode, $Hg \mid Hg_2Cl_2 \mid 3$ M KCl as a reference electrode and a graphite rod as an auxiliary electrode. All potentials referenced in this work were measured against calomel (3 M KCl) reference electrode at 25 °C in 10 mL electrochemical cell, unless otherwise stated. Samples were stirred using an electromagnetic bar stirrer.

Electrode preparation

Graphite rod (spectroscopic purity, 4 mm in diameter and 5 mm long) was inserted into a glass body and sealed with epoxy resin. Formed graphite disc was thoroughly rinsed in acetone, deionised water and finally air dried. A layer of hexacyanometallate of iron(II) and nickel(II) (FeNiHCM) was formed on the surface of the disk by cycling the potential (starting in anodic direction) between –0.1 and 0.9 V at a scan rate of 100 mV/s for 20 minutes in nickel plating solution containing 0.6 M KCl, 2.5 mM $K_3Fe(CN)_6$, 2.5 mM $NiCl_2$ and HCl (to adjust the pH of the solution to 2). Subsequently, another 20 min of potential cycling between –0.1 and 0.9 V at 50 mV/s was done in the same plating electrolyte, but with addition of 1 mM $RuCl_3$. The resulting layer of hexacyanometallate of iron(II) and nickel(II) modified with ruthenium (Ru-FeNiHCM) was finally washed, air dried and an oxalate oxidase enzyme layer was immobilised on it.

Immobilisation of oxalate oxidase was performed by well-known glutaraldehyde bovine serum albumin cross-linking procedure. In a small volume vessel, approximately 1 mg of BSA (w=10 %) was weighted and dissolved in 10 μL of 0.05 M succinic buffer (pH=3.8). The mass of 0.2 mg (0.142 U) of oxalate oxidase was added to this solution. After 30 min this albumin solution was well mixed with equal volume of glutaraldehyde solution (w=2.5 %) and 5 μL of this mixture was deposited on the top of electrode and left to dry for 20 minutes at room temperature in order to allow the formation of a gel. The prepared enzyme electrode was conditioned in succinic buffer solution (pH=3.8) at 6 °C overnight.

Food samples preparation

Three portions of spinach leaves (approximately 80 g) were minced (blended) in a food blender. All three portions were extracted under reflux for 30 min. The first portion was extracted in distilled water, the second in 0.1 M HCl solution and the third portion in 5.4 mM EDTA solution. The volume of extraction solutions was 200 mL for each portion. After extraction each of the three portions of sample solution was decanted, centrifuged and filtered through cellulose nitrate filter (Sartorius, Germany) with pore size 0.45 μm. For oxalate determination aliquots of 20 μL were taken for amperometric measurements.

Samples of beer were used without any pre-treatment. Aliquots of 200 μL of Stella Artois and Ožujsko (Zagrebačka pivovara, Zagreb, Croatia), Karlovačko (Karlovačka pivovara, Karlovac, Croatia), Zlatorog (Pivovarna Laško, Laško, Slovenia) and 250 μL of Tuborg (Panonska pivovara, Koprivnica, Croatia), Heineken (Amsterdam, Holland) and Gässer (Brauerei Gäss, Leoben, Austria) were taken for analysis.

Results

To study the properties of modified graphite electrode several electrodes have been prepared by electrochemical deposition of two types of layers on the surface of electrode:

a) layer of hexacyanometallate of iron(II) and nickel(II) (FeNiHCM) and

b) the same layer modified with ruthenium (Ru-FeNiHCM).

In Fig. 1 first six cyclic voltammograms recorded in nickel plating solution by scanning the potential (starting in anodic direction) between –0.1 and 0.8 V at scan rate 100 mV/s are shown. The current increase in consecutive voltammograms indicates the FeNiHCM layer formation.

After formation of FeNiHCM layer, ruthenium solution was added to the nickel plating solution, and for another 20 min the potential was cycled between –0.1 and 0.8 V at scan rate of 50 mV/s to allow the formation of ruthenium modified FeNiHCM layer. From cyclic voltammograms of finally prepared electrodes, FeNiHCM and Ru-FeNiHCM modified carbon electrode, it can be noticed that cathodic and anodic peak potentials, corresponding to the redox couple $[Fe(II/III)Ni(II)(CN)_6]^{2-/1-}$ are at approximately E_k=555 mV and E_A=635 mV for FeNiHCM electrode, and at E_k=570 mV and E_A=628 mV for Ru-FeNiHCM electrode. The decrease of peak-to-peak separation from 80 mV to 58 mV, together with the decrease of half current peak width, indicates that the voltammetric response of Ru-FeNiHCM electrode shows more reversible electrochemical behaviour and faster electron transfer through the surface layer of the modified electrode. Therefore, compared with FeNiHCM electrode without the inclusion of ruthenium in the surface crystal structure, a more stable and better pronounced catalytic properties of Ru-FeNiHCM electrode would be expected.

Catalytic properties of Ru-FeNiHCM graphite electrode compared to bare unmodified graphite electrode are shown in Fig. 2. Cyclic voltammograms were recorded in 0.6 M KCl at pH=2 and scan rate of 50 mV/s with addition of hydrogen peroxide ($c_{H_2O_2}$=12 mM). From Fig. 2 it is obvious that on graphite electrode, in the potential range between –200 and 800 mV, reduction or oxidation of hydrogen peroxide does not occur. This is in agreement with the results published by Wang *et al.* (*31*) where it was shown that at carbon electrode the oxidation of H_2O_2 starts at +800, while the reduction starts at –300 mV, which is –250 mV more negative than the working potential used in this work. On Ru-FeNiHCM graphite electrode, due to the catalytic effect of hexa-

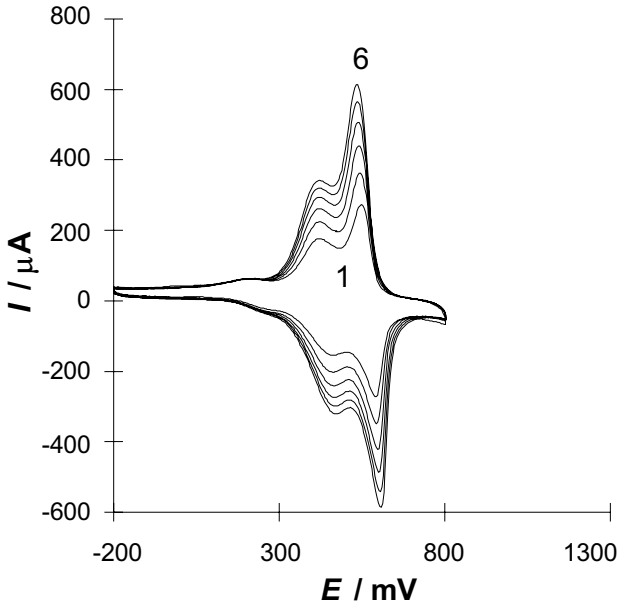

Fig. 1. Cyclic voltammograms of FeNiHCM film formation on graphite electrode; supporting electrolyte: 0.6 M KCl at pH=2 (adjusted with HCl), 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $NiCl_2$; scan rate 100 mV/s

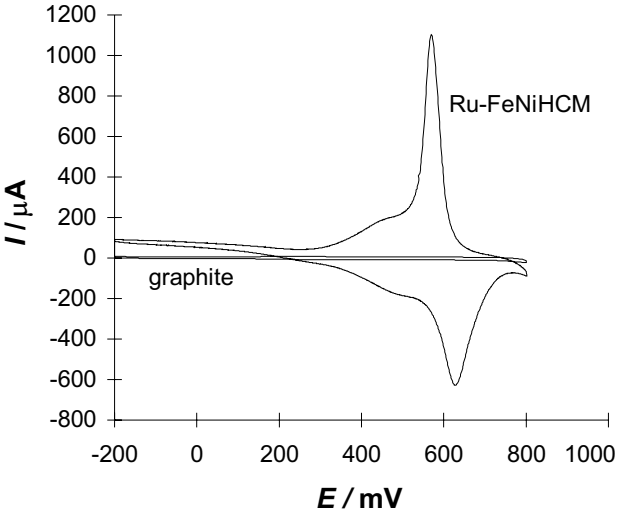

Fig. 2. Cyclic voltammogram of graphite electrode and Ru-FeNiHCM modified graphite electrode in supporting electrolyte: 0.6 M KCl at pH=2, after addition of hydrogen peroxide (c =12 mM); scan rate 50 mV/s

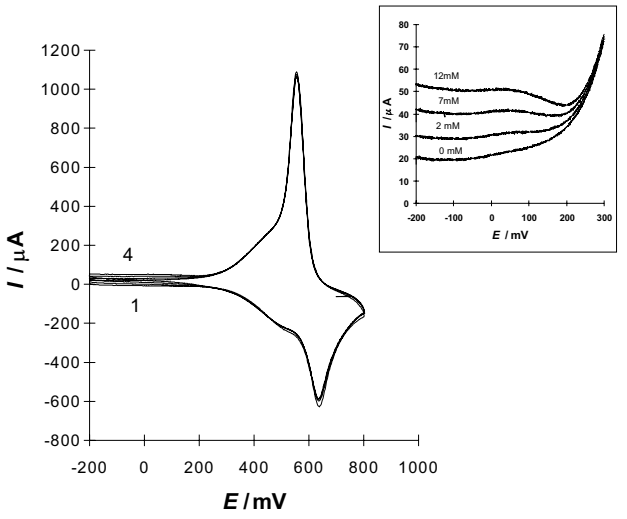

Fig. 3. Catalytic effect of FeNiHCM layer deposited on graphite electrode on reduction of hydrogen peroxide; $c(H_2O_2)$: (1) 0 mM; (2) 2 mM; (3) 7 mM; (4) 12 mM; supporting electrolyte: 0.6 M KCl at pH=2; scan rate 50 mV/s

cyanometallate layer, an increase of current in the potential range between 200 and –200 mV is visible.

In Fig. 2 this effect seems smaller than it really is, due to unfavourable current scale which, at the same time, shows the undergoing redox reaction in the potential range between 400 and 800 mV.

Therefore, in Figs. 3 and 4 a full scale cyclic voltammograms on a FeNiHCM and Ru-FeNiHCM modified graphite electrodes are shown, respectively, for reduction of hydrogen peroxide with the same experimental conditions as in Fig. 2, together with a magnified catalytic response for the reduction of hydrogen peroxide obtained in the favourable range of working potentials between –200 and 200 mV. For both electrodes the increase of current is proportional to the concentrations of hydrogen peroxide (c = 0, 2, 7, and 12 mM), although better linearity and greater sensitivity is obtained using Ru-FeNiHCM modified graphite electrode (Fig. 4). To assure that higher currents obtained for the reduction of hydrogen peroxide in the potential range between –200 and 200 mV at Ru-FeNiHCM modified graphite electrode are not the consequence of larger active surface area of the electrode, the cathodic and anodic peak areas of cyclic voltammograms obtained for both electrodes in the region between 400 and 800 mV are calculated by integration. The calculated cathodic peak areas were 2.36 and 1.93 mC, while anodic peak areas were 2.41 and 2.04 mC for FeNiHCM and Ru-FeNiHCM modified electrodes, respectively. This means that even at lower active surface area of the Ru-FeNiHCM modified electrode higher current response than at FeNiHCM modified electrode is obtained, obviously showing more pronounced catalytic effect of the ruthenium modified electrode. Cathodic and anodic peak areas of FeNiHCM electrode are approximately 20 % higher than those of Ru-FeNiHCM electrode, but the corresponding catalytic current is two times lower compared to the catalytic current with ruthenium modified electrode for the same concentration of hydrogen peroxide. This unambiguously proves that

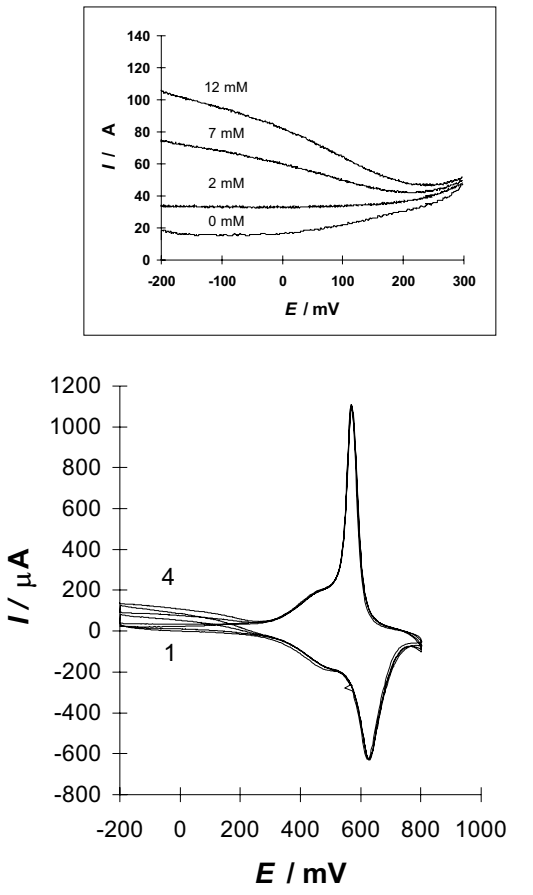

Fig. 4. Catalytic effect of Ru-FeNiHCM layer deposited on graphite electrode on reduction of hydrogen peroxide; $c(H_2O_2)$: (1) 0 mM; (2) 2 mM; (3) 7 mM; (4) 12 mM; supporting electrolyte: 0.6 M KCl at pH=2; scan rate 50 mV/s

the addition of ruthenium enhances the catalytic properties of the electrode and its sensitivity.

The response of Ru-FeNiHCM modified graphite electrode was tested in wide concentration range, between 10^{-1} and 10^{-6} M of hydrogen peroxide (Fig. 5). Linear concentration range was obtained between $0.4 \cdot 10^{-3}$ M and $70 \cdot 10^{-3}$ M of hydrogen peroxide with good correlation coefficient, $R^2 = 0.9983$, and corresponding linear regression equation:

$$\log (I/\mu A) = (72.5 \pm 0.6)\ 10^{-2} \log (c/M) + (-2.62 \pm 0.01).$$

High sensitivity and relatively wide linear concentration range obtained for hydrogen peroxide reduction at Ru-FeNiHCM graphite electrode was the reason why this electrode was used for further immobilisation of the enzyme oxalate oxidase in order to prepare the oxalate biosensor with enhanced sensitivity.

Oxalate oxidase enzyme in the presence of oxygen decomposes the oxalate to carbon dioxide and hydrogen peroxide. The current response of biosensor with immobilised enzyme oxalate oxidase is proportional to the hydrogen peroxide concentration, and thus to the concentration of oxalate according to the Eqs. /1/ and /2/:

$$HOOC\text{-}COOH + Ox_{ox}(FAD) \rightarrow 2CO_2 + Ox_{ox}(FADH_2) \quad /1/$$

$$Ox_{ox}(FADH_2) + O_2 \rightarrow Ox_{ox}(FAD) + H_2O_2 \quad /2/$$

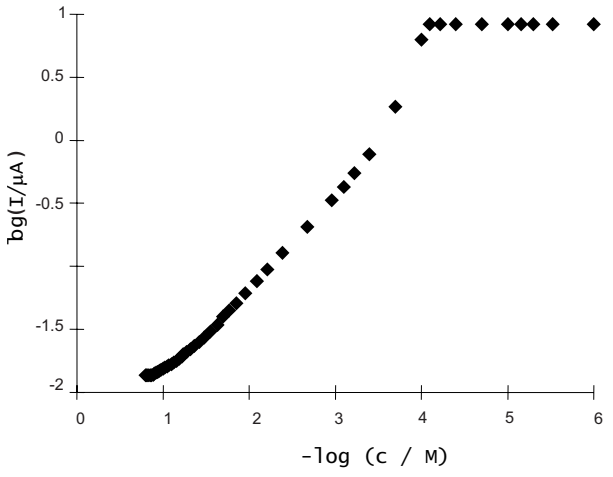

Fig. 5. Calibration diagram for determination of hydrogen peroxide with Ru-FeNiHCM modified electrode in supporting electrolyte 0.6 M KCl at pH=2; working potential was –50 mV. Linear concentration range was between 0.4 and 70 mM of hydrogen peroxide

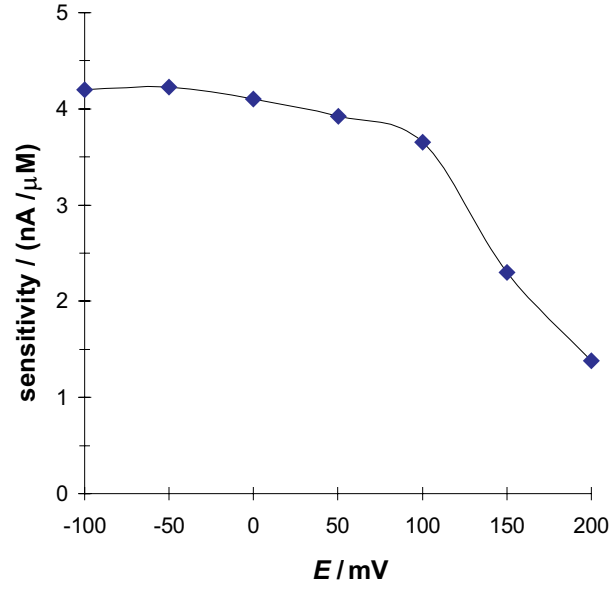

Fig. 6. Sensitivity of oxalate biosensor at different working potentials; supporting electrolyte: succinic buffer pH=3.8, 0.1 M KCl; each point represents the biosensor response after addition of 50 μM of oxalic acid

From the manufacturer literature data (*32*) it is known that the best activity of oxalate oxidase is obtained in pH region between 3.6 and 3.8 in succinate buffer. Succinate buffer, as well as EDTA, acts as the activator of oxalate oxidase activity. The most common inhibitors of oxalate oxidase activity are divalent metal ions (Ca^{2+}, Cu^{2+}, Ba^{2+}, Mn^{2+} and Fe^{2+}), some buffers containing anions such as phosphate, citrate, maleate or acetate, and salt solutions, especially NaCl. Thus supporting electrolyte used in this work was succinic buffer with EDTA and KCl. The addition of potassium chloride as constituent of supporting electrolyte was necessary to enable the proper work of the Ru-FeNiHCM electrode, according to Eq. /3/:

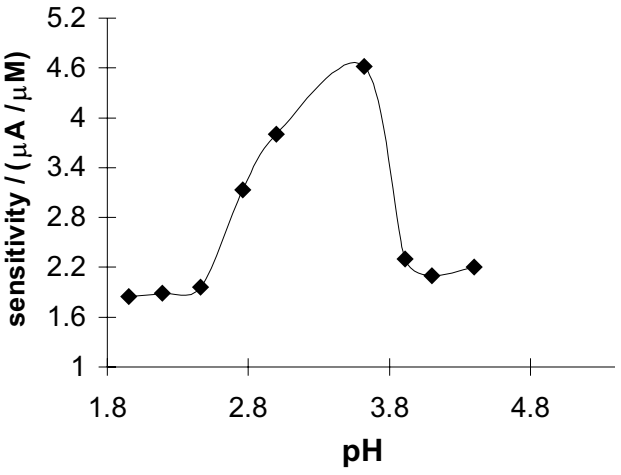

Fig. 7. Sensitivity of oxalate biosensor at different pH; supporting electrolyte: succinic buffer (pH values between 1.9 and 4.6 were changed with addition of HCl or NaOH) and 0.1 M KCl; each point belongs to biosensor response after addition of 70 μM of oxalic acid. Working potential was –50 mV

$$2\ K_2[Fe^{2+}Ni(CN)_6] + H_2O_2 + 2\ H^+ \rightarrow 2\ K[Fe^{3+}Ni(CN)_6] + 2\ H_2O + 2\ K^+ \qquad /3/$$

To determine the optimal working potential of biosensor, current responses obtained at different potentials (from –100 to 200 mV) were measured for 50 μM of oxalic acid in supporting electrolyte containing succinate buffer pH=3.8 and 0.1 M KCl. The results are presented in Fig. 6 showing that at –100, –50 and 0 mV the highest sensitivities of the biosensor were obtained. Potential of –50 mV was chosen as working potential for all further measurements, because at more negative potentials (–100 mV) the response of biosensor was more noisy, while at more positive potentials oxidation of some interferences present in the real samples might occur.

Influence of pH on oxalate biosensor response was checked in this work and the results are presented in Fig. 7. The sensor response was measured in the pH range between 1.9 and 4.6 in supporting succinate buffer electrolyte containing 0.1 M KCl and 70 μM of oxalic acid. The results in Fig. 7 prove that the best sensitivity of biosensor is obtained at pH=3.6, as it was expected according to the specification of enzyme oxalate oxidase activity (*32*). All further measurements were performed at this pH.

Calibration curve obtained for solutions of oxalic acid with Ru-FeNiHCM biosensor is shown in Fig. 8. Measurements at –50 mV were made in supporting electrolyte of pH=3.6 and 0.1 M KCl. In the concentration range up to 100 μM sensor shows good linearity with squared correlation coefficient R^2=0.9974. The average sensitivity (N=9) in the measuring range is 43.2 nA/(μM cm^2). After 11 weeks this sensitivity decreased 15 % of the initial sensitivity indicating that the electrode half lifetime is much longer than three months.

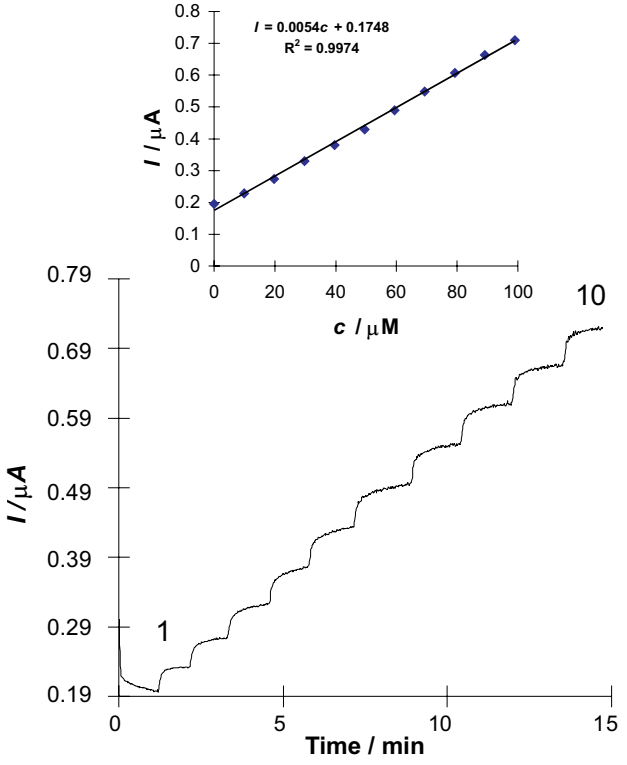

Fig. 8. Calibration diagram for determination of oxalate with Ru-FeNiHCM based oxalate oxidase biosensor; supporting electrolyte was succinic buffer (pH=3.6) with 0.1 M KCl and 5.6 mM EDTA. Ten successive additions of 10 μM of oxalic acid were added. Working potential was –50 mV. Linear concentration range was up to 0.1 mM of oxalate

In order to investigate the biosensor fabrication reproducibility, six biosensors were prepared and the current response reproducibility for the same oxalate concentration varied within few percents.

Wide oxalate concentration measuring range and high sensitivity of biosensor enable the oxalate determination in different matrices.

The influence of some possible interferents on oxalate was investigated. It was found that ascorbic acid (2 mM) lowers significantly the chronoamperometric signal obtained for aliquots of oxalic acid, while HCl (> 0.01 M) increases the background current. Moreover, the presence of any interference changes the shape of oxalate signal, therefore an aliquot of standard oxalate solution is added as a control signal, before sample addition. Constancy or change of the signal is used as an additional criterion to check the possible presence of any interference, because using this procedure immediate presence of the interference is visible from the current response.

In real samples ascorbic acid is present in spinach. However, oxalate from spinach is extracted 30 min at 100 °C with water or 0.1 M HCl or 5.4 mM EDTA, and under these extraction conditions all ascorbic acid decomposes.

In the continuation of this investigation, the proposed biosensor was applied for oxalate determination in real food sample of spinach and different beer. Oxalate was determined in spinach leaves, after extraction in either water or EDTA or HCl solution, using standard addition method at working potential of –50 mV and pH=3.6. An example of the chronoamperogram for oxalate determination in spinach leaves extracted with HCl is presented in Fig. 9. First step on the chronoamperogram is the biosensor response after addition of 20 μM of standard oxalic acid solution, and second and third steps are the responses of 20 μL addition of spinach sample. Next steps (4–6) are the responses of 20 μM of standard oxalic acid solution, respectively. Each sample was analysed in the same manner 3 times. Linear regression lines for all three extractions are presented in Table 1. The concentrations of oxalate in spinach samples after extraction is given in measuring cell, in extracting solution and as the mass fraction in samples, in Table 2. The highest concentration of oxalate was obtained for the sample extracted in HCl, while the concentration in samples extracted with water and EDTA is lower, because only oxalate in soluble form was determined in this two extracts. The difference between the values obtained for the oxalate after extraction in water (free oxalate) and extraction in HCl (total oxalate) gives values for bounded oxalate.

Oxalate in different brands of beer was determined directly in beer samples without any sample pre-treat-

Table 1. Regression lines for the calibration diagrams obtained with oxalate biosensor for determination of oxalate with internal standard addition method in samples of spinach leaves after extraction in water (I_1), EDTA (I_2), and HCl (I_3) and in different samples of beer

Sample	Regression equations	R^2
Spinach	$I_1/\mu A = (76.5 \pm 1.4)\ 10^{-3} + (2.7 \pm 0.4)\ 10^{-3} c/\mu M$	0.9996
	$I_2/\mu A = (73.8 \pm 3.8)\ 10^{-3} + (2.5 \pm 1.0)\ 10^{-3}\ c/\mu M$	0.9966
	$I_3/\mu A = (82.0 \pm 0.1)\ 10^{-3} + (2.5 \pm 0.1)\ 10^{-3}\ c/\mu M$	0.9999
Stella	$I/\mu A = (15.6 \pm 2.6)\ 10^{-3} + (5.9 \pm 0.09)\ 10^{-3}\ c/\mu M$	0.9995
Karlovačko	$I/\mu A = (19.1 \pm 3.9)\ 10^{-3} + (6.4 \pm 0.1)\ 10^{-3}\ c/\mu M$	0.9990
Ožujsko	$I/\mu A = (17.2 \pm 2.0)\ 10^{-3} + (6.2 \pm 0.07)\ 10^{-3}\ c/\mu M$	0.9997
Zlatorog	$I/\mu A = (12.3 \pm 4.2)\ 10^{-3} + (6.5 \pm 0.2)\ 10^{-3}\ c/\mu M$	0.9989
Heineken	$I/\mu A = (1.7 \pm 5.1)\ 10^{-3} + (6.6 \pm 0.3)\ 10^{-3}\ c/\mu M$	0.9983
Gässer	$I/\mu A = (1.0 \pm 0.2)\ 10^{-3} + (6.6 \pm 3.8)\ 10^{-3}\ c/\mu M$	0.9971
Tuborg	–	–

Table 2. Mass fraction (w) of oxalate determined with modified biosensor in samples of spinach leaves after extraction in water, EDTA, and HCl and different brands of beer

Sample	Extraction	$c(\text{ox})_{\text{cell}}$	$c(\text{ox})_{\text{sample}}$	w
		μM	mM	$g \cdot kg^{-1}$
Spinach	water	28.33	14.19	3.27
	EDTA	29.52	14.78	3.38
	HCl	32.84	16.45	3.71
Beer				$\gamma/mg \cdot L^{-1}$
Stella	–	2.67	0.136	12.3
Karlovačko	–	2.97	0.152	13.7
Ožujsko	–	2.90	0.148	13.3
Zlatorog	–	1.87	0.096	8.6
Heineken	–	0.27	0.011	1.0
Gässer	–	0.15	0.006	0.6
Tuborg	–	–	–	< 0.5

ment and the results are given in Table 2. An example of chronoamperogram of oxalate determination in beer Stella Artois, with oxalate biosensor described in this paper, is given in Fig 10. In this figure step 1 indicates the biosensor response to the addition of 20 μM of oxalic acid prior to any measurement. Subsequently, an aliquot (V=200 μL) of beer (step 2) and three standard additions of oxalic acid solution (c=15 μM) were added (steps 3–5). For each sample three biosensor responses, like the example given in Fig. 10, were recorded. The linear regression lines for six different brands of beer were calculated and presented in Table 1. The oxalate mass concentrations found in these beers were between 0.6 and 13.7 mg/L (Table 2). Four beers have oxalate concentrations between 8.6 and 13.7 mg/L, two brands of beer (Heineken and Gässer) have very low oxalate concentration (0.6 and 1.0 mg/L), while the concentration of oxalate in Tuborg is below the detection limit of the biosensor.

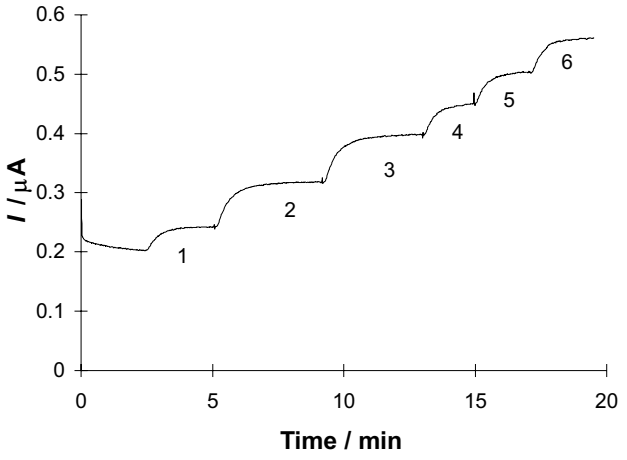

Fig. 9. Determination of oxalate with biosensor in 20 μL aliquots of spinach leaves extracted in HCl, steps 2 and 3, with standard additions of oxalic acid aliquots (c=20 μM), steps 4–6; step 1 indicates the addition of 20 μM of oxalic acid prior to any measurement; working potential was –50 mV. Supporting electrolyte: 0.1 M KCl, succinic buffer pH=3.6 and 5.6 mM EDTA

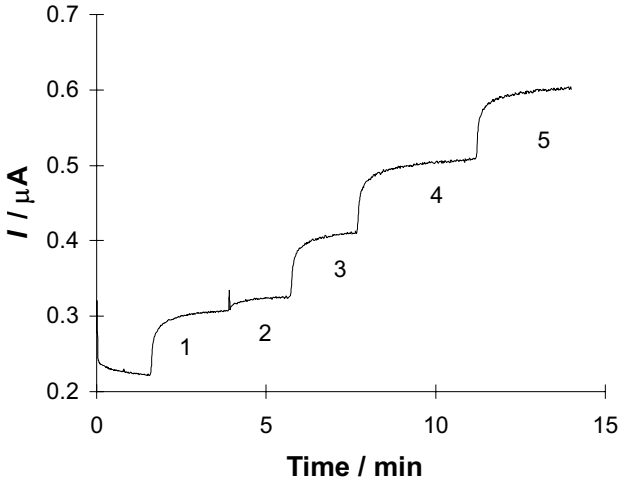

Fig. 10. Determination of oxalate in beer Stella Artois with biosensor; in one aliquot of beer (V=0.2 mL), step 2, three aliquots of oxalic acid were added (c=15 μM), steps 3–5. Step 1 indicates the addition of 20 μM of oxalic acid prior to any measurement. Working potential was –50 mV. Supporting electrolyte: 0.1 M KCl, succinic buffer pH=3.6 and 5.6 mM EDTA

Conclusion

The results presented in this paper indicate that the catalytic current obtained for hydrogen peroxide reduction at ruthenium modified FeNiHCM electrode is higher than at FeNiHCM electrode (75 and 42 μA, respectively, for 7 mM of H_2O_2) and Ru-FeNiHCM electrode shows better electrochemical reversibility.

Therefore, the Ru-FeNiHCM film electrocrystallised on the top of the electrode and modified with oxalate oxidase enzyme can be used as a sensitive biosensor for oxalate determination, based on enhanced catalytic reduction of hydrogen peroxide at –50 mV *vs*. reference electrode in succinic buffer solution at pH= 3.6. The broad linear concentration range from 0.12 to 100 μM makes the proposed biosensor useful for possible technical and medical application. The average sensitivity of the biosensor in the mentioned linear concentration range is 43.2 nA/(μM cm^2).

The biosensor was tested for determination of oxalate in the samples of spinach after extraction either in water or EDTA or HCl, as well as in different brands of beer. In four out of seven brands of beer significant oxalate concentration was found (8.6 to 13.7 mg/L), while three brands of beer had very low oxalate concentrations (from 0.5 to 1.0 mg/L). All concentrations were determined with the standard addition method.

At the end, it has to be stressed out that the sensitivity of biosensor decreased only 15 % of the initial value through the testing period of eleven weeks.

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Osjetljivi amperometrijski biosenzor za određivanje oksalata u hrani

Sažetak

Pripravljen je i opisan biosenzor za određivanje oksalata s kovalentno imobiliziranim enzimom oksalat oksidazom na grafitnoj elektrodi modificiranoj slojem heksacianometalata rutenija, željeza i nikla (Ru-FeNiHCM). Dodatak rutenija sloju FeNiHCM katalizira redukciju H_2O_2 pri pH=2 te povećava stabilnost i osjetljivost biosenzora. Da bi se dobio optimalan odziv biosenzora, ispitan je utjecaj nekoliko eksperimentalnih pokazatelja, kao što su radni potencijal, pH i linearno područje koncentracije. Vodikov peroksid, nastao enzimskom razgradnjom oksalata, mjeren je pri –50 mV prema $Hg \mid Hg_2Cl_2 \mid 3M$ KCl u jantarnom puferu pri pH=3,6. Linearno područje koncentracije izmjereno je do 100 μM H_2O_2 uz kvadrat koeficijenta korelacije R^2=0,9985. Osjetljivost biosenzora u tom koncentracijskom području iznosila je 43,2 nA/(μM cm^2). Oksalat je određen u realnim uzorcima špinata nakon ekstrakcije u vodi, EDTA i HCl, te u različitim vrstama piva. Tijekom 11 tjedana smanjena je osjetljivost biosenzora za svega 15 % od početne osjetljivosti, što znači da se biosenzor može rabiti nekoliko mjeseci.