A novel biamperometric biosensor for urinary oxalate determination using flow-injection analysis

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**A B S T R A C T**

A biosensor for determination of oxalate concentration in urine has been developed by immobilisation of oxalate oxidase and peroxidase on the surface of an interdigitated gold electrode. Enzyme immobilisation was performed using BSA and glutaraldehyde. Biamperometric measurements were made in flow conditions both in aqueous oxalate solutions (tested concentration range between 50 μM and 10 mM) and in real urine samples (tested measuring range between 5 and 100 μM). Optimal working conditions were examined for flow-injection analysis, and good correlation was achieved between added oxalate quantity and the one measured by biosensor in urine matrix ($R^2 = 0.9983$). The influence of some interferences (ascorbic acid, uric acid, paracetamol, acetylsalicylic acid) was also studied using biamperometric measurement mode.

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1. Introduction

Calcium oxalate is the main component of urinary tract stones. Basically, oxalate is a product of protein metabolism and becomes toxic at high concentration due to production of insoluble complex salts with divalent cations (mainly calcium). Determination of oxalates in urine is important for diagnosis of many diseases and mostly indicates the presence of kidney stones. Many methods have been recommended for oxalate determination in clinical laboratory analyses but some of them are time-consuming [1] (as chromatographic and spectrophotometric) while some others need a chemically pre-treated sample [2].

Enzyme-based biosensors are user-friendly devices offering good analytical precision, specificity, sufficiently short response time and durability. Amperometric biosensors have been used routinely for metabolite determination in biochemical laboratories since 1975.

Two enzymes catalyse oxalate degradation: oxalate decarboxylase and oxalate oxidase. Bioreactions of oxalate in the presence of oxalate oxidase can be described by Eq. (1):

\[(\text{COOH})_2 + \text{O}_2 \rightarrow 2\text{CO}_2 + \text{H}_2\text{O}_2\]  

According to Eq. (1) oxalate concentration is proportional to carbon dioxide and hydrogen peroxide concentration. Therefore, some of previously developed oxalate biosensors were based on $p\text{CO}_2$ or $p\text{H}$ measurements [3] ($p\text{H}$ changes proportionally with released $\text{CO}_2$). Amperometric-based oxalate biosensors mainly determine hydrogen peroxide concentration [4]. Some other biosensors are based on measurements of oxygen consumption during oxalate presence [5].

Hansen et al. suggested urinary oxalate determination by oxalate oxidase immobilised on silanised glass beads and chemiluminescence detection of hydrogen peroxide with luminol [6].

An amperometric biosensor for oxalate determination based on immobilisation of oxalate oxidase in gelatine using glutaraldehyde on top of the oxygen probe was reported by Dincçekaya and Telefoncu [7].

Reddy et al. described a biosensor for determination of human urinary oxalates [8]. They co-immobilised oxalate oxidase in bovine serum albumin by glutaraldehyde, between haemodialysis and cellulose acetate membrane, to prevent some chemical and electrochemical interferences.

Recently Capra et al. described an enzymatic electrode for oxalate determination with extended analytical range and better stability [9]. Quantification of oxalic acid in urine by employing an amperometric Clark-type electrode imprinted by spinach tissue layer was suggested by Sezgintürk and Dincçekaya [10].

Bienzymatic amperometric biosensor for oxalate determination was described by Perez et al. [11]. Sotomayor et al. proposed bienzymatic optode as a detection system for oxalate determination [12],
while Pundir offered oxalate bienzymatic biosensor obtained by co-
immobilisation of oxalate oxidase and peroxidase into glass beads [13].

Electrochemical determination of oxalate at pyrolytic graphite electrode was described by Sjiškić et al. [14]. Stefan et al. described an oxalate ion selective electrode for determination of urinary oxalate [15].

Biamperometric detection has been applied in flow-injection analysis because of high selectivity of measurements (low potential imposed between electrodes) and increased sensitivity as a result of low sample dispersion.

Biamperometry is based on detection with two identical working electrodes polarised with a small voltage difference in a solution containing indicating reversible redox couple. Direct biamperometric measurements exploit one part of the redox pair while the second part of the redox pair is produced by chemical or bio-
chemical interaction with the analyte. The most used indicating systems [16], for biamperometric determination include Fe$^{3+}$/Fe$^{2+}$, I$_2$/I$^-$, Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$, and lately DPPH$^+$/DPPH and ABTS$^+$/ABTS redox couple [17,18]. The application of biamperometric detection for flow-injection analy-

sis was proposed by Tougas et al. [19]. Due to high selectivity of the method, biamperometric measurements were used for deter-
mination of various analytes as components of complex biological samples (urine, blood) [20,21].

Application of a microelectrode with interdigitated array is an efficient method for miniaturisation of electrochemical sensors and biosensors because of the features such as rapid rise to steady state, high sensitivity and high current response.

Sanderson and Anderson explained the application of interdigi-
tated electrode (IDE) as an electrochemical detector almost 20 years ago [22].

Carbon-based interdigitated array electrodes utilised for elect-
rochemical measurements in batch mode and in flow system were studied by Morita et al. [23], while application of multi microelec-
trode array of eight different sizes for biological electrochemistry, was presented by Kudera et al. [24]. Application of interdigitated array electrodes for determination of enzyme activity was also described [25].

A detection system consisting of four pairs of thin gold interdigi-
tated electrodes and two auxiliary electrodes fixed on micro-fluidic platform was described as a chip-based detector for rapid detection and quantification of nucleic acids [26]. Intergated ultra micro-
electrode arrays (IDUAs) as transducers in a portable micro-fluidic based biosensor were designed with the aim to maximise signal-
to-noise ratio [27]. A chip with four separated parallel arrays of iridium-made ultra microelectrodes and miniaturized flow device for trace heavy metal measurements in water was proposed by Xie et al. [28].

In the present paper, a two-enzyme (bienzymatic) biosensor based on biamperometric determination of oxalate in urine is described. Oxalate oxidase enzyme immobilised on the surface of an interdigitated electrode converted oxalate to carbon dioxide and hydrogen peroxide according to Eq. (1).

$$\text{H}_2\text{O}_2 + 2\text{Fe(CN)}_6^{3-} + 2\text{H}^+ \xrightarrow{\text{peroxidase}} 2\text{H}_2\text{O} + 2[\text{Fe(CN)}_6^{4-}]$$  \hspace{1cm} (2)

The produced hexacyanoferrate(II) was then reduced at the inter-
digitated electrode as denoted by Eq. (3).

$$[\text{Fe(CN)}_6^{3-}]_2^+ + e^- \rightarrow [\text{Fe(CN)}_6^{4-}]_2^+$$  \hspace{1cm} (3)

Biamperometric current was proportional to hexacyanofer-
rate(III) concentration and thus also to oxalate concentration. Biamperometric measurement mode was used to avoid some electrochemical interferences. Optimisation of pH, flow rate, hexa-

cyanoferrate(II) concentration and thus also to oxalate concentration.

2. Materials and methods

2.1. Chemicals and reagents

Commercially available chemicals of the highest purity were used. Oxalate oxidase (from barley seedlings, lyophilised powder, 0.70 units mg$^{-1}$ solid), peroxidase (147 U mg$^{-1}$), bovine serum albumin (BSA), glutaraldehyde (GLA, mass fraction 25%) were obtained from Sigma–Aldrich (USA). Potassium hexacyanofer-
rate(III) trihydrate, potassium hexacyanoferrate(II), succinic acid, oxalic acid dihydrate, hydrochloric acid, potassium chloride, disodi-
ummethylenediamintetraacetic acid dihydratetra, L (+) ascorbic acid, uric acid and ethanol (96%) were from Kemika, Croatia. Paracetam-
ol (from a Panadol pill) and acetylsalicylic acid (from an aspirin pill) were from Glaxo SmithKline, Croatia.

Deionised water was purified using a Milipore-MilliQ system.

2.2. Electrode preparation

Intergated electrode (IDE) IME 1525.3 FD Au P (ABTCH, Rich-
mond, USA) was employed as a supporting electrode.

Prior to measurement, the interdigitated electrodes were cleaned and preconditioned according to manufacturer’s recommen-
dations. The conditioning was made in 0.8 M sulphuric acid by 3-fold cycling in the potential range between 600 and 1000 mV using 50 mV s$^{-1}$ scan rate. The preconditioning was repeated for each part of the IDE pair using Hg$_2$Cl$_2$/3 M KCl as a reference electrode, and a disc glassy carbon electrode as an auxiliary electrode. Oxalate oxidase and peroxidase were co-immobilised by glutaraldehyde-bovine serum albumin cross-linking procedure on top of the gold interdigitated electrode array. Two-enzyme layer was prepared by mixing 0.3 mg oxalate oxidase and 0.3 mg per-
oxidase into 20 μL 10% bovine serum albumin solution (BSA (M(BSA)) = 45,000 g mol$^{-1}$; solution prepared by dissolving 100 mg BSA in 1 mL of succinic buffer, pH 3.6). The solution was homogenised for 30 min. After 30 min, the albumin–enzyme solution was well mixed with 10 μL 5% glutaraldehyde and finally 2 μL of this mixture were deposited by micropipette on top of IDE and left to dry. Thus, prepared enzyme electrode was conditioned overnight in succinic buffer solution (pH 3.8) at 7 °C.

2.3. Urine samples

The urine samples were taken daily.

Biosensor testing was performed by recovery test (amount added vs. amount found) based on addition of the known concen-

tration of oxalic acid to urine samples with very low natural oxalate content (after dilution, c(oxalate) < 1 μM).

2.4. Apparatus

Electrochemical measurements were carried out on the Poten-
tiotstat 273 A (Princeton Applied Research, USA) connected to the computer for data collection and analysis. Interdigitated electrode (IDE) IME 1525.3 FD Au P (ABTCH, Richmond, USA) was coated
by co-immobilised enzyme layer. The electrode is a microlithographically fabricated sensor chip consisting of two separated working gold electrode arrays on the chip (6.4 mm × 5.5 mm, digit length \( W_a = 2.985 \) mm, digit width \( W_g = 1.5 \) mm, interdigit space \( W_d = 1.5 \) mm, and the number of digit pairs = 25). For current–time measurements, a pair of interdigitated electrodes fixed in a flow-through measurement cell was used. Transportation of the carrier solution in FIA mode (Fig. 1) was made by double tubing peristaltic pump. Sample injection into carrier stream was done by a syringe using injector valve Rheodyne Model 7125 and sample loops of 10 \( \mu \)L and 100 \( \mu \)L. Knitted coil reactor was made using a Teflon tube (1 mm in diameter, 60 cm long).

Homogenisation of enzyme–BSA layer was done using ultrasonic mixer Transsonic 460/H, Elma, Germany, pH electrode (Blueline 17 pH, pH 0–14/−51 000 °C/3 M KCl, SCHOTT, Germany) and pH-meter MA 574O, Iskra, Slovenia.

3. Results and discussion

Fig. 2a shows \( I–E \) curves characteristic for \([\text{Fe(CN)}_6]^{4−}|[\text{Fe(CN)}_6]^{3−}\) redox pair determined in a classic electrochemical cell (batch mode) using the interdigitated electrode in biamperometric measurement mode. 1 mL 20 mM \( K_4[\text{Fe(CN)}_6] \) was added to succinic buffer solution (8 mL 200 mM, pH 3.6) containing 50 mM EDTA and the curve designated as 0 was recorded. Curves 1–4 were recorded after successive addition of 100 \( \mu \)L 10 mM \( K_3[\text{Fe(CN)}_6] \). According to Fig. 2a, there was insignificant current response due to existence of only \( K_4[\text{Fe(CN)}_6] \) in the solution. The addition of \( K_3[\text{Fe(CN)}_6] \) resulted in linear current response confirming that biamperometric response depends linearly on the concentration of \( K_3[\text{Fe(CN)}_6] \), i.e. the response is proportional to that part of redox pair that is present in the solution at lower concentration. Fig. 2b represents calibration curve derived from Fig. 2a for potential of 100 mV. Five-point curve can be described with the following equation:

\[
I = (0.0934 \pm 0.043) \mu A + (7.183 \pm 0.165) \mu A \text{mM}^{-1} \cdot c(\text{K_3[Fe(CN)_6]})\text{mM}; \quad R^2 = 0.9992.
\]

It is also notable that potential changes to higher values are followed by higher current response; however, at potentials higher than 100 mV, the difference in current response is negligible. According to Eq. (1), \([\text{Fe(CN)}_6]^{3−}\) concentration is proportional to oxalate concentration, confirming that IDE is suitable as a supporting electrode for oxalate biosensor construction.

3.1. Optimisation of the measuring system

It is well known that physiological concentration of oxalate is in the range between 50 and 2000 \( \mu \)M. Furthermore, many different species, contained in urine, strongly affect oxalate oxidase and therefore samples must be diluted to almost 20-fold, or even higher to avoid denaturation of the enzyme by constituents of urine matrix [6]. After dilution, oxalate concentrations in samples are in the range between 2.5 and 100 \( \mu \)M. In the case of flow-injection analysis, retention time of sample in the biosensor measuring cell is short, which also decreases the current response. Very low enzyme (oxalate oxidase) activity (production of hydrogen peroxide in small quantity per enzyme mass) causes further decrease of the current response. Thus, optimisation of the measurement system is required to obtain sufficient sensitivity of the developed biosensor.

3.1.1. Optimisation of potassium hexacyanoferrate(II) concentration

Optimal hexacyanoferrate(II) concentration was determined by injection 10 \( \mu \)L of 2 mM oxalate into the carrier stream containing succinic buffer pH 3.6. The applied potential difference to biosensor was 100 mV and the flow rate of 1.28 mL min\(^{-1}\) was used. Optimisa-
Fig. 3. Biosensor current response is shown as a function of potassium hexacyanoferrate(II) concentration. The applied potential was 100 mV, flow rate 1.28 mL min⁻¹, oxalate concentration 2 mM, injection volume 10 μL, carrier solution containing succinic buffer pH 3.6. The squares represent background current; the circles represent biosensor current peaks. The tested potassium hexacyanoferrate(II) concentration was in the range from 0.5 to 20 mM.

The gradual increase in biosensor response was evident in the concentration range between 0.5 and 10 mM. Increasing concentration of potassium hexacyanoferrate(II) above 10 mM (circle) cannot enhance current response (Fig. 3). Therefore, all subsequent experiments were done using 10 mM potassium hexacyanoferrate(II) concentration. The same gradual increase in background current (squares) was expected because concentrated potassium hexacyanoferrate(II) solutions contain an increased level of potassium hexacyanoferrate(III) produced by oxidation from air oxygen.

3.1.2. Optimisation of pH

Biosensor response obtained in succinic buffer solution with different pH values is shown in Fig. 4. Measurement conditions: working potential 100 mV, flow rate 1.28 mL min⁻¹, 10 mM K₄[Fe(CN)₆], oxalate concentration 2 mM, injected volume 10 μL. The tested pH values were in the range from 3.07 to 5.61.

Fig. 4. pH influence on oxalate biosensor response. The squares represent background current, the circles represent biosensor current peaks.

The current peak height–pH graph shows the highest value at pH 3.6 and linear decrease at increased pH values (circles). Background current (squares) decreases linearly with increasing pH because redox air oxygen potential lowers by 59 mV pH⁻¹ reducing the production of [Fe(CN)₆]³⁻. Background current becomes negligible at pH 7. Despite the fact that the background current has no influence on the proper working of a developed biosensor, it can be strongly reduced by simple buffer deoxygenation.

According to literature, optimum pH for peroxidase is in the range between pH 6 and 6.5 and for oxalate oxidase between 3.8 and 4. The highest biosensor response is close to the pH optimum of oxalate oxidase indicating that the produced hydrogen peroxide concentration is low due to low oxalate oxidase activity, and becomes a limiting reagent for further enzymatic reactions with peroxidase. To obtain the highest biosensor response, the pH values of tested urine samples were adjusted to a value compatible with the pH maximum of the immobilised enzymes (pH 3.6). Peroxidase was co-immobilised onto the electrode due to signal amplification because of great sensitivity to H₂O₂ [29], even at very small concentration. Activity of peroxidase is reduced in acidic medium and thus the applied activity of the used peroxidase enzymes was increased (2.94 U/electrode) than the activity of the utilised oxalate oxidase (0.014 U/electrode). Amounts of immobilised enzymes were selected according to enzymatic assay solution for oxalate determination (Sigma, USA) [31].

Succinic buffer and EDTA were used as the oxalate oxidase activators, based on the literature overview of biosensor applications [9]. Concentration of the used cross-linker was previously optimised [30].

3.1.3. Flow rate optimisation

The optimum flow rate was determined by injection of 10 μL 2 mM oxalate into the carrier stream containing succinic buffer (0.1 M) pH 3.6 and 10 mM K₄[Fe(CN)₆]. The working potential imposed to electrode was 100 mV and the tested flow rates ranged between 3.14 and 0.77 mL min⁻¹ (Fig. 5a).

It is evident (Fig. 5b) that there was no influence of the flow rate on the background current (squares). However, the biosensor response rose as the flow rate decreased (circles). Increase of peak current at lower flow rate was the result of extended reaction time between biosensor enzymes and oxalate. A flow rate of 1.24 mL min⁻¹ was chosen for further experiments because it
3.1.4. Optimisation of the working potential

The optimal working potential was determined by injection of 10 μL 2 mM oxalate into the carrier stream containing succinic buffer (0.1 M, pH 3.6) and 10 mM K₄[Fe(CN)₆]. The tested working potentials were in the range between 20 and 180 mV and the used flow rate was 1.24 mL min⁻¹.

As evident in Fig. 6a and b, the increase in potential was followed by a gradual increase in current response (circles). At the potentials higher than 100 mV, further current increase was insignificant and the potential of 100 mV was chosen for further experiments. The background current (squares) should show linear rise with potential according to the Ohm’s law, but changes in the carrier solution resistance ([Fe(CN)₆]³⁻ concentration rises with time) cause some deviation from linearity.

3.1.5. Biosensor calibration

After optimisation of various parameters, as described above, the biosensor was used for FIA calibration. Oxalate standards were prepared by serial dilution of a 0.1 M stock solution using succinic buffer pH 3.6. The testing range of oxalate concentration was between 25 μM and 10 mM.

A series of standard oxalate solutions were injected in triplicates, as shown in Fig. 7a.

The biosensor showed linearity in the range between 50 μM and 10 mM with injection volume of 10 μL. Such small volume of injected substrate caused enzyme saturation at high oxalate concentration, while very small current response was obvious in the urine oxalate range (10–200 μM). Fig. 7b shows the nine-point calibration curve derived from Fig. 7a, and it is represented with the following equation:

\[
\Delta I = (-2.521 \pm 1.156) \text{nA} + (0.019 \pm 3.027 \cdot 10^{-4}) \text{nA} \mu \text{M}^{-1} \cdot c(\text{oxalate}) \mu \text{M}^{-1} \\
R^2 = 0.9992
\]

To obtain the higher current response in the range between 10 and 200 μM, the injection volume of 100 μL was used. Eight-point calibration, made with sample loop of 100 μL, is denoted by equation:

\[
(\Delta I) = (0.653 \pm 0.425) \text{nA} + (0.129 \pm 0.005) \text{nA} \mu \text{M}^{-1} \\
\cdot c(\text{oxalate}) \mu \text{M}^{-1} \\
R^2 = 0.9960
\]

3.1.6. Influence of urine dilution

Achieving good linearity for aqueous standards did not improve the practical application of the developed biosensor for real sample measurements. Oxalate oxidase is inhibited by the sodium salts of chloride, phosphate, citrate and acetate which are the standard constituents of urine. To overcome that inhibitory influence, urine samples should be diluted before the measurement, as previously suggested [2,6].

The results of urine dilution on biosensor response for four dilution ratios (V(urate)/V(buffer): 1:3, 1:7, 1:11 and 1:19) are given in Table 1.

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>I(urate)/I(buffer) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>61.2</td>
</tr>
<tr>
<td>8</td>
<td>86.0</td>
</tr>
<tr>
<td>10</td>
<td>89.0</td>
</tr>
<tr>
<td>20</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Experimental conditions: working potential 100 mV, flow rate 1.24 mL min⁻¹, c(K₄[Fe(CN)₆]) = 10 mM, sample loop 100 μL, c(oxalate) = 50 μM, succinic buffer solution pH 3.6 containing 50 mM EDTA.
which additionally offered 4-fold sample dilution automatically (in total, a 20-fold dilution).

Oxalate determination in the solution prepared by 20-fold diluted urine is shown in diagram (Fig. 8a). Experimental conditions were the same as used before. Each sample was double injected. The first two peaks indicate the biosensor response for pure, 20-fold diluted urine samples, while other peaks designate diluted urine samples with some added oxalate. The two peaks for the same concentration (Fig. 8a) are actually equal in height, however the second peak appears to be higher because of the background current variations.

Double curves (Fig. 8b) show the biosensor response to a series of oxalate solutions prepared in diluted urine. The curves have identical slope, however some differences in intercept are evident. Curve 1 (squares) was obtained by using ΔI as difference between peak current and background current for each tested concentration (y-axis) vs. oxalate urine concentration (x-axis). Curve 2 (circles) was obtained by using ΔI as difference between Δ peak current and Δ peak current obtained in diluted urine (the sample without added oxalate) for all tested solutions.

The current obtained in diluted urine (first two peaks in Fig. 8a) represent the biosensor response for physiological urine oxalate concentration, therefore ΔI (curve 2) represents current response for added oxalate only (circles).

Physiological oxalate concentration for the used urine sample was very low, and it was the same for each of prepared urine solutions. Equations that denote the curves are as follows:

\[ ΔI = (2.4358 ± 0.8406) nA + (0.1296 ± 0.0048) nA \mu M^{-1} \]
\[ c(\text{oxalate}) \mu M; \quad R^2 = 0.9959 \quad (\text{squares}) \]
\[ ΔI = (−0.0642 ± 0.8406) nA + (0.1296 ± 0.0048) nA \mu M^{-1} \]
\[ c(\text{oxalate}) \mu M; \quad R^2 = 0.9959 \quad (\text{circles}) \]

Results of measurements obtained in the experiment (Fig. 8a and b) described above showed linear dependence between oxalate biosensor response and oxalate concentration for prepared urine samples.

For determination of oxalate concentration (added) for each prepared oxalate–urine solution, the equation obtained by three-point calibrating curve was used (three oxalate water standards were injected at the end of urine oxalate sample testing). Table 2 presents the results of concentration established by biosensor measurement (right column) and of analytically prepared concentration (added, left column).

Comparison of the oxalate concentration analytically prepared in urine and the one determined by the biosensor using buffer oxalate standards gave the slope equal to 1, low intercept (0.9596 µM) and the correlation coefficient of 0.9983. Correlation graph is represented by the following equation:

\[ c(\text{det. by bios.}) = (0.959 ± 0.765) \mu M + (1.007 ± 0.017) \]
\[ c(\text{added}) \mu M; \quad R^2 = 0.9983. \]

Good correlation of results over a wide range of urinary oxalate concentration supports the practical application of the developed biosensor for oxalate determination in urine.

The stability of the biosensor was tested for 8 weeks on daily basis for almost 8 h. When not in use, the measurement cell with biosensor was filled with succinic buffer and stored at 7 °C. In the testing period, the sensitivity change from 0.135 nA µM⁻¹ to 0.094 nA µM⁻¹ signified that the half-life time of the electrode was 2 months. The detection limit calculated as LOD = 3 × standard deviation/slope was found to be 4.76 µM in the measurement cell corresponding to 19.04 µM in undiluted samples. To improve the good performance of the developed biosensor, a comparison with two amperometric oxalate biosensors of similar construction was made. The results of comparison between the developed biosensor and amperometric biosensors are given in Table 3. Biosensors [4], without Prussian blue layer and the proposed biosensor showed almost the same sensitivity while biosensor [9], showed significantly lower sensitivity.

### Table 2

<table>
<thead>
<tr>
<th>c(sample, added, µM)</th>
<th>c(sample, found, µM)</th>
<th>c(oxalate in cell, added, µM)</th>
<th>c(oxalate in cell, found, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>14.20 ± 2.40</td>
<td>5</td>
<td>3.55 ± 0.60</td>
</tr>
<tr>
<td>40</td>
<td>36.10 ± 0.75</td>
<td>10</td>
<td>9.02 ± 0.19</td>
</tr>
<tr>
<td>60</td>
<td>64.16 ± 1.51</td>
<td>15</td>
<td>16.04 ± 0.38</td>
</tr>
<tr>
<td>80</td>
<td>79.60 ± 5.24</td>
<td>20</td>
<td>19.90 ± 1.31</td>
</tr>
<tr>
<td>100</td>
<td>94.92 ± 4.49</td>
<td>25</td>
<td>23.73 ± 1.12</td>
</tr>
<tr>
<td>150</td>
<td>150.10 ± 2.99</td>
<td>37.5</td>
<td>37.52 ± 0.75</td>
</tr>
<tr>
<td>200</td>
<td>185.64 ± 3.74</td>
<td>50</td>
<td>46.40 ± 0.94</td>
</tr>
<tr>
<td>400</td>
<td>384.51 ± 2.99</td>
<td>100</td>
<td>96.12 ± 0.75</td>
</tr>
</tbody>
</table>

Column 1—analytically prepared oxalate samples using 5-fold diluted urine, column 2—oxalate concentration determined by the biosensor, column 3—oxalate concentration in the measurement cell after 4-fold sample dilution, column 4—oxalate concentration in the measurement cell determined by the biosensor.
can reduce $K_3[Fe(CN)_6]$ to $K_4[Fe(CN)_6]$), causing significant chemi-
reduced biamperometric current response because ascorbic acid
K_3[Fe(CN)_6]$). Addition of ascorbic acid to the measurement cell
metric measuring mode. The supporting electrolyte contained 8 mL
succinic buffer pH 3.8, 1 mL 20 mM K_4[Fe(CN)_6] and 400
interference study was done in a classic electrochemical cell (batch mode) using interdigitated electrode without enzyme layer connected to a potentiostat in biampero-
working of the biosensor. Interference study was done in a classic
cyclic acid, paracetamol, and uric acid had no influence on the proper
3.1.8. Interferences
Possible interferences in urine are ascorbate, homovanilic acid,
ascorbic acid, acetylsalicylic acid, uric acid and many others. Serious
interferences were observed only for ascorbic acid while acetylsalic-
cylic acid, paracetamol, and uric acid had no influence on the proper
biosensor response [6]. Determination of urine oxalates by the described
biosensor measurement cell together with a reference measure-
response for elimination of interference is in progress.

4. Conclusion
Results of the recovery test demonstrated the reliability of the
proposed bienzyme electrode in biamperometric measurement
mode for urinary oxalate determination. According to literature
there is no published method related to oxalate determination
based on biamperometric measurements by interdigitated elec-
trode.
Application of an interdigitated electrode as a two-electrode
detector makes a reference electrode unnecessary. It is well known
that the reference electrode for continuous flow measurement
must be constructed very carefully, otherwise the reference ele-
trode becomes the weak point of any flow-through system.
Interdigitated electrode as a planar electrode offers some addi-
tional advantages such as small size of the produced biosensor, high
precision and accuracy, small sample volume and reduced cost of
manufacturing.
The applied interdigitated electrode could be part of a micro-
fluidic platform (biochip) offering a new possibility in urinary oxalate
determination. Response time of 172 s (peak forming) for the highest expected oxalate concentration (200 μM) offered
almost 20 analyses per hour. No need for sample preparation
except sample dilution additionally confirmed the high value of
this method for practical application.

Interference of ascorbic acid was noticed while acetylsalicylic
and uric acid did not interfere with the function of the biosensor.

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