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Indirect Determination of Mercury by Inhibition of Glucose Oxidase Immobilized on a Carbon Paste Electrode

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An amperometric biosensor for the indirect determination of mercury was developed by inhibition of glucose oxidase immobilized on a carbon paste electrode bulk modified with manganese dioxide 5% (m:m). Optimum conditions for biosensor were found as: operating potential 0.46 V vs Ag/AgCl, enzyme load 0.56 U/mm^2 electrode surface, supporting electrolyte: 0.1 M citrate phosphate buffer (pH 7). The current response of the biosensor in hydrodynamic amperometry was linearity dependent on the concentration within 2.0 – 32.5 mg/L Hg (II) with a detection limit of 0.5 mg/L. The enzymatic activity could be fully recovered by exposure of the electrode to a solution of EDTA (0.1 M) for 2 minutes. Minor interferences were caused by Cr^{3+} and Zn^{2+}. Analytical results of spiked water sample showed a good correlation with determinations by atomic absorption spectrometry where the difference is not statistically significant at 95% confidence level. The sensor yielded a stable and reproducible response more than 2 weeks when stored dry at 4 °C.

Keywords: Mercuric Detection, Indirect Method, Enzyme Inhibition, Biosensor

1. INTRODUCTION

Mercury is a toxic trace heavy metal. Several analytical methods are widely used for its determination in environmental and biological samples. A standard procedure is cold vapor generation technique using atomic absorption spectrometry (AAS). The method is highly sensitive but time consuming and requires trained personnel and costly apparatuses.

Alternative attention to determine metal ions is currently devoted to the study of biosensors in environmental analysis owing to some advantages, such as rapidity, low cost and good sensitivity.

Most of the biosensors for detecting metal ions are designed on the inhibition of an enzyme. With a constant substrate concentration inhibition leads to a decrease in the activity of the enzyme and as a consequence to a decrease of the response signal which is proportional to the amount of inhibitor in the sample. Glucose oxidase (GOx) biosensors are able to detect mercury (II) [1, 2]. The metal is well known for its ability to react with sulfhydryl groups of proteins, which are frequently responsible for enzyme's active center [3]. In this context, electrochemical sensors and biosensors appear to be an appropriate alternative, or at least a complementary choice to AAS; they are recently very common in the literature for the determination of mercury in polluted environmental samples [1, 4-23].

The aim of this work presented here is to investigated if carbon paste electrodes (CPE) modified with GOx and MnO_2 are useful for the indirect determination of Hg(II) and to investigate and define optimum experimental conditions.

Manganese dioxide is a convenient mediator to reduce the overpotential for the oxidation of hydrogen peroxide at carbon electrode [24]. H_2O_2 is produced by many oxidases as an intermediate; glucose oxidase converts glucose to gluconolactone and uses oxygen as an electron acceptor, which in consequence is reduced to hydrogen peroxide. Thus, MnO_2 is useful as a mediator for the oxidation of the intermediate in biosensors based on oxidases [25, 26], where the current necessary for the re-oxidation of Mn(II, III)-oxides is exploited as analytical signal.

2. MATERIALS AND METHODS

2.1 Reagents

Glucose oxidase from *Aspergillus niger* (GOx, 250 KU) and carbon powder (size < 50 nm) were purchased from Sigma-Aldrich. All other chemicals were of analytical grade (p.a., Merck). The glucose stock solution (10% w/v) was prepared in 0.1 M citrate phosphate buffer pH 7.0 and diluted with buffer solution according to needs; the mercury standard solution (1000 ppm Hg (II) in 1% HNO_3) was diluted correspondingly with deionized water. Ethylenediaminetetraacetic acid (EDTA) solution (0.1 M) was prepared in deionized water before use.

2.2 Instrumentation

Cyclic voltammetric and amperometric measurements were carried out with a potentiostat/galvanostat (Metrohm, PG 11) using a silver/silver chloride reference electrode (3 M KCl) and a platinum wire counter electrode. All potentials mentioned in this work refer to this reference electrode. The working electrode was carbon paste modified with 5% (m:m) manganese oxide in a corresponding holder (diameter 3 cm). All chronoamperometric measurements were carried out in stirred solutions (approx. 300 rpm) of 0.1 M citrate phosphate buffer (pH 7.0).

Analyses with graphite furnace atomic absorption spectrometry (GF-AAS) were performed with an electrothermal atomic absorption spectrometer (AAnalyst^{TM} 800, PerkinElmer).

2.3 Preparation of carbon paste electrodes

Carbon paste was prepared by thoroughly mixing carbon powder (1.000 g) with MnO_2 (50 mg) and paraffin oil (320 μL) until homogenous consistency was achieved. The electrode holder was a plastic syringe tube (diameter 5.8 mm, length 40 mm) and was filled with the modified carbon paste. Electrical contact to the paste was established by inserting a copper wire into the back of the paste. The CPE/MnO_2 electrode surface was polished with weighing paper until a smooth surface was achieved. The GOx/MnO_2/CPE was prepared by dropping glucose oxidase solution in Nafion solution onto the electrode surface. The electrode was dried at room temperature then kept at 4 °C in a refrigerator before use.

2.4 Methods

Amperometric measurements were performed in an electrochemical cell at room temperature. The steady-state current response was recorded in a stirred citrate phosphate buffer (0.1 M, pH 7.0) with a glucose solution (12.2 mM) and an operation potential of 0.46 V vs Ag/AgCl. Signals were evaluated as the difference of the registered current to the current baseline obtained before addition of the analyte.

3. RESULTS AND DISCUSSIONS

3.1 Parameters affecting the enzyme electrode response

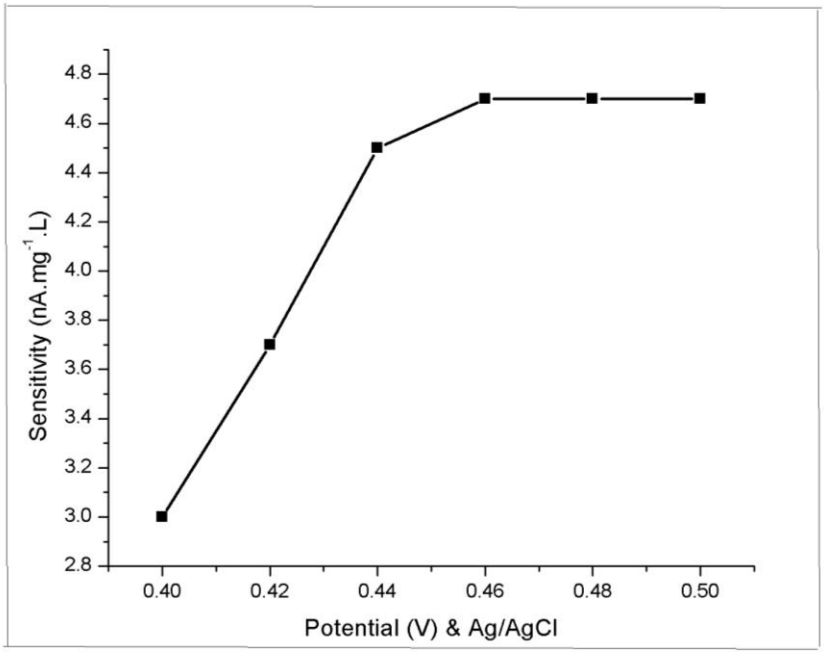

Figure 1. Dependence of the sensitivity of GOx/MnO_2/CPEs on the operating potential; enzyme load 0.56 U/mm^2; stirred phosphate-citrate buffer solution, pH 7.0; slopes from chronoamperometric measurements from 5-15 mg/L glucose.

The carbon paste biosensor with incorporatied manganese dioxide as a mediator and glucose oxidase as a biocomponent was first optimized for the determination of glucose; the experimental conditions obtained were then applied to the determination of mercury by the inhibition of the enzyme activity.

Effect of operating potential**.** The effect of the operating potential on the performance of the biosensor based on GOx/MnO_2/CPE was optimized in citrate phosphate buffer at pH 7.0. At all investigated potentials between 0.4 to 0.5 V a linear relation was found between the current in hydrodynamic amperometric mode and the concentration of glucose ranging from 5-15 mg/L. The dependence of the sensitivity of the electrode (nA L mg^{-1}) in dependence of the operating potential is displayed in Fig.1. The electrode shows increasing sensitivity with increasing potential up to 0.46 V and then levels off. Thus, the latter value was taken the working potential for further studies.

Effect of enzyme load**.** The effect of the enzyme load of the electrode surface on the sensitivity of the chronoamperometric signal is shown in Fig.2. Best sensitivity was found for 0.56 U/mm^2. When the amount of enzyme in the surface becomes higher, the sensitivity decreases due to the increased thickness of the membrane which deteriorates the diffusion of glucose.

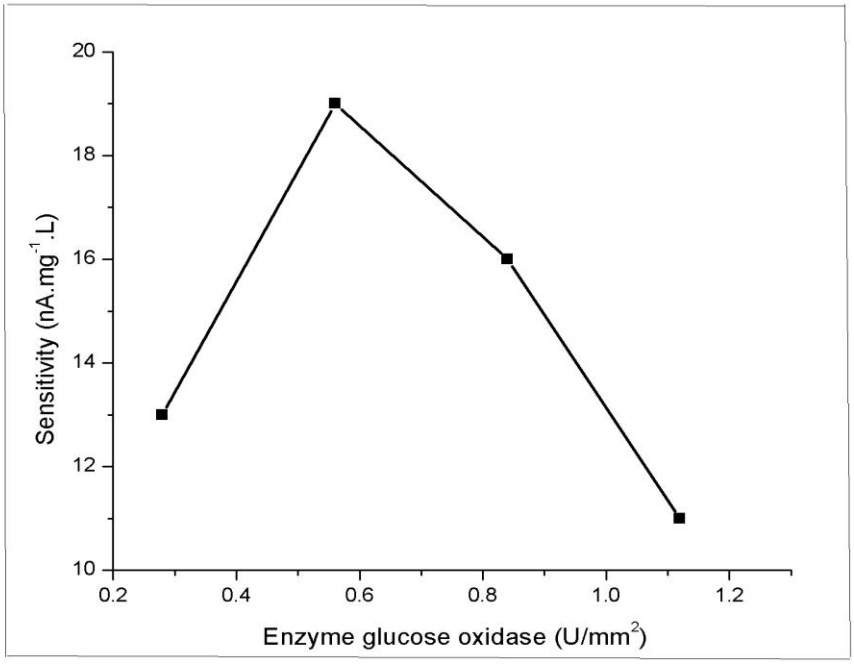

Figure 2. Dependence of the sensitivity of the GOx/MnO_2/CPE on the enzyme load of the electrode surface; operating potential 0.46 V, phosphate buffer pH 7.0, slope of chronoamperometric response of 5-15 mg/L glucose solutions.

Influence of pH. The effect of the pH on the performance of the biosensor is of great importance, because the activity of the enzyme but also the chemical reaction between H_2O_2 and MnO_2 and the electrochemical reconversion of the modifier are dependent on the hydronium ion concentration (e.g., eqns. 1 and 2). To optimize the the latter the pH of the citrate phosphate buffer

solution was varied within 4.0 – 8.0 and the amperometric response recorded (Fig.3). The highest activity was obtained at pH 7.0; this value was chosen for further investigations. The pH optimum found in this work is in accordance with previous investigations where a glucose electrode was used [22].

$$MnOOH \longrightarrow MnO_2 + H^+ + e^- \quad (1)$$

$$MnO + H_2O \longrightarrow MnO_2 + 2H^+ + 2e^- \quad (2)$$

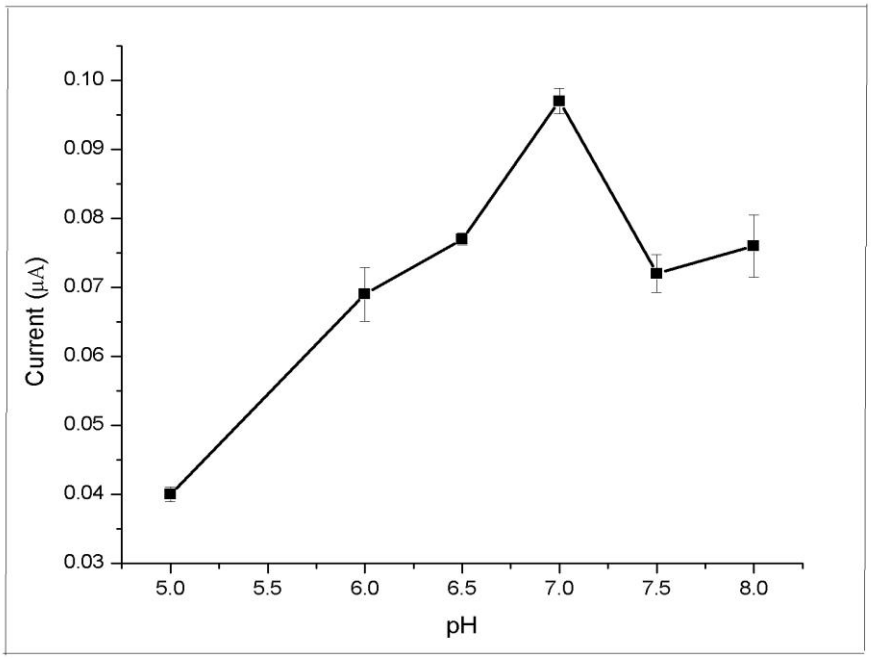

Figure 3. Effect of pH on the amperometric response of GOx/MnO_2/CPEs; citrate phosphate buffer 0.1 M; 500 mg/L glucose; operating potential 0.46 V.

3.2 Inhibition of mercury on the activity of glucose oxidase

For the indirect determination of analytes by inhibition of an enzyme sensor the substrate concentration is held constant, and the decrease of the response signal is monitored after addition of the inhibitor. This system was applied for the determination of mercury (II) with the glucose biosensor operated at 0.46 V using glucose as a substrate. The response current drops when mercury is present in the test solution; the degree of inhibition is proportional to the concentration of Hg(II). Fig.4 displays a typical chronoamperogram of a GOx/MnO_2/CPE biosensor for successive additions of the same amount of mercury under optimized experimental conditions. The anodic current decreases due to the presence of increasing amounts of the heavy metal causing inhibition of the activity of GOx immobilized on the CPE. Thus, the determination of mercury can be realized according to the inhibition degree of glucose oxidase. Mercury is probably bound to sulfhydyl or hydroxyl groups of the glucose oxidase close to or at its active center. In this work, the concentration of glucose solution was fixed as 12.2 mM, whereas the concentration of the inhibitor was increased stepwise by adding

defined amounts of mercury. The relative inhibition, expressed in percents, is evaluated as (I_0 - I) /I $_0$ x 100, where I_0 and I are the current response in the absence and in the presence of the inhibitor, respectively [1, 8, 21]. The corresponding calibration curve with optimized parameters for mercury in the concentration range 2.5 – 30.0 mg/L with time intervals of 100 s is shown in Fig.5. The graph is linear t low concentrations up to 3 mg/L Hg(II) and levels off beyond. In fact, a quasi-linear relation between concentration and current can be approximated for the range 10 to 30 mg/L mercury. The working range for the determination of Hg(II) via chronoamperometric detection is higher than compared to the previous work where flow injection analysis with lower detection limit was used [8].

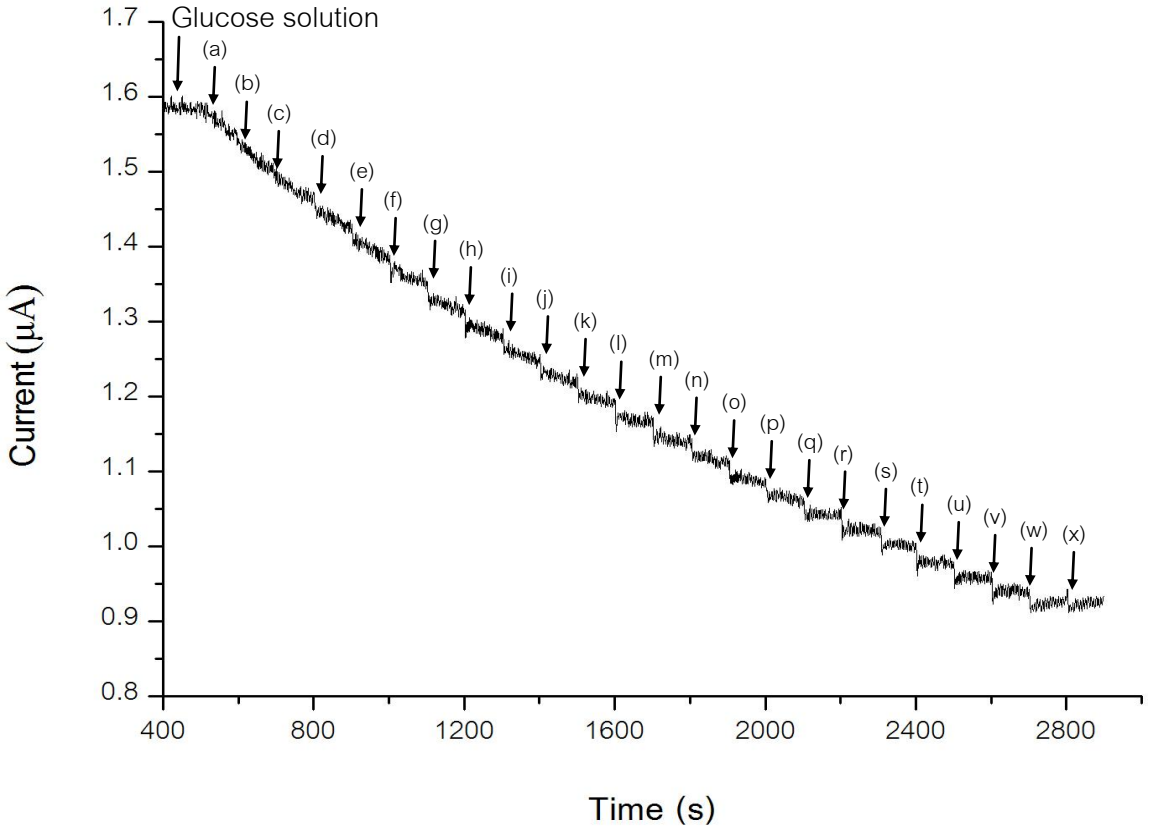

Figure 4. Amperometric response of a GOx/MnO_2/CPE to the addition of mercury; citrate phosphate buffer (0.1 M, pH 7.0), 12.2 mM glucose; operation potential 0.46 V; Hg(II) concentrations: (a) 0.94, (b) 1.17, (c) 2.49, (d) 4.06, (e) 5.56, (f) 7.08, (g) 8.60, (h) 10.10, (i) 11.60, (j) 13.09, (k) 14.56, (l) 16.03, (m) 17.49, (n) 18.94, (o) 20.38, (p) 21.82, (q) 23.24, (r) 24.66, (s) 26.07 (t) 27.47, (u) 28.86, (v) 30.24, (w) 31.62, and (x) 33.00 mg/L.

3.3 Regeneration

After the exposure of the biosensor to mercury, the activity of the enzyme at the electrode surface had to be restored in order to perform multiple measurements with the same electrode. Ethylenediaminetetraacetic acid (EDTA) was found to have a regenerative effect on the glucose-oxidase-based biosensor after contact with the heavy metal. The regeneration efficiency was dependent

on the history of the electrode with respect to mercury exposure. Typically up to 90% or more of the enzyme activity could be restored by the treatment with EDTA. Even heavily-loaded mercury biosensors could be restored with more than 70% efficiency as shown in Fig.6. Short time exposures to solutions of EDTA gave satisfactory results already, which demonstrates that Hg(II) is in fact not very strongly bound to the enzyme.

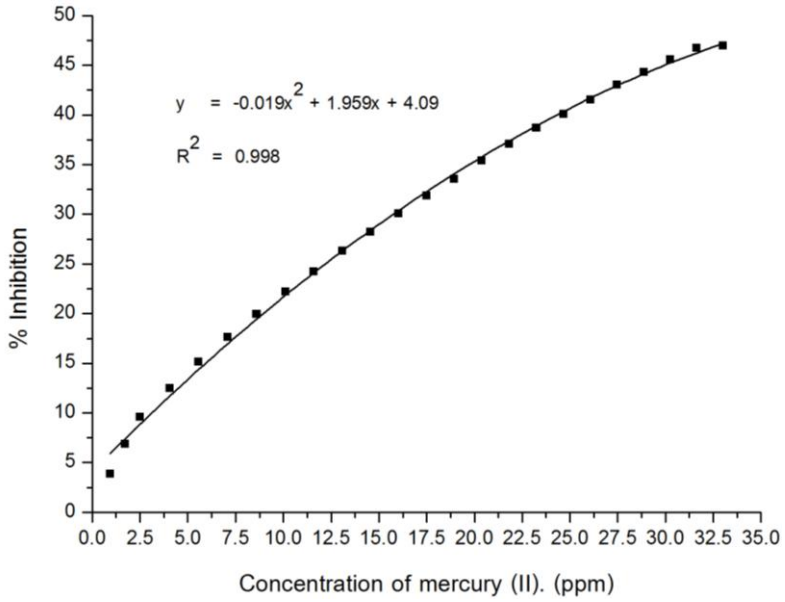

Figure 5. Relative inhibition of a GOx/MnO_2/CPE biosensor by Hg(II); citrate phosphate buffer solution (0.1 M, pH 7.0) containing 12.2 mM glucose; operation potential 0.46 V, chronoamperometric detection.

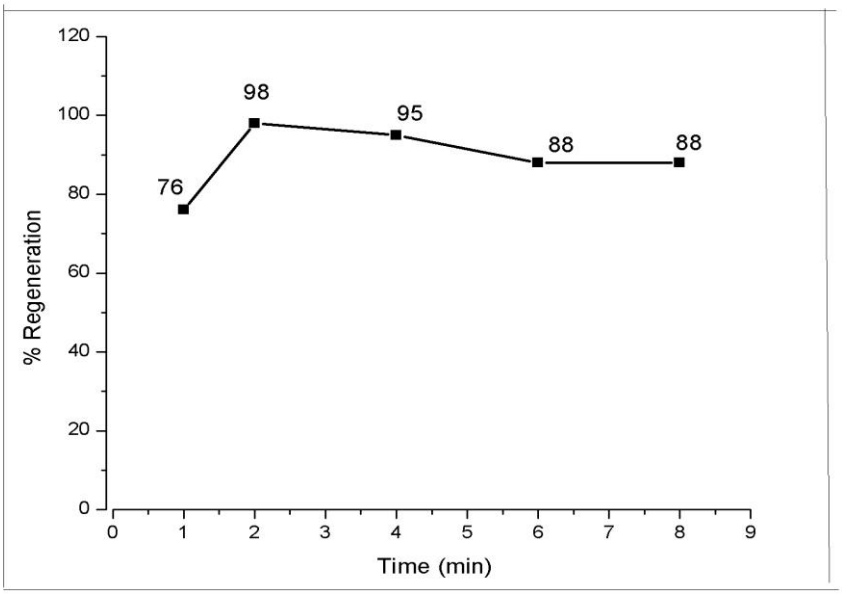

Figure 6. Regeneration of the biosensor in dependence on the regeneration time after exposure to Hg(II) (20 ppm); reagent solution of 0.1 M EDTA; citrate phosphate buffer (0.1 M, pH 7.0) with 12.2 mM glucose solution; operation potential 0.46 with chronoamperometric detection

3.4 Limit of detection

The detection limit (3s) was calculated as 0.5 mg/L using linear section for the calibration graph. The detection limit obtained in this study was higher than in previous works (0.49 µg/L, [8, 22]) but the sensor described here is much easier to prepare compared with the previous one.

3.5 Selectivity

Selectivity is an important aspect for the performance of an inhibition-based enzyme catalytic process. In this study, many heavy metals were investigated on their possible interfering effect on the determination of mercury under the same experimental conditions; the results are summarized in Table 1. Fe(III), Cu(II), Cd(II), Mn(II), Pb(II) and Cr(VI) show practically no inhibition even at concentrations of 250 mg/L. Only Zn(II) and Cr(III) have a significant effect at even lower concentration. In previous works [22] a higher influence was found for the above mentioned metals, which is due to the fact that lower glucose concentrations were investigated. The biosensor described here has some advantages over the latter one whose selectivity is poorer.

Table 1. Relative inhibition of the glucose biosensor by metals; experimental conditions: citrate phosphate buffer (0.1 M, pH 7.0) with 12.2 mM glucose; operating potential 0.46 V with chronoamperometric detection

Interfering ions	Concentration (mg L^{-1})	Inhibition degree (%)
Zn^{2+}	15.0	10%
Cr^{3+}	52.5	10%
Fe^{3+}	250.0	< 4%
Cu^{2+}	250.0	<4%
Cd^{2+}	250.0	<4%
Mn^{2+}	250.0	<4%
Pb^{2+}	250.0	<4%
CrO_4^{2-}	250.0	<4%

3.6 Application to water samples

The biosensor based on GOx/MnO_2 was applied to natural water as a sample matrix. A concentration of about 2 mg/L mercury was spiked to water samples, and the recovery determined. Comparison was made with results obtained by graphite furnace atomic absorption spectrometry (Table 2). The analysis of the statistically significant difference of the two techniques by t-test showed that the results of the GOx/MnO_2 modified carbon paste electrode were in satisfactory agreement with data from GFAAS, indicating that it is promising to apply the GOx/MnO_2 modified carbon paste electrode to determine mercury in water.

Table 2. Determination of mercury (II) ion in spiked natural water with the biosensor and with GFAAS

Sample	Biosensor	GFAAS
Natural water	n.d.	n.d.
Spiked natural water	2.56 ± 0.30	2.20 ± 0.50

n.d. – not detection

3.7 Stability

The long-term stability of the biosensor was monitored during storage at +4 °C in the buffer solution. The biosensor's response to 500 mg/L glucose solution as a function of the storage time showed a loss in activity of around 50 % during 2 weeks but was still useful for performing inhibition experiments.

4. CONCLUSIONS

Inhibition of the enzymatic activity of glucose oxidase immobilized on a MnO_2-modified carbon paste electrode was exploited for the determination of mercury (II). The data shows that the performance of the biosensor in terms of sensitivity, detection limit, linear range and relative standard deviation, facilitate the qualitative and quantitative determination of mercury. The fabrication of the sensor is inexpensive, simple and fast. The biosensor seems promising for being applied in environmental analysis.

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