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Horseradish peroxidase-screen printed biosensors for determination of Ochratoxin A

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ABSTRACT

This work summarizes the manufacturing procedure of Horseradish peroxidase (HRP) based biosensors for the determination of the mycotoxin Ochratoxin A (OTA). The biosensors have been fabricated using the single technology of screen-printing. That is to say, an HRP containing ink has been directly screen-printed onto carbon electrodes, which offers a higher rapidity and simplicity in the manufacturing process of biosensors for OTA determination. The formal redox potential of the Fe(III/II) moiety of HRP has been used to demonstrate the effective loading of enzyme into the ink. The chronoamperometric oxidation current registered has been successfully related to the concentration of OTA in solution from different samples, including beer ones. Under the optimum conditions of the experimental variables, precision in terms of reproducibility and repeatability has been calculated in the concentration range from 23.85 to 203.28 nM. A relative standard deviation for the slopes of 10% ($n = 4$) was obtained for reproducibility. In the case of repeatability, the biosensor retained a 30% of the initial sensitivity after the third calibration. The average capability of detection for 0.05% probabilities of false positive and negative was 26.77 ± 3.61 nM ($\alpha = 0.05$ and $\beta = 0.05$, $n = 3$).

1. Introduction

The necessity of disposable biosensors for simple, rapid and inexpensive analysis in fields such as clinical, environmental or industrial has been highlighted over the past decade. In this way, screen-printed electrodes (SPEs) have been shown as inexpensive and reproducible devices for mass production of miniaturized biosensors [1–4]. These transducers, building by sequential layer deposition on the surface of ceramic or plastic substrates and curing steps, have been conventionally linked to the sensing element by adsorption, cross-linking, electropolymerization or covalent attachment. Bioelements are commonly immobilized after the printing and firing processes, because of the high temperatures reached during the curing step [5]. The immobilization procedure requires maintaining the initial properties of the enzyme intact. Thus, successful developments of biosensors largely rely on the cost and stability of the sensing elements [3].

Even if the above-mentioned immobilization procedures are efficient, they imply additional steps after fabrication of the screen-printed carbon electrodes (SPCEs), which extends the whole biosensor manufacturing. Screen-printing techniques also offer another attractive immobilization procedure consisting of printing the biological material. Enzymes, which are proteins able to catalyse specific chemical reactions in vivo, are by far the most commonly employed bioelements [1]. Enzymes can be integrated into the ink to form the sensing paste, which can be screen-printed resulting in biosensors fabricated by only one technology [5–24]. Undoubtedly, this immobilization procedure, which is known as automated immobilization, is particularly interesting for mass production of disposable biosensors.

This work presents a simple way for preparing SPCEs modified with Horseradish peroxidase (HRP) for the determination of the mycotoxin Ochratoxin A (OTA). The carcinogenic compound OTA has been recently determined using a SPCE-biosensor based on HRP immobilized by pyrrole electropolymerization [25]. The screen-printing of HRP-containing ink offers a higher rapidity and simplicity in the manufacturing process of biosensors for OTA determination.

2. Experimental

2.1. Reagents

Several inks were used in the fabrication of SPCEs, namely C10903P14 (Gwent Electronic Materials, Torfaen, UK), Electrodag

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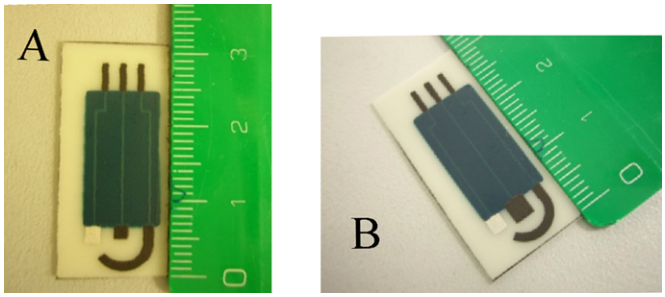


Fig. 1. Picture of (A) a homemade SPCE with a working electrode area of 4 mm^2 and (B) a homemade HRP-SPCE with a working electrode area, 9 mm^2.

6037 SS (Acheson Colloiden, Scheemda, The Netherlands) and D2071120D1 (Gwent Electronic Materials, Torfaen, UK).

All solutions were prepared with water purified with a Milli-Q device, which provided a resistivity of 18.2 MΩ cm.

HRP (EC 1.11.1.7., Sigma, Steinheim, Germany) was used as received.

A stock standard solution of OTA (Sigma, Steinheim, Germany) was prepared by dissolving 5 mg in 1 mL of methanol (Merck, Darmstadt, Germany). Subsequent standards were prepared by its dilution in phosphate buffer.

50 mM phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$, Panreac, Barcelona, Spain) and 100 mM KCl (Merck, Darmstadt, Germany) solutions were used as supporting electrolyte. NaOH (J.T. Baker, Deventer, The Netherlands) was used to adjust the pH value.

2.2. Apparatus and software

SPCEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

Electrochemical measurements were made by a μAutolab electrochemical system with GPES software (Eco Chemie, Utrecht, The Netherlands).

The pH of the solutions was measured with a Crison Model 2002 (Barcelona, Spain) pHmeter.

Data analysis was processed with PROGRESS for the robust regression [26] and DETARCHI [27] for the capability of detection.

2.3. Biosensors manufacturing

A DEK 248 screen-printing system (DEK, Weymouth, UK), screen polyester mesh and polyurethane squeegees were used to fabricate the electrodes. Sequential layer deposition was performed on 0.5 mm thickness polyester films (HiFi Industrial Film, Dardilly, France) obtaining 44 screen-printed configurations of three electrodes each one (working, Ag/AgCl reference and counter electrode) per film (Fig. 1A).

The printing process began with a carbon conducting ink, which is cured at 60 °C for 30 min. This first layer defined not only the conductive tracks, but also the carbon counter and working electrodes. Next, a Ag/AgCl ink was screen-printed over it and cured at the same conditions. In order to prevent the conducting paths from the solution, the dielectric ink was finally screen-printed and cured at 80 °C for 30 min.

HRP containing ink was prepared by thoroughly mixing of the carbon ink with HRP (0.40%, w/w) [21,28]. The mixture was sonicated for 20 min and immediately screen-printed on the working electrodes (Fig. 1B). The resulting biosensors were cured at 40 °C for 1 h in an oven. The biosensors were kept at 4 °C when not in use.

2.4. Measuring procedures of HRP-biosensors

The HRP-biosensors were used without any further pretreatment. Electrochemical measurements were carried out in a batch system, using the modified SPCEs, at room temperature.

Cyclic voltammetric measurements were carried out in a cell containing 5 mL of a 100 mM KCl solution.

Chronoamperometric measurements were made in a cell containing 5 mL of a 50 mM phosphate buffer and 100 mM KCl solution, of pH 5, with constant stirring. An operational potential of −0.3 V was applied vs screen-printed Ag/AgCl reference electrode. Once a stable baseline was registered, hydrogen peroxide was added into the electrochemical cell to a final concentration of 1.4 mM. After reaching a steady-state current again, successive additions of OTA were performed.

3. Results and discussion

3.1. Electrochemical characterization of HRP-SPCEs

The suitability of screen-printed technology for the construction of OTA sensors by immobilization of HRP via pyrrole electropolymerization on SPCEs has been previously shown [25]. During the fabrication process the carbon ink can be contaminated with no conductive substances, which would reduce the final electrode sensitivity. Moreover, the graphite particles could come down to the lower part of the electrode in the curing process, because of the gravity effect, leaving on the upper part a big amount of binder material. Therefore, mechanical (sanding the electrode surface by a thin grain sandpaper) and electrochemical activations (cycling potential between 2 and −2 V vs screen-printed Ag/AgCl reference electrode, scan rate, 100 mV s^{-1}, 20 scans, in a 0.1 M KCl solution) of those SPCEs were carried out before the enzyme immobilization [25]. The cyclic voltammetry of ferricyanide, chosen as a marker to analyze the effect of this pretreatment, shows that the SPCEs fabricated for that purpose required activation in order to enhance their sensitivity (Fig. 2A).

This additional step appears quite inconvenient in the case of biosensors manufactured with enzyme-containing inks. Therefore, a different commercial ink (C10903P14, Gwent Electronic Materials, Torfaen, UK) was tested in the manufacturing of SPCEs according to the procedure described in Section 2.3. The sensitivity of both SPCEs was compared recording cyclic voltammograms of a 1 mM ferricyanide solution in 100 mM KCl (Fig. 2B). It was observed that the carbon ink used in the present work allows the construction of SPCEs with even higher sensitivity without any activation pretreatments than those previously fabricated after activation. Therefore, the combination of the carbon ink marketed by Gwent with the HRP enzyme seems to be very attractive in order to obtain an HRP-containing ink.

The successful HRP immobilization on the SPCEs by screen-printing is confirmed by the cyclic voltammograms registered in a 100 mM KCl solution (Fig. 3). The direct electron transfer behaviour of the immobilized HRP gives rise to a cathodic peak at 0.3 V approximately. This peak corresponds to the reduction of all electroactive HRP-Fe(III) in the film to HRP-Fe(II) on the forward cathodic scan, with full conversion of HRP-Fe(II) back to HRP-Fe(III) on the reversed anodic scan [29].

The long-term stability of the developed HRP-SPCEs was periodically checked by confirming the activity of the HRP immobilized as described above. The biosensors kept their activity after 8 weeks.

3.2. Electrochemical response to OTA at the biosensor

OTA concentration in a solution was related with the anodic peak current corresponding to the chronoamperometric

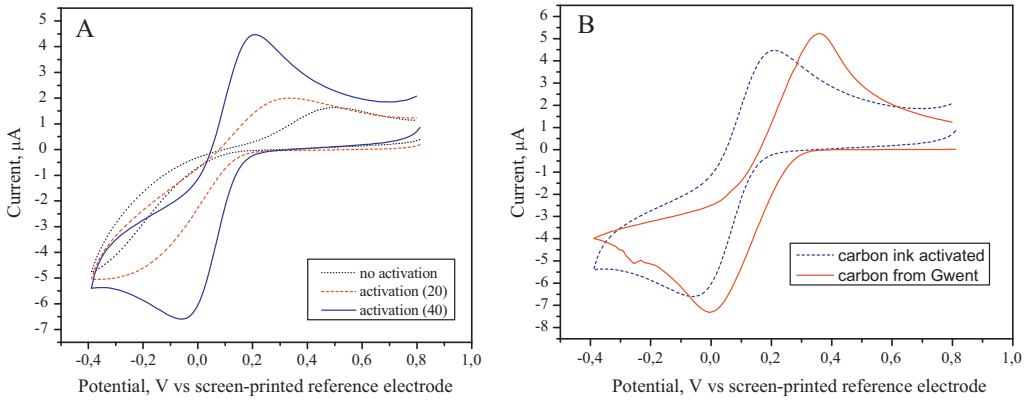
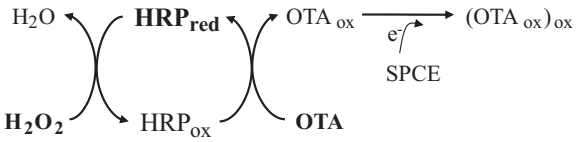


Fig. 2. (A) Cyclic voltammograms recorded for a 1 mM ferricyanide solution in 100 mM KCl using SPCEs with no activation pretreatment (dot line), SPCEs with mechanical and electrochemical activation consisting of 20 scans (dash line) and SPCEs with mechanical and electrochemical activation consisting of 40 scans (solid line). (B) Cyclic voltammograms recorded for a 1 mM ferricyanide solution in 100 mM KCl using SPCEs with electrochemical activation consisting of 40 scans (dash line) and SPCEs fabricated with a carbon ink from Gwent (solid line).

current registered for a new oxidation of the oxidation product of OTA [30], which is formed in the enzymatic reaction with HRP according to the following mechanism [25],



The chronoamperometric current depends directly on experimental variables such as H_2O_2 concentration ($C_{H_2O_2}$), pH of the solution and applied potential (E_{ap}) [25]. Their effect on the current response for OTA, when using HRP modified SPCEs, was simultaneously estimated using the experimental design methodology [25,31–34]. In order to get the maximum chronoamperometric current of a 2.40 μM OTA solution, a 2^3 central composite design was carried out taking the following high (+) and low (−) levels for each factor,

pH (+) = 9.0	E_{ap} (+) = −0.3 V	$C_{H_2O_2}(+) = 1.4 \times 10^{-3}$ M
pH (−) = 5.0	E_{ap} (−) = 0.1 V	$C_{H_2O_2}(-) = 6.0 \times 10^{-4}$ M

From this optimization process, the optimum experimental parameters values that led to a sensitive and selective OTA biosensor were:

pH = 5	E_{ap} = −0.3 V	$C_{H_2O_2}$ = 1.4 mM

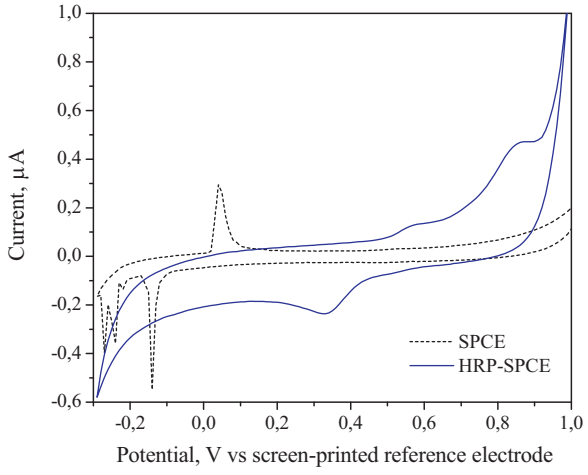


Fig. 3. Cyclic voltammograms of a SPCE (dash line) and an HRP-SPCE (solid line) in a 100 mM KCl solution, at 50 mV s^{-1}.

Fig. 4 shows a chronoamperogram of the biosensor for successive additions of 100 μL of a 1.24 μM OTA solution, registered under the optimum conditions.

The performance of the developed biosensors has been checked using the well-known figures of merit reproducibility, repeatability and capability of detection.

The reproducibility of the HRP-biosensor was investigated by chronoamperometric measurements. Different calibration curves were recorded under the optimum conditions in the concentration range from 23.85 to 203.28 nM, using different biosensors (Table 1). The slopes of the obtained current vs concentration regressions were used to evaluate these parameters in terms of relative standard deviation (RSD). Anomalous points in these regressions would alter intercept and slope values, leading to wrong conclusions. Thus, those points were first detected and eliminated, in such a way the regression parameters would be optimally evaluated [26]. The biosensor showed an acceptable reproducibility with a RSD of 10% (n = 4).

In the same way, repeatability was analyzed. Thus, consecutive calibration curves were recorded under the optimum conditions in the concentration range from 23.85 to 203.28 nM, using the same HRP-SPCE. It was observed that the biosensor retained a 30% of the initial sensitivity after the third calibration (Fig. 5). The lost in the enzyme bioactivity could be attributed to the enzyme fouling

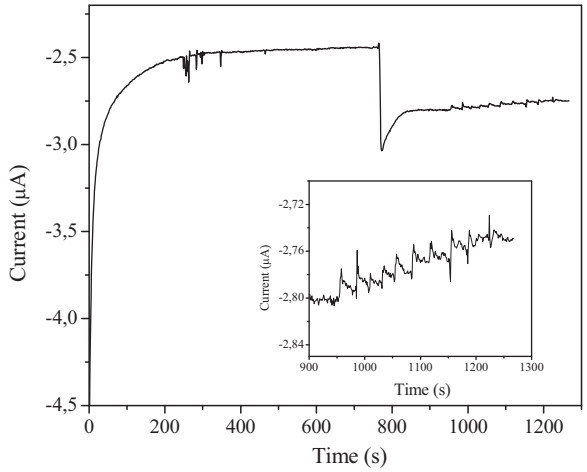


Fig. 4. Chronoamperogram recorded for successive additions of 100 μL of a 1.24 μM OTA solution using an HRP-SPCE under the optimum conditions: pH, 5; operational potential, −0.3 V; hydrogen peroxide concentration, 1.4 mM.

Table 1
Regression parameters corresponding to several calibration curves registered in the concentration range from 23.85 to 203.28 nM of OTA, using different HRP biosensors. Precision, in terms of RSD of the different sensitivities, 10% ($n = 4$).

	Linear range (nM)	Intercept (nA)	Sensitivity $\times 10^{-9}$ (nA M^{-1})	S_{yx}	R^2
Calibration I	23.85–203.28	31.00	1.04	3.29	0.996
Calibration II	46.79–186.00	45.01	0.81	3.86	0.992
Calibration III	68.89–203.28	72.28	0.91	2.38	0.993
Calibration IV	23.85–168.14	−6.17	0.92	5.36	0.990

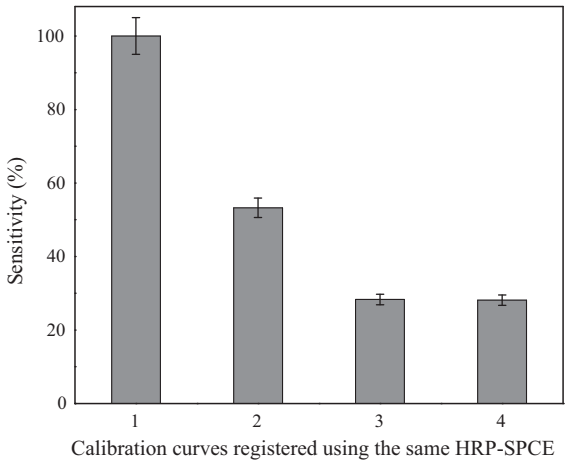


Fig. 5. HRP-SPCE repeatability in terms of sensitivity.

due to reaction products. However, the poor value obtained for the repeatability of the HRP-SPCEs is not significant in the manufacturing of this kind of biosensors, taking into account their disposable character.

This analytical procedure for OTA determination has been also characterized by the calculation of the capability of detection for a given probability of false positive (α) and negative (β) [35,36]. Validated linear regressions between the concentration and the analytical signal were used to calculate this figure of merit, using the DETARCHI program [27]. The average capabilities of detection obtained were 26.77 ± 3.61 nM ($\alpha = 0.05$ and $\beta = 0.05$, $n = 3$).

In order to check the performance of the developed biosensor for OTA determination, beer matrices were analyzed using the standard addition method. The chronoamperograms were recorded under the optimum conditions.

Beer samples were spiked with OTA to provide a 1.24 μM solution. Then, 100 μL of the spiked beer was placed into the electrochemical cell, containing 5 mL of buffer solution and 100 μL of H_2O_2 in a final concentration of 1.4 mM, followed by successive additions of 100 μL of a 1.24 μM OTA solution. The calibration curves were optimally evaluated [26]. The concentration of OTA found was 1.22 ± 0.21 μM ($n = 3$, $\alpha = 0.05$), with an average recovery of 98.70% and a RSD smaller than 7%.

4. Conclusions

The use of HRP based biosensors using SPCEs allows selective and sensitive amperometric determination of OTA. The main advantage of the developed method consists of the enzyme immobilization process. The direct screen-printing of modified ink with the enzyme represents an easier and lower cost procedure in the development of suitable biosensors for the determination of OTA. Moreover, the durability and robustness of the sensor have been improved thanks to this new manufacture process being less time-consuming, avoiding contamination and manipulation processes, which can lead to false analytical results.

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