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Sensitive enzyme-biosensor based on screen-printed electrodes for Ochratoxin A

M. Asunción Alonso-Lomillo[a,*], Olga Domínguez-Renedo[a], Liliana Ferreira-Gonçalves[b], M. Julia Arcos-Martínez[a]

[a] Analytical Chemistry Department, Faculty of Sciences, University of Burgos, Pza. Misael Bañuelos s/n, 09001 Burgos, Spain
[b] Faculty of Sciences, University of Beira Interior, Covilha, Portugal

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ABSTRACT

Horseradish peroxidase has been successfully immobilized in a polypyrrole matrix onto disposable screen-printed carbon electrodes for the selective detection of Ochratoxin A. The chronoamperometric determination of this mycotoxin has been optimized by experimental design methodology, which implied the join evaluation of pH of the buffer solution, applied potential and concentration of H_2O_2. The slopes of the calibration curves built under the optimum conditions of the experimental variables have been used to estimate the reproducibility and the repeatability of the developed biosensor for Ochratoxin A. Relative standard deviation values of 1.9% ($n = 5$ and $\alpha = 0.05$) and 7.1% ($n = 4$ and $\alpha = 0.05$) were obtained for reproducibility and repeatability, respectively. The capability of detection for this method was 0.1 ng mL^{-1} ($\alpha = 0.05$ and $\beta < 0.05$). The viability of the developed biosensor in the determination of Ochratoxin A in spiked beer and in roasted coffee has been shown, yielding average recoveries of 103% and 99%, in that order, with relative standard deviation values less than 5% ($n = 3$ and $\alpha = 0.05$) in both cases.

1. Introduction

Being one of the most abundant food-contaminating mycotoxins, Ochratoxin-A (OTA), which is produced by *Aspergillus ochraceus* and *Penicillium viricatum*, is common in cereals, coffee, spices, dried fruits, beers and wines. It has also been discovered in human and animal fluids (Alarcon et al., 2006; Kaushik et al., 2009; Oliveira et al., 2007; Prieto-Simon et al., 2008; Radi et al., 2009; Turner et al., 2009).

OTA has been classified as a possible carcinogenic compound for humans by the International Agency for Research on Cancer (Turner et al., 2009). OTA causes immunosuppression, immunotoxicity, fertility inhibition, as well as mutagenic and carcinogenic effects (Alarcon et al., 2006; Calcutt et al., 2001; Kaushik et al., 2009; Oliveira et al., 2007; Prieto-Simon et al., 2008; Radi et al., 2009; Turner et al., 2009). Therefore, the European Commission has fixed maximum levels for OTA in foodstuffs, which have been established in 3 μg/kg and 5 μg/kg for cereal products and roasted coffee, respectively (Commission Regulation No. 1881/2006 of 19 December 2006).

Many detection techniques, such as liquid chromatography coupled with immunoaffinity column or solid phase extraction cleanup, have been used for the determination of OTA in different kind of samples (Prieto-Simon et al., 2007; Turner et al., 2009). These methods involve expensive and time-consuming steps. In order to developed sensitive, accurate and fast response methods, electrochemical techniques have been commonly used in recent years (Alarcon et al., 2004, 2006; Calcutt et al., 2001; Fu, 2007; Kaushik et al., 2008, 2009; Khan and Dhayal, 2008, 2009; Liu et al., 2009; Oliveira et al., 2007; Prieto-Simon et al., 2008; Radi et al., 2009; Wang and Wang, 2008). The introduction of the screen-printed technology allows the use of sensors and biosensors in situ or for decentralised test, which is highly desirable, as well as the feasibility of miniaturizing them (Alarcon et al., 2004, 2006; Prieto-Simon et al., 2008; Radi et al., 2009).

Miniaturized transducers have particular relevance nowadays. In the development of bioanalytical devices, the controlled deposition of biomolecules over the working electrode deserves special attention, since it critically influences the performance of the enzyme-modified electrode.

Electropolymerization is an attractive method that allows reproducible, non manual, precise, uniform and thickness-controlled polymer coating without any limitation of the size, area and geometry of the surface (Shang et al., 2009). Pyrrole (Py) appears exceptionally convenient for achieving electropolymerization in the production of electrodes modified by electroactive polymeric films (Cuadrado et al., 1999). It has been intensively used in energy storage systems, biosensors and electronics (Li et al.,

* Corresponding author. Tel.: +34 947258818; fax: +34 947258831.
E-mail address: malomillo@ubu.es (M.A. Alonso-Lomillo).

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2005). Immobilization of a biomolecule, such as Horseradish peroxidase (HRP), in this electropolymerized film is a simple one-step method (Alonso-Lomillo et al., 2009b).

This paper describes an effective electrochemical approach for the selective detection of OTA using HRP-modified screen-printed carbon electrodes (SPCEs). The well-known mechanism of this biosensor involves the oxidation of native HRP by H_2O_2 to an intermediate compound, which is subsequently reduced by a substrate donor, OTA in this case, regenerating the native enzyme (Alonso-Lomillo et al., 2003; ElKaoutit et al., 2008; Serra et al., 2001). Therefore, OTA concentration in a solution can be related with the chronoamperometric current registered.

In order to develop a selective and sensitive sensor, experimental variables that strongly affect to the chronoamperometric response were evaluated by the experimental design methodology (Box et al., 1978). Thus, applied potential (E_{ap}), pH and concentration of H_2O_2 ($C_{H_2O_2}$), were optimized (Box et al., 1978; Lewis et al., 1999). The performance of the developed biosensors was checked in terms of reproducibility, repeatability, capability of detection and application to complex matrices, such as beer and roasted coffee.

2. Experimental

2.1. Reagents

Several inks were used in the fabrication of SPEs, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Acheson Colloiden (Scheemda, The Netherlands).

All solutions were prepared with water purified with a Milli-Q device which provided a resistivity of 18.2 MΩ cm. Nitrogen (99.99%) was used to remove dissolved oxygen.

0.02% (w/v) solutions of HRP (EC 1.11.1.7, Sigma, Steinheim, Germany), 0.05 M Py (Sigma, Steinheim, Germany) and 0.1 M $LiClO_4$ (Aldrich, Steinheim, Germany) were used.

Stock standard solution of OTA (Sigma, Steinheim, Germany) was prepared by dissolving 5 mg in 1 mL of methanol. Subsequent standards were prepared by dilution in water.

0.05 M phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$, Panreac, Barcelona, Spain) and 0.1 M KCl (Merck, Darmstadt, Germany) solution was used as supporting electrolyte. NaOH (J.T. Baker, Deventer, The Netherlands) was used to adjust the pH value.

2.2. Apparatus and software

SPEs 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 with 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) pH meter.

Data analysis was processed with a STATGRAPHICS PLUS software package for the experimental design process (STATGRAPHICS, Copy 1994–2001), PROGRESS for the robust regression (Rousseeuw and Leroy, 1989) and DETARCHI for the capability of detection (Sarabia and Ortiz, 1994).

3. Methods

3.1. SPEs preparation

A DEK 248 screen-printing system (DEK, Weymouth, UK), screen polyester mesh and polyurethane squeegees were used to fabricate the electrodes, as described in previous works (Alonso-Lomillo et al., 2009a,b). Sequential layer deposition has been performed on a polyester film (0.5 mm thickness) obtaining 44 screen-printed configurations of three electrodes each one (working, reference and counter electrode) per film. 4 mm^2 carbon working, 8 mm^2 carbon auxiliary and 6 mm^2 Ag/AgCl reference electrodes were design.

Before modifying the SPCEs a mechanical cleaning was carried out, sanding the counter and working electrode surface by thin grain sandpaper. Then, the working electrode surface is activated by recording 20 cycle voltammograms between 2 and −2 V, scan rate, 100 mV s^{-1}, in a 0.1 M KCl solution.

3.2. Functionalization of SPCEs

The HRP immobilization procedure was carried out according to the described anywhere else (Alonso-Lomillo et al., 2009b). Briefly, a pre-layer of polypyrrole (PPy) (0.1 M $LiClO_4$, 0.05 M Py) was first deposited by scanning the potential between −0.1 and +0.9 V vs screen-printed Ag/AgCl reference electrode, at a scan rate of 10 mV s^{-1}, for one complete cycle, at room temperature.

The HRP/PPy film was subsequently grown onto the carbon working electrode, in a solution containing 0.1 M $LiClO_4$, 0.05 M Py, and 0.02% (w/v) HRP solutions at room temperature. The potential domain was scanned twice between −0.1 and +0.9 V vs screen-printed Ag/AgCl reference electrode, at a scan rate of 10 mV s^{-1}.

After each calibration curve, the biosensor was regenerated by immersing it in an unstirred blank buffer solution for approximately 30 min at 4 °C, in order to wash away the analyte from the polymer film.

4. Results and discussion

The oxidation behaviour of OTA was first checked by cyclic voltammetry in phosphate buffer at different pH. As it has been previously reported (Calcutt et al., 2001; Oliveira et al., 2007), a single irreversible anodic peak at ca. 0.85 V vs screen-printed Ag/AgCl was observed.

HRP-based biosensors can take advance of this oxidation capability, leading to a selective OTA analysis. In this way, the electroanalytical determination of OTA can be carried out by chronoamperometry measuring the anodic peak current corresponding to oxidation of the oxidation product of OTA (Oliveira et al., 2007), which is formed in the enzymatic reaction with HRP (Fig. 1).

Electrochemical experiments carried out by Calcutt et al. (2001) predict that peroxidases would be unable to oxidize protonated OTA, but would be able to oxidize OTA^- to the phenolic radical. The latter undergoes oxidation at potentials considerably lower than phenols. In order to demonstrate the enzymatic mechanism proposed, OTA_{ox} was produced with peroxidase and OTA in solution, and then, the electrochemical oxidation of the product was studied by chronoamperometry using a bare SPCE. It can be seen

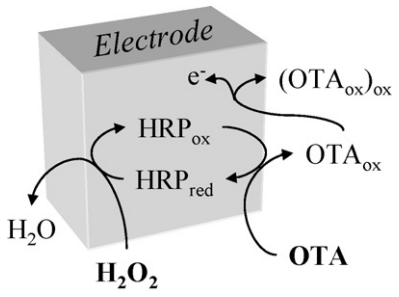

Fig. 1. Mechanism of mediated electron transfer at a HRP-modified SPCE.

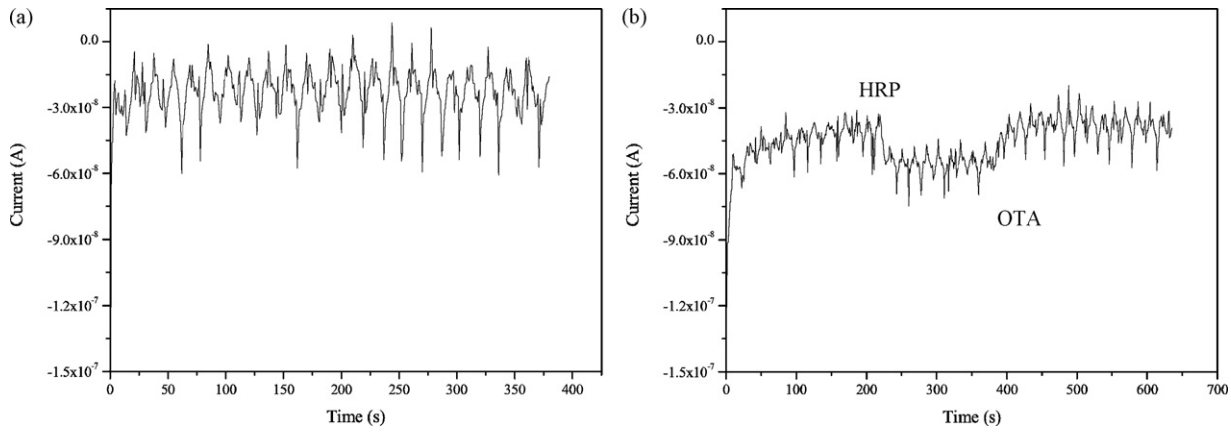

Fig. 2. Chronoamperograms recorded for a 2×10^{-4} M OTA solution, using a bare SPCE, in 3 mL phosphate buffer solution pH 5 (a) and in 3 mL phosphate buffer solution pH 5 after the addition of a HRP solution to a final concentration of 69 μg mL^{-1} (b). Applied potential, −0.3 V.

in Fig. 2b the oxidation current recorded after the addition of a 2×10^{-4} M OTA solution (at 390 s) in a 3 mL phosphate buffer solution pH 5 containing a 69 μg mL^{-1} HRP solution. No current was observed when the experiment was carried out without HRP solution (Fig. 2a).

The electrochemical characterization of this biosensor also included experiments carried out in absence of HRP and H_2O_2, obtaining no chronoamperometric response to OTA additions. Therefore, OTA concentration in a solution can be related with the chronoamperometric current registered.

The chronoamperometric current depends directly on experimental variables such as H_2O_2 concentration, the pH of the solution and the applied potential (E_{ap}). Their effect on the current response was estimated simultaneously. This optimization process tends to be more efficient than the "one-at-a-time", since it also implies the estimation of the interaction between the experimental variables. Therefore, a 2^3 central composite design was planned with the aim of finding the best conditions of these variables to get the maximum chronoamperometric current of a 1.92×10^{-6} M OTA solution. In order to define the experimental region, the values corresponding to the high (+) and low (−) levels, and to the central point (0) for each factor were selected,

pH (+) = 9.0	E_{ap} (+) = −0.3 V	$C_{H_2O_2}$ (+) = 1.4×10^{-3} M
pH (−) = 5.0	E_{ap} (−) = 0.1 V	$C_{H_2O_2}$ (−) = 6.0×10^{-4} M
pH (0) = 7.0	E_{ap} (0) = −0.1 V	$C_{H_2O_2}$ (0) = 1.0×10^{-3} M

Then, the experiments corresponding to the 17 possible combinations, bearing in mind the three replicates in the central point necessary to estimate the residual value, were carried out.

The analysis of the obtained results was carried out using the STATGRAPHICS PLUS software package (STATGRAPHICS, Copy 1994–2001). pH of the buffer solution was obtained not to be a significant variable according to the analysis of the variance. When using the same E_{ap} and $C_{H_2O_2}$, high currents were registered for the experiments carried out at a pH of 5. Therefore, pH 5 was taken as optimum value. The maximum detected by observing the response surface (Fig. 3) at pH 5, led us to select an E_{ap} of −0.3 V. In this way, a higher $C_{H_2O_2}$ was required, although it is well known that it may produce inhibition of the activity of the enzyme (Serra et al., 2003). Thus, the value corresponding as high (+) in the experimental region, 1.4 mM, was selected as optimum for $C_{H_2O_2}$.

It can be seen in Fig. 4a chronoamperogram recorded under the optimum conditions for successive additions of 100 μL of a 1.24×10^{-8} M OTA solution (initial volume of buffer solution into the electrochemical cell, 5 mL). In this way, the chronoamperometric current registered increased up to 8 times in relation to the

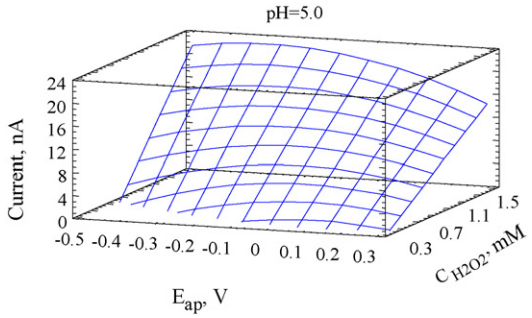

Fig. 3. Response surface for the 2^3 central composite design in the optimization of the experimental variables.

minimum current obtained, leaving out the experimental points where nil response were obtained.

Different calibration curves were recorded under the optimum conditions in the calibration range from 0.24 to 2.06 nM, using different biosensors to check the reproducibility and the same one in the case of repeatability. Current vs concentration regression parameters were optimally evaluated (Alonso-Lomillo et al., 2003; Ortiz et al., 2006; Rousseeuw and Leroy, 1989), which implied the detection and the elimination of the anomalous points that would alter intercept and slope values (Supplementary information, Fig.

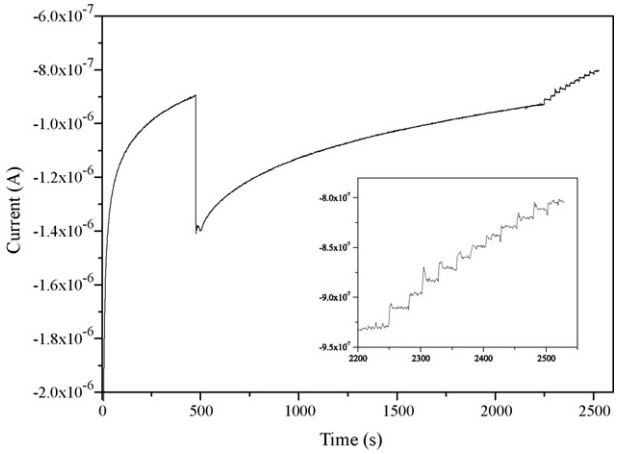

Fig. 4. Chronoamperogram recorded for successive additions of 100 μL of a 1.24×10^{-8} M OTA solution, using an HRP-biosensor, under the optimum conditions: buffer solution pH 5 (5 mL), E_{ap} = −0.3 V and $C_{H_2O_2}$ = 1.4 mM. H_2O_2 was added after 500 s. (Inset) Registered current zoom from 2200 to 2550 s.

Table 1
Minimum detectable concentration (nM) obtained for probabilities of both false positive and negative errors equal to 0.05 in the determination of OTA by chronoamperometry using a HRP-biosensor. Concentration of the lowest calibration standard, 0.24 nM.

	Intercept	Slope	S_{yx}	R^2	Minimum detectable net concentration	
Calibration I	4.75	54.66	1.29	0.998	0.10 nM	0.040 ng mL^{-1}
Calibration II	7.50	54.59	1.14	0.998	0.10 nM	0.040 ng mL^{-1}
Calibration III	11.53	52.35	0.84	0.999	0.09 nM	0.036 ng mL^{-1}

Table 2
Recovery (%), RSD and 95% confidence interval for mean for OTA spiked in beer and for extraction of OTA from coffee, using a HRP-biosensor.

OTA spiked level (M)	OTA detected (M)	Recovery (%)	RSD	95% confidence interval for mean
Beer				
	1.22×10^{-6}	98.38		
1.24×10^{-6}	1.28×10^{-6}	102.99	4.89%	$[1.28 \pm 0.15] \times 10^{-6}$
	1.34×10^{-6}	108.45		
Roasted coffee				
	5.09×10^{-7}	–		
–	5.03×10^{-7}	–	1.28%	$[5.09 \pm 0.16] \times 10^{-7}$
	5.16×10^{-7}	–		
Spiked roasted coffee				
	9.92×10^{-7}	101.11%		
4.72×10^{-7}	9.78×10^{-7}	99.68%	2.08%	$[9.74 \pm 0.50] \times 10^{-7}$
	9.52×10^{-7}	97.06%		

1s). The slopes of these calibrations were used to evaluate these parameters in terms of relative standard deviation (RSD), yielding values of 1.9% ($n = 5$ and $\alpha = 0.05$) and 7.1% ($n = 4$ and $\alpha = 0.05$) for reproducibility and repeatability, respectively.

The capability of detection has been calculated for a probability of false positive (α) and negative (β) (ISO11843), using the DETARCHI program (Sarabia and Ortiz, 1994). The characteristic curves of detection have been constructed using the data from experimental calibrations recorded under the optimum conditions in the calibration range from 0.24 to 2.06 nM, once they had been validated as it has been above-mentioned. It can be seen in Table 1 that the minimum detectable net concentration obtained for $\alpha = \beta = 0.05$ is higher than the corresponding first value of the standards, 0.10 ng mL^{-1}. Therefore, from an analytical point of view, the latter has been taken as the capability of detection, for $\alpha = 0.05$ and $\beta < 0.05$, of this method (Ortiz et al., 2003).

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

Beer was spiked with OTA to provide a 1.24×10^{-6} M 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, following by successive additions of 100 μL of a 1.24×10^{-6} M OTA solution. The calibration curves were optimally evaluated by LMS. Table 2 displays the results obtained in terms of recovery, RSD and 95% confidence interval for mean. The concentration of OTA found was $[1.28 \pm 0.15] \times 10^{-6}$ M ($n = 3$, $\alpha = 0.05$), with an average recovery of 103.27% and a RSD smaller than 5%.

In the case of roasted coffee samples, OTA was first extracted by mixing 25 g of the coffee with 125 mL of chloroform and 12.5 mL of water (Ahmed et al., 2007). The mixture was stirred for 30 min and filtered prior to use. Again, the quantification of OTA was carried out in triplicate by the standard addition procedure. Successive additions of 100 μL of a 1.24×10^{-7} M OTA solution were performed into the electrochemical cell containing the OTA solution extracted from the coffee. The concentration of OTA found was $[5.09 \pm 0.16] \times 10^{-7}$ M ($n = 3$, $\alpha = 0.05$, RSD = 1.28%) as it can be seen in Table 2.

To test the viability of this procedure in the determination of OTA in roasted coffee, recovery studies were also performed with this sample. The total amount of OTA in a spiked sample, 9.81×10^{-7} M, was determined by standard addition in triplicate. The concentration of OTA found was $[9.74 \pm 0.50] \times 10^{-7}$ M ($n = 3$, $\alpha = 0.05$, RSD = 2.08%), with an average recovery of 99.28% (Table 2).

On the basis of these data the method can be considered appropriate to be applied to OTA determination in these kinds of samples.

5. Conclusions

This work shows a new HRP based biosensor for the sensitive determination of OTA in synthetic samples, without any pre-treatment of the sample. PPy matrices have been used to immobilized the enzyme, producing reproducible, 1.9% ($n = 5$ and $\alpha = 0.05$), and repeatable sensors, 7.1% ($n = 4$ and $\alpha = 0.05$), for the quantification of this mycotoxin. These figures of merit have been calculated from the slopes of the calibration curves registered in the calibration range from 0.24 to 2.06 nM under the optimum experimental conditions. These conditions were evaluated using the experimental design methodology, which allows analyze an experimental domain with the minimum number of experiments and takes into account the interaction between variables. The optimum experimental conditions for the chronoamperometric determination, found by the experimental design methodology, have been pH 5, −0.3 V and a concentration of 1.4 mM of a H_2O_2 solution. A significant parameter of an analytical procedure is the capability of detection, which has been calculated taking into account the probability of false positive and negative. The average minimum detectable net concentration obtained mathematically, 0.039 ng mL^{-1} ($n = 3$ and $\alpha = \beta = 0.05$), is slightly higher than the first value of the standards used to built the needed calibration curves for this purpose, so the capability of detection for the proposed method has been set as 0.10 ng mL^{-1} ($\alpha = 0.05$ and $\beta < 0.05$). The biosensors have been finally applied to the determination of OTA in spiked beer samples, without any pre-treatment, and to the OTA extracted from roasted coffee. The performance of the HRP-biosensors has been shown in both cases, yielding average recoveries of 103% and 99%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.024.

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