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Disposable biosensors for determination of biogenic amines

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ABSTRACT

This work reports monoamine oxidase (MAO)/horseradish peroxidase (HRP) and diamine oxidase (DAO)/horseradish peroxidase (HRP) based biosensors using screen-printed carbon electrodes for the determination of biogenic amines (BA). The enzymes have been covalently immobilized onto the carbon working electrode, previously modified by an aryl diazonium salt, using hydroxysuccinimide and carbodiimide. The detection has been performed by measuring the cathodic current due to the reduction of the mediator hydroxymethylferrocene at a low potential, 250 mV vs screen-printed Ag/AgCl reference electrode. The experimental conditions for the enzymes immobilization, as well as for the main variables that can influence the chronoamperometric current have been optimized by the experimental design methodology. Under these optimum conditions, the disposable biosensors have been characterized. A linear response range from 0.2 up to 1.6 μM and from 0.4 to 2.4 μM of histamine was obtained for DAO/HRP and MAO/HRP based biosensors, respectively. The biosensor construction was highly reproducible, yielding relative standard deviations of 10% and 11% in terms of sensitivity for DAO/HRP and MAO/HRP based biosensors, respectively. The capability of detection, 0.18 ± 0.01 μM in the case of DAO/HRP and 0.40 ± 0.04 μM ($\alpha = 0.05$ and $\beta = 0.005$) for MAO/HRP based biosensors, and the biosensor sensitivity towards different BA has also been analyzed. Finally, the developed biosensors have been applied to the determination of the total amine content in fish samples.

1. Introduction

Biogenic amines (BA) are synthesized and degraded during normal metabolism of animals, plants and microorganisms [1–3]. Histamine (His), putrescine (Put), cadaverine (Cad), tyramine (Tyr), tryptamine (Trypt), spermine (Spm) and spermidine (Spd) are considered to be some of the most important BA in food and in human body.

BA detection has been shown to be a valuable tool in different analytical purposes, such as cancer markers [4,5] or for assessing the freshness and quality of a wide variety of foodstuffs [1–4,6–13].

Various methods have been developed for separation and quantification of BA and among then all, electrochemical biosensors [1–17]. Biosensors, which combine biological recognition through enzyme specificity with construction simplicity, have been reported as a good and cheap alternative for the traditional ones. In this way, amine oxidases (AO) based electrodes, which catalyze the oxidative deamination of primary amines, diamines and substituted amines to produce aldehyde, ammonia and hydrogen peroxide, have been reported [2–4,6,8–13,15,18,19]. Measurements of the oxygen consumption or the hydrogen peroxide production are commonly used for the quantification of BA in different samples. The latter implies the application of high operational potentials, which leads to high background currents and to the non-selectivity of the electrodes [14,16,20]. Coupled biosensors, which combine AO and peroxidases, were then developed to avoid these drawbacks [1,4,13,14,16,17,20].

Enzymatic sensors reduce the time needed for analysis and offer a rapid screening method for industrial food quality testing. The use of disposable transducers, such as screen-printed electrodes (SPEs), boosts the intrinsic characteristics of enzymatic sensors. Screen-printing technology, which offers design flexibility, process automatization and good reproducibility in the transducers fabrication, had been shown as a method for mass production of biosensors at low cost [21].

In the case of BA, however, few results have been described in the literature. Chemnitius and Bilitewski [10], as well as Lange and Wittmann [6] have described AO-sensors based on platinum SPEs, using a glutaraldehyde – bovine serum albumin cross-linking immobilization procedure, for the determination of His, Put and Tyr. Both procedures involve the measurement of the H_2O_2 produced by applying a potential higher than 600 mV.

Based on this context, this work reports for the first time monoamine oxidase (MAO)/horseradish peroxidase (HRP) and

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diamine oxidase (DAO)/HRP based biosensors using screen-printed carbon electrodes (SPCEs) for the determination of BA, in order to reduce the above-mentioned high operational potential. These disposable bienzymatic sensors offer the possibility of carrying out sensitive BA determinations in situ.

MAO/HRP and DAO/HRP have been covalently immobilized onto the carbon working electrode, previously modified by an aryl diazonium salt [22,23], using *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC).

The experimental conditions for these enzymes immobilization on the SPCEs, as well as the main variables that can influence the chronoamperometric response have been optimized with respect to histamine using the experimental design methodology [24–26]. Under these optimum conditions, reproducibility, repeatability, capability of detection and biosensor sensitivity towards different BA have been analyzed. Finally, the developed biosensors have been applied to the determination of the total amine content in salted anchovy samples.

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 that provided a resistivity of 18.2 MΩ cm. Nitrogen (99.99%) was used to remove dissolved oxygen.

3 mM solution of 4-nitrobenzenediazonium tetrafluoroborate ($N_2C_6H_4NO_2BF_4$) (Sigma–Aldrich, Steinheim, Germany) was prepared in acetonitrile containing 0.1 M tetrabutylammonium tetrafluoroborate (NBu_4BF_4) (Sigma–Aldrich, Steinheim, Germany).

120 mg mL^{-1} solutions of DAO (EC 1.4.3.6; activity, 0.18 units mg^{-1}; Sigma–Aldrich, Steinheim, Germany) and 5 mg mL^{-1} solutions of HRP (EC 1.11.1.7, activity, 191 purpurogallin units mg^{-1}; Sigma–Aldrich, Steinheim, Germany) in 100 mM phosphate buffer pH 7.4 were used as stock solutions.

5 mg mL^{-1} solutions of MAO (EC 1.4.3.4; activity, 92 units mg^{-1}) were supplied by Sigma (Sigma–Aldrich, Steinheim, Germany) and used as received.

40 mM NHS (Sigma–Aldrich, Steinheim, Germany) and 80 mM EDC (Fluka, Steinheim, Germany) solutions were prepared in 10 mM phosphate buffer pH 6.7, except for the optimization of the experimental factors in the enzymes immobilization procedure.

10 mM stock standard solutions of His (Sigma–Aldrich, Steinheim, Germany), Put (Sigma–Aldrich, Steinheim, Germany), Cad (Sigma–Aldrich, Steinheim, Germany), Tyr (Fluka, Steinheim, Germany), Spd (Across Organics, Geel, Belgium) and Spm (Across Organics, Geel, Belgium) were prepared by dissolving the appropriate amount in water. Trypt solutions (Across Organics, Geel, Belgium) were prepared in ethanol–water (10:90, v/v).

50 mM phosphate buffer (Panreac, Barcelona, Spain) and 100 mM KCl (Merck, Darmstadt, Germany) solution were used as supporting electrolyte. 1 M 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).

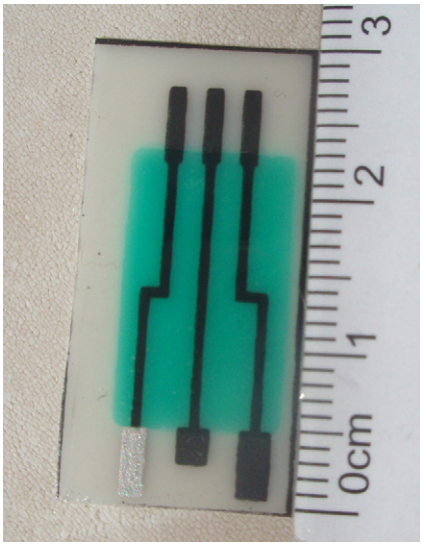


Fig. 1. Picture of a homemade SPCE used to build the AO/HRP based biosensors. 4 mm^2 carbon working (centre), 8 mm^2 carbon auxiliary (right) and 6 mm^2 Ag/AgCl reference electrodes (left) were design.

Electrochemical measurements were made with a μAutolab II 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 [27], PROGRESS for the robust regression [28] and DETARCHI for the capability of detection [29].

3. Methods

3.1. Manufacturing of SPCEs and electrochemical pre-treatment

Three electrode system consisting of carbon working electrode, carbon counter electrode and Ag/AgCl reference electrode constitute the biosensors configuration used (Fig. 1). 44 screen-printed configurations were printed by sequential layer deposition on 0.5 mm thickness polyester films (HiFi Industrial Film, Dardilly, France). The printing process began with a carbon conducting ink, which is cured at 120 °C for 20 min. This first layer defined not only the conductive tracks, but also the carbon counter and working electrodes. Next, Ag/AgCl ink is 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 in an ultraviolet oven.

An electrochemical pre-treatment of the carbon working electrode was carried out prior the use of the SPCEs. First, a mechanical cleaning was performed sanding the electrode surface by thin grain sandpaper. Then, the working electrode surface is activated by recording 20 cycle voltammograms between 2 and −2 V vs screen-printed Ag/AgCl reference electrode, scan rate, 100 mV s^{-1}, in a 100 mM KCl solution [22,30].

3.2. SPCEs surface modification

Electrochemically pretreated electrodes were immersed in a 3 mM solution of $N_2C_6H_4NO_2$ BF_4. The grafting process was conducted by sweeping the potential between +800 and −400 mV vs screen-printed Ag/AgCl reference electrode at a scan rate of 200 mV s^{-1} [22,23]. Two sequential scans were performed.

Then, the aryl diazonium salt modified SPCE was immersed in a deoxygenated 9:1 water/ethanol solution, 0.1 M KCl. Then, the potential was swept between +200 and −1800 mV vs screen-printed Ag/AgCl reference electrode at a scan rate of 200 mV s^{-1}, recording two scans [22,23].

The covalent immobilization of AO and HRP was carried out according to the procedure described anywhere else [22,23,31]. In the case of DAO/HRP, the working electrode was first washed using 10 mM phosphate buffer pH 6.7. Then, 3 μL of a solution DAO:HRP (1:3.35, v/v) was dropped over it. Next, 2 μL of a 40 mM NHS solution and 2 μL of a 80 mM EDC solution in 10 mM phosphate buffer pH 6.7 were added. It was left to react for 90 min at 4 °C. The same conditions were used for MAO/HRP biosensors, except for the enzymes solution that was MAO:HRP (1:4, v/v).

Phosphate buffer pH and enzymes concentration ratio used in this immobilization procedure have been optimized using the experimental design methodology.

The biosensors were finally washed in a stirred 50 mM phosphate buffer and 100 mM KCl solution at pH 9.3 for 1 min, in order to eliminate the enzyme not covalently attached. The biosensors were stored in a blank buffer solution at 4 °C.

3.3. Measuring procedure of the enzyme SPCEs sensors

Electrochemical measurements were carried out in a batch system, by chronoamperometry using the modified SPCEs. All measurements were made at room temperature in a cell containing 5 mL of a 50 mM phosphate buffer and 100 mM KCl solution, of the desired pH, with constant stirring.

The working electrode operated at 250 mV vs screen-printed Ag/AgCl reference electrode, except for the experimental variables optimization process. A volume of 100 μL of a 1.24 mM or 1.00 mM hydroxymethylferrocene (HOMeFc) solution in the case of DAO/HRP or MAO/HRP based biosensors, respectively, was added after reaching a stable baseline (normally after 15 min). Then, the corresponding sample was added.

Characterizations of the biosensors, except for substrate specificity determinations, were done using His.

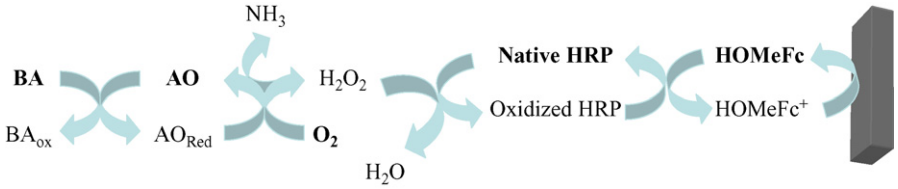
3.4. Real samples preparation

Salted anchovy samples were obtained from a local market. The extraction has been carried out using phosphate buffer according to the procedure described by Carelli et al. [2]. Thus, 2 g of the previously homogenized real sample and 4 mL of phosphate buffer pH 9.3 were mixed in a vortex sample for 1 min. The mixture was placed in an ultrasound bath for 10 min, and then, it was centrifuged at +4 °C for 10 min at 15,000 rpm. The supernatant was recovered. The procedure was repeated and the extracts were gathered and made up to volume with phosphate buffer into a 10 mL flask.

4. Results and discussion

4.1. Optimization of the biosensor working conditions

BA can be easily quantified using an AO/HRP biosensor by chronoamperometry, measuring the cathodic current due to the reduction of the mediator used in the enzymatic reaction, HOMeFc, according to the following scheme [6,13,14],



The construction of such biosensors implies the fabrication and functionalization of the SPCEs to be used as transducers, as well as the immobilization of the enzymes on the working electrode.

The functionalization of the carbon working electrode by aryldiazonium salts resulted on amino groups extended away from the surface (Section 3.2). These groups, which were easily transformed to –NH_2 in a water/ethanol solution (Section 3.2), provide a physical and/or electrical connection with the enzymes to be immobilized. Amide bonds between these groups and the carboxylic groups in the AO/HRP enzymes surface led to the covalent immobilization. NHS and EDC were used as amidization accelerators [32].

The most influence experimental factors in order to obtain a successful enzymes immobilization have been shown to be the ratio of enzymes (V_{AO}/V_{HRP}) and pH of the 10 mM phosphate buffer in which the NHS and EDC solutions were prepared [22]. In order to arrange these factors and their interactions according to their influence on the His chronoamperometric response, as quickly and as surely as possible with the best possible precision, a 2^2 central composite design was carried out [24–26]. Then, 11 experiments were carried out according to the combinations between high (+), low (−) and central (0) level of each factor,

$$\frac{V_{AO}}{V_{HRP}}(+) = 5 \qquad \mathrm{pH}(+) = 8.0$$

$$\frac{V_{AO}}{V_{HRP}}(-) = 1 \qquad \mathrm{pH}(-) = 4.0$$

$$\frac{V_{AO}}{V_{HRP}}(0) = 3 \qquad \mathrm{pH}(0) = 6.0$$

The current corresponding to a 19.2 μM His solution in phosphate buffer pH 8, at a constant applied potential (E_{ap}) of 50 mV (vs screen-printed Ag/AgCl reference electrode), was taken as the response. An 0.1 mM solution of HOMeFC was used.

The relationship obtained between the experimental variables and the chronoamperometric response, registered by the His biosensor, has been represented by a mathematical model [25]. Fig. 2 shows the changes in the response within the zone of interest obtained for this experimental design in terms of contours of estimated response surface.

From this optimization process, the optimum values for the experimental variables in the enzyme immobilization were 10 mM phosphate buffer pH 6.7 and V_{DAO}/V_{HRP} = 3.35 or V_{MAO}/V_{HRP} = 4 in the case of DAO/HRP or MAO/HRP biosensors, respectively.

Once the AO/HRP biosensors have been developed, they were applied to the electrochemical determination of BA. The chronoamperometric determination of His involves the optimization of the experimental variables that can define the selectivity of these biosensors. Therefore, pH of the buffer solution, E_{ap} and HOMeFc concentration ($C_{mediator}$) were optimized by means of a 2^3 central composite design, taken as response the current corresponding to a 19.2 μM His solution. In this step, the experimental domain was defined by the values corresponding to the high (+) and low (−) levels, and to the central point (0) for each factor,

$$\mathrm{pH}(+) = 8.0 \qquad E_{ap}(+) = 200\,\mathrm{mV} \qquad C_{mediator}(+) = 19.6\,\mu\mathrm{m}$$

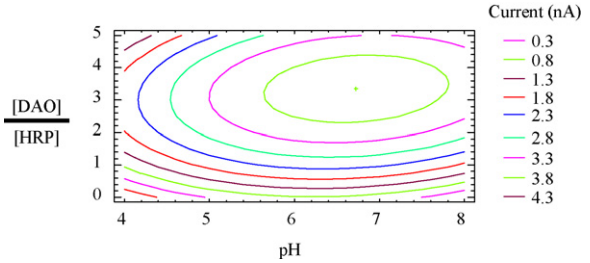


Fig. 2. Contours of estimated response surface for the 2^2 central composite design performed for the optimization of the experimental variables in the DAO/HRP immobilization procedure.

$$\mathrm{pH}(-) = 4.0 \qquad E_{ap}(-) = 100\,\mathrm{mV} \qquad C_{mediator}(-) = 5.9\,\mu\mathrm{m}$$

$$\mathrm{pH}(0) = 6.0 \qquad E_{ap}(0) = 50\,\mathrm{mV} \qquad C_{mediator}(0) = 12.7\,\mu\mathrm{m}$$

The 17 experiments carried out were evaluated in terms of analysis of variance for the reduction current registered. The combination of factors levels that maximizes the response over the experimental region was,

$$\mathrm{pH} = 9.3 \qquad E_{ap} = 250\,\mathrm{mV} \qquad C_{mediator} = 24.3\,\mu\mathrm{m}$$

in the case of DAO/HRP biosensors and,

$$\mathrm{pH} = 9.3 \qquad E_{ap} = 250\,\mathrm{mV} \qquad C_{mediator} = 19.6\,\mu\mathrm{m}$$

for MAO/HRP biosensors.

It can be clearly seen the similarity in the optimization results for the immobilization and the chronoamperometric procedure. This fact is understandable taking into account the analogous nature of DAO and MAO enzymes. Obviously, the experimental parameter that has been shown the biggest different between both biosensors was the ratio of enzymes. It can be explained considering the low activity shown by DAO, which led us to use a large amount of this enzyme (120 mg mL^{-1}) [2] comparing to MAO (5 mg mL^{-1}). The optimum operational potential reached, 250 mV vs screen-printed Ag/AgCl reference electrode, highlights these successful bienzymatic procedures in comparison to the previously screen-printed based biosensors reported [6,10].

Control experiments were carried out under the optimum conditions using bare electrodes, as well as modified electrodes according to the described procedure without enzymes. No analytical signal was obtained.

4.2. Biosensors characterization

In the optimized conditions, the proposed biosensors showed a linear range from 0.2 up to 1.6 μM for the DAO/HRP based SPCEs and 0.4–2.4 μM in the case of MAO/HRP based SPCEs. Several calibration curves were carried out in these linear ranges in order to check the biosensors performance. Precision, in terms of reproducibility and repeatability, and capability of detection were assessed.

Current vs concentration regression parameters were optimally evaluated. In order to detect the anomalous points that would alter intercept and slope values, the PROGRESS program [28] was implemented. These points were eliminated from the calibration set and an ordinary least squares (OLS) regression was carried out with the remaining data.

The variability of the slopes of the calibration curves recorded using different biosensors in the same operational conditions (Table 1) yielded relative standard deviation (RSD) values closed to 10% ($n = 5$, $\alpha = 0.05$). These results indicate a very good reproducibility in the sensors construction.

Table 1
Parameters of the OLS regressions without outliers obtained in the determination of His using AO/HRP based biosensors, under the optimized conditions.

	Intercept (nA)	Sensitivity (nA μM^{-1})	S_{yx}	Coefficient of determination (R^2)
DAO/HRP based biosensors				
Calibration I	2.01	19.68	1.19	0.990
Calibration II	1.17	17.09	0.47	0.997
Calibration III	0.92	16.25	0.51	0.996
Calibration IV	1.02	15.63	0.44	0.997
Calibration V	5.34	19.08	0.29	0.997
RSD ($\alpha = 0.05$)		10%		
MAO/HRP based biosensors				
Calibration I	21.56	32.77	0.22	0.999
Calibration II	6.67	32.03	1.07	0.997
Calibration III	5.70	33.09	1.44	0.996
Calibration IV	15.46	26.72	0.27	0.999
Calibration V	19.71	26.57	1.98	0.991
RSD ($\alpha = 0.05$)		11%		

Likewise, repeatability was estimated by recording successive calibration curves, in the same operational conditions, by a single AO/HRP based biosensor. Fig. 3 shows the lost in sensitivity obtained for both biosensors. It can be seen the DAO/HRP biosensor kept a 60% of sensitivity after the fifth calibration curve. In the case of MAO/HRP biosensors, the precision in terms of repeatability was poorer since <40% of sensitivity was kept after the third calibration curve.

Bearing in mind the disposable character of the SPCEs, it can be concluded that the AO/HRP biosensors can be developed according to the described procedure with high precision.

This analytical procedure for BA determination has also been characterized by the calculation of the capability of detection for a given probability of false positive (α) and negative (β) [33,34]. The parameters of the validated linear regressions between the concentration and the analytical signal were the required data to calculate this figure of merit, using the DETARCHI program [29]. The average capabilities of detection obtained were 0.18 ± 0.01 and 0.40 ± 0.04 μM ($\alpha = 0.05$ and $\beta = 0.005$) in the case of DAO/HRP and MAO/HRP based biosensors, respectively.

4.3. Substrate specificity determination

Due to the broad substrate specificity of DAO, several amines can give a current signal using the DAO/HRP biosensor, which has shown a better performance towards His detection. Put, Cad, Tyr, Trypt, Spm and Spd were tested. Several calibration curves were carried out in the concentration range from 0.2 up to 1.6 μM, under the optimized conditions for His. The biosensor response to the amines (Fig. 4), normalized to 100 for Put, agreed with previous ones since DAO preferentially oxidized diamines [9,11]. The highest sensitivity was obtained for Put (100%), Cad (95%) and His (80%), while the reactions involving the other amines were 73% for Tyr, Spm and Spd and 23% in the case of Trypt.

4.4. Application in anchovy samples

DAO/HRP based biosensor was applied to the determination of BA in salted anchovy samples. The extraction of amines was attempted according to the procedure described in Section 3.4, which minimized the sample pre-treatment. This procedure avoids the use of acid solutions that would require an additional step to neutralize the sample in order to measure at the optimum pH and, moreover, would produce the formation of electroactive substances which gave an interfering additional current [11].

Then, the total amine content of the sample, expressed in His equivalent units [16], was analyzed using the standard addition method.

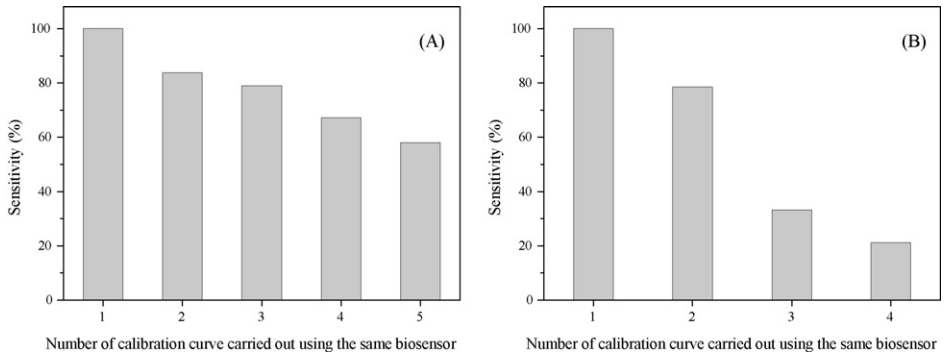


Fig. 3. DAO/HRP (A) and MAO/HRP (B) biosensors repeatability in terms of sensitivity.

Chronoamperograms were recorded under the optimum conditions (Fig. 5). A volume of 100 μL of the extracted BA was placed into the electrochemical cell, containing 5 mL of buffer solution pH 9.3 and HOMeFc in a final concentration of 24.3 μM, following by successive additions of 100 μL of a 10 μM His solution. Current vs concentration regression parameters were optimally evaluated as described-above, using the PROGRESS program [28]. The concentration of His found was [11.5 ± 1.4] μM (n = 4, α = 0.05), with a RSD of 7.5%.

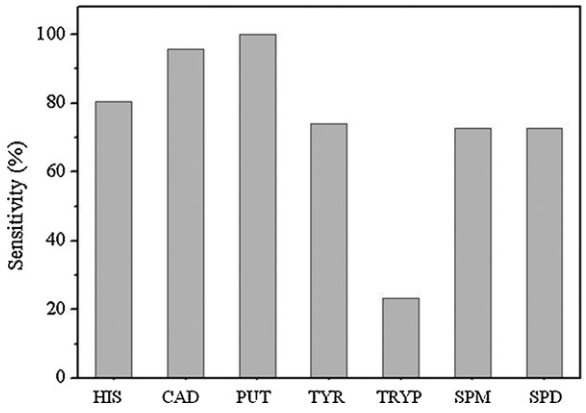


Fig. 4. Percentage response of the DAO/HRP based biosensor for different substrates using His, Put, Cad, Tyr, Trypt, Spm and Spd in the concentration range from 0.19 up to 1.64 μM.

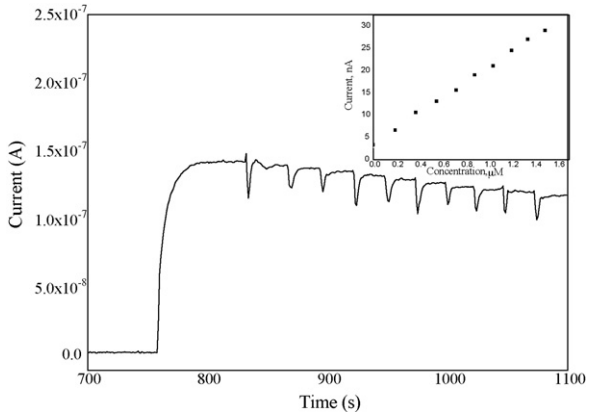


Fig. 5. Chronoamperogram recorded for the determination of His in anchovies samples. The first addition (780 s) corresponds to the HOMeFc solution. Then, the anchovy sample (834 s) was added and successive additions of 100 μL of a 10 μM His solution were made. pH of the buffer solution, 9.3; E_{ap} = 250 mV and $C_{mediator}$ = 2.43 μM. Inset: relationship between the responses and His concentration.

Table 2
Recovery (%), RSD and 95% confidence interval for mean for extracted His from anchovy samples, using DAO/HRP based biosensors.

His spiked level (μM)	His detected (μM)	Recovery (%)	RSD	95% Confidence interval for mean
–	11.5	–		
	12.2	–	7.5%	[11.5 ± 1.4]
	12.0	–		
	10.3	–		
Spiked anchovy sample				
29.5	37.7	92.0%		
	44.0	107.3%	7.7%	[40.8 ± 7.8]
	40.8	99.4%		

To test the viability of this procedure in the determination of His in anchovy samples, recovery studies were also performed with this sample. The total amount of HIS in a spiked sample, 41.0 μM, was determined by standard addition in triplicate. The concentration of His found was [40.8 ± 7.8] μM (n = 3, α = 0.05, RSD = 7.7%), with an average recovery of 99.6% (Table 2).

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

5. Conclusions

This work reports for the first time disposable bienzymatic biosensors for BA determination, using SPCEs modified by aryl diazonium salts that provide amine groups for the subsequent linkage of the enzymes. This key stage in the biosensors construction has been easily achieved by the formation of amide bonds between these amino groups and the carboxylic groups of the enzymes surface, using NHS and the EDC.

The optimized experimental conditions for the enzymes immobilization, as well as for the main variables than can influence the cathodic current due to the reduction of HOMeFc, shows a substantial similarity. This fact is understandable taking into account the analogous nature of DAO and MAO enzymes. The application of a potential of 250 mV has been found out as the optimum value of this experimental variable. This fact represents an important advantage from the previously described procedures based on disposable monoenzymatic screen-printed biosensors, which used potentials higher than 600 mV.

The biosensor construction is highly reproducible, allowing to obtain sensors with very similar sensitivities (RSD = 10%). The biosensors present a linear response range from 0.2 up to 1.6 μM and from 0.4 to 2.4 μM of His for DAO/HRP and MAO/HRP modified SPCEs, respectively.

The suitability of the chronoamperometric method for monitoring the BA content in salted anchovy samples has been demonstrated. Minimal sample pre-treatment and simple instru-

mentation of the analytical system make this approach a viable alternative as analytical method to determine the BA content in samples.

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