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Analytical Methods

Amperometric biosensor for the determination of histamine in fish samples

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ABSTRACT

A bienzymatic biosensor employing diamine oxidase (DOx) and horseradish peroxidase (HRP) for the detection of histamine in fish samples has been developed and optimized in this work. These enzymes have been co-immobilized into a polysulfone/carbon nanotubes/ferrocene membrane by means of phase inversion technique onto screen-printed electrodes. The electrochemical measurements have been carried out in phosphate buffer solution at pH 8.0 in batch mode and low applied potential (−50 mV vs. Ag/AgCl, KCl 0.1 M) to minimize the interferences. Developed biosensor exhibits high sensitivity (1.9×10^7 nA $^{-1}$), low limit of detection (1.7×10^{-7} M), high storage stability and excellent reproducibility, obtaining a linear interval range from 3×10^{-7} to 2×10^{-5} M.

Finally, applicability of the biosensor to the estimation of histamine content in different fish samples has been assessed; obtaining a good correlation between results obtained with the biosensor and those obtained with the reference method (ELISA) in case of sardines, mackerel and greater weever.

1. Introduction

Some biogenic amines (BAs) are considered indicators of an earlier microbial decomposition in fish or shellfish, although they are also present in a variety of food (Halasz, Barath, Simonsarkadi, & Holzapfel, 1994; Lange & Wittmann, 2002; Silla Santos, 1996; Stratton, Hutkins, & Taylor, 1991) and beverage (Di Fusco, Federico, Boffi, Macone, Favero, & Mazzei, 2011; Lopez, Tenorio, Gutierrez, Garde-Cerdan, Garijo, Gonzalez-Arenzana, et al., 2012) such as meat, eggs, chocolate, cheese or wine. BAs are low-molecular-weight organic bases with different structures, formed primarily by the decarboxylation of their precursor amino acids. The most important BAs related with spoilage in food are histamine (His), putrescine (Put) and cadaverine (Cad); being histamine one of the most biologically active compounds from those (Stratton et al., 1991). Therefore, it is crucial to determine its level since it causes scombroid syndrome without altering the fish normal appearance and odour (Wantke, Hemmer, Haglmuller, Gotz, & Jarisch, 1996). When consumed, this compound affects the normal functions of heart, smooth muscle, motor neurons and gastric acid secretion (Lehane & Olley, 2000; Shalaby, 1996; Stratton et al., 1991).

Histamine appears in fish such as mackerel, tuna, bonito, sardines and anchovies, as a result of inappropriate refrigerated handling or preserving after being caught (Carelli, Centonze, Palermo, Quinto, & Rotunno, 2007). For this reason, it is important to develop rapid and low-cost methods for the determination of histamine as an alternative to the classical ones, which mainly involve the use of chromatographic techniques such as the reversed phase high-performance liquid chromatography (RP-HPLC) (Alberto, Arena, & de Nadra, 2002; Lange, Thomas, & Wittmann, 2002), cation-exchange chromatography (CEC) (Triki, Jimenez-Colmenero, Herrero, & Ruiz-Capillas, 2012), gas chromatography (GC) (Awan, Fleet, & Thomas, 2008; Fernandes & Ferreira, 2000) or thin layer chromatography (TLC) (Lapa-Guimaraes & Pickova, 2004). In addition, due to the low optical absorbance of aliphatic amines in the ultraviolet (UV) region, HPLC methods need the usage of a pre- or post-column derivation step to facilitate their detection; requiring long time analysis and increasing its cost. Alternatively, enzymatic determination of BAs by means of amperometric (Boka, Adanyi, Virag, Sebela, & Kiss, 2012), spectrophotometric (Pessatti, Fontana, & Pessatti, 2004), fluorimetric (Cortacero-Ramirez, Arraez-Roman, Segura-Carretero, & Fernandez-Gutierrez, 2007) or chemiluminometric (Zhao, Yong, Shi, & Liu, 2009) detection methods have also been carried out to solve these drawbacks. In this context, biosensors offer low price, rapid and short time analysis methods (Asuncion Alonso-Lomillo, Dominguez-Renedo, Matos, & Julia Arcos-Martinez, 2010; Male, Bouvrette, Luong, & Gibbs, 1996). They are mainly based on the use of amine oxidases, which convert the analyte to the corresponding aldehyde, NH_3 and H_2O_2 (Eq. (1)).

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

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The consumption of O_2 (Hernandez-Cazares, Aristoy, & Toldra, 2011) or the generation of H_2O_2 (Draisci, Volpe, Lucentini, Cecilia, Federico, & Palleschi, 1998; Tombelli & Mascini, 1998) are usually monitored to measure the advance of the reaction, and therefore to quantify BAs. Nevertheless, the detection of hydrogen peroxide requires the application of high potential; which may lead to have other compounds that can act as interferences. Hence, with the combination of peroxidase enzymes and amine oxidases or using redox mediators (Asuncion Alonso-Lomillo et al., 2010), it is possible to detect H_2O_2 at lower applied potentials, a common strategy for the construction of amperometric biosensors.

The immobilization of enzymes is a crucial step in the development of biosensors. In the recent years, phase inversion technique has been used due to its easy and fast incorporation of biomolecules into membranes (Cetó, Céspedes, Capdevila, & del Valle, 2011; Pérez, Sánchez, & Fàbregas, 2012; Sánchez, Roldan, Pérez, & Fàbregas, 2008). Some polymers such as polysulfone (PS) allow the use of this approach, which consist in depositing a thin film of polymer solution (diluted in a solvent) onto the electrode surface and then immerse it into an aqueous solution (non-solvent) that contains the biomaterial. Then, there is an exchange of the solvent by the non-solvent, which causes the precipitation of the membrane, and consequently, biomolecules present in the non-solvent are trapped and incorporated into the polysulfone membrane taking advantage of this process. This material presents highchemical, biological and thermal stability and also high resistance at extreme pH values. Moreover, its porosity can be controlled by varying the bath conditions as the temperature, which allows modulating the active surface of the membrane, or even by changing its composition. Polysulfone is a non conducting polymer, thus, the introduction of a conducting material (Choi, Jegal, & Kim, 2006; Pérez et al., 2012) is a required step for the development of amperometric biosensors. In this way, multiwalled carbon nanotubes (MWCNT) (Iijima,1991) offer excellent electrochemical properties since they provide high current response, large surface-to-volume ratio, great chemical stability and minimization of the surface fouling onto electrochemical devices. All these properties make CNTs an excellent choice to be considered as a transducer.

Herein, we present a new approach to develop an amperometric screen-printed biosensor for the determination of histamine based on the immobilization of diamine oxidase and horseradish peroxidase into a MWCNT/PS/ferrocene membrane by phase inversion technique. To this aim, experimental conditions have been optimized in order to find the optimal conditions that enhance its response characteristics. Finally, developed biosensor has been tested in the determination of histamine level in different real fish samples to assess its applicability, comparing the obtained values with the ones using a reference method.

2. Materials and methods

2.1. Materials

Diamine oxidase (DAO) from plant (526 units mL^{-1}) was supplied by MoLiRom (Roma, Italy). Peroxidase Type II from horseradish (HRP) (188 units mg^{-1}), histamine dihydrochloride, sodium phosphate dibasic anhydrous, potassium chloride, L-trytophan, L-lysine, L-tyrosine, L-histidine and multiwalled carbon nanotubes (MWCNT; length 0.5–200 μm and outer diameter 30–50 nm) were purchased from Sigma–Aldrich© Chemie (Steinheim, Germany). Polysulfone (PS) was obtained from BASF (BASF Ultrasons S 3010 natur, Frankfurt, Germany). N,N-dimethylformamide (DMF) and nitric acid were purchased from Panreac (Barcelona, Spain) and Ferrocene (Fc) from Alfa Aesar (Germany). Veratox® kit ELISA for histamine quantification was supplied by Nirco Diagnóstico & Investigación (Barcelona, Spain).

Deionised water obtained from PURELAB® with Ultra Laboratory Water Purification Systems was used to prepare all solutions. Measurements were carried out in a phosphate buffer solution (0.1 M $HPO_4^{-2}/H_2PO_4^-$, 0.1 M KCl). MWCNTs were previously purified by stirring them in 6 M nitric acid for 2 h and dried at 80 °C (Pérez et al., 2012).

2.2. Apparatus

Voltammetric measurements were taken with a μStat 200 Bipotentiostat from Dropsens (Oviedo, Spain), using the DropView software package to control the instrument, register and perform the analysis of the results. Amperometric measurements were carried out either with the Dropsens Bipotentistat or a LC-4C amperometric detector (BAS Bioanalytical System Inc., U.S.). Optical measurements were performed on a TECAN Sunrise microplate reader. Sonication was carried out with an "Ultrasons 3000683" and centrifugation with Angular 6 Centrifuge, both from J.P.Selecta (Barcelona, Spain).

Carbon screen-printed electrodes (SPEs) consist in a carbon working electrode (4 mm diameter), a carbon counter electrode and a silver reference electrode; in the case of dual screen-printed carbon electrodes (DSPEs), the same configuration was used, but with two carbon working electrodes. All of them supplied by DropSens (Oviedo, Spain): references 110 and C1110, respectively.

2.3. Biosensor preparation

Prior to the membrane deposition onto the carbon working electrode, screen-printed electrodes (SPEs) were electrochemically activated by means of cyclic voltammetry as described in previous work (Pérez et al., 2012). This step increases signal-to-noise ratio (S/N) and improves the stabilization of the current signal in further experiments. In this way, only 5 cycles in 0.05 M potassium ferricyanide ($K_3[Fe(CN)_6]$) solution prepared in phosphate buffer 0.1 M $H_2PO_4^-/HPO_4^{-2}$ and 0.1 M KCl at pH 7.5 were enough to carry out this activation step. For this, potential was swept between −0.6 V and +0.6 V vs. screen-printed Ag/AgCl reference electrode with a scan rate of 100 mV s^{-1} and a step potential of 9 mV (Pérez et al., 2012).

The immobilization of diamine oxidase (DAO) and horseradish peroxidase (HRP) into the polymeric membrane was achieved by means of the Phase Inversion technique (PI) (Pérez et al., 2012). First of all, 84 mg of PS were dissolved in 1 mL of DMF. Then, 100 μl of this solution were mixed with 5 mg of ferrocene and 1 mg of MWCNT. As is well known, MWCNT usually tend to aggregate into bundles because of the Van der Waals forces, thus to avoid that, the mixture was sonicated for 1 h in order to get a stable (ca. 2 days) and homogeneous dispersion; obtaining the membrane paste.

For the membrane formation, 0.6 μl of PS/MWCNT/Fc/DMF paste were placed onto the SPE working electrode and immediately, 5 μl of the phase inversion solution containing both enzymes were added over the paste, causing the coagulation of the membrane and the entrapment of the biomolecules at the same time; in only five minutes. Lastly, a washing step of 5 extra minutes in a stirred buffer solution was necessary to ensure the elimination of any remaining DMF.

The same procedure was followed in the case of dual screen-printed electrodes (DSPE), where both working electrodes were modified with the PS/MWCNT/Fc membrane. In this case, PI process took place employing the enzymatic solution as the precipitating solution for one of the electrodes, whereas for the other one, only phosphate buffer was used. Taking into account that the area

of the working electrodes is minor, only 0.3 μl of PS/MWCNT/Fc/DMF and 2.5 μl of phase inversion solution were employed for the membrane coagulation process. In all the cases, prepared biosensors were stored in phosphate buffer solution (pH 7.5) at 4 °C when not in use.

2.4. Amperometric measurements

As shown in Scheme 1, DAO in presence of amines or diamines and O_2 catalyzes its decomposition to the corresponding aldehyde, ammonia (NH_3) and peroxide. Then, generated peroxide is reduced to H_2O through the action of HRP enzyme, and finally, the current signal detected at the electrode surface corresponds to the reduction of ferrocenium generated.

Amperometric measurements were carried out in batch mode by using the modified SPEs, in an open vessel at room temperature. The first step was to immerse the biosensor in an electrochemical cell containing 10.0 mL of phosphate buffer solution (0.1 M $H_2PO_4^-$/HPO_4^{-2} and 0.1 M KCl at pH 7.5), under constant stirring and applying a potential of −50 mV vs. screen-printed Ag/AgCl reference electrode, except for its optimization study where it was varied. After 3 min, which were required to reach a stable current intensity baseline, additions of histamine were done. Biosensor response time was only 20 s, where taken readings correspond to the steady-state signal from the average of the last 5 s (10 measurements with an interval of 0.5 s). In this manner, all measurements were done in replicate and the given results are the averages of the measurements with their corresponding Relative Standard Deviations (RSD).

The same procedure was also followed in the case of DSPEs, where the obtained biosensor signal (working electrode containing both enzymes in its membrane) was corrected by the one from the second working electrode (blank membrane). In this way, it was possible to eliminate the biosensor response towards any electrochemical active compounds present in real samples (background signal) which could interfere in the histamine determination.

2.5. ELISA measurements

Real samples were also measured with a reference method to assess the reliability of the developed biosensor. As the reference method for the quantification of histamine in fish samples, Neogen's Veratox kit ELISA for histamine was used. It is a competitive direct enzyme-linked immunosorbent assay which allows quantifying histamine in the interval range from 2.5 to 40 ppm. The assay is based on the competition of enzyme-labeled histamine (conjugate) with free histamine in the samples, or the one of the controls when building the calibration plot, for the antibody binding sites. After a washing step, enzyme substrate was added, which reacts with the bounded conjugate producing a change of colour (from red to blue). Then, the test plate was read in a microwell reader at 620 nm to yield optical densities. Finally, obtained readings were interpolated into the calibration plot built with the controls through a log–logit transformation, extracting the concentration in each sample. The entire assay was carried out at room temperature.

2.6. Real samples preparation

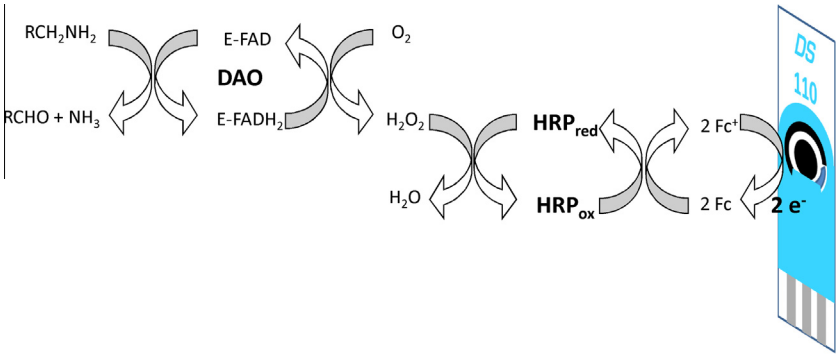
Different fish samples such as anchovies, tuna, sardines, mackerels, shrimps and greater weever, which have been studied in order to test the developed biosensor, were acquired from a local fishmonger's shop. Prior to carry out the amperometric and ELISA measurements, samples were pretreated to extract the biogenic amines. Therefore, they were firstly cleaned and eviscerated, cutting thick slices from back of the pectoral fin, halfway and posterior to the vent. Then, they were blended to obtain a homogenous mixture. 10 g of this mixture plus 90 mL of water were added to an extraction bottle and shaked during 20 s to suspend the fish tissue and improve the extraction process. After approximately 5 min, this procedure was repeated 2 more times, and later the extract was centrifuged at 4 °C for five minutes. Subsequently, the supernatant was recovered and filtered through a Neogen filter syringe (cellulose), being fish samples then suitable for analysis.

3. Results and discussion

3.1. Optimization of the biosensor working conditions

In order to establish which was the optimal working potential to perform the determination of histamine, a study of the obtained signal when varying the applied potential in the electrochemical system was carried out. As previously mentioned, the obtained signal is due to the H_2O_2 produced in the second catalytic reaction, which normally requires the application of high potentials. Herein, the developed approach is based on the bienzymatic system (DOx/HRP) together with the incorporation of ferrocene as a redox mediator, which allows reducing this potential to −50 mV vs. Ag/AgCl, as can be observed in Fig. 1A. This value was selected as the optimal working potential because it showed the combination of the highest and the major stable current response to histamine (0.2 mM), comparing with the signal obtained in phosphate buffer solution at the same working potential.

Given the importance of the pH in the enzymes activity, a study of the effects of the pH of the phosphate buffer solution was also carried out. For this, its influence in the signal towards a 5 μM histamine solution at different pH values (from 6.5 to 8.5) was investigated. As shown in Fig. 1B, it was found that there was a steeply



Scheme 1. Schematic diagram of the reactions involved in the bienzymatic system for the determination of histamine.

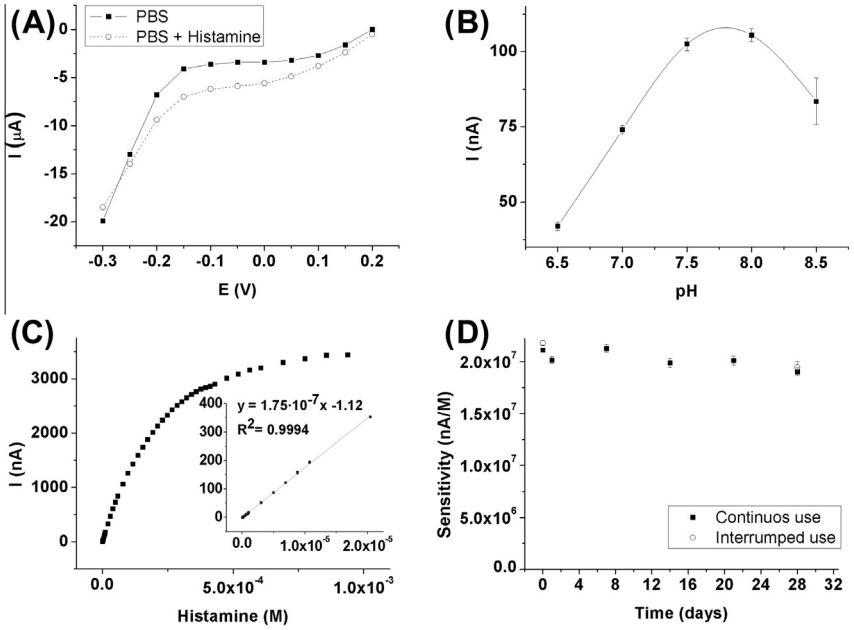


Fig. 1. (A) Study of the working potential. Batch measurements were carried out in (○) phosphate buffer solution at pH 7.5 (0.1 M $H_2PO_4^-/HPO_4^{-2}$/) and (■) a 0.2 mM histamine solution in the same buffer, varying the working potential. The phase inversion solution to prepare the biosensor contained 250 U mL^{-1} of DOx and 3760 U mL of HRP. (B) Study of the influence of the pH in the biosensor response towards a 5 μM histamine solution in phosphate buffer (0.1 M $H_2PO_4^-/HPO_4^{-2}$/). Measurements were carried out at −50 mV in duplicate. The biosensor was prepared using a solution of 250 U mL^{-1} of DOx and 3760 U mL^{-1} of HRP as the phase inversion solution. (C) Study of the linear interval range of the biosensor from histamine calibration curves. Measurements were carried out in duplicate in phosphate buffer solution at pH 8.0. (D) Evaluation of the storage stability of two histamine biosensors during one month by comparison of its sensitivity. When not in use, biosensors were stored at 4 °C in buffer phosphate solution at pH 8.0.

increase in the biosensor response as the pH increased, until reaching its maximum at pH 8.0. Then, from 8.0 to 8.5 there was a decline in its response combined with a huge decrease in the reproducibility. Hence, taking into account this response profile, pH 8.0 was chosen as the optimal one to perform further experiments.

Finally, another crucial factor to study was the amount of enzymes (Scheme 1) required to carry out the phase inversion process. In order to determine their optimal concentrations, and considering that the recorded signal is a combination of both enzymes action, optimization of their amount was performed separately in two steps. For this, several biosensors were constructed varying the concentration of the enzymes in the phase inversion solution, evaluating then their response towards histamine.

First, the influence of HRP amount on the PI solutions was considered. Hence, using a constant concentration of DOx (250 U mL^{-1}), HRP amount on the PI solution was varied from 0 to 9400 U mL^{-1}. After construction of the different biosensors, its characterization was carried out, and sensitivity and RSD values extracted from the calibration plot were used to choose the optimum value (Table 1). As can be observed, there is an increase of the sensitivity if comparing the response of biosensors when they contain or not HRP. Moreover, as can be seen in Table 1, results showed lower RSD values for the biosensors corresponding to a HRP concentration of 940 and 4700 U mL^{-1} in the PI solution. Hence, as a compromise between obtained RSD and in order to guarantee that enough amount of HRP will remain even in case it could be lost from the PS membrane, 4700 U mL^{-1} was selected as the concentration in the phase inversion solution.

As before, a second test for the optimization of DOx was done; but in this case, keeping constant the HRP concentration (the one previously chosen) and varying DOx amount. As expected, calibrations showed that sensitivity increased as DOx amount did, rising values practically equal over 250 U mL^{-1}. Consequently, this was chosen as the optimum concentration of DOx in the PI solution. Hence, for the preparation of new biosensors to perform further experiments, PI solution had 4700 U mL^{-1} of HRP and 250 U mL^{-1} of DOx.

3.2. Characteristics of the biosensor

Histamine biosensors were further characterized with regard to its linear interval range, storage and operational stability and its reproducibility. Fig. 1C shows the study of the linear interval range

Table 1
Optimization of HRP and LOx concentration in the phase inversion solution for the biosensors preparation.

HRP (U mL^{-1})[a]	0	470	940	4700	9400
Sensitivity (nA/M)	1.65×10^7	2.05×10^7	1.78×10^7	1.96×10^7	1.80×10^7
Standard deviation (nA M^{-1})	1.7×10^6	2.4×10^6	1.0×10^5	1.7×10^5	1.2×10^6
RSD%	10.4	11.8	0.6	0.9	6.8
DOx (U mL^{-1})[b]	5.26	27.8	105	250	375
Sensitivity (nA M−1)	1.88×10^6	7.3×10^6	1.31×10^7	1.87×10^7	1.94×10^7
Standard deviation (nA M^{-1})	5.7×10^4	3.2×10^5	2.8×10^5	1.0×10^5	6.4×10^5
RSD%	3.0	4.4	2.1	0.5	3.3

[a] (DOx = 250 U mL^{-1}).
[b] (HRP = 4700 U mL^{-1}).

carried out by means of duplicate measurements after additions of different amounts of histamine in the electrochemical cell, obtaining a calibration curve from 3×10^{-7} to 9×10^{-4} M. Results indicated that the interval range where the enzymatic system works linearly was almost two orders of magnitude, from 3×10^{-7} to 2×10^{-5} M, as can be seen in the amplification of the first points of the calibration plot.

Storage stability of the developed biosensor was established in two different ways: continuous and interrupted use. For this, two sensors were prepared in the same conditions and their sensitivity in histamine calibrations was evaluated during a month. While the response of the first biosensor was studied periodically by triplicate in different days during this time, the response towards histamine of the second was only evaluated after its preparation and the last day of the study. Biosensors were stored at 4 °C and immersed in phosphate buffer solution at pH 7.5 when not in use. Results represented in Fig. 1D showed the evolution of this sensitivity, demonstrating excellent and similar storage stability in both cases, since the decrease was only 9.9 and 10.7%, respectively. Besides, the operational stability was also examined after 35 consecutive calibrations in one day. In this case, it was observed that sensitivity difference between the first and the last calibration was reduced in 19%, an excellent value taking into account that usually SPEs are thought to be used in a disposable manner.

Lastly, in order to test the reliability of the developed biosensor, it was also important to evaluate its reproducibility. On the one hand, it was interesting to demonstrate the reproducibility of the biosensor construction, which was evaluated by comparison of the histamine calibrations between 2.5×10^{-6} and 1.5×10^{-5} M for 5 different sensors. On the other hand, repeatability of the biosensor response after 5 consecutive calibrations using the same one was studied, again under the same experimental conditions as the previous test. In both cases a high reproducibility was obtained, with values of 6.5% and 5.6%, respectively, calculating these values from the slopes of histamine calibration in the indicated concentration interval range. Moreover, the Limit of Detection (LOD) towards histamine was 1.7×10^{-7} M, defined as the concentration corresponding to three times the blank standard deviation. Alternatively, it could be taken the Standard Error of Estimate (SEE) as an estimation of the blank standard deviation, that it is the standard deviation of the residuals from the regression calculation. Therefore, in our case, LOD was calculated from the regression of the five calibration curves.

3.3. Study of interferences

The study of the principal interfering compounds is a crucial step to evaluate the response of the biosensor prior to the analysis of real samples to ensure its applicability. For this reason, biosensor response towards some of the potentially interfering substances in fish was studied, namely, amino acids involved in the biosynthesis of amines such as hystidine, tyrosine, tryptophan and lysine.

The effect of this type of substances was examined by comparison of the signal obtained for a 2.5×10^{-6} and a 5×10^{-6} M histamine standard solution vs. the signal obtained for solutions of the same concentration of those amino acids. As can be seen in Fig. 2, results demonstrate that these amino acids have a slightly influence in the histamine determination since lysine was the only one that produces some noticeable amperometric response at these concentrations levels and under selected measurement conditions, representing only a 5.9% of the histamine response when compared. However, it should be taken into account that the amount of these substances may vary in each type of fish, and in addition, it also evolves in time (Silla Santos, 1996); hence, in some special cases, it could interfere in major or minor grade to the analysis.

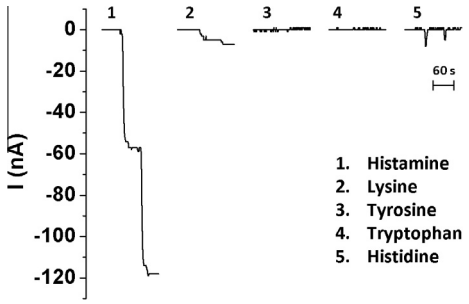


Fig. 2. Study of interferences. Comparison between histamine and potentially interfering amino acids: lysine, tyrosine and tryptophan. Chronoamperometric measurements after two additions of standard solutions (every 60 s) for each compound were performed in phosphate buffer solution at pH 8.0. Final concentrations after each addition correspond to 2.5×10^{-6} and 5×10^{-6} M.

3.4. Assay in fish samples

Different types of fish samples were analysed with the developed biosensor. The main purpose of this variety was to evaluate in which fish species the biosensor was able to quantify the amount of histamine with good reliability because of depending on the sample, the production of histamine and other BAs, namely cadaverine, putrescine or other interfering compounds may vary. Hence, their concentration levels are different and can influence in histamine determination. In this sense, it should be said that DOx also catalyzes the reaction of these other BAs, consequently, biosensor samples analysis give us the total biogenic amines content.

The quantification of histamine was carried out with the two modified biosensors, SPEs and DSPEs. Also, in both approaches, its quantification was performed first by means of direct interpolation in the histamine calibration plot and secondly, the standard addition method was also used in order to counteract any possible matrix effect. Under the optimum established conditions, measurements were carried out in an electrochemical cell containing 10.0 mL of phosphate buffer at pH 8.0 and under an applied potential of −50 mV vs. Ag/AgCl. Moreover, a dilution step, which may vary depending on the sample, between 1:25 and 1:400 was required to fit in the biosensor linear interval range. All amperometric measurements were done in triplicate; and in the case of the direct interpolation, recalibration of the biosensor was done every 3 samples to guarantee biosensor performance.

As can be seen in Table 2, there are no high differences in general between both methods (direct interpolation and standard addition), concluding that the matrix effect was not so critical. Besides, no significant differences were found between the two types of biosensors employed (SPEs and DSPEs) in the histamine determination, inferring that no background signal correction was required given the low potential applied. However, in the case of tuna, histamine analysis was not possible by means of SPE biosensor since some oxidative currents that interfere with the determination were registered. Hence, by using DSPE biosensor and due to the fact that the responsible compounds are directly oxidized by the transducer, without being the enzymes involved at all in that process, DSPEs offered the advantage of correcting this background signal, allowing carrying out the correctquantification of histamine. As an additional verification of the proposed approaches, a Student's paired samples *t*-test was performed between the results provided by the amperometric biosensor and the ones of the ELISA kit. On the one hand, in the case of the differential measurements by interpolation and standard addition, obtained experimental *t*

Table 2
Concentrations of histamine in different fish samples determined by means of SPEs, DSPEs and ELISA kit. Results obtained by amperometric measurements are expressed with their interval of confidence ($n = 3$, confidence level of 95%).

Sample	(μg Histamine g^{-1} sample)				
	Differential measurements		No differential measurements		ELISA
	Interpolation	Standard addition	Interpolation	Standard addition	
Sardine (fresh)	94 ± 15	98 ± 11	100 ± 16	87 ± 3	83
Sardine (24 h)	97 ± 20	97 ± 15	94 ± 7	93 ± 5	87
Sardine (30 h)	108 ± 31	110 ± 23	109 ± 8	107 ± 6	102
Sardine (96 h)	151 ± 16	134 ± 25	184 ± 8	176 ± 16	134
Sardine (168 h)	190 ± 38	190 ± 28	208 ± 19	186 ± 16	152
Mackerel (fresh)	44 ± 3	41 ± 3	41 ± 3	34 ± 7	38
Greater weever (fresh)	23 ± 2	23 ± 1	27 ± 4	23 ± 2	23
Anchovy (fresh)	26 ± 3	25 ± 2	26 ± 1	23.1 ± 0.9	20
Anchovy (24 h)	43 ± 2	42.5 ± 0.5	42 ± 1	41 ± 3	28
Defrosted shrimp	89 ± 8	87 ± 3	86 ± 9	96 ± 19	201
Tuna (fresh)	26 ± 8	24 ± 8	–	–	125

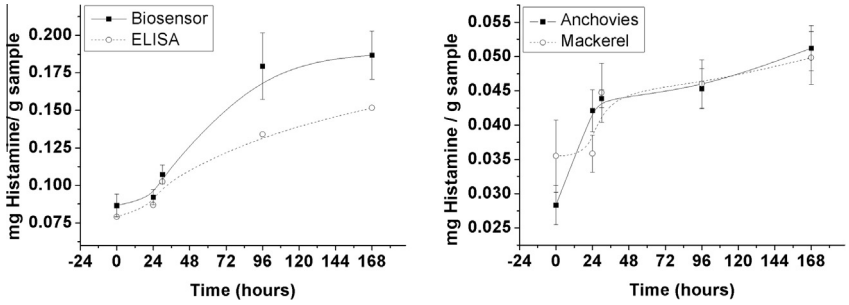


Fig. 3. Evolution of total biogenic amines content in (A) sardines and (B) anchovies and mackerel samples. Samples were stored at 4 °C and determinations were carried out by direct interpolation of the measurements in a histamine calibration plot employing developed SPE biosensor. In case of sardines, values of histamine concentration were compared with those obtained with the ELISA kit.

values were 0.632 and 0.749, respectively, while the critical tabulated t value was 2.228 (95% confidence level and 10 degrees of freedom). On the other hand, in no differential measurements experimental t values were 0.333 and 0.015, lower than the critical t value in both cases (2.262 at 95% confidence level and 9 degrees of freedom). Therefore, it could be concluded that there are no significant differences between the concentrations found with the amperometric method and the ELISA kit.

Moreover, the evolution of histamine in fish samples, when those were stored at 4 °C, was also studied during one week. The evolution of the process of degradation in terms of total biogenic amines was evaluated for sardine, anchovies and mackerel samples. Obtained results are shown in Fig. 3, and as can be seen, in the three types of samples there was a clear tendency in the increase of BAs content, demonstrating in this way, an increment of toxicity as the time of storage increases. As said, histamine and other BAs concentration in fish increases after being caught due to an inappropriate refrigerated handling or simply because fish goes bad. Hence, its concentration levels could be used as an indicator of fish quality or freshness, and its control is very important to avoid scombroid syndrome, which results from eating spoiled fish.

Finally, results obtained with the amperometric method were compared with those obtained with the ELISA kit, which was used as the reference method and that quantifies only free histamine as explained in Section 2.5. On the one hand, developed biosensor shows a good agreement with the reference method in the case of sardines, mackerel and greater weever. However, the results of the evolution of histamine levels in sardines sample when stored at 4 °C, which was studied during several days with the biosensor and with the ELISA kit, started to differ after 96 h between both methodologies, as can be observed in Fig. 3A. This could be explained by the fact that histamine is the first biogenic amine that appears during the degradation process; hence, it could be assumed that this discrepancy corresponds to the production of other BAs that might be also detected by the biosensor. On the other hand, anchovies, shrimp and tuna samples do not present such a good correlation with the results obtained by the immunoassay. This fact can also be attributed to the production of different biogenic amines. Therefore, it can be conclude that the developed biosensor can be employed to quantify histamine in sardines, grater weever and mackerel with high reliability.

4. Conclusions

The present work reports the development, optimization and application in fish samples of a histamine bienzymatic biosensor based on PS/MWCNT/Fc membrane, where DOx and HRP enzymes have been immobilized by means of phase inversion technique. This easy and fast methodology allows the development of a compact biosensor that contains all the elements required to carry out the amperometric measurement, namely enzymes, transducer and the redox mediator.

The developed biosensor exhibits advantages over other methods such as short time analysis, low detection limit, high sensitivity, storage stability, as well as good reproducibility and repeatability with acceptable RSD values. In addition, the biosensor offers a lower cost per analysis in comparison to ELISA kit or chromatographic methods, usually employed as reference methods.

Finally, obtained results suggest that this approach is a reliable alternative method for the quantification of histamine in samples such as sardines, mackerel and great weever.

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