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ORIGINAL PAPER

Electrochemical enzyme sensor arrays for the detection of the biogenic amines histamine, putrescine and cadaverine using magnetic beads as immobilisation supports

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Abstract Electrochemical biosensors based on diamine oxidase (DAO) conjugated to magnetic beads (MBs) were developed for the detection of histamine (Hist), putrescine (Put) and cadaverine (Cad), the most relevant biogenic amines (BAs) related to food safety and quality. DAO-MBs were immobilised on Co(II)-phthalocyanine/carbon and Prussian Blue/carbon electrodes to obtain mono-enzymatic biosensors, and on Os-wired HRP-modified carbon electrodes to obtain bi-enzymatic biosensors. The three sensor have low working potentials (+0.4 V, −0.1 V and −0.05 V *vs* Ag/AgCl, respectively), a linear range of two orders of magnitude (from 0.01 to 1 mM BA), good reproducibility (variability lower than 10 %), high repeatability (up to 8 consecutive measurements), limits of detection in the μM concentration range for Hist and in the sub-μM concentration range for Put and Cad, and no response from possible interfering compounds. The DAO-MB conjugates display excellent long-term stability (at least 3 months). The biosensor has been applied to the determination of BAs in spiked and naturally-spoiled fish, demonstrating its suitability both as screening tool and for BAs quantification. The use of MBs as supports for enzyme immobilisation is advantageous because the resulting biosensors are simple, fast, stable, affordable, and can be integrated into array platforms. This makes them suitable for high-throughput analysis of BAs in the food industry.

Keywords Diamine oxidase · Os-wired HRP · Prussian Blue · Co(II)phthalocyanine · Screen-printed carbon electrode · Magnetic particles · Electrode array · Fish analysis · High-throughput analysis

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Introduction

Biogenic amines (BAs) are low-molecular-weight organic bases mainly produced by microbial decarboxylation of amino acids. BAs may be found in variety of foods, such as fish, meat, cheese, vegetables and wines, as a consequence of inappropriate processing and storage conditions and subsequent microbial contamination [1, 2]. The most important BAs related with spoilage in food are histamine (Hist), putrescine (Put) and cadaverine (Cad), which can be considered appropriate food freshness indicators. Among them, Hist is considered one of the most relevant due to its biological toxicity, whose harmful effect is enhanced in the presence of Put and Cad, which inhibit the intestinal histamine-metabolizing enzymes [3]. The European Regulation establishes Hist limits of 100 $mg{\cdot}kg^{-1}$ (average) and 200 $mg{\cdot}kg^{-1}$ (individual) in fishery products from fish species associated with high histidine amounts, while for products which have undergone enzyme maturation treatment in brine, the afore mentioned limits rise to 200 and 400 $mg{\cdot}kg^{-1}$ [4]. Nowadays, chromatographic techniques coupled to different instrumentation for the detection are the most commonly used analytical methods for the detection of BAs [5]. However, alternative methods are needed to achieve rapid, accurate, reproducible and low-cost detection. In this direction, some electrochemical biosensors have been reported for BAs detection. Most of them are based on the reaction between amine oxidases and BAs, which generates NH_3 and H_2O_2, the later being subsequently monitored directly, through redox mediators or combined with HRP in

order to lower the applied potentials and increase the selectivity of the biosensor.

Regarding enzyme immobilisation, it is well-known that this step is crucial in the development of biosensors. In most of the reported biosensors, amine oxidases have been immobilised directly on electrodes by adsorption, cross-linking [6–10] and/or entrapment into gels, polymers or membranes [11–17]. These methods are simple but, depending on the immobilisation strategy, they may suffer from enzyme leakage, enzyme inactivation or diffusion limitations. Over the last few years, micro/nanomaterials have begun to be explored as biomolecule supports, with the aim to provide advantages in terms of larger electroactive surface area, promoted electron transfer, enhanced electrochemical signals and lower limits of detection (LODs) [18–20]. However, the method used for the immobilisation of the micro/nanomaterial on the electrode certainly plays an important role in the biosensor performance, and techniques such as adsorption or entrapment may also compromise the biosensor performance. The use of magnetic beads (MBs) with diameters in the micro/nm range as biomolecule supports allows a stable immobilisation by simply applying a magnetic field, while presenting a large surface area available for biomolecule immobilisation, the possibility to regenerate the electrode surface or the easy integration into microfluidic devices. The combination of functionalised MBs with electrochemical detection constitutes a powerful and efficient strategy for the development of biosensors [21–23]. However, most MB-based electrochemical biosensors reported up to date are based on antibodies or DNA probes and the use of MBs as enzyme immobilisation supports has been much less explored and still presents some challenges [24].

In this work, diamine oxidase (DAO) has been conjugated to MBs and immobilised on electrodes for the development of electrochemical biosensors for the detection of Hist, Put and Cad. After optimisation of the experimental parameters, the analytical performance of the DAO-MB biosensors has been evaluated. The high storage stability of the conjugates and the possibility to transfer them to multiplexed systems has been demonstrated. The DAO-MB biosensors have been applied to the determination of BAs contents in spiked and naturally-spoiled fish samples, demonstrating their promising use as a simple, stable, easy-to-use and inexpensive tool for high-throughput bioanalysis of BAs in routine food industry monitoring programs.

Experimental

Reagents and materials

Diamine oxidase (DAO) from plant (120 $U \cdot mL^{-1}$) was supplied by MoLiRom (Roma, Italy, www.molirom.com). Peroxidase from horseradish Type XII (HRP) (250–330 $U \cdot mg^{-1}$) was obtained from Sigma-Aldrich (Tres Cantos, Spain, www.sigmaaldrich.com). Horseradish Peroxidase wired with Os-PVP redox polymer (Os-wired HRP) was purchased from from Bioanalytical Systems, Inc. (West Lafayette, USA, www.basinc.com). Dynabeads® M-270 Carboxylic Acid (2×10^9 beads$\cdot mL^{-1}$) were obtained from Invitrogen by Life Technologies, S.A. (Alcobendas, Spain, www.thermofisher.com).

Histamine dihydrochloride (Hist), putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad), dopamine hydrochloride (DA), tyramine hydrochloride (TA), L-histidine (His), L-lysine (Lys), L-arginine (Arg), L-tyrosine (Tyr), DL-tryptophan (Trp), hydrogen peroxide 30 % solution (H_2O_2), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), N-methylphenazonium methyl sulphate (MPMS), 2-(N-morpholino)ethanesulfonic acid hydrate (MES), *o*-dianisidine dihydrochloride (ODA), potassium phosphate monobasic and potassium chloride were supplied by Sigma-Aldrich (Tres Cantos, Spain, www.sigmaaldrich.com). All solutions were prepared using Milli-Q water (Millipore, Bedford, USA, www.merckmillipore.com).

Screen-printed electrodes (carbon DRP-110, single-walled carbon nanotubes/carbon DRP-110SWCNT, graphene/carbon DRP-110GPH, Co-phthalocyanine/carbon DRP-410, Prussian Blue/carbon DRP-710, gold DRP-220AT and an array of eight carbon electrodes DRP-8X110), boxed connectors (DRP-DSC and DRP-CAST8X) and magnetic supports (DRP-MAGNET and DRP-MAGNET8X) were provided by Dropsens S.L. (Oviedo, Spain, www.dropsens.com). Individual screen-printed carbon electrodes consist of a carbon working electrode (some electrodes are modified with electrochemical mediators or nanomaterials) of 4 mm in diameter, a carbon counter electrode and a silver reference electrode. Individual screen-printed gold electrodes consist of a gold working electrode of 4 mm in diameter, a gold counter electrode and a silver reference electrode. The array consists of 8 carbon working electrodes of 2.5 mm in diameter, each one with its carbon counter electrode and silver reference electrode.

Conjugation of DAO to MBs

A HulaMixer® Sample Mixer from Invitrogen (Leek, The Netherlands, www.thermofisher.com) was used in the conjugation of DAO to carboxylic acid-modified MBs. Magnetic separation was performed using a Z5342 MagneSphere® Technology Magnetic Separation Stand (for 12 1.5-mL tubes) from Promega (Madrid, Spain, www.promega.com). The protocol was as follows: (1) 100 μL of MB suspension were added to a 1.5-mL tube and washed twice with 100 μL of 25 mM MES, pH 5, for 10 min with

vigorous mixing; for the washing steps, the tube was placed on the magnetic separation stand and the washing solutions were removed; (2) 50 μL of 50 mg·mL^{-1} EDC solution and 50 μL of 50 mg·mL^{-1} NHS solution (prepared immediately before use in cold 25 mM MES), pH 5.0, were added and incubated for 30 min with slow tilt rotation; (3) the activated MBs were washed twice with 25 mM MES, pH 5.0; (4) 200 μL of DAO (from 1/8 to 1/256 dilution) in 25 mM MES, pH 5.0, were added and incubated for 1 h with slow tilt rotation; (5) the DAO-MB conjugates were washed three times with 0.1 M potassium phosphate buffer, pH 7.2. All steps were performed at room temperature. For higher MB amounts, volumes were adjusted proportionally.

Biosensor preparation

For the biosensor preparation, 10 μL of DAO-MB suspension were placed on Co(II)-phthalocyanine/carbon, Prussian Blue/carbon or Os-wired HRP-modified carbon working electrodes, where modified MBs were trapped with a magnetic support at the back side. The supernatant was then removed. Os-wired HRP-modified carbon electrodes were previously obtained by placing 1 μL of Os-wired HRP stock solution on the DRP-110 working electrode and allowing to dry for 30 min.

DRP-8X110 electrodes were used for multiple analysis (eight samples). In this case, 5 μL of a 1/10 Os-wired HRP dilution from stock solution were placed on each working electrode and allowed to dry for 30 min. Then, 5 μL of DAO-MB suspension were placed on the working electrodes, modified MBs were trapped with a magnetic support and the supernatant was removed.

Storage stability of DAO-MB conjugates

A DAO-MB conjugate pool was prepared, and aliquots were stored at 4 °C and −20 °C without and with 10 % of glycerol. The initial activity of the conjugates (reference value) and the activity after 1, 2, 7, 15, 30, 60 and 90 days was measured as explained in "Electrochemical protocols" section.

Fish samples and BAs extraction

Sea bass samples were obtained from Alfacs bay in Ebre Delta (NW Mediterranean Sea). Fish samples were first 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. Samples were stored at 4 °C and at room temperature (25 °C) during 3 days.

For BAs extraction, 2 g of the homogenized sample were vigorously mixed with 4 mL of phosphate buffer, pH 7.2 for 1 min in a vortex. The mixture was then centrifuged at 4 °C for 10 min at 15,000 rpm and the supernatant was recovered. The procedure was repeated and the two extracts were gathered, adjusting the volume to a final concentration of 25 mg·mL^{-1} with the same buffer.

Electrochemical protocols

Cyclic voltammetry and amperometric measurements were performed with an AUTOLAB PGSTAT128N potentiostat (Utrecht, The Netherlands, www.metrohm-autolab.com) and a PalmSens potentiostat connected to an 8-channel multiplexer (MUX8) (Houte, The Netherlands, www.palmsens.com). Data were collected and evaluated by General Purpose Electrochemical System (GPES) and PalmSensPC software. Screen-printed electrodes were horizontally-positioned on the magnetic supports. All measurements were performed at room temperature.

Cyclic voltammetry was used to characterise the response of the DAO-modified electrodes towards BAs in mono-enzymatic and bi-enzymatic configurations. The protocol was as follows: 45 μL of 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2, were placed on the electrode and cyclic voltammograms were recorded between −0.2 and +0.8 V vs. Ag (potential window depended on the electrode and electrochemical transduction approach) at 5 mV·s^{-1}; 5 μL of 10 mM Hist, Put or Cad solution were then added and, after 10 min, a cyclic voltammogram was recorded again. This protocol, using Co(II)-phthalocyanine/carbon electrodes and 50 mM Hist, was applied for the optimisation of the concentration of DAO to use in the conjugation to MBs.

Amperometry was used to construct the calibration curves, to evaluate the storage stability of DAO-MB conjugates and to determine the BAs content in fish samples. The protocol was as follows: 25 μL of 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2 were placed on the electrode and a potential of +0.4 V (vs. Ag) for Co(II)-phthalocyanine/carbon electrodes, −0.1 V (vs. Ag) for Prussian Blue/carbon electrodes or +0.05 V (vs. Ag) for Os-wired HRP-modified carbon electrodes was applied; the current intensity was recorded for 60 s; 50 μL of sample were then added and, after 10 min, the current was recorded again for 120 s. The difference in current intensity between before and after sample addition was measured. Experiments were performed in triplicate ($N = 3$). For the BA calibration curves, the samples consisted of Hist, Put or Cad solutions at different concentrations ranging from 1 μM to 4 mM and the three type of electrodes were used. In the study of the DAO-MB storage stability, the sample was 10 mM Put and Prussian Blue/carbon electrodes were used. In the analysis of fish samples, the sample was sea bass extract at 25 mg·mL^{-1} and Prussian Blue/carbon electrodes were also used.

The amperometric multiplexed detection was used to construct a Hist calibration curve and to perform an interferences study. The protocol was similar to the one with individual electrodes, but adjusting the volumes to the smaller area of

the working electrodes of the array. In this case, 20 μL of 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2 were placed on the electrodes and a potential of +0.05 V (vs. Ag) was applied; the current intensity was recorded for 60 s; 40 μL of sample were added on each electrode and, after 10 min, the current was recorded again for 120 s. The difference in current intensity between before and after sample addition was measured. Experiments were also performed in triplicate ($N = 3$). For the Hist calibration curve, concentrations ranging from 1 μM to 4 mM Hist were used. In the study of interferences, 1 mM Hist, DA, TA, His, Lys, Arg, Tyr or Trp were used.

All experiments reported were performed using a different electrode for each measurement. However, consecutive measurements were performed with the same electrode to study the repeatability of the DAO-MB biosensors. In this case, Prussian Blue/carbon electrodes were used and the protocol was as follows: 25 μL of 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2 were placed on the biosensor and a potential of −0.1 V was applied. When the current was stabilised, 50 μL of 1 mM Hist were added. Once the current was stabilised again, the difference in current intensity between before and after Hist addition was measured. The supernatant was then removed and the biosensor was washed with 50 μL of 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2. The same protocol was repeated up to 8 consecutive times. Repeatability studies were performed in quadruplicate ($N = 4$).

Colorimetric enzyme assay

For the colorimetric enzyme assay, 100 μL of Hist at concentrations ranging from 0.01 to 1 mM or sea bass extracts at 25 $mg{\cdot}mL^{-1}$ were added into microtiter wells containing 20 μL of 5 $mg{\cdot}mL^{-1}$ ODA, 20 μL of 0.01 $mg{\cdot}mL^{-1}$ HRP and 60 μL of 1/100 DAO dilution or 0.1 M potassium phosphate buffer, pH 7.2. After 15 min incubation with gentle shaking at room temperature, absorbance at 440 nm was measured using a Synergy HT microplate reader (Winooski, United States, www.biotek.com). Assays were performed in duplicate ($N = 2$).

Statistical analysis

The linear regression model was used to evaluate the correlation between the expected and the determined Hist equivalent contents in spiked fish samples. In the comparison of the analysis of naturally-spoiled fish samples by the electrochemical biosensor and the colorimetric assay, data were first tested for normality. Since data were non-normally distributed, the Wilcoxon matched-pairs signed-ranks test was used to evaluate the differences in the Hist equivalent contents determined by the two methods. SigmaStat 3.1 software package was used for the linear regression and the Wilcoxon matched-pairs signed-ranks test. In both analyses, differences in the results were considered statistically significant at the 0.05 level.

Results and discussion

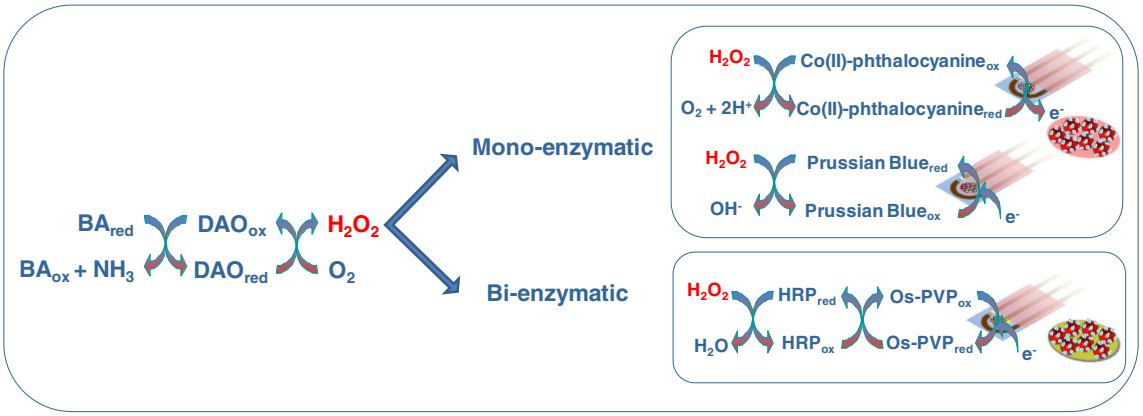
Optimisation of the DAO-MB biosensors

The detection of Hist, Put and Cad was performed following the strategies shown in Schema 1. DAO specifically recognises the biogenic amines, oxidising them and producing H_2O_2 in the enzyme reaction. The H_2O_2 is then detected directly on the electrode surface or through redox mediators (mono-enzymatic biosensor) or through reaction with mediated HRP (bi-enzymatic biosensor). An exhaustive comparison of electrode supports and electrochemical transduction strategies for the detection of H_2O_2 was previously performed. Results are presented in the Electronic Supplementary Material (ESM). Co(II)-phthalocyanine/carbon and Prussian Blue/carbon electrodes and Os-wired HRP-modified carbon electrodes were selected for the subsequent development of mono-enzymatic and bi-enzymatic biosensors, respectively.

Although HRP is known to have a higher enzyme activity when working at lower pH, potassium phosphate buffer, pH 7.2, the optimal buffer for DAO enzyme activity, was used. Unlike HRP, which is present in excess, DAO activity may become limiting and thus, it was prioritised regarding the pH.

For the optimisation of DAO concentration to use in the conjugation to MBs, conjugates synthesised with different DAO dilutions were tested on Co(II)-phthalocyanine/carbon electrodes by cyclic voltammetry. The conjugation of DAO to MBs is based on a stable amide bond between the activated carboxylic acid group of the beads and an amine group of the enzyme. The oxidation peak intensity due to the presence of a saturated concentration of Hist was constant from 1/8 to 1/128 DAO dilution, and started to decrease at 1/256 DAO dilution (*results not shown*), suggesting that the 1/128 DAO dilution was enough to completely coat the MBs. As a preventive measure, a 1/100 DAO dilution was chosen for subsequent experiments. This experiment demonstrated not only the efficiency of the immobilisation method, but also the retention of the functionality of the enzyme despite having been immobilised through one of its amine groups.

Cyclic voltammetry was performed to compare the response from DAO-MB conjugates to different BAs. Addition of the BA on the mono-enzymatic biosensors incorporating DAO-MB conjugates and Co(II)-phthalocyanine (Fig. 1a) and Prussian Blue (Fig. 1b) mediators resulted in an increase of the oxidation and reduction current,



Schema 1 Enzymatic mechanisms and electrochemical transduction strategies of DAO-MB mono- and bi-enzymatic biosensors

respectively. A higher affinity of DAO towards Put and Cad compared with Hist was observed, as it happened when measuring the activity of DAO in solution by the colorimetric assay. Similar results were obtained with the bi-enzymatic biosensor using DAO-MB conjugates on Os-wired HRP-modified carbon electrodes (Fig. 1c). Well-defined osmium oxidation and reduction peaks were observed before BAs addition. In the presence of BAs, bioelectrocatalytic currents were obtained and differences between BAs were clearly observed.

Characterisation of the DAO-MB biosensors

Amperometric detection of BAs was performed applying the potentials selected from the characterisation by cyclic voltammetry. For Prussian Blue/carbon and Os-wired HRP-modified carbon electrodes, working potentials of −0.1 V and +0.05 V were chosen, respectively, to guarantee a complete reduction of the mediator oxidised after the BA addition, and thus to obtain high current intensities in amperometry. However, the potential of the oxidation peak with Co(II)-phthalocyanine/carbon electrodes was too high (+0.6 V) and compromise between high current intensities and interferences avoidance had to be undertaken. Consequently, calibration curves were obtained for Hist, Put and Cad applying +0.4 V to Co-phthalocyanine/carbon electrodes, −0.1 V to Prussian Blue/carbon electrodes and +0.05 V to Os-wired HRP-modified carbon electrodes. The biosensor variability, expressed as the relative standard deviation (RSD) for the triplicate measurements, was always less than 10 % in all configurations, showing appropriate intra-day reproducibility. Despite enzyme adsorption generally results in weak immobilisations that could lead to enzyme leaching, the configuration with Os-wired HRP-modified carbon electrodes presented similar biosensor variability than the other configurations. This can be well explained by the high adsorptive properties of the Os-wired HRP due to the presence of the PVP redox polymer.

Table 1 summarises the results obtained from the calibration curves. Analytical parameters were very similar across strategies, with linear measuring ranges from 0.01 up to 1 mM BA. Higher sensitivities and lower LODs (LOD = blank +3 SD) and limits of quantification (LOQs) (LOQ = blank +10 SD) were always attained for Put and Cad, as expected from the cyclic voltammetry measurements. However, it should be mentioned that the bi-enzymatic approach presented higher intercept values compared to the mono-enzymatic approaches. This fact may be explained by the presence of Os-wired HRP adsorbed on the modified electrode and a change on the kinetics of the overall enzymatic reaction [25]. Although some works have reported that bi-enzymatic approaches result in a better performance compared to mono-enzymatic approaches [26], in this work the bi-enzymatic biosensor did not present a higher sensitivity. Taking into account the results obtained in the H_2O_2 detection optimisation (*see ESM*), we hypothesise that the amount of immobilised redox mediator may be limiting the bioelectrocatalytic current. Nonetheless, the three strategies present enough sensitivity to detect Hist at lower concentrations than the regulation levels and even at much lower concentrations for Put and Cad, as well as high reproducibility, low-cost and rapid detection. Any of the three approaches can be used to conduct the subsequent experiments.

Lower LODs may be attained applying a higher working potential when using Co(II)-phthalocyanine/carbon electrodes, but this can be detrimental because matrix compounds often interfere. In case of electrodes modified with Os-wired HRP, higher amounts of immobilised redox mediator may also improve the LODs, but this would raise the price of the assay. Consequently, although feasible, these improvements in the LOD may compromise the practical applicability. Table 2 summarises the electrochemical biosensors for Hist, Put or Cad detection reported since 2010. As it can be observed, the LODs obtained in this work are similar to those attained with other enzymatic electrochemical biosensors. When

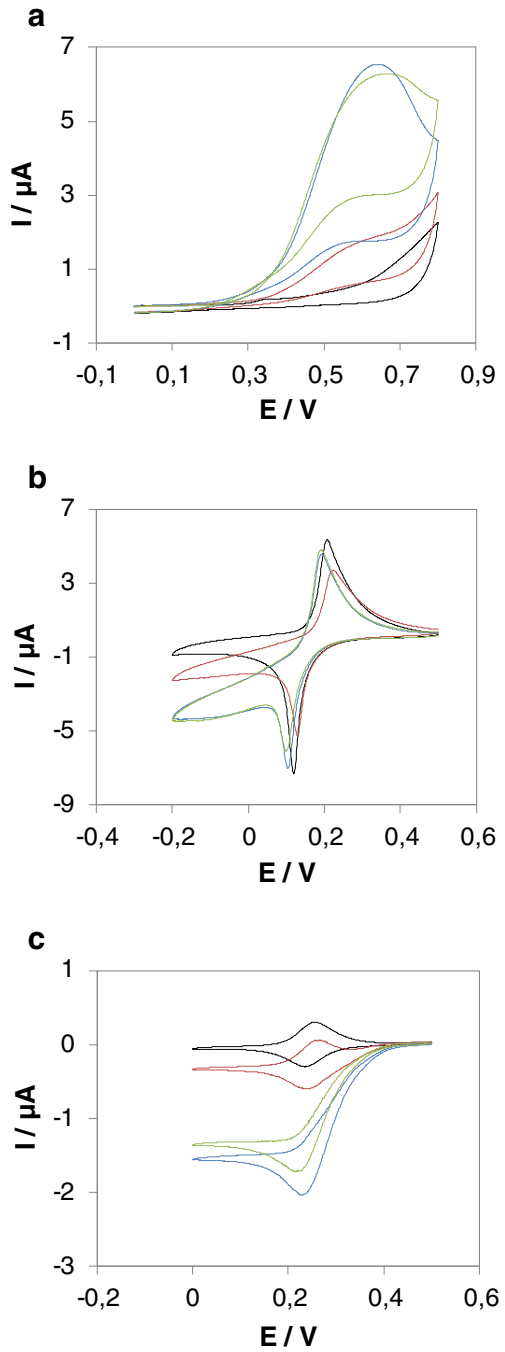


Fig. 1 Cyclic voltammograms performed in 0.1 M potassium phosphate buffer, 0.1 M KCl, pH 7.2 at 5 $mV \cdot s^{-1}$ in the presence of DAO-MB conjugates before (*black*) and after the addition of 10 mM Hist (*red*), Put (*blue*) and Cad (*green*) using: **a** Co(II)-phthalocyanine/carbon; **b** Prussian Blue/carbon; and **c** Os-wired HRP-modified carbon electrodes

comparing the DAO-MB electrochemical biosensors to the optical biosensor that also uses magnetic beads as DAO immobilisation supports, the LODs obtained are even two orders of magnitude lower [27]. The use of MBs as enzyme immobilisation supports allows the direct modification of the electrode surface in a simple and fast way, and provides high stability to the biosensor.

Due to the low cost, ease construction and high reproducibility, DAO-MB biosensors were originally designed for single use. However, a repeatability study was performed using Prussian Blue electrodes by consecutive 1 mM Hist additions. Results demonstrated that the biosensor can be used at least 8 times consecutive without any loss of activity (>95 % amperometric response). The possibility to reuse the same biosensor further reduces the cost of the assay and highlights another advantage of the use of MBs in terms of biosensor regeneration.

Storage stability of DAO-MB conjugates

To investigate the possibility to store the DAO-MB conjugates until use, their stability at 4 °C and −20 °C during 90 days was evaluated. Glycerol was added to MB-DAO suspensions to study if the addition of a stabiliser improved the retention of the activity of the conjugates. Amperometric recordings on Prussian Blue/carbon electrodes were constant with time, clearly demonstrating that DAO-MB conjugates were stable for at least 3 months, regardless the temperature and the presence of glycerol (Fig. S3). A high robustness of the conjugates under different storage conditions was observed (RSD between 3 and 10 %, depending on the measurement day). Additionally, the measurements showed an appropriate inter-day reproducibility (RSD = 8 %). To the best of our knowledge, the high stability of the DAO-MB conjugates provides the most stable enzymatic electrochemical biosensor for Hist, Put or Cad detection reported in recent years (Table 2).

As observed in previous works [28], the immobilisation of the enzyme on MBs increases the stability of the enzyme activity. The good storage stability of the conjugates demonstrates additional advantages of using MBs as enzyme immobilisation supports in the biosensor development. On the one hand, as there is no need to prepare the conjugates immediately prior the analysis, the protocol is simplified and the assay time is drastically shortened. On the other hand, the ability to use a same conjugate pool for the analysis of different samples on different days provides even higher reproducibility to the system.

Multiplexed detection

To demonstrate the straightforward integration of the DAO-MBs in a multiplexed system, a calibration curve for Hist was performed using an array of 8 Os-wired HRP-modified carbon screen-printed modified electrodes connected to the potentiostat through an 8-channel multiplexer. The array configuration presented a sensitivity of 0.47 $nA \cdot \mu M^{-1}$, with a linear range from 0.06 mM to 1 mM Hist and an LOD of 8.25 µM. Despite the smaller surface area of the working electrodes of the array compared to the single electrodes

Table 1 Analytical parameters of the DAO-MB electrochemical biosensors on Co(II)-phthalocyanine/carbon at +0.4 V (vs. Ag), Prussian Blue/carbon at −0.1 V (vs. Ag) and Os-wired HRP-modified carbon electrodes at +0.5 V (vs. Ag) for Hist, Put and Cad determination

		Intercept (nA)	Sensitivity (nA·μM^{-1})	LOD (μM)	LOQ (μM)	R^2
Co(II)-phthalocyanine	Hist	6.07	0.13	5.13	49.46	0.998
	Put	5.83	2.11	1.03	3.16	0.999
	Cad	6.22	3.03	0.60	2.07	0.995
Prussian Blue	Hist	7.27	0.57	4.80	38.12	0.995
	Put	7.09	3.22	0.90	6.80	0.997
	Cad	7.93	3.12	0.67	6.70	0.999
Os-wired HRP	Hist	31.70	0.31	4.50	38.48	0.999
	Put	30.07	3.31	0.90	3.90	0.997
	Cad	31.85	2.61	0.47	4.27	0.999

(2.5 mm in diameter in front of 4 mm) and the lower amount of DAO-MB conjugates used in this configuration (5 μL in front of 10 μL), the sensitivity is slightly higher (0.47 nA·μM^{-1} in front of 0.31 nA·μM^{-1}). However, the LOD is higher than in the single approach (8.25 μM in front of 4.50 μM). This increase in the LOD may be explained by a lower accuracy of the portable PalmSens potentiostat (±0.5 %), additionally connected to the 8-channel multiplexer, in comparison with the AUTOLAB PGSTAT128N potentiostat used to measure the single electrodes (±0.2 %). Nevertheless, it remains at the same order of magnitude than the previous configurations and it is still low enough to detect Hist at values lower than those established by the European Regulation or other agencies.

The feasibility to use lower amounts of MBs and reduce the cost of the biosensor, together with the possibility to perform multiple measurements in a compact and fast way, makes the use of DAO-MBs on array electrodes clearly suitable for high-throughput analysis of BAs.

Study of interferences

Histidine (Hist), lysine (Lys), arginine (Arg), tyrosine (Tyr) and tryptophan (Trp) are amino acids involved in the biosynthesis of BAs, and frequently found in fish. Dopamine (DA) and tyramine (TA) are biogenic amines similar to Hist, Put and Cad that may show electrochemical interferences [29, 30]. To ensure the applicability of the developed biosensor to the determination of Hist, Put and Cad content in fish samples, the possible interference from these amino acids as well as DA and TA should be evaluated. As can be seen in Fig. 2, all amperometric responses were lower than 6 % of the response towards Hist. Results demonstrate that all these related compounds are not detected by the biosensor and thus do not interfere in the determination of BAs contents. Moreover, as this study was conducted with the electrode array, it demonstrates again the suitability of the approach for the analysis of multiple samples.

Analysis of fish samples

To evaluate the applicability of the DAO-MB electrochemical biosensor to the determination of BAs contents in fish samples, spike recovery studies and analysis of naturally-spoiled sea bass samples were performed on Prussian Blue/carbon electrodes. In the recovery experiments, different Hist/Put/Cad combinations were spiked and the final BAs contents of the samples were expressed in Hist equivalent contents (Put and Cad concentrations were converted into Hist equivalents by interpolation using the calibration curves). For the calculation of the recovery, the initial BAs content present in the sample (combination 1) was subtracted. As it can be seen in Table 3, results obtained were satisfactory, recovery being always between 97.0 and 102.2 %. Comparing the whole set of combinations, statistical analysis revealed that were not significant differences between the expected and the determined Hist equivalent contents ($y = 0.983\times + 1.761$, $R^2 = 0.998$, $p < 0.001$).

DAO-MB biosensors were also used to analyse sea bass samples stored for 3 days at 4 °C and 25 °C to follow natural spoilage (Table 4). As expected, sea bass samples presented low initial BAs contents, which increased with storage time, the effect being much more evident with samples stored at 25 °C. In some samples, BAs were detected at concentrations below the LOQ. Although quantifications at these levels were not accurate and showed high relative standard deviation values, they have been provided to show the increase of BAs concentrations in fish with time and/or under inadequate storage conditions. Concentrations above the LOQ provide relative standard deviations not higher than 10 %.

BAs contents determined with the biosensor were compared to those obtained by the colorimetric enzyme assay. Statistical analysis revealed that were not significant differences between the results determined by the amperometric biosensor and those provided by the colorimetric enzyme assay ($W = 53$, $p = 1$). These results demonstrate the applicability of the developed biosensor to the analysis of fish samples

Table 2 Enzymatic electrochemical biosensors for the determination of Hist, Put or Cad reported since 2010

Biogenic amines	Enzyme	Immobilisation	Mediator	Potential (mV)	LOD	Stability	Sample	References
Hist, Put, Cad	DAO, DAO/HRP	Covalent binding with carboxylic acid groups of magnetic beads	Co(II)-phthalocyanine Prussian Blue Osmium	+400 −100 +50	4.5–5.13 μM Hist 0.90–1.03 μM Put 0.47–0.60 μM Cad	3 months	Sea bass	This work
Hist PUT	HMD PUO	Crosslinking with GA and BSA on SPE	Tetrathiafulvalene	+130 +300	8.1 μM Hist 10 μM Put	–	Octopus Zucchini	[6]
Put	PUO	Crosslinking with GA and BSA on SPE	Tetrathiafulvalene	+300	10.1 μM Put	–	Octopus Zucchini	[7]
Hist	DAO	Entrapment on CeO_2-polyaniline nanoparticles using a Nafion solution	–	−100	48.7 μM Hist	18 days	Prawns	[18]
Hist	DAO/HRP	Immobilisation into a polysulfone/carbon nanotubes/ferrocene membrane	Ferrocene	−50	0.17 μM Hist	1 month	Fish	[11]
Put, Cad	MAO	Crosslinking with GA and BSA on SPE or an array modified with AuNPs	Tetrathiafulvalene	+250	9.9 μM Put 19.9 μM Cad	–	Octopus	[8]
Put	MAO	Crosslinking with GA and BSA on SPE	TTF	+250	17.2 μM Put	–	Zucchini Anchovies	[9]
Put, Cad	PSAO	Entrapment into a Nafion membrane	MnO_2	+400	6.8 μM Put 12.6 μM Cad	–	Chicken	[12]
Put, Cad	PSAO	Entrapment into a Nafion membrane	MnO_2	+400	49.6 μM Put 57.1 μM Cad	–	Fish sauce	[13]
Hist	DAO	Entrapment into a photocured poly (2-hydroxyethyl) metacrylate film	Potassium ferricyanide	+350	3.5 μM Hist	–	Prawns	[14]
Put	PUO/HRP	Entrapment into PEGDE	Osmium	+50	5 μM Put	7–10 days	Beer	[15]
Hist Put	DAO/HRP PUO/HRP	Entrapment into PEGDE	Osmium	-50 +50	5 μM Hist 5 μM Put	–	Fish Meat	[16]
Put	DAO	Crosslinking on FeO_3 nanoparticles	–	+400	0.65 nM	6 days	–	[19]
Put	DAO	Entrapment into a polyazetine prepolymer	–	+600	1.24 μM	–	Wine Beer	[17]
Hist	MAO/HRP DAO/HRP	Crosslinking on SPE using an aryl diazonium salt	Ferrocene	+250	0.4 μM Hist 0.18 μM Hist	–	Anchovies	[10]

DAO diamine oxidase, *HMD* histamine deshydrogenase, *HRP* horseradish peroxidase, *MAO* monoamine oxidase, *PSAO* pea seedling amine oxidase, *PUO* putrescine oxidase, *SPE* screen-printed electrode

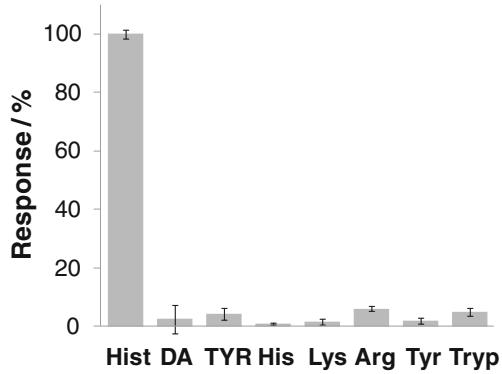


Fig. 2 Study of interferences of DAO-MB biosensors using Os-wired HRP-modified carbon electrode arrays. Comparison between 1 mM histamine (Hist) and 1 mM of potentially interfering compounds: dopamine (DA), tyramine (TA), histidine (His), lysine (Lys), arginine (Arg), tyrosine (Tyr) and tryptophan (Tryp)

and their use as both screening tool at the regulatory level and quantification analysis technique.

Conclusions

The present work reports low-cost and easy-to-use amperometric biosensors for BAs determination using MBs as DAO immobilisation supports. This immobilisation technique is simple and efficient, provides stable conjugates and allows direct contact of the enzyme with the analyte without diffusion barriers. Moreover, the immobilisation of DAO on MBs increases the stability of the enzyme activity.

The use of MBs as immobilisation supports allows the direct modification of any type of electrode surface by only placing the electrode on a magnetic support. By this method, Co(II)-phthalocyanine and Prussian Blue monoenzymatic biosensors and Os-wired HRP-modified bienzymatic biosensors have been developed, presenting in all cases LODs for Hist, Put and Cad in the submicromolar to micromolar range, a broad linear range of two orders of magnitude, good reproducibility, high repeatability and no interferences from other related compounds. A clear advantage presented by the use of DAO-MB conjugates over other BAs electrochemical biosensors is their excellent storage stability, which allows having ready-to-use conjugates available any time. This fact, together with their easy transfer to multiplexed systems to perform multiple measurements, reduces the analysis time and drastically simplifies the protocol. Given the demonstrated suitability of the three approaches for monitoring the BAs content, DAO-MBs constitute an excellent tool to foster the applicability of electrochemical biosensors to fast, easy, low-cost and high throughput detection of BAs in the fish industry in particular and in the agro-food sector in general. Efforts focused on the increase of bio/electrocatalytic currents may lead to lower LODs and LOQs, providing more accurate and precise BAs quantifications at lower concentrations. The integration of these DAO-MB biosensors into microfluidics systems may also result in more sensitive and faster analysis devices, which would also be automated and portable.

The described approaches, applied to DAO as enzyme and BAs as analytes, are versatile and can be easily used with other oxidases and target compounds of interest in food control, but also in environmental and medical applications.

Table 4 Hist equivalent contents ($mg \cdot kg^{-1}$) in sea bass samples determined by the DAO-MB electrochemical biosensor using Prussian Blue/carbon electrodes and the colorimetric enzyme assay

	DAO-MB electrochemical biosensor		Colorimetric enzyme assay	
0 h	23 ± 10		36 ± 9	
	4 °C	25 °C	4 °C	25 °C
24 h	57 ± 17	212 ± 35	89 ± 29	170 ± 9
48 h	149 ± 17	301 ± 28	87 ± 35	425 ± 40
72 h	162 ± 36	1050 ± 110	138 ± 20	1188 ± 16

Table 3 BAs spiking combinations and recovery percentages from sea bass samples with the DAO-MB electrochemical biosensor using Prussian Blue/carbon electrodes

Spiking combinations	Hist	Put	Cad	Expected [Hist]eq (μM)	Determined [Hist]eq (μM)	Recovery (%)
1	−	−	−	–	–	–
2	+	−	−	62.50	61.45 ± 0.11	98.3
3	−	+	−	66.01	64.03 ± 0.77	97.0
4	−	−	+	44.86	45.70 ± 0.14	101.9
5	+	+	−	128.51	125.06 ± 0.53	97.3
6	+	−	+	107.36	107.24 ± 0.89	99.9
7	−	+	+	110.86	113.25 ± 0.17	102.2
8	+	+	+	173.36	175.94 ± 0.65	101.5

The + symbol indicates 62.5 μM for Hist and 15.6 μM for Put and Cad; the − symbol indicates 0

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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