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Determination of biogenic amines with an electrochemical biosensor and its application to salted anchovies

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This paper describes the assembling and optimization of an electrochemical biosensor for the determination of biogenic amines (putrescine, cadaverine, histamine, tyramine, spermidine, spermine, tryptamine) commonly present in food products, and its application to salted anchovy samples. Variations of the amine content in anchovies during ripening time were measured both with the biosensor and ion chromatography with integrated pulsed amperometric detection (IC–IPAD). The probe is based on a platinum electrode which senses the hydrogen peroxide produced by the reaction catalysed by the enzyme diamine oxidase (DAO), purified from commercial seeds of cicer and immobilized on the electrode surface. Parameters such as enzyme immobilization and pH have been studied and optimised in order to obtain similar sensitivity for all the amines tested. The immobilization of the enzyme on a nylon-net membrane, using glutaraldehyde as cross-linking agent, and phosphate buffer at pH 8.0 were selected. The detection limit was 5×10^{-7} mol litre^{-1}. The linear range common to the amines tested was observed from 1×10^{-6} to 5×10^{-5} mol litre^{-1}. The effect of potentially interfering compounds was also evaluated. Underivatized biogenic amines such as putrescine, cadaverine, histamine, tyramine and spermidine were also detected with the IC–IPAD method. Changes in the concentration of biogenic amine content in salted anchovy samples, measured with the biosensor and IC–IPAD methods, exhibited the same trend and demonstrated that the biosensor is a useful tool to monitor the variation of the total amine content in fish during storage.

INTRODUCTION

Biogenic amines are aliphatic, alicyclic and heterocyclic organic bases of low molecular weight. These substances are ubiquitous in biological matrices. They are not only biosynthesized in animal and plant cells but also produced by microbial decarboxylation of amino acids. The amount and type of amine formed is therefore strongly influenced by the food composition, microbial flora and by several parameters which allow bacterial growth during food storage, such as food treatment prior to storage, food additives, temperature, moisture, ripening and packaging (Halasz *et al.*, 1994). The importance of the estimation of the levels of biogenic amines in food and beverages is related to their impact on human health and food quality.

Biogenic amines have various toxicological implications.

1. Histamine poisoning, which results from ingestion of food with high levels of histamine, produces a variety of gastrointestinal (nausea, vomiting, diarrhea, abdominal cramps), cutaneous (rash, urticaria, edema), hemodynamic (hypotension) and neurological (flushing, itching, burning, tingling, headache) symptoms (Merson *et al.*, 1974; Arnold and Brown, 1978; Murray *et al.*, 1982; Taylor, 1986). The histamine toxicity is increased by the presence of other amines, such as putrescine and cadaverine, that inhibit the intestinal enzymes (diamine oxidase and histamine-*N*-methyl-trans-

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ferase) that metabolize it (Ienistea, 1973; Stratton *et al.*, 1991).
2. Tyramine can exert vasoactive effects; a typical phenomenon is the 'cheese reaction', usually caused by high levels of tyramine in cheese (Rice *et al.*, 1976).
3. In the presence of nitrites, biogenic amines produce compounds that can be endogenous precursors of *N*-nitrosamines (Heisler *et al.*, 1974).
4. Finally, ingestion of biogenic amines may provoke hypertensive crises in patients being treated with monoamine oxidase inhibitor drugs (Ponto *et al.*, 1977).

However, these amines are not considered a grave risk for humans if they are present in low levels in foods.

Since the concentration of biogenic amines can change during food processing and storage, their formation has been proposed as an index of chemical quality (Mietz and Karmas, 1977).

Analytical determination of biogenic amines is usually carried out by HPLC methods. Almost all the proposed methods use indirect detection following precolumn derivatisation to form dansyl (DNS) derivatives (Spinelli *et al.*, 1974; Hui and Taylor, 1983; Lehtonen, 1986; Rosier and Peteghem, 1988), benzoyl derivatives (Yen and Hsieh, 1991) or *o*-phthalaldehyde (OPT) derivatives (Veciana-Nogues *et al.*, 1995). Recently we developed a method based on ion exchange chromatography with integrated pulsed amperometric detection (IC–IPAD) for simultaneous determination of underivatized biogenic amines (Draisci *et al.*, 1993). However, chromatographic methods are quite complex and require long analysis times and expensive instrumentation.

Electrochemical enzyme probes, based on oxygen electrodes using monoamine oxidase (MAO) and diamine oxidase (DAO), have been reported (Toul and Macholan, 1975; Karube *et al.*, 1980). A plant tissue biosensor for analysis of biogenic amines was also reported (Botrè *et al.*, 1993).

The aim of this work was the characterization and analytical optimization of a H_2O_2 electrochemical probe based on the enzyme DAO, to evaluate variations of the total amine content in salted anchovies during the ripening process. The concentration of the single amines in the anchovy samples was also determined by IC–IPAD.

MATERIALS AND METHODS

Materials

Diamine oxidase-specific activity 25 U mg protein^{-1}, was obtained from cicer seedlings grown 8–10 days in vermiculite, in the dark, at 25°C following the procedure of Padiglia *et al.* (1991) for the isolation of the enzyme. The activity of the enzyme was determined polarographically by a Gilson oxygraph equipped with a Clark electrode. The measurements were carried out at 37°C in 1 ml phosphate buffer, 0.1 mol litre^{-1}, pH 7.0, as reported by Padiglia *et al.* (1991).

Putrescine, cadaverine, tyrosine, tyramine were from Novachimica, Milan, Italy. Spermidine, spermine, histamine, histidine and tryptamine were from Sigma Chemical Co., St Louis, MO, USA. For HPLC analysis, water was purified with a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Cellulose acetate membrane, 100-DA cut-off, was prepared in our laboratory as reported in the literature (Taylor *et al.*, 1977; Palleschi *et al.*, 1986; Mascini and Mazzei, 1987).

Polycarbonate membrane, 0.03-μm pore size, was from Nucleopore, Pleasanton, CA, USA; Immobilon® Affinity Membrane was from Millipore Co. (Bedford, MA, USA); Nylon Net 120 mesh cm^2, 100 μm thickness was from A. Bozzone (Appiano Gentile, Milan, Italy). A commercially available nyon net, 60 mesh cm^2, 100 μm thickness, obtained from a local shop, was also used as support for the enzymatic mixture.

Apparatus

The H_2O_2 sensor consisting of a platinum electrode poised at +650 mV vs a built-in silver–silver chloride was from Universal Sensors (Metairie, LA, USA). The current output was monitored with an Amperometric Biosensor Detector (ABD) from Universal Sensors and recorded with an Amel Mod. 868 recorder (Milan, Italy).

For chromatographic analysis, a model 4500i liquid chromatography (Dionex Co., Sunnyvale, CA, USA) was used. IPAD was performed with a model ED40 electrochemical detector (Dionex Co.), comprising a thin-layer electrochemical cell equipped with a gold working electrode, a stainless-steel counter electrode and a combined pH-Ag/AgCl reference electrode. A Dionex Reagent Delivery Module (RDM) was used for post-column addition of sodium hydroxide. Samples were injected using a Rheodyne model 9126-038 injection valve with a PEEK sample loop of 25 μl (Cotati, CA, USA). The system was interfaced via an Advanced Computer Interface, ACI, module (Dionex Co.) to an EL-35 80386X based personal computer (Epson, Sesto S. Giovanni, Italy). Chromatographic software AI-450 (Dionex Co.) was used for data acquisition and instrument control.

An Ultra-Turrax T25 blender (Janke & Kunker Ika Labortechnik, Staufen, Germany) and a model ALC 4237 centrifuge (ALC International, Milan, Italy) were used for sample preparation.

Biosensor development

Enzyme immobilization procedure

Three different membranes were used for the immobilization of diamine oxidase: (i) Immobilon® Affinity

membrane (hydrophilic polyvinylidene difluoride) chemically derivatized to allow covalent protein immobilization through the ε-amino groups of lysines; (ii) nylon net membrane (nylon 66) activated according to the method described by Mascini *et al.* (1983); and (iii) nylon net commercially available used only as membrane support with glutaraldehyde as cross-linking agent.

Enzymatic solution (8 μl) was pipetted onto one side of the Immobilon membrane and allowed to react for 90 min. The membrane was then washed with phosphate buffer, 0.1 mol litre^{-1}, pH 7.0.

In the case of the nylon net membrane, 80 μl of DAO was pipetted onto one side of the membrane which was left in a Petri dish for 2 h at room temperature and then at 4°C for 24 h. The membrane was first extensively washed with phosphate buffer (0.1 mol litre^{-1}, pH 7.0) containing NaCl 1 mol litre^{-1} and then with glycine 0.1 mol litre^{-1} for 30 min.

For the immobilization of the enzyme on the nylon net used only as support, 16 μl of enzymatic solution was mixed with 4 μl of glutaraldehyde 0.25% in a well using a glassy stick. The mixture was put on the membrane and dried for 20 min with a cold draught. Then it was extensively washed (30 min) with glycine 0.1 mol litre^{-1} to eliminate the excess of glutaraldehyde.

Biosensor assembling

The probe was assembled by placing the following membranes on an inverted jacket in the given order: cellulose acetate membrane (which protects the platinum electrode from electrochemical interferences), enzymatic membrane and a polycarbonate membrane which protects the enzyme from large molecules or bacteria. These membranes were then secured with an O-ring. The electrode jacket was filled with a solution of 0.1 mol litre^{-1} potassium chloride, the electrode was then inserted into the jacket and screwed down until the tip of the platinum was firmly in contact with the membranes.

Preparation of standard solution and samples

For HPLC analysis, biogenic amine stock solutions containing 1000 μg/ml^{-1} of each compound were prepared in perchloric acid 0.375 mol litre^{-1}. The solutions were stored refrigerated at +4°C and were stable for at least 15 days. Standard solutions in the range 0.05–10 μg ml^{-1} were prepared daily by diluting the stock solutions with 0.375 mol litre^{-1} perchloric acid. Sample preparation for HPLC analysis was as follows: 30 ml perchloric acid, 0.375 mol litre^{-1}, were added to 10 g of homogenized sample. Samples were then homogenized (1500 rpm, 5 min) and quantitatively transferred into a 100-ml volumetric flask, rinsing carefully all glassware with perchloric acid, 0.375 mol litre^{-1}. In order to remove the lipid fraction, 5 ml of the solution was extracted with 2.5 ml of *n*-hexane and centrifuged at 3000 rpm for 10 min, discarding the supernatant. Finally, the aqueous phase was filtered through a 0.2-μm filter and injected for chromatographic determination.

For biosensor detection, 1 g of sample was homogenized with 10 ml of phosphate buffer, 0.1 mol litre^{-1} pH 8. After filtration the homogenate was injected directly into the buffer solution for the analysis.

Amine analysis

Biosensor method

The generic reaction, catalysed by diamine oxidase, is as follows:

$$R - CH_2 - NH_2 + O_2 \rightarrow R - CHO + H_2O_2 + NH_3.$$

The content of amines in samples was measured as follows: the assembled biosensor was allowed to equilibrate in 3 ml of phosphate buffer (pH 8) at room temperature and under magnetic stirring until a steady current baseline was reached. This took about 10 min. Aliquots of samples (200 μl) or amine standard solutions were then injected and a current change due to the H_2O_2 production was recorded and related to the amine concentration.

IC–IPAD method

Analysis was carried out following the method of Draisci *et al.* (1993) with some modifications. Amine separations were performed on a 250×4 mm IonPack CS10 cation exchange analytical column (Dionex Co.) coupled with an IonPack CG10 guard column (Dionex Co.) packed with 8.5 μm solvent compatible ethylvinylbenzene/divinylbenzene 55% XL substrate, agglomerated with 175 nm cation exchange latex for a cation exchange capacity of approximately 80 μeq column^{-1}.

Isocratic separation was performed with 1 mol litre^{-1} sodium perchlorate, 0.375 mol litre^{-1} perchloric acid, water (81:5:14, v/v/v) at 1 ml min^{-1} flow rate. The waveform time-potential used for Integrated Pulsed Amperometric Detection is reported (Toul and Macholan, 1975). To achieve a basic pH for the IPAD on Au electrode, NaOH 0.25 mol litre^{-1} at 0.8 ml min^{-1} was added to the column eluate via the RDM.

RESULTS AND DISCUSSION

Biosensor optimization

Due to the broad substrate specificity of DAO, several amines can give a current signal using this enzyme electrode. The amines selected in this work were those most commonly found in fish samples (Rosier and Peteghem, 1988; Lebiedzinska *et al.*, 1991; Yen and Hsieh, 1991) and those which could increase in anchovies during ripening time.

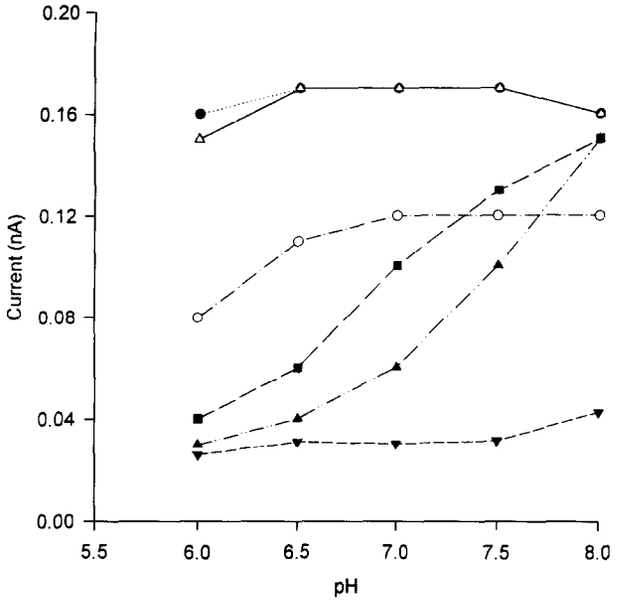

Fig. 1. pH profile of DAO immobilized on the Immobilon membrane; (●) putrescine, (△) cadaverine, (■) histamine, (○) spermidine, (▲) spermine, (▼) tryptamine.

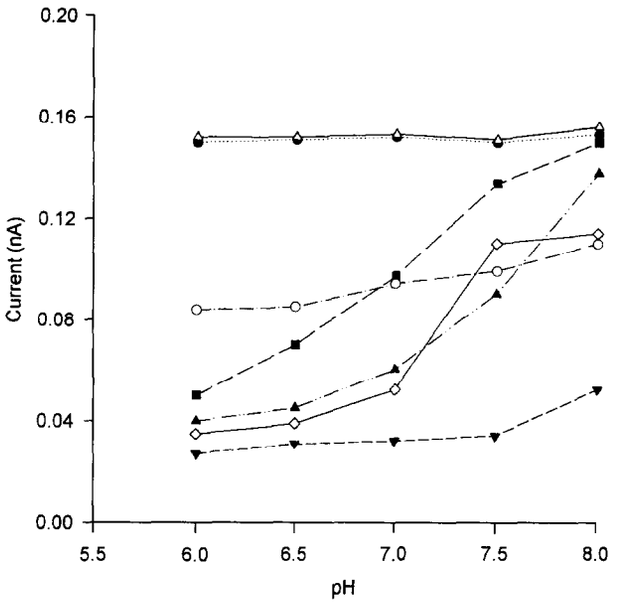

Fig. 3. pH profile of DAO immobilized on the nylon net using glutardehyde as cross-linking agent; (●) putrescine, (△) cadaverine, (■) histamine, (○) spermidine, (▲) spermine, (◇) tyramine, (▼) tryptamine.

In order to optimize the biosensor response to all the amines selected, each amine was studied using phosphate buffer at different pHs and different enzyme immobilization procedures. The response time was also studied and optimized.

pH/immobilization studies

The pH study was carried out using phosphate buffer 0.1 mol litre^{-1} in a pH range of 6–8. The concentration for each amine tested was 5×10^{-6} mol litre^{-1}. Phosphate buffer was selected because it was reported as the best for this enzyme (Padiglia *et al.*, 1991).

The pH profile of diamine oxidase immobilized on the Immobilon membrane is shown in Fig. 1. The high response for putrescine and cadaverine at pH 6, which remained almost constant in the pH interval studied, is shown.

At pH 6, spermidine gave a lower response than the first two amines, then the current signal increased and levelled off from pH 6.5 to 8.0. At the same pH value the current variations for histamine and spermine were almost four times lower than putrescine and cadaverine. However, this signal increased with pH, so that their

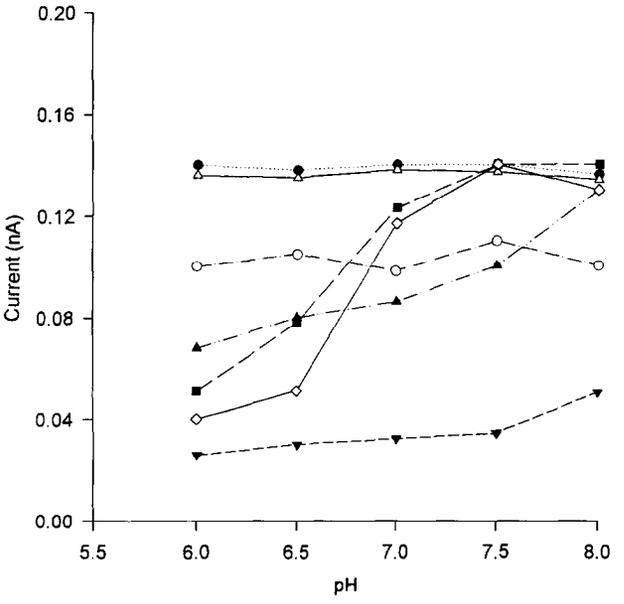

Fig. 2. pH profile of DAO immobilized on the preactivated nylon net; (●) putrescine, (△) cadaverine, (■) histamine, (○) spermidine, (▲) spermine, (◇) tyramine, (▼) tryptamine.

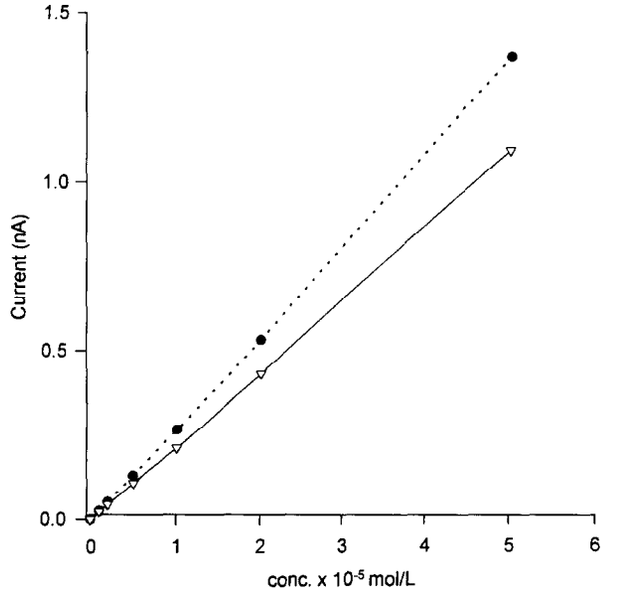

Fig. 4. Calibration curves in the linear range 10^{-6}–5×10^{-5} mol litre^{-1}; (●) putrescine, (▽) equimolar mixture of amines.

Table 1. Linear range, detection limit, and repeatibility for each amine in the optimized experimental conditions

Amines	Linear range (mol litre^{-1})	Detection limit (mol litre^{-1})	RSD (%)
Putrescine	10^{-6}–$2{\cdot}0\times10^{-3}$	$5{\cdot}0\times10^{-7}$	1·8
Cadaverine	10^{-6}–$2{\cdot}0\times10^{-3}$	$5{\cdot}0\times10^{-7}$	2·0
Histamine	10^{-6}–$5{\cdot}0\times10^{-5}$	$5{\cdot}0\times10^{-7}$	3·0
Spermidine	10^{-6}–10^{-3}	$5{\cdot}0\times10^{-7}$	2·4
Spermine	10^{-6}–$5{\cdot}0\times10^{-5}$	$5{\cdot}0\times10^{-7}$	2·8
Tyramine	10^{-6}–$5{\cdot}0\times10^{-5}$	$5{\cdot}0\times10^{-7}$	3·1
Tryptamine	$3{\cdot}5\times10^{-6}$–10^{-4}	$1{\cdot}8\times10^{-6}$	3·4

sensitivity at pH 8.0 was almost the same as putrescine and cadaverine. Tryptamine gave a response similar to histamine and spermine at pH 6.0, but the current value remained almost costant in the pH range considered. The response time was about 3 min for all the amines studied.

When tyramine was tested, 10 min was required to attain a current steady-state. Although this current variation was not negligible, the signal recorded after 3 min (response time for all the other amines tested) was negligible compared with the signal due to the other amines so it was not reported in Fig. 1.

The immobilization of DAO on preactivated nylon net and on nylon net used as support only (with glutaraldeyde), exhibited a similar trend in function of pH (Figs 2 and 3) As shown, cadaverine and putrescine gave a high and stable current signal while for the other amines the response increased with pH.

However, using the nylon-based enzyme membranes, the steady state for tyramine was obtained in 5 min with respect to 3 min for the other amines.

It is interesting to note that for all the immobilization procedures, pH 8 was the best for the detection of all the amines with similar sensitivity.

Even though the preactivated nylon membrane exhibited a more homogeneous amine response at pH 8 (Fig. 2), the glutaraldehyde-based procedure was selected because it was simple and required a lower amount of enzyme.

Table 2. Percentage response of the biosensor to the amines tested

Amines	Response (%)
Putrescine	100
Cadaverine	100
Histamine	98
Spermine	91
Tyramine	73
Spermidine	71
Tryptamine	30

Evaluation of the biosensor response to the amines

Detection limit, linear range and repeatibility ($n=5$, at 5×10^{-6} mol litre^{-1}) for each amine with the optimized experimental conditions, are reported in Table 1. A detection limit (defined as a signal three times higher than the noise of the current background) of 5×10^{-7} mol litre^{-1}, corresponding to 0.04 μg ml^{-1} (putrescine)–0.10 μg ml^{-1} (spermine), was observed for all the amines tested except for tryptamine. These values are comparable with the detection limit for the IC–IPAD method of 0.05–0.10 μg ml^{-1} for all the amines.

Table 3. Recovery study (average of three extractions) performed by adding standard solutions of putrescine to a fish sample (time from death = 0 days)

Putrescine added (mol litre$^{-1}\times10^{-5}$)	Amines found in the sample before spiking (mol litre$^{-1}\times10^{-5}$)	Expected value (mol litre$\times10^{-5}$)	Found value (mol litre$^{-1}\times10^{-5}$)	Recovery (%)	RSD (%)
0·2	0·22	0·42	0·43	102	3·2
2·0	0·22	2·22	2·09	94	2·8
5·0	0·22	5·22	4·97	95	2·3

Table 4. Recovery study (average of three extractions) performed by adding standard solutions of an equimolar mixture of amines to a fish sample (time from death = 0 days)

Equimolar mixture of amines added (mol litre$^{-1}\times10^{-5}$)	Amines found in the sample before spiking (mol litre$^{-1}\times10^{-5}$)	Expected value (mol litre$^{-1}\times10^{-5}$)	Found value (mol litre$^{-1}\times10^{-5}$)	Recovery (%)	RSD (%)
0·2	0·29	0·49	0·50	102	4·0
2·0	0·29	2·29	2·12	93	3·3
5·0	0·29	5·29	4·91	93	2·6

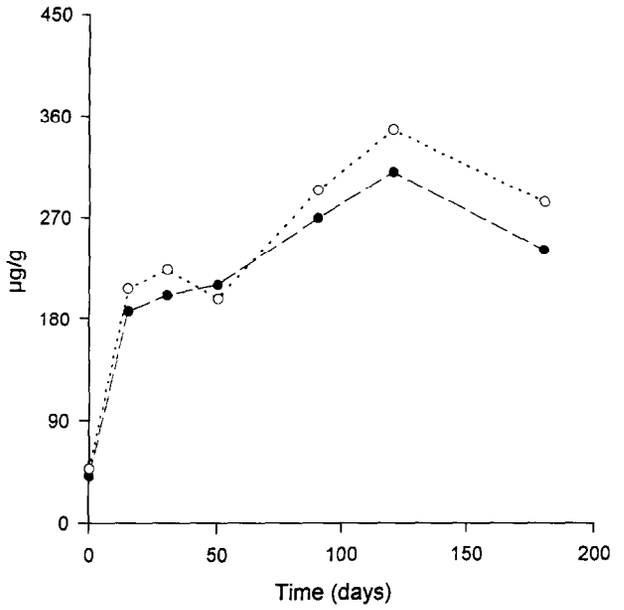

Fig. 5. Total amine variations during ripening time, monitored with the biosensor (●) and IC–IPAD method (○).

Although a linear response from 10^{-6} to 2×10^{-3} mol litre^{-1} was observed for putrescine and cadaverine, the linear range common to all the amines tested, except for tryptamine, was 10^{-6}–5×10^{-5} mol litre^{-1}. This range was selected for application studies since in fish samples the tryptamine content is negligible when compared with the total amine concentration (Yen and Hsieh, 1991).

The biosensor response to the amines, normalized to 100 for putrescine and cadaverine, is reported in Table 2.

Table 5. Total amines in salted anchovy samples expressed as equivalent of putrescine obtained with the biosensor

Time (days)	$\mu g\,g^{-1}$	RSD (%)
0	41	3·2
15	187	2·5
30	201	1·8
50	210	3·7
90	269	3·1
120	310	2·6
180	240	2·0

A good sensitivity was also observed for histamine, spermine, tyramine and spermidine.

Two calibration curves, in the range 10^{-6}–5×10^{-5} mol litre^{-1}, are given in Fig. 4. Curve A was obtained with standard solutions of putrescine and curve B with a equimolar mixture of all amines. The difference in current observed between the two curves was about 20%. This value was expected because the average of the results in Table 2 for all the amines showed that the current signal was about 80% compared to putrescine. This demonstrated that the sensitivity of the biosensor did not change using a pool of amines instead of single amine standard solutions.

Interference studies

Potentially interfering substances such as amino acids involved in the biosynthesis of amines (histidine, tyrosine, tryptophan, lysine, glutamine, ornithine, arginine) (Halasz *et al.*, 1994) were evaluated.

Analysis was carried out by comparison of 10^{-5} mol litre^{-1} of putrescine standard with 10^{-4} mol litre^{-1} of amino acid standard solutions. In fact, previous experimental studies, carried out with IC–IPAD (results not published) have shown that anchovy samples contain concentrations of istidine and tyrosine 10 times higher than the amines. Only lysine gave an interference of 15%.

Analysis of anchovy samples

The biosensor was applied to the determination of biogenic amines in anchovy samples during transformation process under salt. The purpose was to verify the trend of these amines during ripening time and to compare the results with those obtained using the IC–IPAD.

For the biosensor analysis, the extraction of amines was first attempted using perchloric acid 0.375 mol litre^{-1}. This extraction had two drawbacks: an additional step was required to neutralise the sample in order to measure at the optimum pH; moreover, such a sample treatment produced the formation of electroactive substances which gave an interfering additional current. This interference was of an electrochemical nature because we observed the same current increase when the probe was used with the enzyme previously denaturated.

Table 6. Concentration of single amines in salted anchovy samples performed with IC–IPAD

Time (days)	Putrescine ($\mu g\,g^{-1}$)	Cadaverine ($\mu g\,g^{-1}$)	Histamine ($\mu g\,g^{-1}$)	Tyramine ($\mu g\,g^{-1}$)	Spermidine ($\mu g\,g^{-1}$)
0	34	5	7	2	0
15	140	38	16	13	0
30	151	24	11	23	15
50	183	16	nd	nd	nd
90	268	16	nd	nd	nd
120	294	53	nd	nd	nd
180	264	19	nd	nd	nd

nd, not detected.

The extraction was then carried out with 0.1 mol litre^{-1} phosphate buffer, pH 8. This procedure was tested for recovery. Standard solutions of putrescine were added to 1 g of fish sample (time from death = 0 - days) to obtain a final concentration of 2×10^{-6} mol litre^{-1}, 2×10^{-5} mol litre^{-1}, 5×10^{-5} mol litre^{-1}. Another aliquot of anchovy was used as blank. Recovery (average of three extractions) and precision data are reported in Table 3. The same procedure was repeated using a mixture of amines (Table 4). In both cases a recovery from 93 to 102% was observed.

Considering the satisfactory recovery and precision obtained, the analysis of salted anchovies was carried out. Samples stored under salt for 15, 30, 50, 90, 120, 180 days and kept under 70 g cm^{-2} pressure at a temperature of 22°C were analysed with the biosensor and IC–IPAD.

The concentrations of the total amines (mean of three determinations) expressed as equivalent of putrescine and the RSD% obtained analysing the anchovy samples with the biosensor are reported in Table 5. The concentrations of single amines determined with HPLC procedure are given in Table 6.

It can be observed that the predominant amine present in samples with a time from death = 0 days and during the ripening process was putrescine.

The total changes in the concentration of biogenic amines during time monitored with biosensor and IC–IPAD method are shown in Fig. 5. In both cases a sharp increase from 0 to 15 days was observed. A further increase from 50 to 120 days was observed followed by a slight decrease from 120 to 180 days.

CONCLUSIONS

A simple and rapid method for the determination of biogenic amines was developed. This method, based on an H_2O_2 probe with the enzyme diamine oxidase immobilized on the electrode surface, was used for the determination of total biogenic amine variations in salted anchovies during the ripening process. The comparison of the biosensor results with those obtained with IC–IPAD method indicated that both techniques are useful to monitor the variations of amine content in fish during time. The IC–IPAD technique gives information on the individual amine content while the biosensor procedure allows the measurement of the total amines, with advantages such as low cost, short analysis time, simplicity of use. In view of these facts, the biosensor can be considered a valuable candidate for the quality control of fish processing and storage.

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