Development of a Voltammetric Amine Oxidase-Modified Biosensor for the Determination of Biogenic Amines in Food

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A novel, stable biosensor for the detection of biogenic amines (BAs) was designed by immobilizing pea seedling amine oxidase (PSAO) on MnO_2 modified carbon paste electrodes (CPEs). Hydrogen peroxide – one of the products of the enzymatic reaction - was the electrochemically active intermediate and was detected at + 400 mV vs. Ag/AgCl with differential pulse voltammetry (DPV). Immobilization of the enzyme was achieved with a Nafion® membrane, which provides excellent biocompatibility as well as good fixation properties. The biosensor was tested with different substrates, such as cadaverine, putrescine, tyramine, and histamine to obtain information about substrate specificity. A linear correlation was shown from 30 – 88 µg/mL for cadaverine and 24 – 67 µg/mL for putrescine. The limits of detection (LOD) were determined to be 10 µg/mL for cadaverine and 8 µg/mL for putrescine and the limits of quantification (LOQ) were calculated to be 30 µg/mL and 24 µg/mL respectively. Immobilized PSAO was inhibited by the irreversible non-competitive inhibitor semicarbazide to prove that the observed signals were enzymatically generated. Common interfering agents such as uric acid, acetylsalicylic acid, and 4-acetamidophenol were checked on their influence on the performance of the sensor. The sensor was used to quantify BAs in commercial fish sauce. The accuracy of the results was verified using the standard addition method.

1. INTRODUCTION

BAs are aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine) or heterocyclic (histamine) organic bases of low molecular weight. They are generated during the bacterial degradation of amino acids. When ingested in high amounts, BAs can cause rash, dysfunctional blood pressure, tachycardia, headaches and nausea [1]. High levels of BAs can be found in processed food such as red wine, ripened meat products, and fermented food like soy

sauce or in food during putrefaction. BAs are thermally stable and are therefore important marker substances for the freshness of food. The European Union fixed the statutory limit of histamine to 200 mg/kg in fresh fish (Comission Regulation (EC) 2073/2005) but there is still no limit for other BAs although it can be assumed that one BA can intensify the toxicological effects of another [2].

During the past years different analytical methods were developed to quantify BAs in biological samples such as thin-layer chromatography [3], capillary electrophoresis [4, 5], gas chromatography [6, 7] and - most commonly - high performance liquid chromatography [8–10]. Most of these analytical methods require sample preparation like pre-column derivatization which is often time-consuming. In addition various biosensors for detecting BAs have been reported [11, 12]. A great advantage of bio-sensing systems − aside from their cost effectiveness − is, that there is no need for a preceding sample preparation. Especially enzyme modified biosensors have attracted enormous attention in electroanalysis during the past years. They provide several advantages compared to other analytical systems like the generation of a direct signal, high sensitivity, rapid detection, and economic analysis.

In bio-sensing systems the immobilization of the enzyme is the crucial step. The ideal immobilization method should provide a good fixation of the enzyme and conserve its activity as long as possible. Literature suggests different possibilities: adsorption [13], microencapsulation [14], covalent attachment [15, 16], crosslinking with glutaraldehyde [17], immobilization in gel-sol films [18, 19], and entrapment via polymers and/or nanostructured carbon materials [20]. Immobilization of PSAO has been performed via adsorption [21], crosslinking [22, 23] and entrapment with poly(ethylenglycol) (400) diglycidyl ether (PEGDGE) [12, 24].

The stabilization via the polymer Nafion[®] is well approved in the design of glucose oxidase biosensors and is a valuable system to detect enzymatically generated hydrogen peroxide [25] but it has never been tried with PSAO. Nafion[®] is an ionomeric derivate of Teflon[®] and was the first synthetic polymer with ionic characteristics [26]. Nafion[®] allows an unspecific entrapment of the enzyme and therefore minimizes interferences between the bio-component and the immobilization device.

Pea seedling amine oxidase (PSAO) is a herbal diamine oxidase with high substrate specificity for cadaverine, putrescine and mediocre specificity for tyramine and histamine. It can be used as a molecular detection agent for BAs. PSAO belongs to the class of copper amine oxidases (CuAOs) and selectively catalyzes the oxidative deamination of BAs in the presence of oxygen and water as shown in the following reaction equation [27].

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2$$
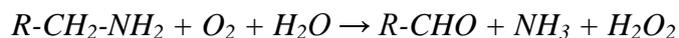
As a typical CuAO, PSAO is a homodimer built from an A and a B chain. Each contains Cu (II) and a 2,4,5-trihydroxyphenylalanine quinine (TPQ) as an organic cofactor [28]. PSAO is not membrane bound but shows apoplastic localization [29] and therefore displays high stability. CuAOs can be found in almost all organisms where they are responsible for different tasks like detoxification in mammals or wound-healing in plants [28]. Semicarbazide, a derivate of urea, is a non-competitive,

irreversible inhibitor of all CuAOs, therefore they are also called semicarbazide-sensitive amine oxidases (SSAOs).

The enzymatic reaction shown above displays several electrochemical active reactants. Hydrogen peroxide appears to be the most appropriate electrochemical measuring agent as it offers high response, low LODs, accuracy, and high reproducibility.

It is well known that the oxidation of hydrogen peroxide requires a large overpotential at a bare electrode surface. Therefore the coupling of the enzymatic entity with an appropriate electrocatalytic mediator is necessary. CPEs with manganese dioxide as mediator yielded excellent results for the determination of hydrogen peroxide [14, 30]. The mediator MnO_2 is reduced by hydrogen peroxide to Mn^{3+} which is detected and re-oxidized at the electrode. Due to the presence of MnO_2, hydrogen peroxide can be detected at a potential of + 400 mV vs. Ag/AgCl [31].
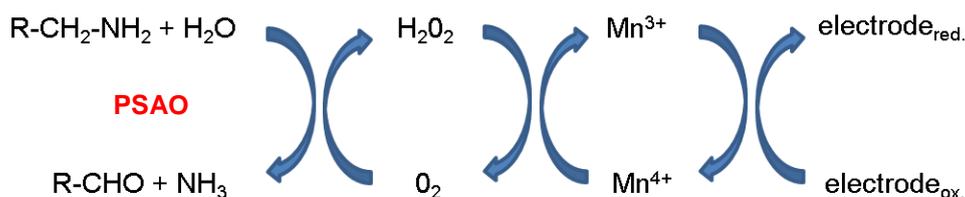

Figure 1. Working principle of the novel PSAO biosensor showing the redox reactions between analyte, mediator and CPE.

This paper describes the design and optimization of a novel PSAO modified biosensor as an analytical tool for the quantification of BAs in food.

2.EXPERIMENTAL PART

2.1 Apparatus and Electrodes

All electrochemical measurements were carried out with a VA Stand Metrohm 694 and a VA Processor Metrohm 693 electrochemical workstation from Metrohm Schweiz AG. Data were analyzed with VA Database 2.2 software by Metrohm Schweiz AG using the tangent method. The carbon paste working electrode was prepared as described in section 2.3. A Ag/AgCl/3 M KCl electrode and a platinum electrode were used as reference and auxiliary electrode respectively.

2.2 Reagents and Solutions

PSAO EC 1.4.3.6 (specific activity: 2.44 IU/mg) was a generous gift from Bio-Research Product, Inc. Carbon powder of highest purity grade was purchased from Ringsdorff-Werke (type RW-B, Bad Godesberg, Germany). Manganese dioxide (85-90 %), paraffin oil (Uvasol®), KH_2PO_4 (99 %),

$Na_2HPO_4 \cdot 2H_2O$ (99.5 %), ethanol (96 %), and EDTA solution (0.02 M) were purchased from Merck. Nafion® (5 % m:m solution), sucrose, cadaverine di-hydrochloride, tyramine hydrochloride and histamine di-hydrochloride were purchased from Sigma Aldrich. Semicarbazide hydrochloride and glycerol purum (>98 %) were obtained from Fluka Chemie AG. Putrescine, acetylsalicylic acid (99 %), uric acid (>99 %), and 4-acetamidophenol (98 %) were purchased from Acros Organics. Fish sauce was purchased at a local store.

All used chemicals and reagents were of analytical grade.

All aqueous solutions were prepared with highly pure water, freshly prepared in the lab by a catridge system (Milli-Q).

Sørensen phosphate buffer (66 mM, pH 7.5) was prepared by mixing a 66 mM KH_2PO_4 solution and a 66 mM $Na_2HPO_4 \cdot 2H_2O$ solution until the required pH was achieved.

The enzyme immobilization buffer was prepared by dissolving sucrose 85.6 g/L, glycerol 50 g/L, and 5 mL/L EDTA solution (0.02 M) in Sørensen phosphate buffer (66 mM, pH 7.5). The buffer was stored at 4 °C and used for not longer than one month.

PSAO itself was dissolved in Sørensen phosphate buffer (66 mM, pH 7.5) with a concentration of 10 mg/mL, aliquoted and stored at -20 °C to avoid frequent freeze-thaw cycles.

All substrate, inhibitor, and interference solutions were prepared by dissolving the reagents in Sørensen phosphate buffer (66 mM, pH 7.5), stored at 4 °C and prepared freshly every two days.

Nafion® was neutralized with ammonia before use. The enzyme immobilization solution was prepared by carefully mixing 7.5 µL enzyme immobilization buffer, 10 µL PSAO solution (10 mg/mL), 2 µL Nafion®, and 8 µL ethanol in that order. This solution was sealed with Parafilm®, stored at 4 °C until use and prepared freshly every two days.

2.3 Preparation of the PSAO modified biosensor

Carbon paste, modified with 4 % (m:m) MnO_2 as mediator, was used as electrode material. 0.136 g carbon powder, 0.056 g paraffin oil and 0.008 g MnO_2 were thoroughly mixed to a paste-like consistency. The paste was brought into the holder (diameter 3 mm) and polished carefully on a Teflon® plate until a smooth surface was obtained.

3 µL of the enzyme containing Nafion® film solution was drop-coated on the electrode's surface and dried at room temperature. Before use, the electrode was rinsed with buffer to remove non-immobilized enzyme.

2.4 Measuring procedure

Electrochemical measurements were performed with DPV with a classic three-electrode system containing a carbon paste working electrode, a Ag/AgCl reference electrode and a platinum auxiliary electrode. 1.3 mL of Sørensen phosphate buffer were used as measurement medium. The analyzer was operated under the following parameters: RDE; mode, DPV; potential ramp, 0.00 to + 0.80 V;

potential step, 20 mV/s; pulse amplitude, 50 mV; pulse duration, 40 ms; measurement duration, 20 ms; rotation speed, 200 rpm.

All measurements were carried out at room temperature.

2.5 Inhibition of immobilized PSAO with semicarbazide

A semicarbazide solution with a concentration of 470 µg/mL was used to inhibit the bio-component. First, a cadaverine solution with a concentration of 88 µg/mL was measured as reference signal. After that, the media was changed and 60 µL of the semicarbazide solution 470 µg/mL were added (final concentration in the vessel: 22 µg/mL). A voltammogram of the semicarbazide solution was recorded. Subsequently, cadaverine was added into the same batch (final concentration in the vessel 88 µg/mL) and a voltammogram was recorded. After a change of media, cadaverine (88 µg/mL) was measured again.

2.6 Analysis of the fish sauce

For the determination of BAs in biological matrix, a commercial fish sauce was used as an analyte. The results were verified using the standard addition method. Thus cadaverine was added to the sample solution, resulting in four solutions, containing 0, 15, 30, and 45 µg cadaverine. All four solutions were measured consecutively with the same electrode. The results were calculated in cadaverine equivalents.

3. RESULTS AND DISCUSSION

3.1 Immobilization of PSAO

The enzyme containing film composition was optimized to provide good biocompatibility as well as high fixation abilities. The sulphonated tetrafluorpolymer Nafion® immobilizes the bio-component in a non-specific way. Nafion® forms a net-like structure that avoids the leach out of big molecules like the bio-component PSAO but still allows small molecules like substrates or inhibitors of the enzyme to reach the electrode surface. Immobilization via covalent binding between bio-component and immobilization agent can reduce enzyme activity. An unspecific immobilization like the entrapment in Nafion® films avoids this problem.

The addition of glycerol and ethanol facilitates the formation of a smooth, fully-functional film without cracks as well as an ideal distribution of the enzyme on the electrode surface. Table 1 shows the optimized composition of the PSAO containing Nafion® film.

Table 1. Composition of PSAO containing Nafion® film.

Components	[µL]	[%]
Enzyme immobilization buffer	7.5	27.3
PSAO solution 10 µg/mL	10.0	36.3
Nafion®	2.0	7.3
Ethanol	8.0	29.1

The Nafion® PSAO biosensor was stable for up to 30 runs, although a slight increase of the baseline after about ten runs could be observed. The developed PSAO modified biosensor showed sharp peaks at + 400 mV due to the mediator MnO_2.

Figure 2 shows a typical voltammogram of cadaverine (red line). The current is caused by the catalytic oxidation of hydrogen peroxide due to the mediator MnO_2 according to figure 1.
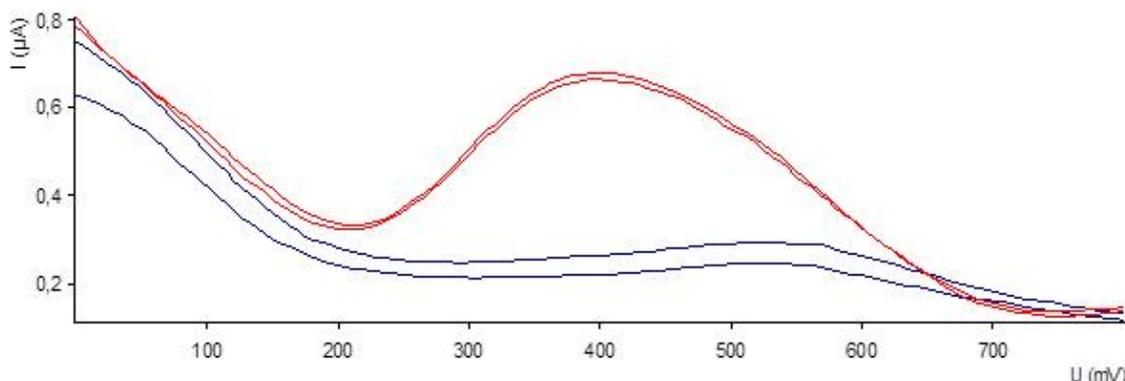

Figure 2. Typical voltammogram of cadaverine 143 µg/mL in Sørensen phosphate buffer (66 mM, pH 7.5); scan rate 20 mV/s. ● Baseline (n = 2), ● Cadaverine 143 µg/mL (n = 2).

3.2 Substrate specificity

Hydrogen peroxide was chosen as electrochemical measurement agent and was detected at + 400 mV. The PSAO biosensor was tested with the substrates cadaverine, putrescine, tyramine, and histamine to investigate the substrate specificity. Cadaverine and putrescine turned out to be the best substrates for the designed biosensor. For cadaverine, a linear correlation from $30 - 88$ µg/mL (I [µA] = 0.0021c [µg/mL] + 0.0330; correlation coefficient $R^2 = 0.9904$) was observed. Putrescine showed a linear correlation from $24 - 67$ µg/mL (I [µA] = 0.0027c [µg/mL] + 0.0340; correlation coefficient $R^2 = 0.9809$). The diamine histamine could not be detected with the designed biosensor. Herbal amine oxidases like PSAO usually do not have to deal with the deamination of histamine and therefore have only little substrate specificity [32].

The monoamine tyramine did not show a detectable signal in the examined concentration range either $(10 - 500$ µg/mL). These results are in good accordance to literature since tyramine is deaminated only at higher substrate concentrations (1 mM = 137 mg/mL) [21, 33].

3.3 Analytical parameters

The LODs for the substrates cadaverine and putrescine were determined by substrate solutions of different concentrations. According to ICH Q2 (R1), the substrate solutions with the lowest concentrations generating a clearly visible peak were set as LOD. For cadaverine, the LOD was 10 µg/mL, whereas for putrescine it was 8 µg/mL. The LOQs were estimated to be LOD x 3, which equals 30 µg/mL and 24 µg/mL respectively.

The intra-electrode repeatability of the PSAO biosensor was tested by analyzing a cadaverine solution (45 µg/mL) with the same electrode. The media was changed after every run. The standard deviation of the repeatability was calculated to be 2.9 % (n = 6). The standard deviation of the inter-electrode repeatability was calculated to be 6.1 % (n = 6). Table 2 shows an overview of all analytical parameters investigated.

Table 2. Analytical parameters investigated on the PSAO modified biosensor. For determinations besides substrate specificity, cadaverine was set as standard substrate.

Analytical parameters	Cadaverine	Putrescine
Linear correlation	30 – 88 µg/mL	24 – 67 µg/mL
Correlation coefficient R^2	0.9904	0.9809
LOD	10 µg/mL	8 µg/mL
Intra-electrode repeatability	2.9 %	/
Inter-electrode repeatability	6.1 %	/

3.4 Inhibition of immobilized PSAO with semicarbazide

Semicarbazide is a non-competitive irreversible inhibitor of all copper-dependent amine oxidases such as PSAO. Thus semicarbazide inhibits the enzymatic reaction and therefore the formation of hydrogen peroxide. So even high amounts of substrate should not generate a signal. Therefore, the addition of semicarbazide is a valid and easy method to prove that the observed signals are in fact enzymatically generated and not caused by interferences.

A cadaverine solution with a final concentration of 88 µg/mL showed the typical peak at + 400 mV. After that the media was changed and a semicarbazide solution was added (concentration in the batch 22 µg/mL). A cadaverine solution added to the same media still showed a signal, owed to the fact that the inhibitor needs some time to bind to all enzyme molecules. After another change of media and a third addition of cadaverine (final concentration 88 µg/mL) no hydrogen peroxide signal occurred due to complete inhibition of the enzyme (figure 3). These results demonstrated that the observed signals were solely induced by the reaction product hydrogen peroxide.
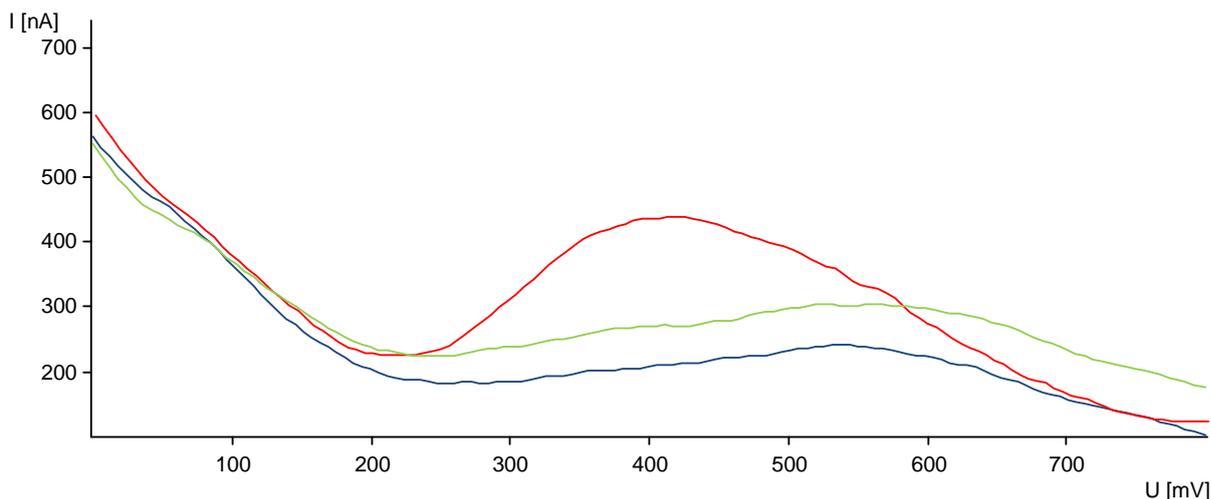
Figure 3. Irreversible inhibition of the PSAO sensor with semicarbazide 22 µg/mL in Sørensen phosphate buffer (66 mM, pH 7.5); scan rate 20 mV/s. ● Baseline; ● Cadaverine (88 µg/mL) before inhibition with semicarbazide; ● Cadaverine (88 µg/mL) after inhibition with semicarbazide.

3.5 Interferences

Interfering agents are model substances to test the specificity of the developed biosensors. Uric acid, acetylsalicylic acid, and 4-acetamidophenol represent chemical structures that have similar impact on the electrode as substances that can be found in biological samples (e.g. blood). Testing interfering agents gives important information about how the sensor can be affected by biological matrices.

The electrochemical behaviour of all interfering agents together with cadaverine was tested on the PSAO modified biosensor. For that purpose, a solution of each interfering agent was measured together with cadaverine in different ratios, where the concentration of the latter was 88 µg/mL in the batch. After the addition of the substrate the typical hydrogen peroxide peak was observed. The impact of the interferent on the cadaverine signal was detected. The ratios between substrate and interfering agent were 1:0.1; 1:1; and 1:2.

Uric acid increased the cadaverine peak already at the ratio of 1:0.1 by about 20 %, whereas 4-acetamidophenol had an impact on the signal at the ratio 1:1 by about 25 %. Acetylsalicylic acid did not show any interfering capacities even at the ratio of 1:2.

3.6 Real sample

The applicability of the sensor was tested with a commercial fish sauce. Typical voltammograms of the sample before and after addition of the standard cadaverine are shown in figure 4. In order to prove that the standard addition method can be unambiguously applied, the matrix was also measured after inhibiting the enzyme of the biosensor. The complete inhibition of the enzyme did

not yield a signal with the sensor under the experimental conditions which proved that there was no background contribution from the matrix of the sample. Thus it can be concluded that fish sauce does not contain substances interfering with the method.
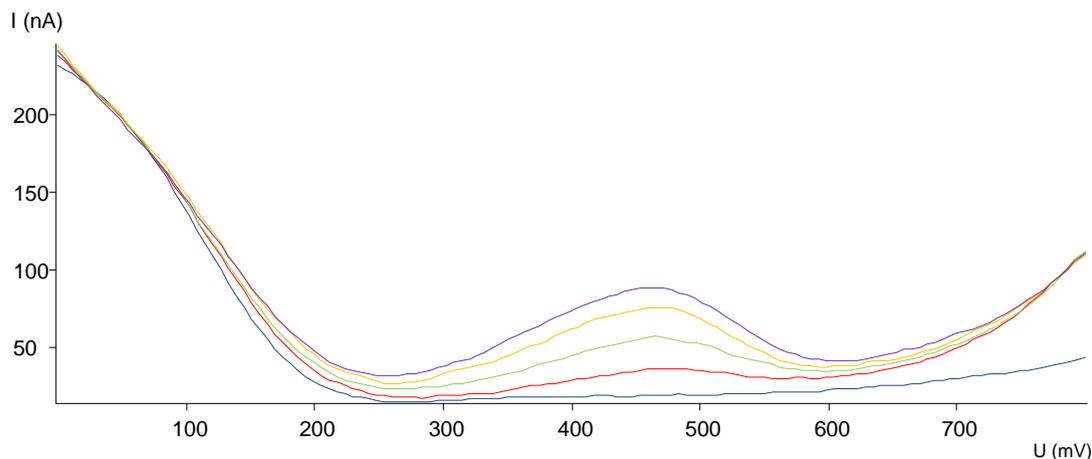

Figure 4. DPV Voltammogram of fish sauce by using the standard addition method in Sørensen phosphate buffer (66 mM, pH 7.5); scan rate 20 mV/s. ● Baseline; ● Fish sauce; ● Fish sauce/cadaverine 15 µg; ● Fish sauce/cadaverine 30 µg; ● Fish sauce/cadaverine 45 µg.

The measurements were performed six times on six different electrodes and the signals of the solutions 1-4 were averaged, a standard deviation was calculated and a linear correlation was established (figure 5). The linear correlation coefficient R^2 was calculated to be 0.9998. According to the linear equation drawn from the standard addition, the overall concentration of BAs in the fish sauce was calculated to be 737 mg/L cadaverine equivalents with a standard deviation of 6.1 %.
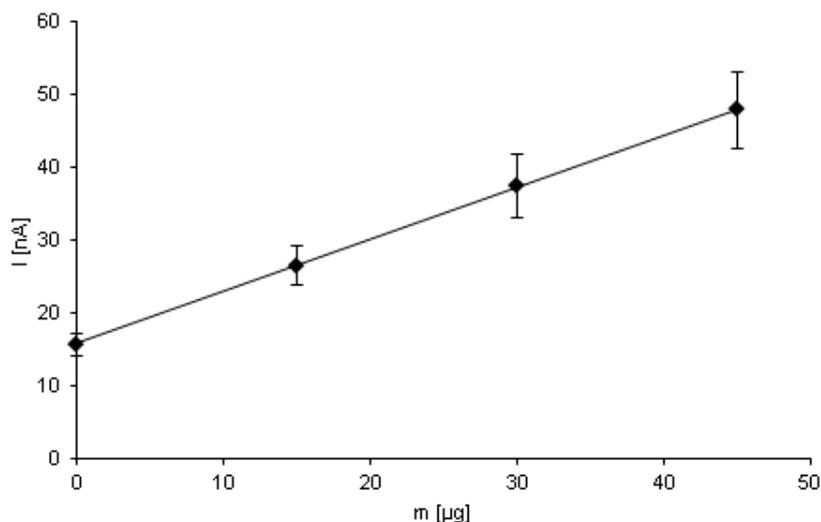

Figure 5. Determination of BAs in commercial fish sauce and verification of the results via standard addition method (n = 6) in Sørensen phosphate buffer (66 mM, pH 7.5); scan rate 20 mV/s. The x-axis represents the total mass of the added cadaverine in the batch, in which the data point m = 0 µg shows the current obtained from the fish sauce.

4. CONCLUSION

This paper describes the development of a novel voltammetric biosensor with the herbal amine oxidase PSAO as bio-component, which shows diamine oxidase-like characteristics. The working electrode is made of carbon paste, modified with the mediator MnO_2. Since the immobilization of the enzyme is the crucial step in the design of biosensors, special attention was paid to optimize this parameter. With the development of a Nafion$^®$ containing film, a stable, non-covalent fixation method could be established that displays excellent biocompatibility. The analytical parameters of the sensor were determined and the specificity of the electrode was tested by measuring the model interfering agents uric acid, acetylsalicylic acid and 4-acetamidophenol.

By inhibiting PSAO with the irreversible, non-competitive inhibitor semicarbazide, it was proved that the yielded signals were enzymatically generated.

The sensor was used to quantify BAs in commercial fish sauce and the accuracy of the obtained results was verified with the standard addition method.

The developed PSAO biosensor is a rapid, simple and economic tool for the quantification of BAs in biological matrix and could be a useful analytical device in food chemistry to detect spoilage.

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