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Characterization of an Amperometric Biosensor for the Determination of Biogenic Amines in Flow Injection Analysis

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An amperometric biosensor using pea seedling amine oxidase (PSAO) as molecular recognition element for the determination of biogenic amines was characterized in flow injection analysis (FIA). All measurements were performed with Sørensen phosphate buffer 66 mM, pH 7.5 as mobile phase. As working electrode a bulk electrode made of carbon paste modified with manganese dioxide as mediator was used. PSAO was immobilized with a Nafion® containing film which displayed unspecific entrapment of the bio-component. With its net-like structure the film provided high biocompatibility and excellent fixation properties even in permanent flow. The enzymatically produced hydrogen peroxide was chosen as electrochemical measuring agent and the working potential was fixed at +400 mV vs. Ag/AgCl. Several biogenic amines (BAs) were tested as substrates. Putrescine, cadaverine and tyramine are marker substances for food freshness and were well detected by the biosensing FIA system. A linear correlation from 5.0 – 100.0 µg/mL was shown for cadaverine and putrescine and from 25.0 –1000.0 µg/mL for tyramine. The limit of detection (LOD) was determined to be 1.1 µg/mL for putrescine and 2.2 µg/mL for cadaverine. For tyramine the LOD was measured to be 6.2 µg/mL. The application of the biosensing FIA system for the detection of biogenic amines was successfully tested in chicken meat samples.

Keywords: biosensor; pea seedling amine oxidase; flow injection analysis; amperometric detection

1. INTRODUCTION

Dietary biogenic amines are products of the bacterial degradation of amino acids. They are formed during ripening and putrefaction. Therefore they are important marker substances for the

freshness of food products [1]. Especially in sensitive persons high BA levels can cause toxic effects such as flush, headaches, rash, tachycardia and hypertonia [2]. Besides ripened food like red wine, chocolate, ripened cheese and fermented food especially dairy products, fish and meat products are risky to contain critically high amounts of BAs. Therefore a precise, economic analytical tool for the determination of BAs in food products is of great interest for food chemistry.

Different approaches have been made to quantify BAs in biological matrices, such as thin-layer chromatography [3], capillary electrophoresis [4, 5], gas chromatography [6, 7], and - most commonly - high performance liquid chromatography [8 - 10].These methods are well approved. However most of them require the derivatization of the analytes to enable their determination. These preceding procedures are not only time-consuming but also bear the risk of an only partial detection due to an incomplete derivatization.

Biosensors display some important advantages in comparison to conventional analytical methods like HPLC. They do not require time-consuming sample derivatization and preparation and they generate a direct signal. Above that they are much more economic than most other analytical methods. By choosing the right bio-component the biosensor can be highly specific for the required analytes. Amine oxidases (AO) selectively catalyze the oxidative deamination of BAs in the presence of oxygen. The enzymatically produced substances are the corresponding aldehyde, ammonia and hydrogen peroxide. Several publications quantifying BAs with enzyme based biosensors are available, using either PSAO [11, 12] or diamine oxidase (DAO) from porcine kidney [13, 14] as bio-component. Most of that systems use a second enzyme, such as horseradish peroxidase for detecting the biogenic amines and therefore they are rather complex systems. Also different immobilization methods for PSAO and DAO have been used so far, such as adsorption [15], crosslinking [16], embedding in polymers, such as poly(ethylenglycol)(400)diglycidyl ether [17], or covalent attachment [18].

In this group basic voltammetric investigations in batch systems were carried out with a PSAO modified biosensor using a Nafion® containing film as immobilization device [19]. This PSAO biosensor based on the detection of enzymatically produced hydrogen peroxide at a carbon paste electrode with manganese dioxide as mediator [20, 21].

The purpose of this work was now to characterize the performance of the PSAO biosensor that has proven to be a valuable analytical tool in batch systems in FIA systems. Due to the basic set-up of the FIA system itself, it offers several important advantages compared to batch systems. The measuring chamber is incomparably smaller, avoiding further dilution of the analyte (except for dispersion). Due to the permanent flow, the contact time between the analyte and the working electrode is shorter than in batch systems, which requires a sensitive working electrode on the one hand but prolongs the working electrode's shelf life on the other hand due to an immediate wash out of the analyte.

Other important advantages of FIA systems are their fast response characteristics and their excellent hydrodynamic properties [22]. Last but not least automatization of the FIA system is possible. This is an important point when applying the system in routine analysis.

2. EXPERIMENTAL PART

2.1 Apparatus

All electrochemical measurements were made with a Hewlett Packard 1049. An electrochemical detector using a Merck Hitachi D-6000 interface and a Merck Hitachi L-6000 HPLC pump. Data was analysed with D-7000 Multi-HSM Manager software. The interpretation of the collected data was performed with MS Excel.

The electrochemical cell consists of a carbon paste working electrode (preparation described in section 2.3), a Ag/AgCl/3 M KCl reference electrode, and a stainless steel electrode as auxiliary electrode. A spacer with a size of 127 µm was used to generate a measuring chamber. The electrochemical cell was put into a Perkin-ElmerLC-17 Faraday cage to prevent noise signals.

For the extraction of the meat samples a common household blender shaft was used for homogenization. The samples were extracted with a Transsonic 660/H; Elma® ultrasonication bath and a Haereus Instruments Labofuge 400; d = 110 mm centrifuge was used to separate the residue.

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 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 dihydrochloride, and tyramine hydrochloride were purchased from Sigma Aldrich. Semicarbazide hydrochloride and glycerol purum (>98 %) were purchased from Fluka Chemie AG. Putrescine, acetylic salicylic acid (99 %), uric acid (>99 %), and 4-acetamidophenol (98 %) were purchased from Acros Organics. All used chemicals and reagents were of analytical grade. Chicken meat was purchased at a local store.

All aqueous solutions were prepared with highly pure water, freshly prepared in the lab by a cartridge 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.

PSAO immobilization buffer was prepared by dissolving sucrose 85.6 g/l, glycerol 50 g/l, and EDTA solution (5 mL, 0.02 M) in Sørensen phosphate buffer. The PSAO immobilization buffer was stored at 4 °C and used for not longer than one month. PSAO 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 PSAO immobilization buffer, 10 µL PSAO solution 10 mg/mL, 2 µL Nafion®, and 16 µL ethanol in that order. This solution was sealed with Parafilm®, stored at 4 °C until use and prepared freshly every two days [19].

2.3 Preparation of PSAO modified biosensor

Carbon paste modified with 4 % (m:m) MnO_2 as mediator were prepared by thoroughly mixing 0.136 g carbon powder, 0.056 g paraffin oil and 0.008 g MnO_2 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 without scratches was obtained. 5 µL of the enzyme containing film solution was dropcoated on the electrode's surface and dried at room temperature.

2.4 Electrochemical measurements

All electrochemical measurements were performed with Sørensen phosphate buffer as mobile phase. The buffer was degased with vacuum for about 30 min. Usually a flow rate of 1.00 mL/min was applied except for the injection of the semicarbazide solution where a flow rate of 0.01 mL/min as well as a flow rate of 0.50 mL/min showed the best results. All measurements were performed at a potential of +400 mV.

Prior to every run the electrode was rinsed with mobile phase at the working potential of +400 mV until a stable baseline had established (noise-drift-test < 10 nA/min). During the analysis 20 µL of the sample were injected into the carrier current.

When not in use the biosensor was left in the electrochemical detector and set to a circulating permanent flow at 0.02 mL/min.

2.5 Analytical parameters

Substrate stock solutions with a concentration of 1 mg/mL respectively were prepared. For the determination of the calibration curves, substrate solutions of different concentrations were diluted out of the stock solutions. Each concentration was injected three times and the signals were averaged. Six different runs were performed respectively. The signals were averaged again and a regression curve was calculated.

LOD was determined to be peak/noise ratio is 3:1 and LOQ was estimated to be LOD x 3.

Intraday repeatability was investigated by a five times repeated injection of cadaverine 50 µg/mL solution (medium range) on the very same electrode.

Interday repeatability was determined by comparing the peaks of six injections of cadaverine 50 µg/mL solution each on two different electrodes.

2.6 Inhibition with semicarbazide

A semicarbazide solution 0.47 mg/mL [23] was prepared. The inhibitor solution was injected at a flow rate of 0.01 mL/min. This step was followed by a three times injection of cadaverine solution 2 mg/mL at a flow rate of 0.50 mL/min.

2.7 Interfering agents

Solutions containing cadaverine and the respective interfering agent such as 4-acetamidophenol, uric acid and acetyl salicylic acid in the ratios 1:0.1; 1:1; and 1:2 were prepared.

The measurements were performed as following: Every solution was injected six times beginning with cadaverine (5 µg/mL) as a reference, followed by a pure solution of the respective interference substance (5 µg/mL) and the mixed solutions of both.

2.8 Determination of BAs in fresh chicken meat samples

The extraction was performed according to the method proposed by Carelli et al. [14]. Briefly fresh chicken meat, purchased at a local store was homogenized with a common household blender shaft. 2.0 g meat were weighed in a centrifuge tube and 5.0 ml Sørensen phosphate buffer were added. The sample was homogenized by vortexing for 1 min. The sample was extracted by ultrasonication for 10 min at room temperature. After that the tube was centrifuged at 3500 rpm for 10 min at room temperature. The supernatant was transferred to a 20 mL volumetric flask and the extraction was repeated two more times. The volumetric flask was filled up to the mark with phosphate buffer and an aliquot was filtered through a 0.22 µm SCFA filter.

The method was calibrated using the standard addition method adding different amounts of cadaverine to the homogenized meat before the first extraction step. Three extracts (I-III) from each meat sample (pure meat, pure meat with added standards) were prepared. Considering the added cadaverine four solutions containing (a) 0, (b) 5, (c) 10, and (d) 15 µg/mL cadaverine were investigated.

Each of the resulting 12 samples was determined with FIA two times. The results were calculated in cadaverine equivalents.

3. RESULTS AND DISCUSSION

3.1 Electrochemical measurements

The PSAO biosensor that has proved to be accurate and stable in basic voltammetric investigations by this group [19] was tested in amperometric FIA system. Flow systems as already mentioned provide several advantages in comparison to batch systems. The advantages of FIA devices lie among others in the basic set-up of the FIA apparatus itself, the significantly higher sensitivity and the possibility of automatization when carrying out routine analyses [22]. Therefore the developed PSAO biosensor was investigated for its applicability in flow systems.

Preceding results [19] have shown that the peak maximum of enzymatically produced hydrogen peroxide at the MnO_2 modified carbon paste electrode occurred at + 400 mV and so it was set as working potential. Sørensen phosphate buffer (66 mM, pH 7.5) was chosen as mobile phase due to its physiological properties with a flow rate of 1 mL/min. Under these conditions the obtained

hydrogen peroxide peaks were well shaped without any tailing and the used immobilization method with Nafion® containing films provided excellent fixation properties even in permanent flow.

Due to the permanent flow and thus the short contact time between analytes and working electrode the life-circle of the biosensor could be increased. Depending on the substrate investigated, one electrode could be used from one to two weeks even in permanent flow. Detailed studies showed the varying impact of the respective substrates. Tyramine caused a significant decrease of sensor activity after about one week whereas cadaverine and putrescine produced reproducible signals for about two weeks.

Compared to other biosensors aiming at the same target the designed PSAO biosensor is characterized by a simple yet very effective set-up. Since the quantification of BAs is performed via hydrogen peroxide no coupling with another enzyme, such as horse radish peroxidase is necessary [11, 18]. The immobilization of PSAO via Nafion® films on the electrodes for the use in FIA is novel and has proved to be a very valuable immobilization method.

3.2 Analytical parameters

The determination of the linear correlation between peak height and concentration of the analyte was performed with three different substrates: cadaverine, putrescine and tyramine. Cadaverine showed a linear correlation from 5 – 100 µg/mL. A typical FIA diagram displaying a concentration row of cadaverine (5 -100 µg/mL) is given in Figure 1. The results of the measurements were averaged and a regression curve was calculated (correlation coefficient $R^2 = 0.9960$).

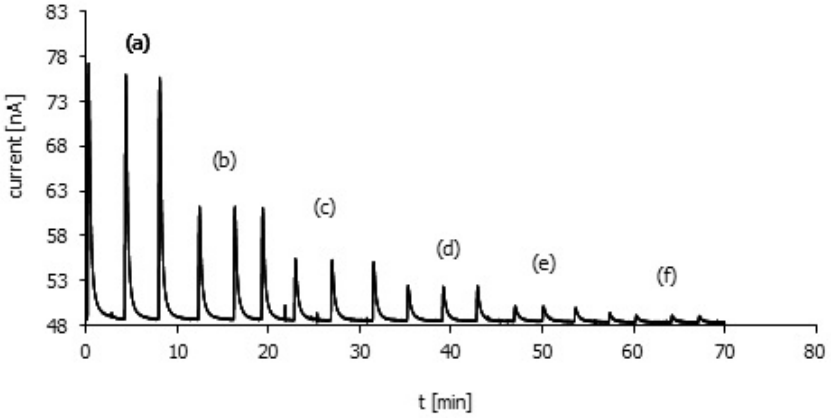

Figure 1. Flow injection amperogram of a typical concentration series of cadaverine. (a) 100, (b) 50, (c) 30, (d) 20, (e) 10, and (f) 5 µg/mL; mobile phase Sørensen phosphate buffer (66 mM, pH 7.5), working potential + 400 mV, injection volume 20 µL; flow rate 1 mL/min.

Putrescine showed the same linear range as cadaverine (5 -100 µg/mL) with a correlation coefficient of $R^2 = 0.9994$. However, tyramine displayed a linear correlation in a significantly higher concentration range from 25 - 1000 µg/mL with a correlation coefficient of $R^2 = 0.9915$.

These results proved that cadaverine and putrescine are ideal substrates for the PSAO modified biosensor. The analytical parameters for putrescine, cadaverine and tyramine can be seen in Table 1.

Table 1. Analytical parameters of the investigated substrates putrescine, cadaverine and tyramine

Analyte	Linear range [µg/mL]	Slope	R^2	LOD [µg/mL]	LOQ [µg/mL]
Cadaverine	5 - 100	0.4057	0.9960	1.1	5.0
Putrescine	5 - 100	0.5091	0.9994	2.2	5.0
Tyramine	25 - 1000	0.0963	0.9915	6.2	25.0

Intraday repeatability was determined by the comparison of six injections of cadaverine 50 µg/mL (medium range) on the very same electrode. For the investigation of the interday repeatability the signals of cadaverine 50 µg/mL injected on six different days (measured on two different electrodes) were compared. The standard deviation of the intraday repeatability was determined to be 3.9 %, whereas the standard deviation of the interday repeatability was determined to be 4.2 %.

3.3 Inhibition with semicarbazide

PSAO is irreversibly inhibited by semicarbazide, therefore it is also called as semicarbazide sensitive amine oxidase. To prove that the detected peaks were enzymatically generated hydrogen peroxide signals, the bio-component PSAO was irreversibly inhibited with this compound. The investigations had shown that it was necessary to allow the inhibitor an adequate time to bind to the protein molecules at the electrode's surface. For that purpose flow rates from 1.00 to 0.01 mL/min were investigated to ensure a complete inhibition of the enzyme. Total inhibition was achieved only at the lowest flow rate of 0.01 mL/min after a double injection of semicarbazide. In a consequence of the irreversible inhibition of PSAO even an exceptional high concentration of cadaverine (2.0 mg/mL) did not show any signals (injected at a flow rate of 0.5 mL/min). These results demonstrated that all signals were due to enzymatically generated hydrogen peroxide and can be rated as a proof of principle.

3.4 Interfering agents

Uric acid, acetylsalicylic acid, and 4-acetamidophenol were tested as interfering agents. They display chemical structures that have similar impact on the electrode as substances that can be found in biological samples. Therefore these investigations are important in order to obtain information about how the sensor can be influenced by biological matrices.

Table 2 gives an overview if and how the tested inference substances affect the sensor's performance using cadaverine as substrate. The tested interfering agents had either no impact on the

signal height or increased them. Table 2 expresses the increase of peak heights at the respective ratios in percentage.

Table 2. Impact of the interference substances uric acid, 4-acetamidophenol, and acetylsalicylic acid on cadaverine (5 µg/mL) at different ratios.

Interfering agent	1:0.1	1:1	1:2
Uric acid	+ 14 %	+ 20 %	+ 77 %
4-acetamidophenol	+ 0 %	+ 0 %	+ 15 %
Acetylsalicylic acid	+ 0 %	+ 0 %	+ 0 %

As can be seen from the table above, uric acid is the only interference agent tested that increases the BA signal already at low concentrations. The other ones have either no impact at all (acetylsalicylic acid) or increase the signal only at high concentrations (4-acetamidophenol).

3.5 Determination of BAs in fresh chicken meat samples

The biosensor's applicability in complex biological matrix was tested using chicken meat samples. The calibration was performed using the standard addition method. For that purpose known concentrations of a well approved analyte like cadaverine were added to the meat sample and measured with FIA. Every sample was injected twice, beginning with the three samples without cadaverine (pure meat extracts), followed by the samples with 5, 10, and 15 µg/mL cadaverine. Figure 2 shows a typical amperogram for such a measurement. The average standard deviation of the repeatability of the signals was about 2 %.

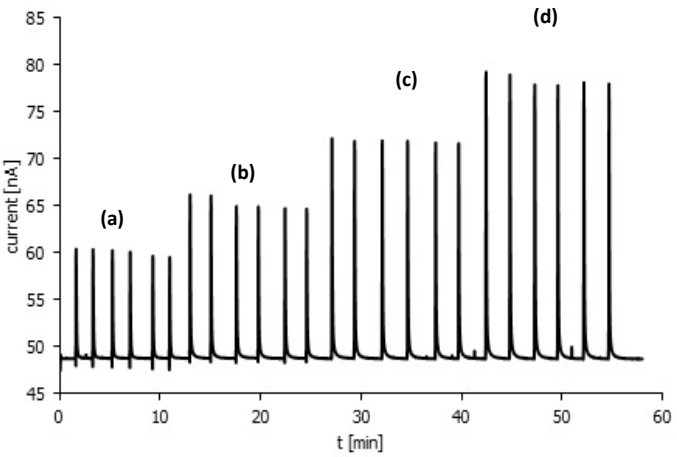

Figure 2. Flow injection amperogram of chicken meat sample by using the standard addition method. Injection of (a) pure meat extracts I, II, and III; (b) meat extracts with 5 µg/mL cadaverine I, II, III; (c) meat extracts with 10 µg/mL cadaverine I, II, and III; and (d) meat extracts with 15 µg/mL cadaverine I, II, and III. (n = 2); mobile phase Sørensen phosphate buffer (66 mM, pH 7.5), working potential + 400 mV, injection volume 20 µL; flow rate 1 mL/min.

The signals of each cadaverine concentration (from 0 to 15 µg/mL) were averaged and a regression curve was calculated (Firgure 3). The linear correlation was calculated to be I [nA] = 1.28c [µg/mL] + 12.46 with a correlation coefficient of $R^2 = 0.9977$. Based on the calibration curve, the total amount of BAs in the chicken meat sample was calculated to be 194 mg/kg (± 3 mg/kg) cadaverine equivalents. The determination of a second sample series extracted 24 hours later revealed an increase of the BA concentration in the chicken meat by almost 50 %.

The total amount of BAs in chicken meat found with the PSAO sensor is in the same range as the once published for similar food products (fish, pork, cheese) varying from 70 - 300 mg/kg [11, 14].

As the standard addition method showed a linear dependence of the signal on the concentration and as the complete inhibition of the enzymatic activity of the biosensor gave a void signal with the sample, it may be concluded that (i) the measured signals arise from enzymatic substrates of the enzyme (BAs) only, and that (ii) the standard addition method is a proper procedure to evaluate the contents of BAs in chicken meat as equivalents of the added standard, in this case of cadaverine. Thus, as the contents of BAs, evaluated as cadaverine equivalent, depends on the enzyme used (corresponding to its activity spectrum towards amino compounds) it can be regarded only as a relative sum parameter; therefore we decided deliberately not to compare with a standard reference method (such as HPLC) because of incompatibility of the results.

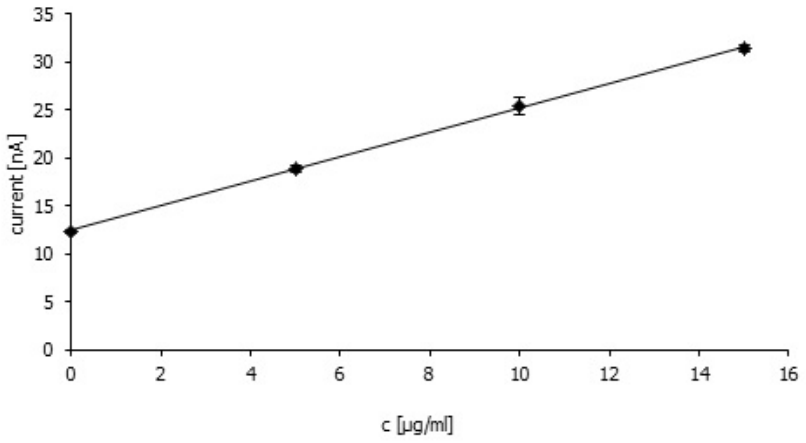

Figure 3. Determination of BAs in chicken meat and verification of the results via standard addition method (n = 3); mobile phase Sørensen phosphate buffer (66 mM, pH 7.5), working potential + 400 mV, injection volume 20 µL; flow rate 1 mL/min.

4. CONCLUSION

The developed PSAO modified biosensor is a valuable tool for the determination of BAs and is highly suitable for the application in FIA. A profound determination of analytical parameters for three substrates - cadaverine, putrescine and tyramine - was performed, including linearity, LOD and LOQ. These experiments showed a good sensitivity of the sensor as well as a broad linear range for the respective substrates.

Cadaverine was set as a standard substrate to investigate further analytical parameters. Intraday and interday repeatability showed excellent stability of the sensor. One electrode could be used for up to two weeks, depending on the investigated substrate. Although a good repeatability could be achieved, the use of an internal standard seems to be recommendable to balance the variability caused by the manual production of the electrode and the enzyme containing film.

With a simple yet effective experiment it was proved that the detected signals were exclusively enzymatically generated. PSAO was inhibited with the irreversible inhibitor semicarbazide. Subsequent injections of cadaverine did not generate any visible signals.

Since the sensor was intended for the use in complex biological matrix it was necessary to test its specificity by determining the impact of so-called model interference agents: acetylsalicylic acid, uric acid and 4-acetamidophenol. Although an increase of the signals could be observed for uric acid and 4-acetamidophenl, the impact was comparatively moderate and the sensor seems to be quite specific for BAs.

The optimized sensor was tested for its applicability in biological matrix (fresh chicken meat) by using the standard addition method with cadaverine as standard. The detailed analytics and the experiments with biological matrix make the developed PSAO biosensing FIA system a robust, sensitive and economic tool for the determination of biogenic amines in food samples.

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