Anal Bioanal Chem (2002) 372 : 276–283
DOI 10.1007/s00216-001-1130-9

SPECIAL ISSUE PAPER

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Enzyme sensor array for the determination of biogenic amines in food samples

Received: 15 June 2001 / Accepted after revision: 25 August 2001 / Published online: 15 December 2001

Abstract An enzyme sensor array for the simultaneous determination of the three biogenic amines (histamine, tyramine and putrescine) by pattern recognition using an artificial neural network and its application to different food samples is described. A combination of a monoamine oxidase, a tyramine oxidase and a diamine oxidase (with specific activities sufficient for rapid detection) are immobilised each on a separate screen-printed thick-film electrode via transglutaminase and glutaraldehyde to compare these cross-linking reagents with regard to their suitability. To calculate the amount of a specific biogenic amine, the raw data from multichannel software were transferred to a neural network. The sensor array takes 20 min to complete (excluding statistical data analysis) with only one extraction and subsequent neutralisation step required prior to sensor measurement. The lower detection limits with the enzyme sensor were 10 mg/kg for histamine and tyramine, and 5 mg/kg for putrescine with a linear range up to 200 mg/kg for histamine and tyramine and 100 mg/kg for putrescine. The application area of the enzyme sensor array was tested from fish to meat products, sauerkraut, beer, dairy products, wine and further fermented foods and compared with the data of conventional LC analyses (mean correlation coefficient: 0.854).

Keywords Biogenic amines (histamine, putrescine, tyramine) · Enzymes (diamine oxidase, monoamine oxidase, tyramine oxidase) · Enzyme sensor · Food samples · Liquid chromatography (LC) · Neural network · Pattern recognition

Dedicated to Professor Dr. Rolf D. Schmid, University of Stuttgart, on the occasion of his 60th birthday

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Abbreviations *TAO* Tyramine oxidase · *PAO* plasma amine oxidase · *HRP* horseradish peroxidase · *DAO* diamine oxidase · *BSA* bovine serum albumin · *ABTS* 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) · *TMB* 3,3'5,5'-tetramethylbenzidine · *His* histamine · *Tyr* tyramine · *Spd* spermidine · *Spm* spermine · *Cad* cadaverine · *Put* putrescine · *Agm* agmatine · *TCA* trichloroacetic acid · *TEA* triethylamine · *DMSO* dimethyl sulfoxide · *OPA* *o*-phthalaldehyde

Introduction

During recent years biogenic amines often appear in conjunction with food intoxication. Scombroid fish poisoning (or histamine fish poisoning) is described as a food-borne chemical intoxication which presents with symptoms such as skin rash, epigastric pain, disturbances of the gastrointestinal tract, and is treated with antihistamines [1]. Therefore, certain biogenic amines could be used as an indicator for food quality and hygiene during food processing. Biogenic amines are generated by microbial spoilage of food high in protein content or through processing, ripening and storage of fermented foodstuff, e.g. cheese, fish and meat products, wine, beer, and sauerkraut yielding sometimes high amine amounts [2, 3]. The significance of histamine is well known. Persons being highly sensitive to histamine often develop pseudoallergic symptoms shortly after histamine ingestion [4]. The biogenic amine content of food depends on the biotechnological processes involved in the production procedures as far as food ripening in red wine, sausages, cheese and sauerkraut is concerned [5]. Diamines such as histamine, putrescine and cadaverine are decomposition products of histidine, ornithine and lysine. Histidine is present in abundance in dark-fleshed fish. Consumption of high levels of histamine can lead to scombrotoxicosis while the presence of other biogenic amines is described to potentiate the effects (e.g. nausea, respiratory distress, heart palpitations and hyper- or hypotension) as decribed above [6]. For healthy individuals, the diamines putrescine or cadaverine are not

considered to be toxic. Therefore, in general, dietary polyamines at levels normally present in food are nontoxic, while biogenic amines, particularly histamine, are toxic at high intakes. Histamine itself is not removed by cooking. To prevent histamine poisoning, in Germany maximum limits are set. In fish and fish products, the maximum permissible concentration (prescribed by the "Fischhygieneverordnung") for histamine is 200 mg per kg of the food product whereas in Canada, Finland and Switzerland the limit set is only 100 mg/kg.

Traditionally histamine has been measured by derivatisation with fluorescent reagents followed by chromatographic separation (e.g. HPLC in most cases) [7, 8, 9]. As the HPLC analysis of biogenic amines is tedious with regard to sample clean-up prior to the analysis and it requires trained personnel in combination with quite expensive equipment further analysis methods have been described such as capillary electrophoresis [10], immunochemical methods as ELISA [11], and even some gas chromatographic methods were studied for this purpose [12]. To reduce the time needed for analysis and to offer a rapid screening method for industrial food quality testing, some enzymatic methods [13] and several enzyme sensors have been described so far [14, 15, 16, 17]. Biosensor applications, in general, exhibit various advantages such as allowing a more rapid analysis with less sample treatment being required [18]. Therefore, our aim was the development of a rapid assay suitable for application in the food industry as a main part of the quality assurance system. Using an enzyme sensor for this purpose on the basis of immobilised amine oxidases (cf. Fig. 1 showing the scheme for the enzyme detection via hydrogen peroxide as colour reaction in the enzymatic assay and the respective electrode reaction) has another drawback that the majority of the amine oxidases that could be used do not react with only a single biogenic amine but with different amines to various extents. To solve this problem, we investigated a neural network for pattern recognition [19]. Finally, with the enzyme array developed, its suitability to measure a wide range of different foods was to be checked to offer rapid analysis for food producing companies and, in addition, to governmental survey laboratories.

Materials and methods

Materials

Chemicals. Tyramine oxidase (TAO) from *Arthrobacter spp*. (EC 1.4.3.4, 3.2 units/mg solid, Sigma & Aldrich, Deisenhofen, Germany; T-0905); plasma amine oxidase (PAO) (suspension in 2.0 M $(NH_4)_2SO_4$; EC 1.4.3.6, 90 units/g protein, Sigma, M-4636); peroxidase from horseradish (HRP) (EC 1.11.1.7, 883 units/mg solid, Sigma); were used as received. Diamine oxidase (DAO) from pea seedlings (EC 1.4.3.6, 5.45 units/mg protein) was obtained following the procedure of Güvenilir and Deveci [20].

Bovine serum albumin (BSA), 3-aminopropyltriethoxysilane, and glutaraldehyde (25% aqueous solution) were purchased from Sigma, 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) from Roche Diagnostics (Mannheim, Germany), and 3,3',5,5'-tetramethylbenzidine (TMB) was from Merck (Darmstadt, Germany). Transglutaminase (Ajinomoto Co's transglutaminase ACTIVA™ EB (binding effect)) was obtained as free sample from Ajinomoto, Tokyo, Japan. The amine standards (as free bases or as hydrochlorides) histamine (His), tyramine (Tyr), spermidine (Spd), spermine (Spm), cadaverine (Cad), putrescine (Put), and agmatine (Agm), the OPA-reagent (purified and concentrated), trichloroacetic acid (TCA), mercaptoethanol (FLUKA) and triethylamine (TEA) were purchased from Sigma & Aldrich (Deisenhofen, Germany). Boric acid, the HPLC grade solvents methanol, isopropanol, *n*-butanol, *n*-heptane, tetrahydrofuran, and acetonitrile were from Mallinckrodt Baker (Griesheim, Germany).

Food samples. A wide range of different food samples was investigated, several food samples were provided by the local food industry. The food samples are listed in brief as follows: fish: salmon, herring, cod; cheese: Roquefort, Gouda, Tilsiter; meat products: salami, ham, onion sausage; vegetables: olives and tomatoes in oil, canned sauerkraut; and liquid food samples: white and red wine, beer. All food samples were homogenised or in the case of the cheese samples first grated, and then mixed thoroughly. All the homogenised samples were divided into subsamples; one of each analysed immediately and the other subsample stored deep frozen (–20 °C) either prior or subsequent to the extract preparation.

Methods

Apparatus for sensor measurement. Electrochemical measurements were carried out with an Autolab PGSTAT 10 and GPES (General Purpose Electrochemical System) software (both Deutsche Metrohm, Filderstadt, Germany). With the Multichannel version of GPES it is possible to determine the electrochemical behaviour of a maximum of six working electrodes in one cell.

Apparatus for HPLC analyses. HPLC system HP 1100 series (Hewlett Packard, Waldbronn, Germany) comprised the following modules: high pressure gradient pump (binary or quaternary), on-line vacuum degasser, autosampler, thermostated column compartment, diode array detector and was, in addition, equipped with the ChemStation software.

Screen-printed electrodes. The screen-printed thick-film electrodes were obtained from the Forschungszentrum Biosensorik (Greifswald, Germany) and, in principle, could be purchased from BCS Bio- und Chemosensoren GmbH (Greifswald, Germany). Figure 1 shows the layout of the electrodes to detect hydrogen peroxide at a potential of +700 mV. They consisted of a round platinum working electrode and a Ag/AgCl counter electrode. The working electrode and the connecting wire are coated with an insulating layer. The preparation of the screen-printed thick-film electrodes and their characterisation via cyclic voltammetry is described elsewhere [21]. To achieve an improved selectivity to hydrogen peroxide, a cellulose acetate layer was formed on the electrode surface. For this purpose, 0.2 g of cellulose acetate was dissolved in a mixture of 3 mL acetone and 2 mL cyclohexanone. The electrodes were dipped into the polymer solution, then removed and dried on air overnight. The electrodes are contacted by a clip with the electrode cable and an additional adapter has to be installed to address every single working electrode.

Enzyme characterisation. As histamine (for its maximum limit set in the "Fischhygieneverordnung") and tyramine are the most common biogenic amines present in food these two were our main target analytes. In addition, we chose putrescine as the third analyte and an indicator for spoiling.

One problem was to use amine oxidases with a specific activity being sufficiently high for a rapid detection. The three enzymes investigated were TAO, PAO, and DAO. Due to the indications of the suppliers, the specific activity for TAO was 3 units/mg solid in that one unit will oxidise 1.0 µmol of tyramine to *p*-hydroxyphenylacetaldehyde per min at pH 7.5. The specific activity for PAO was given with 90 units/g protein where 1 unit will oxidise 1.0 µmol of benzylamine to benzaldehyde per min at pH 7.4 at 25 °C. To check the specific activity for the DAO from pea

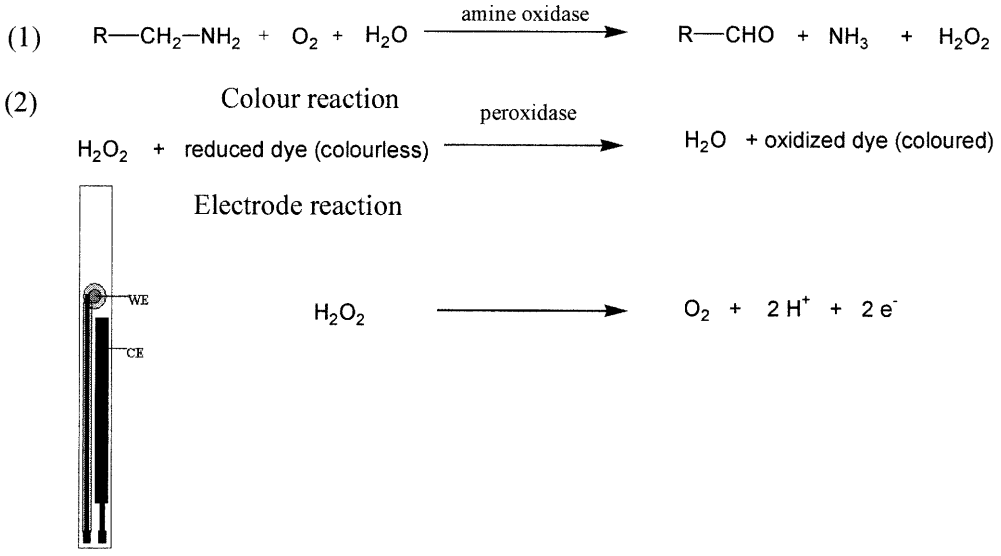


Fig. 1 Enzymatic reaction and detection scheme for the enzymatic assay, and detection principle for the enzyme sensor including layout of screen-printed thick-film electrodes investigated (size: 0.5×5 cm ceramic substrate) with a platinum working electrode (WE, diameter: 0.2 cm) and a Ag/AgCl counter electrode (CE, size: 0.2×2.3 cm). The electrodes were fabricated and obtained from Forschungszentrum Sensorik (Greifswald, Germany)

seedlings, the hydrogen peroxide generated by the enzymatic conversion was measured spectrophotometrically using ABTS as the chromogenic reagent according to the procedure as described by Chemnitius et al. [22]. One unit is defined to catalyse the generation of 1 μmol of hydrogen peroxide per minute using putrescine as the substrate.

Another problem was the fact that most amine oxidases are not monospecific but can react with different substrates to various extents. The so called "cross-reactivities" of the three enzymes (TAO, PAO, DAO) used were characterised in an enzyme assay. For this purpose, the following amines were used: histamine, putrescine, agmatine, cadaverine, spermine, spermidine, and tyramine. A standard series was prepared first in distilled water and, in a second step, in a cheese extract (cf. sample pretreatment). The latter standard series was utilised additionally for the training of the neural net. The standard series comprised the following concentrations: 0 – 1 – 10 – 25 – 50 – 100 – 1000 mg amine per L of distilled water or liquid cheese extract. The enzyme assay was carried out in microtitre plates or glass tubes. A 0.1 M potassium phosphate buffer, pH 6.5, was used to prepare the "enzyme mix". The "enzyme mix" consisted of 50 μL of the respective amine oxidase solution, 50 μL of a standard, 50 μL TMB solution, and 50 μL peroxidase solution. To prepare the enzyme solution for each amine oxidase, 0.005 unit was inserted in the assay within the 50 μL. The TMB solution was prepared by dissolving 6 mg TMB in 1 mL dimethyl sulfoxide (DMSO). From this TMB stock solution, 400 μL were added to 25 mL of the phosphate buffer and 50 μL taken from this dilution for the assay. In addition, in the assay, 4–5 units of peroxidase were used. The enzyme reaction was stopped after 5 min by the addition of 50 μL 2 M H_2SO_4 and the absorption values were measured with an ELISA reader or a spectrophotometer at 450 nm. The middle of the test was determined in a way analogous to the procedure frequently used in ELISA techniques [23]. For application to the neural network the obtained cross-reactivity values of the three enzymes were used (cf. Table 1).

Enzyme immobilisation. First, we used transglutaminase for enzyme immobilisation. For this purpose, we investigated a certain quality obtained by Ajinomoto. As there was no information available about the ideal transglut-aminase concentration, preliminary experiments were performed using 5%, 10%, and 50% transglutaminase solutions. It turned out finally that a 20% solution gave the best results. The following protocol was applied for immobilisation via transglutaminase: 50 μL of 20% transglutaminase in either distilled water or 0.1 M phosphate buffer, pH 6.5, were mixed with 50 μL of the respective amine oxidase and incubated overnight at 4 °C. Then 50 μL of 10% BSA solution was added, mixed and allowed to incubate for one further hour. Finally, 5 μL of this mixture was dropped onto the working electrode and allowed to dry for 1 h. The electrode was washed with the phosphate buffer and stored at 4 °C until use. Unfortunately, when this lot of transglutaminase was finished difficulties occurred with the regular quality. Therefore, conventional immobilisation procedures were investigated. The amine oxidases were immobilised each on a separate working electrode by a method based on glutaraldehyde-albumin cross-linking. A 10 μL volume of the enzyme and 10 μL of 5% BSA solution were well mixed, then 10 μL of 5% glutaraldehyde were added. After rapid mixing, 5 μL of the mixture were dropped onto the electrode with a micropipette and allowed to dry for 1 h. Finally, the electrode was washed with the 0.1 M potassium phosphate buffer, pH 6.5, and stored at 4 °C until inserted for analysis.

Table 1 Cross-reactivities of the amine oxidases used

Amine oxidase	Cross-reactivities (in %)
TAO	100% tyramine, <1% histamine, putrescine, cadaverine, spermine, spermidine, agmatine
PAO	100% histamine, 90% tyramine, 13% spermidine, <5% putrescine, cadaverine and spermine, <1% agmatine
DAO	100% putrescine, 50% spermidine, 8% spermine, 6% tyramine, 2% histamine and cadaverine, <1% agmatine

To increase the amount of enzyme that could be immobilised in one step on the electrode surface, a silanisation step with a 10% solution of 3-aminopropyltriethoxysilane in 0.1 M potassium phosphate buffer, pH 6.5, preceded the enzyme immobilisation step. To achieve this, the electrode was immersed in total in the silanising reagent for at least 15 min.

Biosensor assembling. A four-electrode system was employed. For the amperometric measurement system, the four electrodes (three enzyme (TAO, PAO, DAO) sensors and one with a reagent blank with only the reagents being immobilised and without any enzyme) were connected to the Autolab PGSTAT 10 system. Every working and counter electrode was connected via an adapter to the Autolab system in such a way that every working electrode could be addressed separately. The working electrodes were all polarised at +0.7 V against the counter electrodes.

The enzyme electrodes were then put together into a glass beaker with 30 mL 0.1 M potassium phosphate buffer. After the output current had reached a stable baseline, at first a standard series for histamine, putrescine, and tyramine was measured by dipping the electrodes into the respective amine concentration and calculation of the calibration data. For the real sample measurements the electrodes were put into a beaker with the neutralised food extract.

Electrochemical measurements. All electrochemical measurements were carried out at room temperature with the Autolab PGSTAT 10. Chronoamperometric analysis at a constant potential (+700 mV) was performed and the current data was sampled. Although the response time in our case was less than 5 s it appeared, in addition, more suitable to study a steady-state measurement of the current. The original signal (*t* vs. *A*) was elaborated using the GPES software, the data points were collected as ASCII files and then first transferred to Excel files and the data amount reduced. Then the files could as such be read and analysed by the modified SPSS neural network software.

Pattern recognition analysis/neural network. SPSS neural network software (SPSS Neural Connection 2.1, SPSS Inc. Chicago, USA) was applied with some modifications performed with respect to learning methods.

One of the major advantages of neural nets is their ability to generalise [24]. This means that a trained net could classify data from the same class as the learning data that it has never seen before [25]. The first step was to find a suitable network topology. A feedforward net with 3 input and 3 output units was the basis to find out the ideal topology. Tested were one and more hidden layers and additional shortcut connections in subsequence. The mean and maximum deviation for the backward propagated output values were compared. With this topology different learning methods: backpropagation, variations of backpropagation (with momentum term, flash-spot elimination and with weight decay), and Rprop, were investigated. For each learning method different learning parameters were compared.

Sample treatment and procedure of analysis. Each food sample (10 g, with the exception of the cheese samples) was extracted twice with 25 mL 5% TCA, centrifuged (10 min at 4000 rpm), the collected supernatants were made up with 5% TCA to 50 mL and used directly for the enzyme sensor array after adjusting the pH to 6–7 (neutralisation with 1 M NaOH). For the LC method a further purification step prior to the analysis is reqired, consisting of either a liquid-liquid extraction with butanol/*n*-heptane or a solid-phase separation using BakerBond cartridges (equilibrated with methanol) with isopropanol/potassium citrate buffer.

The cheese samples were extracted according to the procedure published by Vale and Gloria [26], which is briefly described here: 10 g of grated cheese were suspended in 20 mL 0.1 M HCl and mixed in a Vortex for 5 min. Subsequent to a centrifugation step (6000 rpm for 30 min at room temperature) the supernatant was collected. The solid residue was extracted three more times with 20 mL HCl by the same procedure. The supernatants were combined and stored at 4 °C to crystallise most of the fat. The agglomerated fat layer was removed, and the supernatant was filtered.

HPLC conditions. A precolumn OPA (*o*-phthalaldehyde)-amine derivatisation was applied to reduce the time needed for analysis on one hand and to increase the number of amines and amino acids which could be analysed. A method modified from the one described by Petridis and Steinhart [27] was used and the conditions for HPLC measurement are given in Table 2.

Table 2 HPLC conditions

Column	LiChrospher RP18, 250×4 mm, 5 μm	
Precolumn	LiChrospher RP18, 4×4 mm, 5 μm	
Eluent	Phase A: 20 mM sodium acetate, 0.01% triethylamine (pH 7.2 adjusted with 1–2% acetic acid), 0.3% tetrahydrofuran Phase B: 20% 100 mM sodium acetate (adjusted to pH 7.2 with 1–2% acetic acid), 40% acetonitrile, 40% methanol	
Flow rate	0.9 mL/min	
Gradient	Time (min)	Mobile phase B (%)
	0	30
	5	70
	16	100
	22	100
	23	30
Injection	1 μL sample (mixed precolumn in seat with OPA and borate buffer: 5 μL borate buffer – 1 μL OPA – 0.1 μL water – 1 μL sample – mixed in seat)	
Total analysis time	35 min (post time: 5 min)	

Results

An enzyme sensor array for the simultaneous detection and discrimination between histamine, tyramine and putrescine was developed and optimised. In a second step, the optimised enzyme sensor array was applied to the measurement of various real food samples.

Array development and optimisation

The specific activity of all enzymes used here (especially of the plasma amine oxidase) should have been higher in order to allow for an easier chronoamperometric measurement. Instead with the plasma amine oxidase sensor, in some cases, steady-state measurements had to be performed. Furthermore, the stabilities of the enzyme electrodes could be guaranteed only for 1 week (remaining activity: 80% after 7 days) if the electrodes were stored at 4 °C between the measurements. After at a maximum 7 days the enzyme sensors had to be renewed.

The immobilisation of the amine oxidases worked best with the sample of transglutaminase but as soon as it was finished difficulties mainly in terms of reproducibility occurred. Therefore, it was switched to the conventional immobilisation procedure via glutaraldehyde.

Figure 2 shows the standard curves for the three biogenic amines as obtained with the amine oxidase exhibiting the highest specificity for the respective amine (e.g. the tyramine calibration curve was obtained using the tyramine oxidase sensor). The lower detection limits (2σ) were calculated to be 10 mg/kg for histamine and tyramine, and 5 mg/kg for putrescine. The measuring range was linear up to a concentration of 200 mg/kg for histamine and tyramine, and 100 mg/kg for putrescine. In terms of controlling the maximum permissible histamine concentration (200 mg/kg) in fish and fish products, the measuring range for histamine (10–200 mg/kg) turned out to be ideal as no dilution steps were necessary. With regard to food intolerances following the ingestion of tyramine amounts as low as 5 mg/kg, the measuring range for tyramine (10–200 mg/kg) and even putrescine (5–100 mg/kg) should exhibit a higher sensitivity for these compounds. In contrast, lower detection limits and an extended measuring range was obtained by HPLC for the majority of the biogenic amines and several amino acids which could

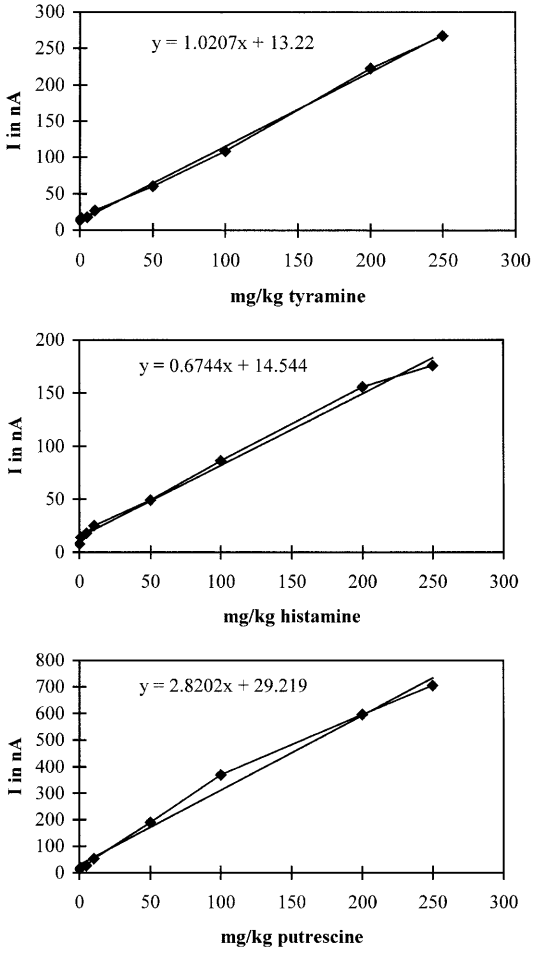


Fig. 2 Standard curves for tyramine (**a** with tyramine oxidase, TAO), histamine (**b** with plasma amine oxidase, PAO), and putrescine (**c** with diamine oxidase, DAO) as obtained by measuring the currents with an Autolab PGSTAT 10 applying a potential of +700 mV

be analysed in parallel (e.g. for histamine a detection limit of 0.5 mg/kg was determined, and 1.5 mg/kg for tyramine with the HPLC analysis).

With the neural network, the best discrimination was observed for tyramine containing samples (91% correct samples), the worst was the situation with the spiked samples containing putrescine (only 57% of the samples were correctly determined) with histamine lying between these two values with 75% of the samples being correctly detected. In total, 12% false positive results and 25% false negative results were obtained. The false positive results were mainly obtained for putrescine, which was absent in all real food samples (as detected by HPLC). The false negative results could, in the majority of cases, be explained by the lower detection limits for histamine and tyramine that could be reached by HPLC analyses, contrary to the detection limits of 10 mg/kg (a factor of 10 or 20 higher) with the enzyme sensors.

Analysis of food samples

Different food samples to a major extent provided by the local food producing industry were analysed with the enzyme sensor array. The results are shown for some of the original food samples in Table 3. Putrescine could not be detected in all the samples analysed via HPLC.

With the real sample measurements a recalibration was necessary every five samples carried out with at least one standard concentration (in most cases 50 mg/kg). From Table 3 it is obvious that the samples containing only small amounts of histamine and/or tyramine were not detected (e.g. ham, cod, and white wine) by the enzyme sensor array and must be taken into account as false negatives. On the other hand, for the canned sauerkraut sample, histamine and tyramine were detected although this result could not be confirmed by HPLC analysis data. In this case, experiments performed with a food matrix spiked with increasing amounts of ascorbic acid showed that a signal was obtained, i.e. the determination might be disturbed if the sample contained ascorbic acid in a concentration higher than 120 mg/kg. In our case, the sauerkraut had a vitamin C content of 20 mg/100 g as measured by a redox titration. Underestimations for histamine and tyramine (as in the case of the Roquefort cheese sample) were quite rare. In general, only a few fish and cheese samples analysed exceeded the 200 mg/kg value exhibiting higher histamine amounts (mostly in the case of the fish products) or higher tyramine contents (e.g. the Roquefort cheese sample). In the latter case, spiking the Roquefort

Table 3 Results of the enzyme sensor array

Food	Enzyme sensor array		HPLC	
	Histamine (mg/kg)	Tyramine	Histamine (mg/kg)	Tyramine
Ham	<10	<10	<0.5	3.5
Herring	30	<10	22	<1.5
Salmon	<10	<10	<0.5	<1.5
Cod	<10	<10	0.75	2
White wine	<10	<10	<0.5	5
Gouda cheese	15	34	12	22
Roquefort cheese	<10	137	<0.5	288
Canned sauerkraut	68	85	<0.5	<1.5

Table 4 Recovery rates for fish samples spiked with [a] 100 mg/kg histamine and [b] 100 mg/kg histamine+100 mg/kg tyramine

Food sample spiked	Recovery rate as measured by HPLC (%)	Recovery rate as analysed with the enzyme sensor array (%)
Salmon[a]	97.2	85
Salmon[b]	98.7	56 histamine, 90 tyramine
Cod[a]	101.6	89
Cod[b]	100.3	170 histamine, 95 tyramine
Herring[a]	98.6	120
Herring[b]	103.4	130 histamine, 180 tyramine

cheese with 100 mg/kg tyramine gave more reliable results with the HPLC analyses than by the enzyme sensor array measurements. From Table 4 it can be assumed that over- and underestimations occurred quite frequently for the enzyme sensor array measurements in comparison to HPLC analyses but to a higher extent if a further discrimination between histamine and tyramine by the enzyme sensor array is required.

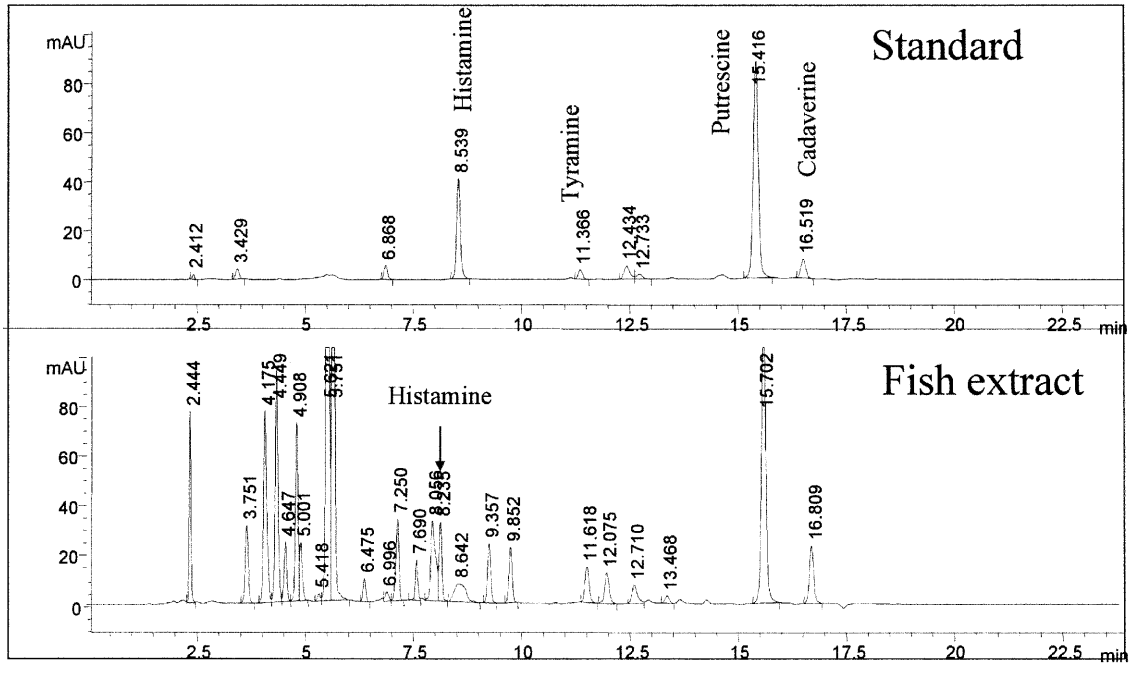
For the conventional LC analysis, a precolumn derivatisation is required. Figure 3 shows the LC chromatograms of a standard and a fish extract, in this case a cod sample, where 0.75 mg/kg histamine was found. On one hand, a great advantage of the LC method is the lower detection limit and the fact that besides the different biogenic amines the respective amino acids could be analyzed in the same LC run. On the other hand, as could be seen in this Figure, the HPLC analysis results had to be confirmed by LC/MS analysis to document that only histamine and not tyramine, putrescine and cadaverine, respectively, were present in the sample. This confirmation could not be carried out by spectral analysis alone but an MS identification was absolutely necessary here.

Fig. 3 HPLC chromatograms of a histamine, tyramine, putrescine and cadaverine standard (concentrations: 1 mg/kg, each amine) and a fish extract (cod: containing 0.75 mg/kg histamine). The presence of histamine and absence of tyramine, putrescine, and cadaverine was confirmed by LC/MS analysis (LC conditions cf. Methods section)

Discussion

An enzyme sensor array for the analysis of histamine, putrescine and tyramine in parallel was described which could, in principle, be applied to the analysis of these three biogenic amines in different food samples as documented by the results obtained.

If one critically compares different aspects of the analysis with the optimised enzyme sensor array and classical HPLC for this purpose some of the criteria listed in Table 5 should be carefully considered. A disadvantage of the classical HPLC analysis in general is the long and tedious sample pretreatment as subsequent to the extraction and neutralisation step a further clean-up either via liquid-liquid or solid-phase extraction is absolutely necessary to get useful chromatograms. In addition, the analysis time is longer for HPLC as compared to the enzyme sensor array although a precolumn derivatisation is already included in the HPLC analysis. A further principal drawback of HPLC is the requirement for organic solvents (sometimes harmful and therefore with further requirements to handle them), mostly of HPLC grade quality, whereby the costs for their disposal had to be taken into consideration. With regard to automation, a general drawback at the present stage with the enzyme sensor array was that handling was laborious and could only be performed sequentially for every sample. In addition, with the enzyme sensor array, a



recalibration with at least one standard concentration was necessary following the analysis of every five samples. Taking into account the number of analytes that could be determined at one time, there is a clear preference for the HPLC method as a great number of analytes (amines and in addition several amino acids) could be detected simultaneously in combination with a higher sensitivity. Because lower detection limits were reached with HPLC by comparison with the enzyme sensor array described here, HPLC is better suited to study biogenic amine concentrations (especially tyramine) in food for obtaining a data set for people with food intolerances [28, 29, 30]. This seemed to be possible with some biosensor developments described in the literature [31, 32, 33], where a higher sensitivity for histamine was reached although with the biosensors more than one single amine was detected. Furthermore, the linear range of the HPLC method is broader, which is advantageous as no further dilution step is required for the majority of the samples. The number of false positive results (which was calculated by setting the HPLC data as correct results) is quite high although this fact is not as problematic as the high number of false negative results planning an application as screening method. This was mainly due to the detection limits reached by the enzyme sensor array being a factor of 10 or 20 higher than with the HPLC in combination with the fact that the majority of the food samples contained only small amounts of histamine and tyramine. But even with HPLC analysis a very few number of false positive results may occur, requiring a final confirmation by an identification of the original compound behind a peak using LC/MS analysis for this purpose. Reproducibility of the enzyme sensor array was sometimes not so high depending on several factors, e.g. if the same enzyme electrode was used or if one enzyme sensor had to be replaced by a new one. The application area is rather an important criterion for the food industry the same as for governmental survey laboratories. So far, the enzyme sensor array could only be applied although to a broad range of different food samples but rather as a rapid screening method.

Conclusions and outlook

To conclude, at the present stage for a rapid screening a microtitre plate based enzymatic array is generally favoured by the food industry. In addition, for a rapid quantitative analysis the method of choice could be capillary electrophoresis in future, as at least, in principle, some results for histamine determination look quite promising [34] unless an inline biosensor would become achievable. As an outlook, to reach the stage of an inline sensor, for several critical points a solution has to be found. A disadvantage of biosensor application to the analysis of biogenic amines in food as the matrix generally is that at least some sample pretreatment has to be carried out (i.e. in our case an extraction and further neutralisation step) that prevents the system being applied as an online sensor. In principle, the sample pretreatment and the analysis should be combined to end up with an online or better an inline sensor. For this purpose, there is a high demand for a precise and easy liquid handling, e.g. using a flow injection analysis system [35] to become automated and eventually directly integrated in the biosensor assembly, e.g. as kind of a lab-on-a-chip [36]. One further aspect still remains an improvement of the biological compound of the biosensor system, i.e. in our case the supply of enzymes exhibiting high specific activities and, additionally, either a single

Table 5 Method comparison

Aspect	Enzyme sensor array	HPLC
Sample pretreatment	Extraction and neutralisation required	Extraction+neutralisation+liquid-liquid extraction or solid phase extraction required (membrane filtration of the extracts prior to LC injection)
Correlation with HPLC	Coefficient: 0.854	–
Analysis time	20 min+additional time for data calculation (external: 3 min per sample)	Analysis time (excluding sample pretreatment/ clean-up): 30 min
Lower detection limit	10 mg/kg for histamine and tyramine, 5 mg/kg for putrescine	0.5–2 mg/kg depending on the amine or amino acid
Linear range	Up to 200 mg/kg for histamine and tyramine, up to 100 mg/kg for putrescine	Up to 1000 mg/kg
Analytes	Histamine, putrescine, tyramine	Histamine, tyramine, cadaverine, putrescine, spermin, spermidine, agmatine, and further biogenic amines and several amino acids
Validity of analysis data	12% false positive results, 25% false negative results	(<1% false positive results as registered by LC/MS confirmation analyses)
Reproducibility	High if using the same electrode	Very high
Application area	Limited because of sample pretreatment	Availability of equipment

substrate specificity or nearly the same specificity for a broad range of different substrates, which could become achievable at least in theory by tailor-made enzymes using site-directed mutagenesis.

Acknowledgements We gratefully acknowledge financial support from the BMBF (grant 1700498). We thank Dr. Peter U. Abel and his colleagues (Forschungszentrum Sensorik Greifswald e. V. and BCS GmbH) for providing us with the screen-printed thick-film electrodes. We are grateful to Mr. Friedrich from Hochschule Wismar for the support in modifying the SPSS Neural Network software and the help in data transfer techniques. In addition, several regional companies supplied us with food samples for analysis of the biogenic amines.

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