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AMPEROMETRIC DETERMINATION OF SARCOSINE WITH SARCOSINE OXIDASE ENTRAPPED WITH NAFION® ON MANGANESE DIOXIDE-MODIFIED SCREEN-PRINTED ELECTRODES

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A simple biosensor, constructed by bulk-modification of carbon ink with manganese dioxide as an electron mediator and sarcosine oxidase enzyme as a biocomponent entrapped with Nafion® was developed and investigated for its ability to serve as amperometric detector of sarcosine in flow injection analysis (FIA) mode. The dependence of the response on the applied potential, the pH of

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phosphate buffer, the concentration and the flow rate were studied. Factors influencing the amperometric response were studied in detail. The sensors could be operated at optimized conditions which were found as follow: flow rate, 0.2 ml min^{-1}*; carrier, 0.1 M phosphate buffer (pH 8.25); injection volume, 150 µl; operation potential, 0.38 V vs. Ag/AgCl. The sensor exhibited a linear increase of amperometric signal with the concentration of sarcosine in the range of 10 – 250 ppm (*R^2 *= 0.9992) with a detection limit (evaluated as 3σ) of 2.5 mg* l^{-1}*. The developed biosensor exhibited a good selectivity over other biogenic amines. Its application in food analysis was also examined.*

Introduction

Sarcosine belongs to the family of biogenic amines. According to the structure, they represent aliphatic, alicyclic and heterocyclic organic bases with low molecular mass that possess biological activity [1,2]. Their occurrence is extremely widespread in a variety of food products ranging from fish and seafood to meat products, milk products, beer, wine, sauerkraut, grapes, olives, *etc.* [3,4]. Biogenic amines are generated by microbial spoilage of food high in protein content or through processing, ripening and storage of fermented foodstuff. Most amines in food originate from the corresponding amino acids which have undergone decarboxylation by putrefactive bacteria (mainly yielding putrescine, cadaverine and histamine) or lactic acid bacteria (yielding tyramine) [5].

Consumption of a high level of biogenic amines can lead to health diseases. Histamine and tyramine have deleterious psychoactive and vasoactive effects, putrescine, spermidine and spermine are considered as constituents affecting cellular metabolism, putrescine and cadaverine may amplify vasoactive effects of other amines. In addition, in the presence of nitrites, biogenic amines produce compounds that can be endogenous precursors of *N*-nitrosamines [6].

Analytical determination of biogenic amines is usually carried out by methods of high performance liquid chromatography (HPLC), mainly as dansyl-, benzoyl- or *o*-phthalaldehyde derivatives. Electrochemical enzyme probes based on the oxygen consumption or the hydrogen peroxide production are also widely used. Amine oxidases are ubiquitous water-soluble enzymes, which catalyze the oxidative deamination of amines to the corresponding aldehydes, ammonia and hydrogen peroxide according to Eq. (1) [7]

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + H_2O_2 + NH_3 \qquad (1)$$

Hydrogen peroxide as an intermediate of such enzymatic oxidation can react chemically with manganese dioxide producing manganese species of lower oxidation states, which can be electrochemically reoxidized to MnO_2; the oxidation current can be directly related to the H_2O_2 concentration [8–11]. In combination with a biocatalyst, such a basic sensor unit can be utilized in the development of biosensors (corresponding chemical and electrochemical reactions occurring in the recognition layer are described elsewhere [12,13]).

The aim of this work was the development and optimization of an amperometric biosensor for biogenic amines, in particular for sarcosine, based on sarcosine oxidase as the biochemically active entity and on manganese dioxide as a mediator for the detection of hydrogen peroxide.

Experimental

Apparatus

The flow-injection system consisted of an HPLC pump (model 620, Waters), a sample injection valve, and a self-constructed thin layer electrochemical cell. The latter consisted of a steel plate with an inlet and an outlet for the carrier and a drilling for the reference electrode; the plate acted also as the counter electrode. The working electrodes up to 10 in parallel arrangement along the flow stream were placed over the plate with a spacer (0.5 mm) and fixed with a simple locking mechanism. An Ag/AgCl electrode (3M KCl, model RE-6, BAS) served as the reference.

MnO_2 bulk-modified carbon ink was prepared by thoroughly mixing 1.9 g carbon ink (C50905DI, Gwent, Pontypoll, UK) and 0.1 g manganese dioxide (Merck). The modified ink was sonicated for 30 min. The electrodes were screen-printed on inert laser pre-etched ceramic supports (total sensor area 40 × 10 mm, 3 rows à 10 sensors per plate; Coors Ceramic GmbH, Chattanooga, TN, USA). The preparation consisted of applying thick layers (0.1 mm) of the ink (effective electrode area 34 × 3 mm) onto the substrates with the screen printing device (SP-200, MPM, Ma, USA). The plates were then dried at 60 °C for one hour. The enzyme (sarcosine oxidase, 1 mg) was dissolved in a mixture of 20 µl phosphate buffer and 20 µl 2,5% the Nafion® neutralized with concentrated ammonia. A volume of 5 µl of this solution was applied onto the surface of the above screen-printed electrode.

Chemicals and Reagents

Sarcosine oxidase (from *Bacillus sp.*, specific activity 35 U mg^{-1} protein), sarcosine, cadaverine, tryptamine, tyramine, histamine and benzylamine were purchased from Sigma.

Deionized water was distilled twice in a quartz still and then purified using an ion-exchange system (Nanopure, Barnstead). Phosphate buffer (0.1 mol l^{-1}, pH 8.25) was prepared by mixing 0.1 M aqueous solutions of disodium hydrogen phosphate and sodium dihydrogenphosphate (both from Fluka) to achieve the desired pH.

Procedure

Flow injection analysis (FIA) was performed with an applied potential of 0.38 V vs. Ag/AgCl. The carrier was phosphate buffer with a typical flow rate of 0.2 ml min^{-1}; the injection volume was 150 µl. The responses were evaluated by their peak heights.

Food Samples

Three samples of cheese (smoked), meat (beef), and fish (salmon) bought from a local shop were stored in a refrigerator at 4 °C. For analysis, a sample (≈ 5 g) was homogenized with 50 ml of phosphate buffer and, after centrifugation and filtration, the homogenate was injected directly into the buffer carrier stream.

Results and Discussion

Optimization of Operational Parameters

All operational parameters were optimized. The operating potential is a critical parameter for the amperometric response. The dependence of both the peak current and the background current on the potential was measured in the potential range of 0.34 – 0.48 V. As can be seen in Fig. 1, the current increased with increasing potential. It was also observed that at potentials below 0.36 V, the background current became negative. This phenomenon can be attributed to possible electrochemical reduction and consequent destruction of the modifier MnO_2 which also influences negatively the reproducibility. The optimum analyte signal was obtained at potentials within 0.36 and 0.38 V; the latter value was chosen for further measurements.

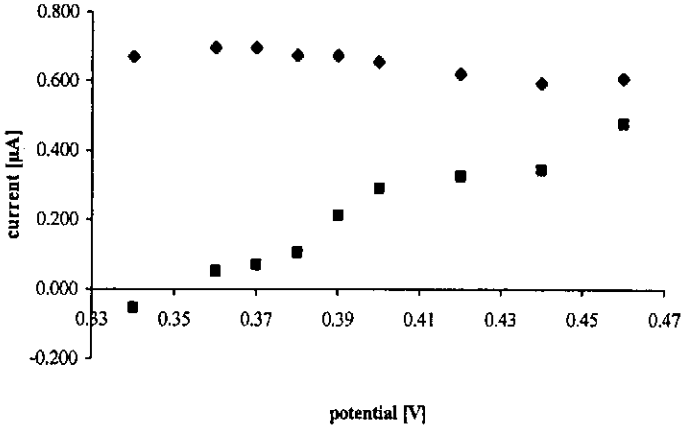

Fig. 1 Dependence of the FIA peak current response on the applied potential: Flow rate, 0.2 ml min^{-1}; injection volume, 150 µl; carrier, 0.1 M phosphate buffer (pH 8.25); sarcosine concentration, 500 ppm. Symbols: squares – background current, diamonds – response

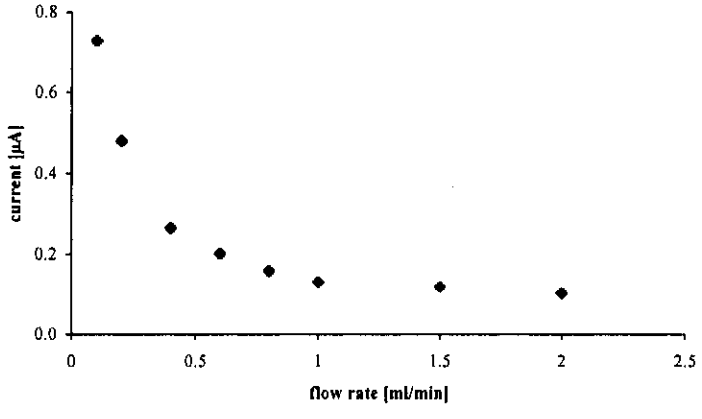

Fig.2 The dependence of the FIA peak current on the flow rate: Operating potential, 0.38 V vs. Ag/AgCl; injection volume, 150 µl; carrier, 0.1 M phosphate buffer (pH 8.25); sarcosine concentration, 500 mg l^{-1}

The flow rate plays a significant role for the height of the amperometric signal. As shown in Fig. 2, the peak current decreases strongly with increasing flow rate, which indicates that the enzymatic reaction is relatively slow and plays the major role in the reaction sequence taking place in the recognition layer. Additionally, the dispersion of the analyte in the carrier multiplies the effect as

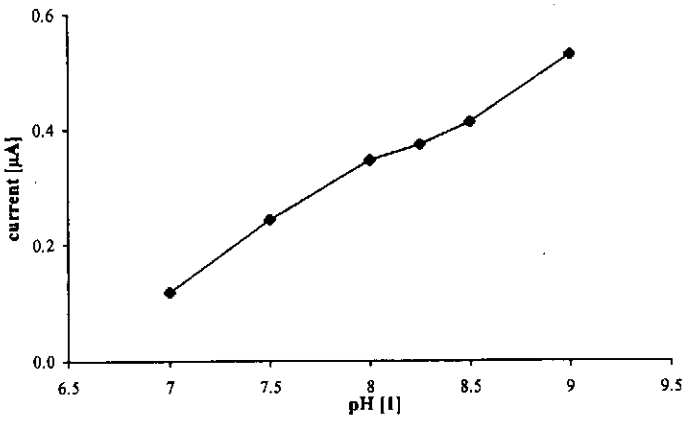

Fig.3 The dependence of the FIA peak current on the buffer pH value: Operating potential, 0.38 V vs. Ag/AgCl; injection volume, 150 µl; carrier, 0.1 M phosphate buffers (pH 7.0 – 9.0); flow rate, 0.2 ml min^{-1}; sarcosine concentration, 500 mg l^{-1}

well. The highest response occurred with a flow rate of 0.1 ml min^{-1}. In order to avoid too long analysis time, a flow rate of 0.2 ml min^{-1} was chosen as a reasonable compromise.

The dependence of the peak current on pH in phosphate buffers is shown in Fig. 3. The graph displays a signal increase with increasing pH value. However, the background current also increases unfavourably together with a significant decrease of reproducibility, and also decomposition of hydrogen peroxide may occur in more alkaline solutions (pH > 8.5). Due to that, a pH of 8.25 of the carrier (phosphate buffer) was chosen as best suitable, also with respect to the optimum pH-range of the enzyme activity.

Calibration, Analysis of Real Samples

A linear relationship between the current and concentration of standards was found for sarcosine in the range of 10 – 250 mg l^{-1}. Above the latter concentration, one can see a small deviation from the linearity. The detection limit for sarcosine (estimated as 3σ values) was found to be 2.5 mg l^{-1} (R^2 = 0.9992).

Because some enzymes may react with more than one substrate of similar structures, the selectivity of the sensor was also characterized. Relative specific activities were calculated by setting the response towards sarcosine as 100 % and evaluating the responses of other amines relative to it. The values are summarized in Table I: it could be concluded that sarcosine oxidase was practically specific to

Table I The relative specific activity of the sacrosine oxidase to biogenic amines

Substrate	Relative specific activity, %
Sarcosine	100
Cadaverine	2.8
Tryptamine	0.9
Tyramine	10.4
Benzylamine	6.4
Histamine	5.5

Concentration of biogenic amines, 2000 mg l^{-1}

Table II Changes in the found sarcosine content in food samples (filtered homogenate)

Sample	Not spiked			Spiked*		
	Starting value	After 3 days	After 5 days	Starting value	After 3 days	After 5 days
Fish[a]	b.l.[c]	22.8	33.8	4.4	31.8	67.6
Cheese[a]	5.0	b.l.[c]	23.9	17.6	5.8	29.9
Meat[b]	2.5	11.8	19.5	19.9	14.8	19.9

*) Spiked with sacrosine, [a]) 20 mg l^{-1}; [b]) 50 mg l^{-1}
[c]) Below detection limit

sarcosine. Nevertheless, it has also some affinity to tyramine (higher that 10 %), whereas the influence of other biogenic amines is quite low.

The optimized biosensor was employed for analyses of real food samples (fish, meat, and cheese, see Table II). After pre-treatment described in Experimental, each of the samples was divided into two parts. The first one was measured directly, the second was spiked with sarcosine. The dependence of the sarcosine concentration on the storage time was studied. In samples not spiked, a time dependence resulting in increase of the sarcosine content was observed during the study. Particularly pronounced was this effect with fish, but also meat showed the same trend. On the other hand, spiked samples from meat and cheese indicated some irregularities, which could be explained by possible side reactions with food additives (mainly nitrites). Nitrites added to meat and cheese in higher concentrations prevent the foodstuff from infection by *Clostridium botulinum*, but can react chemically to the corresponding nitrosamines, which are not converted by the amine oxidase anymore. Anyway the data look very promising, especially for determining the freshness of fish, but also of other foodstuff, probably after

some initial pre-treatment to destroy nitrite (and also nitrate which may be converted to nitrite by microorganisms [14]). Therefore, the results presented here should be considered as preliminary but subsequent studies are already in progress.

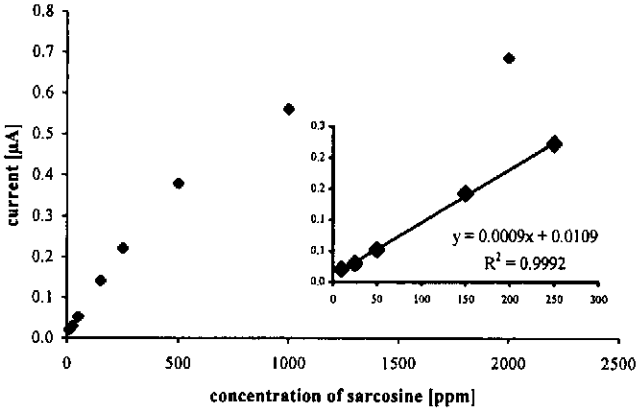

Fig.4 Calibration curve of sarcosine: Operating potential, 0.38 V vs. Ag/AgCl; injection volume, 150 μl; carrier, 0.1 M phosphate buffer (pH 8.25); flow rate, 0.2 ml min^{-1}

Conclusion

It should be noted that some comments expressed above could be disputable until the production of nitrites *via* microorganisms is verified. In many countries (including the Czech Republic), nitrites are not used as meat additives, meat is processed in abattoirs only and if stored in cooling plants, it may be treated with diluted solutions of organic acids (usually lactic acid; acetic acid is applied for spray only). In case that microorganisms are produced by microbial way, the samples would be heavily contaminated by them. Or, meat would be stored for a very long time to make the detection of nitrites possible. Thus, it can be concluded that in such cases, simultaneous treatment of food samples ought to be highly desirable (studies including an influence of fats, albumens, microbial contamination, etc.). However, such a treatment needs some time, which is usually longer than the lifetime of corresponding biosensors.

Acknowledgments

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