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# Enantioselective screen-printed amperometric biosensor for the determination of D-amino acids

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**Abstract**

D-amino acids are generally considered to be important markers of bacterial contamination of food products. A screen-printed amperometric biosensor for the detection of D-amino acids has been constructed by the immobilization of D-amino acid oxidase on a graphite working electrode of a screen-printed strip modified with Prussian Blue and Nafion layers. Enzyme immobilization was then carried out by cross-linking of a mixture of the enzyme and bovine serum albumin with glutaraldehyde. As a result of the mediator addition and because of the multi-layer construction of the biosensor, including a polymer layer to avoid the interferences, the limit of the detection of the developed biosensor was two orders of magnitude improved in comparison to other screen-printed biosensors, as far as the determination of amino acids is concerned. Additional modification of the graphite electrode with carbon nanotubes led to a significant enhancement of the signal magnitude. A fast linear response of the developed biosensor was subsequently observed in static measurements for D-alanine in the concentration range from 5 to 200 μM. Excellent enantioselectivity towards D-amino acids was discovered. During the experiment, D-amino acids were detected in fruit juices and some milk samples. The complex matrix of natural milk samples had no influence on the response of the biosensor. The results were in good agreement with those obtained by capillary electrophoresis measurements.


*Keywords:* Chiral sensors; Enzymatic; Amperometry; Screen-printed biosensor; D-amino acids; D-alanine

## 1. Introduction

The ratio of enantiomers of food constituents is an indicator of the quality of food products, the progress in food technology, the pollution of food products and the ageing processes. Among other food constituents, increased attention has recently been focused on amino acids, commonly present in food products of plant and animal origin. D-amino acids form a part of the staple diet for animals and humans. Hence, their presence in food products is of great significance because of their important role in particular tissues or physiological fluids, including their therapeutic function [1]. D-alanine is an essential building component of bacterial cell walls, which, among others, is a crucial factor in the operation of certain antibiotics [2]. The increased level of D-alanine may indicate the presence of bacteria in the analyzed sample. The exact control of the content of bacteria in food products is of special significance to the monitoring of the fermentation processes, for instance in cheese production. The increased content of D-alanine in cow milk stored at 4 °C might be the evidence of a bacterial uptake from infected cattle [3]. The presence of more than 1 ppm D-alanine in fruit juice may indicate contamination with yeast and implies low quality [4].

Besides numerous chromatographic and electrophoretic methods developed for the determination of amino acids [5], including also chiral determinations [6], a method for efficient determination of amino acids of particular chiral configuration can be developed employing stereoselective enzymes, such as amino acid oxidases. D-amino acid oxidase (DAAOx) is a flavoprotein that catalyzes oxidation of D-amino acids to corresponding imino acids, which are quickly hydrolyzed to appropriate α-keto acids and ammonium ions [7]. In yeast cells DAAOx is involved in basic metabolism where the presence of

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D-amino acids is a key factor in cell growth, while its role in higher organisms is not yet fully understood. For instance, in the kidneys DAAOx is probably involved in the detoxification of residual D-amino acid, whereas in the brain it acts as a regulator for neurotransmitters [8]. The amount of DAAOx enzymes and their properties are different in various classes of organisms, the differences concerning the structure of the active site of the enzyme that results in varying degrees of chiral specificity.

Numerous examples of enzymatic biosensors for amino acids have already been described in analytical and biochemical literature, differing as to the kind of enzyme used, the method of enzyme immobilization employed and the type of transducers utilized. A specific multienzyme biosensor for L-alanine has been developed with the use of alanine dehydrogenase combined with salicylate hydrolase and pyruvate oxidase on a Teflon membrane covering a Clark amperometric oxygen sensor [9]. More commonly, however, amino acid oxidases are employed for this purpose. DAAOx has been immobilized in teflonized graphite paste with peroxidase and ferrocene as a mediator [10,11]. In the development of a D-alanine flow-through sensor for the monitoring of the fermentation, the selectivity of biosensing has been obtained by the combination of DAAOx immobilized in a flow-through reactor and pyruvate oxidase. [12]. A linear response for this determination was reported in the range 0.1–1.0 mM. DAAOx immobilized in a Prussian Blue layer by co-deposition resulted in the biosensor exhibiting the linear range of D-alanine response from 70 μM to 14 mM with the limit of detection (LOD) 30 μM [13]. Amino acid oxidases immobilized on a protein membrane with glutaraldehyde were employed for detecting D- and L-alanine in the concentration range 0.2–3.0 and 0.1–3.0 mM respectively [14]. The obtained biosensors examined in terms of selectivity for different amino acids exhibited a negligible response for enantiomers opposite than the enzyme employed. For the biosensor based on L-amino acid oxidase, the incorporation of carbon nanotubes into sol–gel used for enzyme immobilization turned out to be favourable in terms of long-term stability [15]. Besides numerous electrochemical enzymatic sensors for amino acids, some optical probes have been reported in the literature, as well. A DAAOx-based fiber optic fluorimetric sensor with coimmobilized horseradish peroxidase has been reported for the determination of D-amino acids in a serum [16], while a dual enzyme electrode with optical sensitivity consisting of two platinum disks modified with LAAOx and DAAOx was developed for the determination of the enantiomers of amino acids in a FIA system [17]. Numerous other designs of chiral enzymatic sensors have been reviewed recently [18].

The aim of the current project was to develop a screen-printed biosensor with immobilized DAAOx that could be used for amperometric determination of D-alanine in some food products. As it was shown in the case of apple juice concentrate, in HPLC determinations with enzymatic detection employing amino acid oxidases, D-alanine was the only D-amino acid detected [19]. Screen-printed electrodes (SPE) have been already reported for the use in the determination of amino acids. SPE with carbon working electrode was examined in terms of sensitivity towards cysteine and tyrosine using linear sweep and hydrodynamic voltammetries without any biocatalytic step [20]. In the developed SPE amino acid biosensor based on amino acid oxidases the working electrode contained the immobilized enzyme and rhodinised carbon to facilitate hydrogen peroxide oxidation at a decreased operating potential [21]. In the present experiment, a DAAOx-based SPE was examined with an enzyme immobilized on a working carbon electrode together with a mediating layer of Prussian Blue. The application of DAAOx in amperometric biosensing using Prussian Blue as a mediator was based on the sequence of reactions outlined below. DAAOx catalyzes the oxidation of the amino acid by oxygen to pyruvic acid and hydrogen peroxide, and the latter oxidizes the reduced form of the mediator to hydroxyl ions. The electrode process of Prussian Blue reduction is a direct source of electric current necessary for amperometric detection Scheme 1.

Besides the aspects of the optimization of biosensor construction and the conditions of functioning, the developed biosensor was also examined with regard to the determination of D-alanine in samples of fruit juices. The outcome of the experiment was then compared with the results of capillary electrophoretic determinations.

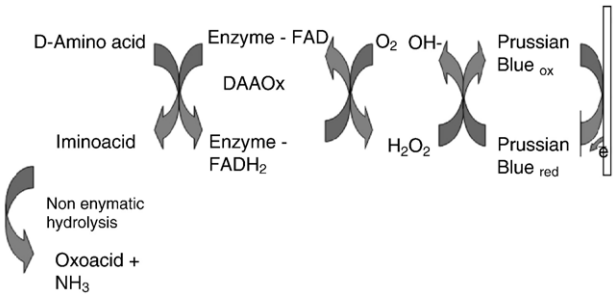
## 2. Experimental

### 2.1. Reagents

The following reagents were purchased from Sigma: D-amino acid oxidase 1.4.3.3 preparations (DAAOx) of activity 2.8 and 7.5 U/mg, glutaraldehyde (GA), flavoadenine dinucleotide (FAD), ferric chloride, ferric hexacyanoferrate, potassium dihydrophosphate, Nafion solution, bovine serum albumin (BSA), single walled carbon nanotubes (SWCNT) (1.2–1.5 nm inner diameter and 2–5 μm long), multi walled carbon nanotubes MWCNT, preparation A (10–30 nm, ID 5–10 nm, 0.5–500 μm long) and carbon nanofibres (NF). MWCNT preparation B was obtained as a gift from the laboratory of Professor Robert C. Haddon at the Department of Chemistry, University of California at Riverside, USA.

### 2.2. Instrumentation

Electrochemical measurements for the optimization of biosensor construction and the conditions of measurement




Scheme 1.

were carried out with the use of a potentiostat Autolab model PG Stat 10 from Eco Chemie (Utrecht, The Netherlands). Analytical measurements with developed biosensors were carried out with an HPLC electrochemical detector model 55 from Amel (Milan, Italy) and a potentiostat model 830B from CH Instruments (Austin, USA).

All electrophoretic measurements have been carried out using a capillary electrophoresis instrument Beckman P/ACE System 2050 equipped with a LIF detector (Fullerton, CA, USA). The excitation was carried out with an argon ion laser at the wavelength of 488 nm; the light was focused on the capillary by a fiber optic connection. The band-pass filter 520 nm was used for emission. All separations were carried out using a fused-silica capillary (Polymicro Technologies, Germany) of 58 cm total length (50 cm effective length) and 50 μm I.D. The capillary was maintained at 20 °C. Hydrodynamic injection mode with a pressure of 0.5 psi was applied for 3 s. Data processing and instrument control were performed using Beckman Station software version 1.2.

### 2.3. The preparation of the screen-printed biosensor

#### 2.3.1. The preparation of SPE with Prussian Blue

The preparation of the SPE modified with Prussian Blue (PB) was carried our according to the procedure published earlier [35]. Prior to the deposition of the PB on the working electrode, the SPE was pretreated by a 3 min anodization in the solution of 50 mM phosphate buffer pH 7.4, containing 0.1 M KCl at 1.7 V, in order to oxidize the traces of organic contaminants on the surface. 10 μl of solution obtained by *in situ* mixing of equal volumes of 0.1 M solution of potassium hexacyanoferrate(III) in 10 mM HCl and 0.1 M ferric chloride in 10 mM HCl was then deposited on the dried surface of the working electrode. The layer of PB on the surface of the working electrode was formed within 10 min; afterwards the reacting solution was washed with 5 ml of 10 mM HCl and the electrode was left to dry for 1 h at 100 °C. The Nafion layer for protection of the Prussian Blue film was prepared by the evaporation of 3 μl of Nafion solution on the working electrode surface.

#### 2.3.2. The dialysis of DAAOx

In order to remove Tris salts contained in the commercial enzyme preparations, it was necessary to dialyze the purchased samples. A solution containing 10 mg DAAOx was dialyzed in 10 mM phosphate buffer of pH 7.4 in a Microdialyzer system at a volume ration 1:1000 for 2 h; the procedure was repeated twice. The dialyzed samples were stored frozen before further use.

#### 2.3.3. The immobilization of DAAOx

8 μl of enzyme solution was deposited on the surface of the carbon working electrode of the SPE modified with PB and covered with a Nafion layer. The solution was obtained by mixing 30 μl of a dialyzed DAAOx solution, 10 μl 40 mg/ml of BSA and 10 μl 2.5% of GA. The biosensor was left overnight in a refrigerator to dry at 4 °C.

## 3. Results and discussion

### 3.1. The optimization of the biosensor configuration

Two basic factors, crucial to the success of the construction of the biosensor, are: the method of immobilization of the enzyme and the selection of the most suitable signal transducer. In the amperometric biosensors with immobilized amino acid oxidases reported earlier, the enzymes have been immobilized by a number of different methods. The appropriate selection of the immobilization method requires reaching a compromise between the stability of the bound enzyme and the biocatalytic activity towards the substrate obtained in the given conditions. Usually the stronger the binding of enzyme to a given support the lower its catalytic activity, and the lower the sensitivity of produced biosensor. Amino acid oxidases have been immobilized in graphite paste [22], in a composite-carbon layer [23], in a graphite–Teflon matrix [11] or throughout a polytyramine membrane electrodeposited on a platinized glassy carbon electrode [24]. DAAOx was also incorporated into a Prussian Blue (PB) film during its electrochemical growth process [13]. In several designs, surface immobilization methods have been employed by covalent binding to various supports, such as pre-activated polyamide [25] or nylon [26] membranes, on a glassy carbon electrode surface modified with $CuPtCl_6$ complex [27], or on the graphite working electrode of SPE modified with a mixture of hydroxyethylcellulose and polyethylenimine [21].

A surface immobilization of DAAOx in the layer of cross-linked BSA with the enzyme was also employed in this study. The purchased commercial lyophylisate of DAAOx from porcine kidney required preliminary dialysis in order to remove any traces of Tris salts, which would bind a part of the glutaraldehyde (GA) during the cross-linking process. Apparently, biosensors prepared from a non-dialyzed enzyme exhibited 10–20-fold lower amperometric signals, than those with a dialyzed one.

As it was discussed by many authors (cf. e.g. [28]), the graphite working electrode of SPE strips require some pretreatment in most cases and for this purpose in present work a few minute anodic polarization in phosphate buffer was employed.

The essential improvement of the properties of biosensors with immobilized oxidases in terms of selectivity and signal magnitude can be obtained by adding dissolved or immobilized mediators of electron transfer to the system. In the reported biosensors with amino acid oxidases, ferrocene [11], tetrathiafulvalene [29] and benzoquinone [30] (to name a few) were used for this purpose. In order to lower the potential of the working electrode and provide a better selectivity of response, catalytic properties of some additives have been used in the construction of amino acid biosensors, such as rhodinised carbon [21], iridium-dispersed carbon paste [22], metallic palladium incorporated into composite ceramic-carbon [23], platinized glassy carbon [24] or glassy carbon modified with $CuPtCl_6$ [27]. It was mentioned with reference to the biosensor reported above with DAAOx incorporated into a Prussian Blue film, but PB was also successfully employed as a catalyst in the screen-printed biosensor with immobilized oxidases [31], and the

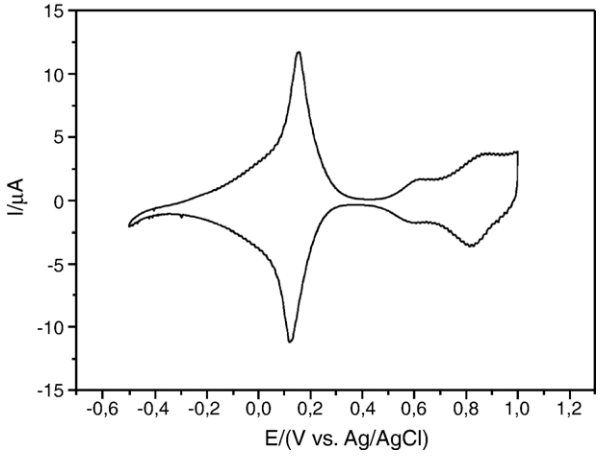



Fig. 1. Cyclic voltammogram recorded for screen-printed electrode with graphite working electrode modified with Prussian Blue by *in–situ* precipitation on the electrode surface and silver–silver chloride as reference electrode. Measurement was made in 50 mM phosphate buffer of pH 7.4 containing 0.1 M KCl with scan rate of 50 mV/s.

examined enzymes were immobilized by cross-linking with GA in a mixture of BSA and Nafion. The enzyme layer obtained using the latter method was too brittle and instable, so the cross-linking was carried out only for the mixture of BSA and the enzyme, and the Nafion layer was deposited beforehand, forming a protective layer for the mediator after the evaporation. The procedure is commonly employed in designing enzyme biosensors (cf. [32]), including also the immobilization of enzymes (cf. [33]), and has been reported for SPE biosensors, as well (cf. [34,35]).

Fig. 1 below shows an example cyclic voltammogram for a graphite working electrode, pretreated with anodization, and with a PB layer deposited according to the procedure described above in the Experimental. The diagram shows two pairs of oxidation and reduction peaks of PB, which is in good agreement with earlier reports [13]. 50 ml of the measured solution was placed with a pipette on a horizontal SPE biosensor. An additional enhancement of the long-term stability of the biosensor response was observed in the presence of enzyme cofactor FAD in the measured solution. As the pH value of measured solutions affects the stability of the PB layer, changes of the signal magnitude of the SPE modified with PB were examined in 0.1 mM solutions of hydrogen peroxide at −0.05 V. A series of measurements was carried out for a single electrode, while the pH was being changed. The obtained values of electrical current in successive measurements are displayed in Fig. 2. The optimum pH for catalytic activity of immobilized DAAOx towards D-amino acids was previously reported to be 8.3 [14]. However, 7.4 was established as the optimum pH value for the measurements with a complete DAAOx biosensor, which is close to the value used by other authors (cf. [13,21,27]). The dependence of the magnitude of the amperometric signal of the SPE biosensor for D-alanine on a polarizing potential in the range from −02 to +0.05 V was examined. As a compromise between the signal magnitude, the noise amplitude and the rate obtained of a stable measured current, the polarizing voltage −0.05 V was selected for further measurements.

Another method recently employed for the improvement of the properties of electrochemical sensors and biosensors, such

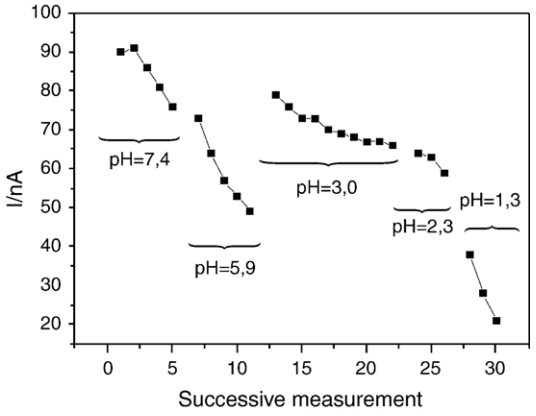



Fig. 2. Effect of pH on stability of response of SPE with working electrode modified with Prussian Blue in successive measurements obtained for 0.1 mM $H_2O_2$ solution in 50 mM phosphate buffer of pH 7.4 containing 0.1 M KCl at −0.05 V.

as the response magnitude, the reversibility of electrode processes and the long-term stability, is to modify the working electrode surface with carbon nanotubes (CNT) [36], also recently used for the modification of working electrodes in SPE sensors [37] and biosensors [38]. A composite material based on CNT has also been used for the preparation of a biosensor with immobilized L-amino acid oxidase [39,40]. Some attempts to modify a working electrode of SPE with CNT and carbon nanofibre (CNF) were also undertaken in this project, using commercial preparations of CNF, SWCNT, MWCNT and a laboratory made MWCNT obtained as gift from the University of California at Riverside. Each of these preparations was deposited on a working electrode surface as 1 μl of 5 mg/ml suspension in dimethylformamide, and then left for 24 h to evaporate in room temperature. Thus modified, the electrodes were covered by a PB layer and used for recording of CVs in phosphate buffer with an addition of 0.1 M KCl and 10 μM FAD. The modification with CNF did not improve the signal magnitude, but as it is shown in Fig. 3, modification with CNT

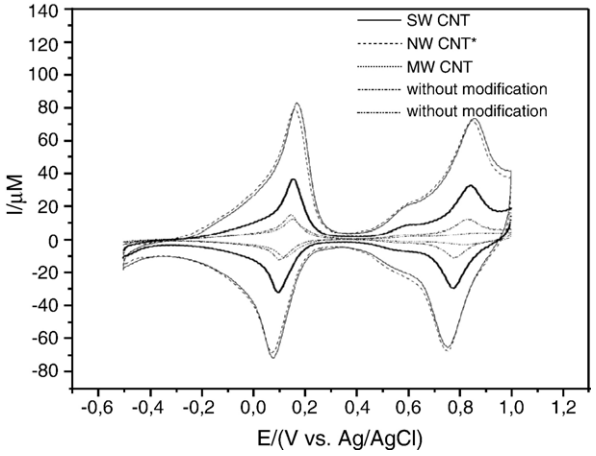



Fig. 3. Cyclic voltammograms obtained for SPE with working electrode with deposited layer of Prussian Blue without and with additional modification with various preparations of carbon nanotubes obtained and silver–silver chloride reference electrode in solution of 50 mM phosphate buffer of pH. 7.4 with 0.1 M KCl at −0.05 V.

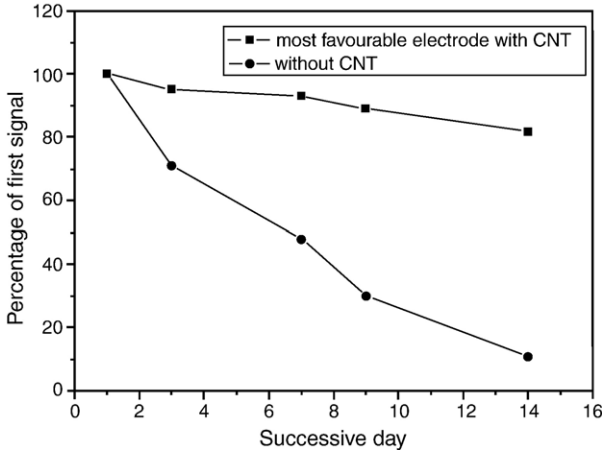



Fig. 4. Comparison of long-term stability of SPE biosensors with immobilized DAAOx in for 0.1 mM D-alanine in 50 mM phosphate buffer of pH 7.4 containing 0.1 M KCl and 10 μM FAD for biosensors without (●) and with (■) working electrode modified with multi walled carbon nanotubes.

was effective. The best results of signal enhancement were obtained with the use of SWCNT, and one of the MWCNT preparations. As for complete biosensors with CNT-modified electrodes, a better stability of response in time was discovered (Fig. 4). The modification of a working electrode with CNT for the preparation of a biosensor is, however, associated with a significant increase of noise amplitude and worse repeatability of the preparation for a larger number of sensors. Therefore, before a further optimization of CNT-based biosensors, biosensors without CNT were employed for further measurements.

### 3.2. Characteristics of optimized biosensor

With the optimum configuration, in a few months' time span and from various sheets of screen-printed electrodes, 15 biosensors were assembled and used for the final evaluation and example application reported below. The response of the biosensors in the concentration range examined here was

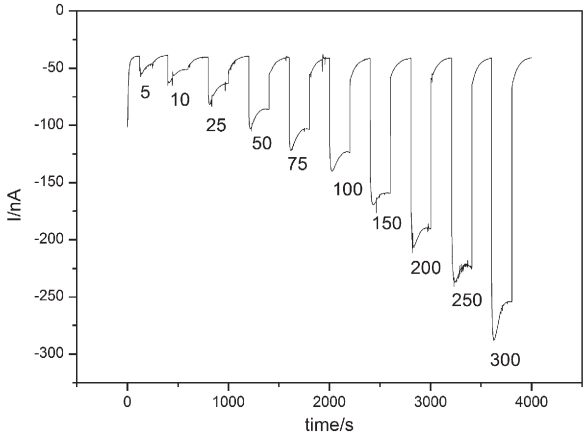



Fig. 5. The current response recorded for SPE biosensor for various concentration of D-alanine in 50 mM phosphate buffer of pH 7.4 containing 0.1 M KCl and 10 μM FAD at −0.05 V.

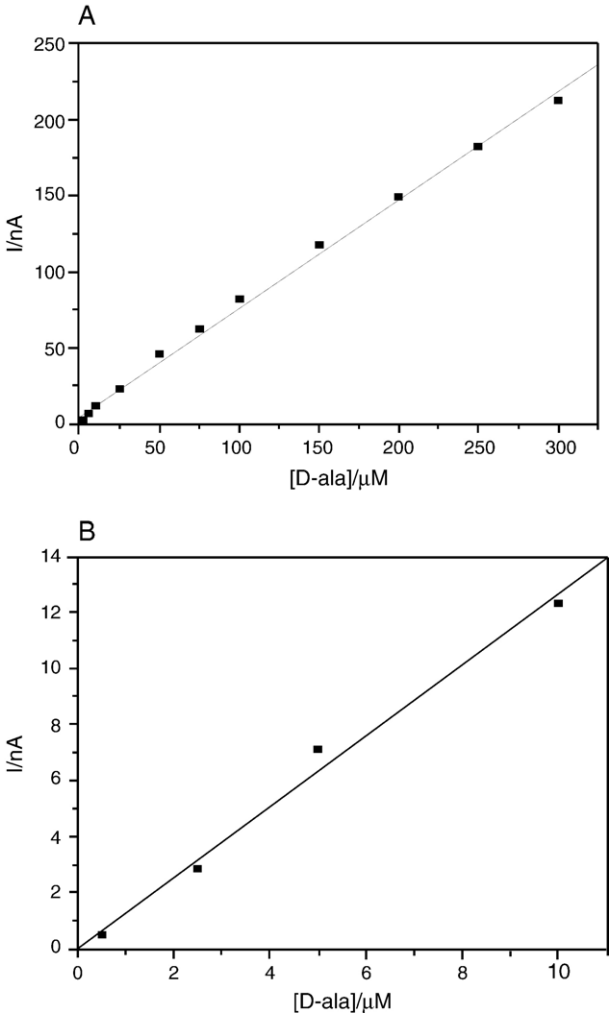



Fig. 6. The calibration plots for D-alanine obtained for recording shown in Fig. 5, 6B is showing response to smallest measured concentrations of D-alanine.

usually stabilized after 3 min, and an example recording of a calibration measurement for the concentration range from 0.5 to 300 μM of D-alanine obtained for a single biosensor is shown in Fig. 5. Between successive measurements, the biosensor was washed with 3 portions of phosphate buffer of pH 7.4. Between measuring runs the biosensor was kept in a refrigerator at 4 °C, in a buffer solution containing 50 μM FAD. The calibration curves for recording in Fig. 5 are plotted in Fig. 6, where 6B is showing the response in the lowest range of the signal magnitude. Depending on the particular biosensor and potentiostat used for the given part of the experiment, the observed noise amplitude ranged from 0.2 to 2.0 nA, while the sensitivity of response to D-alanine — from 0.2 to 0.7 nA/μM. These data allow to evaluate the limit of detection (LOD) for $S/N=3$ as 1 μM for the lowest noise and the largest sensitivity and for 30 μM in the worst case.

In the concentration range up to 300 μM, the biosensor did not exhibit any measurable response for L-alanine. The selectivity of response to some other D-amino acids was also examined. A much lower signal was recorded for D-serine, a comparable one for D-valine, and the strongest one for D-

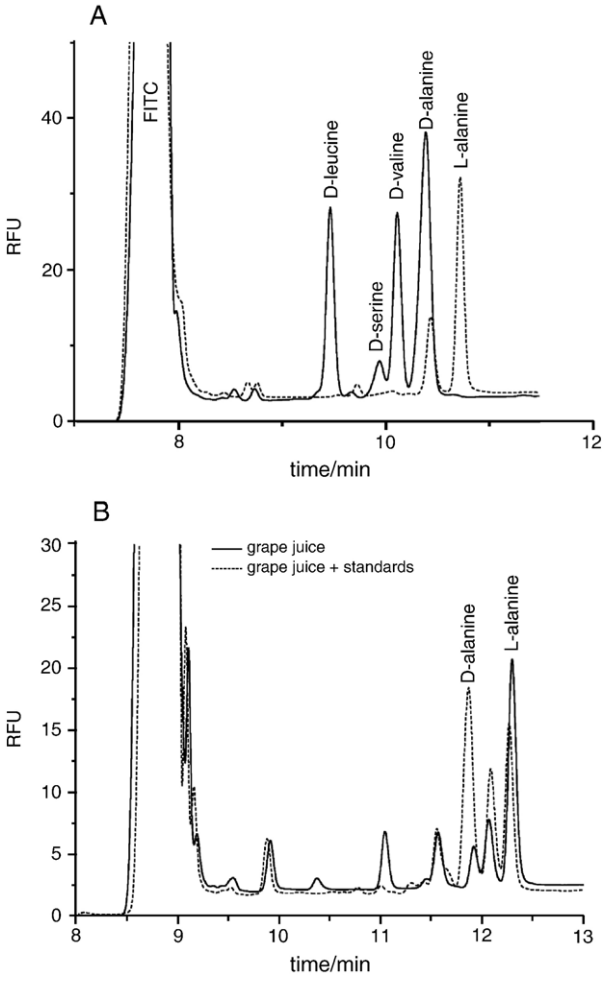



Fig. 7. Capillary electropherograms obtained in chiral determinations with laser-induced fluorescence detection for mixtures of amino acids (A) and grape juice (B) used for determination of D-amino acids with SPE biosensor. Conditions of measurement as described in Experimental. Recorded curves: in (A) solid dark line — mixture of 5 μM D-alanine, D-serine, D-leucine and D-valine, gray line — mixture of 1.7 μM D- and 5 μM L-alanine; in (B) solid dark line — grape juice diluted 1:500 with water, gray line — grape juice diluted 1:500 with water spiked with 10 μM D- and L-alanine.

leucine. For other biosensors with immobilized DAAOx reported above, the relative signal magnitude for various D-amino acids is different. It was also proved that, for the same enzyme lot, the relative signal magnitude depends on the method of immobilization [39]. In the earlier work on SPE biosensors with DAAOx, a comparable signal was observed for D-alanine and D-valine, a 50% weaker for D-leucine, and even lower for D-serine [21].

### 3.3. Application in the analysis of fruit juices and milk

As it was mentioned in the Introduction, the determination of D-amino acids, and especially D-alanine, is of great importance in food technology of dairy products, beverages and juices and in fermentation processes. Numerous biosensors with DAAOx developed earlier were applied in analyses for that purpose, including the use for monitoring milk ageing [21], for the determination of D-amino acids in the samples from different stages of brewing [14], and the determination of D-alanine in fish sauces. With the use of optical biosensors with DAAOx, D-amino acids were determined in human serum samples [16,41].

Table 1

| Sample (dilution) | Determined D-amino acids, mM | Recovery in spiked sample*, % | Determined by CE, mM |
|---|---|---|---|
| Apple juice A | 0.69 | 66 | 0.4 |
| (1:10) | 2.08** | 47 | n.a. |
| Apple juice B | 0.33 | 91 | n.a. |
| (1:20) | | | |
| Apple juice C | 1.96 | 69 | 2.08 |
| (1:10) | | | |
| Grape juice | 0.21 | 80 | n.a. |
| (1:20) | 0.96** | 52 | 0.68 |
| Apple–grape juice | 0.83 | 66 | 0.60 |
| (1:10) | | | |

*raw samples spiked with 1 mM D-alanine.
**after 6 month storage frozen in refrigerator.
n.a. — not analyzed.

D-amino acids are important constituents of fruit juices, where they are considered as molecular markers of bacterial activity, heat treatments and shelf life. It was shown that significant amounts of free D-alanine were only found in juices affected by bacterial contamination [4]. For the determination of D-amino acids in juices, gas chromatography [4] and HPLC methods [4,19,42] are mainly used, but recently also capillary electrophoresis has been employed for this purpose [43,44]. In analyses of orange juice it was found that besides D-alanine, even larger quantities of D-arginine can be found, and smaller quantities of D-glutamate [43], while from the results of analyses of about 90 samples of different fruit juices, mainly grapefruit and pear, it was concluded that of all determined D-amino acids, D-aspartate and D-glutamate were always in smaller quantities than D-alanine [4]. The content of D-alanine was, in most samples, 3 to 6 times lower than that of L-alanine. It was concluded that content of D-alanine >1 ppm is an indicative of bacterial contamination of the juice. In HPLC determinations

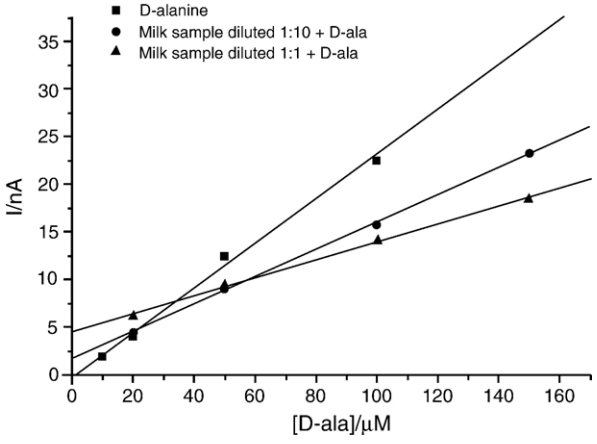



Fig. 8. The calibration plots for D-alanine (■) and milk samples determined with method of standard addition, (●) — milk sample diluted 1:10, (▲) — milk sample diluted 1:1 in 50 μM phosphate buffer of pH 7.4 containing 0.1 M KCl and 10 μM FAD.

Table 2

| Sample, percentage of fat (dilution) | Determined D-amino acids, mM |
|---|---|
| Milk A, n.d. (1:10) | 0.13 |
| Milk B, 1.5% (1:10) | 0.15 |
| Milk C, 3.2% (1:10) | 0.13 |
| Milk D, 2% (1:10) | 0.12 |
| Milk E*, 5.6% (1:100) | 0.41 |

*milk from the cow with mastitis.
n.d. — not determined.

with derivatization of analytes in a flow-through reactor with immobilized DAAOx, in apple juice concentrate mainly D-alanine was detected, and in 32 analyzed fruit concentrates its content amounted from non-detectable to 135.1 mg/l [19]. It was also admitted in conclusions that a natural occurrence of D-amino acids in certain fruit juices cannot be excluded.

As an example application of the developed SPE biosensor, a possibility of its application for the determination of D-alanine (or rather total content of D-amino acids) in several samples of commercial fruit juices was examined. The content of D-alanine in these samples was determined using capillary electrophoresis with laser-induced fluorescence detection after derivatization and with the use of chiral selectors [45]. In these measurements, both satisfactory separation of alanine enantiomers and a separation of D-alanine from other D-amino acids can be obtained (Fig. 7A). An example recording for grape juice sample is shown in Fig. 7B. The obtained results of the determination based on calibration of the biosensor for D-alanine in 5 juices of various producers are shown in Table 1. However, based on the literature data one can assume that measured values are the total concentration of D-amino acids present in the samples. In freshly opened juices, the measured concentrations are within the range of D-alanine concentration reported earlier [19] and the recover of spiking is generally above 70%. For two samples stored in the fridge for 6 months, an evident increase of D-amino acids concentration was observed, and worse recovery was noted. In comparison to the CE results for one of the sample satisfactory agreement was achieved, while for two others, the results obtained were about 30% higher, which can be attributed to the presence of D-amino acids than D-alanine.

In the measured samples of milk, it was necessary to use the method of standard addition. Although the complex milk matrix decreases the biosensor sensitivity (Fig. 8), it does not affect the further response of the biosensor in consecutive measurements. The results of measurements in several of the milk samples with different content of fat and a comparable level of D-amino acids are shown in Table 2. The determined content of D-amino acids in examined samples is significantly higher than the one recently reported in milk samples tested with the CE method [46]. The amount of D-amino acids in the milk from a cow with mastitis, as expected, is significantly higher than in commercially available milk.

## 4. Conclusions

For a developed SPE biosensor several important experimental factors have been examined, including the preparation of commercial enzyme preparation, pretreatment and modification of working electrode, formation of enzyme layer and selection of the optimum conditions of measurements. Due to the enantioselectivity of the enzyme a selective response to D-alanine and several other D-amino acids has been confirmed. A limit of detection down to 1 μM has been obtained for successful preparation of biosensor, which is about 2 orders of magnitude lower than that reported earlier for a different configuration of the SPE biosensor with immobilized DAAOx [21], and better also compared to some conventional biosensors [24,26], but it has to be also admitted that for some biosensors with immobilized amino acid oxidases a sub-micromolar LODs have been reported [25,41]. The repeatability of the preparation of biosensors can be considered as satisfactory, as well as their dynamic properties. The developed SPE biosensors are not a single-use disposable devices but their long-term stability in continuous use is limited to about 10 days. The results of their preliminary applications in the analysis of fruit juices show that they can be promising as portable screening devices for control of D-amino acids content in food products. Further studies should include some attempts to improve long-term stability of response and their testing in analysis of larger amount of different real sample, including also dairy products and fermentation processes.

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