DEVELOPMENT OF A NEW ETHANOL BIOSENSOR WITH ELECTROPOLYMERISED MELDOLA BLUE AS MEDIATOR

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abstract: An amperometric biosensor for ethanol was developed based on immobilization of alcohol dehydrogenase on screen-printed electrodes modified with electropolymerised Meldola Blue carried out by cyclic voltammetry in the range from –0.6 to +1.4 V vs. Ag/AgCl. The calibration plots for ethanol in 0.1 mol.L-1 phosphate buffer pH 8.5 containing 0.1 M KCl shows linearity in the range from 0.1x10-3 to 25x10-3 mol.L-1 ethanol The biosensor showed no decrease in sensitivity after 5 hours of continuous use..

Introduction

The measurement of alcoholic compounds, particularly of ethanol, plays an important role in the quality control of alcoholic beverages such as beer, wines and spirits. A variety of methods had been reported for the determination of this analyte, such as spectrophotometry [1], gas chromatography [2], liquid chromatography with amperometric detection [3,4]. These methods are expensive, slow, need well trained operators and in some cases, require steps of extraction or sample pretreatment, increasing the time of analysis. The food and drink industries need rapid methods to determine compounds of interest [5].

An alternative to facilitate the analysis in routine of industrial products is the biosensors development. Some advantages of this promising tool for food analysis are high selectivity and specificity, relative low cost of construction and storage, potential for miniaturization, facility of automation and simple and portable equipment construction for a fast analysis and monitoring. [6].

In the case of ethanol a number of enzymes based electrochemical devices have been developed either with alcohol oxidase [4,7-9] or alcohol dehydrogenase [10,11]. In the case of biosensors for ethanol based on alcohol oxidase the oxygen consumption or hydrogen peroxide production is monitored [8,12,13]. The alcohol dehydrogenase has the advantage to be more stable and more specific for ethanol but also the disadvantage to be dependent on the coenzyme NAD^+ which has to be added to the assay. Additionally, electrochemical detection of NADH requires overpotential of about 1 V for oxidation and at this potential a number of other substances present in food samples, is also oxidized and can interfere in

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the measurement. This disadvantage may be overcome by adding a mediator in order to improve the electron transfer kinetics and lower the applied electrode potential and effectively regenerate NAD^+ [14]. Biosensors based on quinones [15], oil-soluble mediators based on phenothiazinium chloride [16], phenoxazines [17], organic dyes [18-21]

In last years the NADH detection based on screen-printed electrodes has attracted growing interest of researchers as these electrodes can be mass-produced at low cost making them suitable for commercial purposes. Immobilization of mediators on screen-printed electrodes was successfully conducted by different way: inclusion in different matrices at the electrode surface [12,22], inclusion of the mediator in the screen-printing ink [20,21] or by electrochemical polymerisation [23-25].

We have chosen as electron transfer mediator, Meldola Blue (7-dimethylamino-1,2-benzophenoxazine), having a fast rate of electron transfer with NADH. This mediator allows to achieve high sensitivity for the amperometric determination of NADH and to detect as low as $2x10^{-6}$ mol.L^{-1} [25], with good selectivity since the measurements could be made in an "ideal" potential window (from 0 V to –0.200 V *vs.* SCE) where electrochemical interferences are minimal.

In order to improve the stability of the mediator modified electrodes and to avoid the mediator leakage, different strategies were reported [26-28]. The characteristics of poly(MB) screen printed sensors are superior to those previously reported for detectors based on the same screen-printed graphite electrodes containing Meldola Blue-Reinecke salt [28]. In terms of sensitivity, the poly(MB) sensors [31] are superior to the other detectors already reported based on the same mediator [32]. The NADH detection limit attained by screen-printed electrodes modified by the electropolymerisation of Meldola Blue is close to the best value reported for screen-printed sensors ($2.0x10^{-6}$ mol.L^{-1}) [21].

In this paper an amperometric biosensor for ethanol was developed based on immobilization of alcohol dehydrogenase on screen-printed electrodes with electropolymerised Meldola Blue carried out by cyclic voltammetry in the range from –0.6 to +1.4 V *vs*. Ag/AgCl. The characteristics of screen-printed electrodes with poly Meldola Blue were already reported. [25,29].

Experimental

The enzyme alcohol dehydrogenase ADH (EC 1.1.1.1.) from baker's yeast 264 U/mg solid, *β*-nicotinamide adenine dinucleotide, in its oxidized form of NAD^+ and reduced form NADH, ethanol were purchased from Sigma Chemical Co. Meldola Blue was from Aldrich.

The supporting electrolytes used in this work were Sörensen 0.1 mol.L^{-1} phosphate buffers *p*H 8.5. In amperometry determination the buffer contained also 0.1 mol/L KCl in order to insure proper functioning of the screen-printing pseudo Ag/AgCl reference. All NADH solutions were prepared right before use. Screen-printed graphite electrodes with a geometric area of 0.17 cm^2 were fabricated at University of Perpignan, France according to a previously described procedure [28] and were kindly provided by Prof. Jean-Louis Marty.

All the experiments were carried out with a BAS 100B/W Electrochemical Workstation (BioAnalytical System Inc., West Lafayette, USA). Data display and recording were

supported by BAS electrochemical software version 3.2. In CV experiments the reference was an Ag/AgCl (3M KCl, BAS) electrode while a platinum wire was used as counter electrode, all BAS, USA.

Biosensor preparation

Sensors were prepared by electrochemical polymerization of Meldola Blue (MB) on screen-printed graphite electrodes. After film deposition, the electrodes were rinsed with distilled water and kept dry at room temperature until use. Immobilization of alcohol dehydrogenase was achieved by cross-linking with glutaraldehyde by depositing 3µL of a mixture containing 20 IU/µL ADH, 1% (m/v) bovine serum albumin and 0.5% (m/v) glutaraldehyde on the surface of screen-printed electrodes modified with poly(MB). The sensors were left to dry at least 24 h at 4°C and kept at this temperature until use.

Results and discussions

Cyclic voltammetry experiments have been performed in the absence and the presence of NADH in order to establish the catalytic ability towards the oxidation of NADH of poly Meldola Blue (Fig. 1).

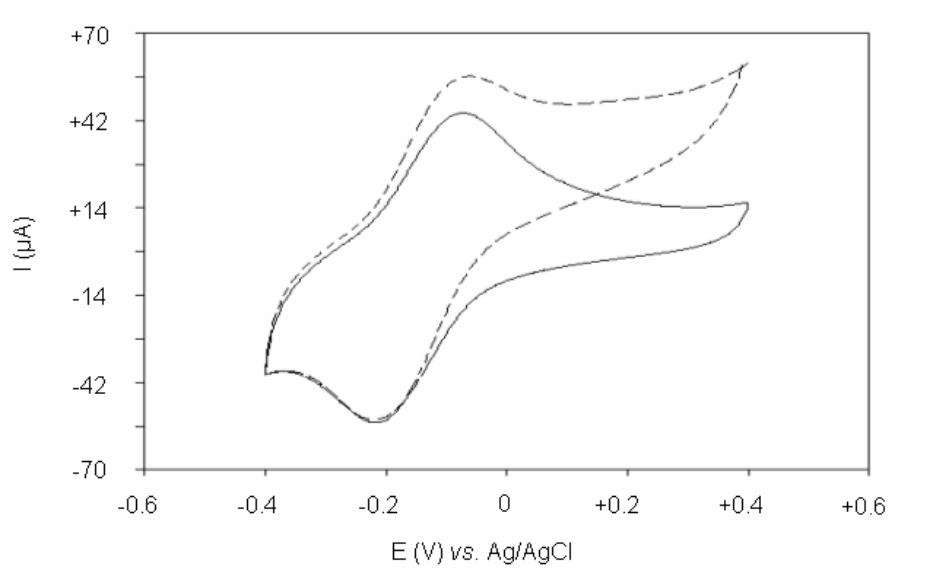

Fig. 1. *Cyclic voltammogramms at poly(MB) electrodes*
in the absence (——) and the presence (----) of 5.10^{-3} mol.l^{-1} NADH.

The sensors screen-printed sensors with polyMB show catalytically ability for NADH oxidation with a larger increase in the anodic current in the presence of NADH $5x10^{-3}$ mol.L^{-1} and a superior sensitivity compare to other modified screen-printed electrodes reported previously [31,32]. The results show that the screen-printed electrodes modified with polyMB can catalyze the oxidation of NADH that is generated from the reaction of NAD^+ and ethanol catalyzed by alcohol dehydrogenase, as schematized in Fig. 2.

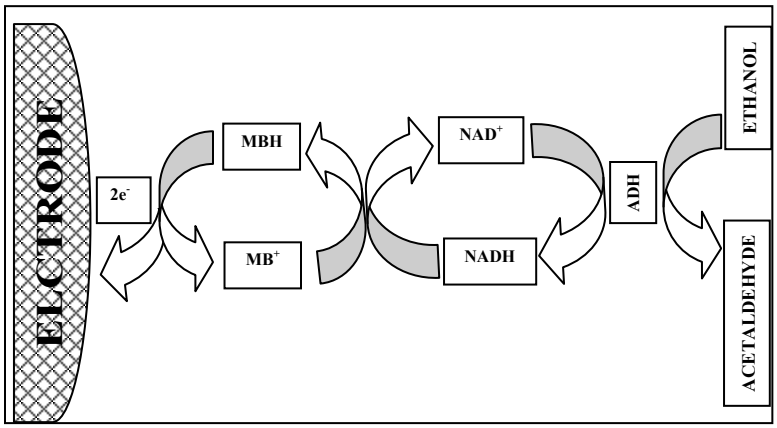

Fig. 2. *Schematic representation of the mechanism of response of mediated ethanol biosensor*

An amperometric biosensor for ethanol was developed based on immobilization of alcohol dehydrogenase on screen-printed electrodes with electropolymerised Meldola Blue, Electropolymerisation of MB represents the best approach for obtaining sensors with good operational stability and remarkable analytical performances for the amperometric detection of NADH [25].

Preliminary experiments with this sensor aimed to the stable immobilization of the enzyme by cross-linking with glutaraldehyde. Several tests were run to illustrate the influence of enzyme loading and the cofactor concentration on the performances of the alcohol detector.

Optimization of cofactor concentration

In a first approach, the concentration of NAD^+ cofactor was varied from 4.10^{-4} to 2.10^{-3} mol.L^{-1} and the response of a biosensor with 12 IU alcohol dehydrogenase was recorded for ethanol concentrations up to 12.10^{-3} mol.L^{-1}. The amperometric determinations were carried out at 0.05 V *vs*. Ag/AgCl in 0.1 mol.L^{-1} phosphate buffer *p*H 8.5. It was shown that cofactor concentrations above 8.10^{-4} mol.L^{-1} did not significantly improve the sensitivity or the detection limit but increased the magnitude of the linear range for the detection of ethanol. A concentration of $1.6.10^{-3}$ mol.L^{-1} NAD^+ was considered as optimum that allowed a detection limit of 3.10^{-4} mol.L^{-1} (S/N=3) and a linear range up to $7.5x10^{-3}$ mol.L^{-1}. This biosensor was characterized by a rather narrow linear range and a low sensitivity (8.54 μA.L.mol^{-1}).

Effect of enzyme loading on the biosensor sensitivity

To emphasize the effect of enzyme loading on the sensitivity of the biosensor, three different volumes (2.5, 3 and 3.5 μL) of the same biocatalytic mixture were deposited on the surface of poly (MB) electrodes. Comparison of the resulted biosensors presented in Table 1 revealed a substantial increase in sensitivity. High enzyme loadings thus appear as necessary in order to obtain biosensors with a satisfactory sensitivity.

Table 1. Effect of enzyme loading on the sensitivity of the biosensor

Enzyme loading (IU)	Biosensor sensitivity (μA.L.mol^{-1} ± SD*)
12.5	4.7 ± 0.6
15.0	11.0 ± 0.9
17.5	31.0 ± 2.5

* standard deviation for five measurements.

Calibration of the biosensors

The calibration plots for ethanol in 0.1 mol.L^{-1} phosphate buffer *p*H 8.5 containing 0.1 M KCl shows linearity in the range from 0.1x10^{-3} to 25x10^{-3} mol.L^{-1} ethanol. the analytical curve is describe by the equation I (nA) = 36.46 (±0.02) + 204.3 (±0.07) $C_{ethanol}$, R^2=0.9993 for *n*=21. Detection limits around 0.008 mM ethanol could be estimated considering Signal/Noise=3. The overall coefficient of variation for the determination of ethanol using described biosensor was 5.4% (*n*=30). This coefficient was found to be mainly induced by the screen–printed procedure, differences in the responses of the sensors being caused by small differences in area of modified graphite layer.

The response time was measured as the time required reaching 95% of the saturation current and is 90 s.

Operational stability

The operational stability of alcohol dehydrogenase sensors was tested by performing 10 consecutive series of calibration with the same biosensor. The concentration of cofactor in the electrochemical cell was 1.6.10^{-3} mol.L^{-1} and the calibration was performed by adding different volumes of a 5.10^{-2} mol.l^{-1} ethanol solution until reaching saturation. No significant decrease in sensor sensitivity was observed at the end of this 5-hour experiment, which represents the more important result obtained for the ethanol biosensor.

Additionally, the described biosensor shows the potentialities of poly(MB)-electrodes as detectors associated with NAD^+-dependent dehydrogenases that catalyse reactions with very low equilibrium constants (K_{eq}= 8.10^{-5}mol.l^{-1} for ethanol transformation at *p*H 7). In these cases sensitive detection of NADH is particularly necessary, especially if amplification systems or coupled reactions that drive the equilibrium to the product side are not taken into consideration.

Analysis of alcoholic beverages

The performance of the ethanol biosensor allowed its application for the direct monitoring of ethanol concentration in different beverages: gin, beer, red wine and Romanian palinca. All the samples were diluted with phosphate buffer solution (1:200 for wine and 1:500 for the other samples), in order to avoid the interferences effect and to fit the analyte concentration within the linear range of the calibration curve. Table 2 presents the values found and the recoveries obtained with the biosensor.

The quantification method was standard addition. As can be observed the results show excellent agreement between the biosensors results and those certified by the suppliers following the Official Method of the European Community consisting of a distillation.

Table 2. Recovery percentages in different samples obtained with ethanol biosensor

Beverage	Added ethanol ($m.mol.L^{-1}$)	Found ethanol ($m.mol.L^{-1}$)	Recovery* %
Gin	3.0	2.95 ± 0.12	99.5 ± 2 98.7 ± 2
Beer	3.0	2.78 ± 0.11	99.3 ± 3
Wine	3.0	2.87 ± 0.10	99.14 ± 3
Romanian palinca	3.0	2.96 ± 0.12	99.4 ± 2

* Average of five measurements

Conclusions

The biosensor for alcohol has a good operational stability and stands for an example of application of poly (MB) detectors with NAD^+-dependent dehydrogenases, especially demanding with respect of sensitivity towards NADH. Another common feature for the detection of NADH is the improvement obtained by Meldola Blue polymerization over other procedures for electrode modification with the mediator like incorporation of Meldola Blue or Meldola Blue-Reinecke salt in the screen-printing ink. This improvement refers to detection limit, sensitivity and operational stability.

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