DISPOSABLE ALCOHOL BIOSENSOR BASED ON ALCOHOL DEHYDROGENASE AND SCREEN-PRINTED ELECTRODES

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abstract: A disposable biosensor for alcohol based on alcohol dehydrogenase and screen-printing electrodes is described. The mediator of the electron transfer is Meldola Blue included in the graphite ink. The applied potential is + 50 mV *vs.* Ag/AgCl. The biosensor was tested with very good results for ethanol determination in beverages.

Introduction

The control of food quality and freshness is of growing interest for both consumer and food industry. In the food industry, the quality of a product is checked using conventionally techniques as, chromatography, spectrophotometry and others. 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 [1].

An alternative to facilitate the analysis in routine of industrial products is the biosensors development. Biosensors are analytical devices composed of a biological recognition element (such as enzyme, antibody, receptor or microorganisms) coupled to a chemical or physical transducer (electrochemical, mass, optical and thermal). These devices represent a promising tool for food analysis. Some advantages as 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 in platforms of raw material reception, quality control laboratories or some stage during the food processing [2].

The determination of alcoholic compounds, particularly of ethanol, is relevant to the food industry, especially in alcoholic beverages such as beer, wines and spirits. In the case of ethanol a number of enzyme-based electrochemical devices have been developed. For this purpose there are two enzymes: alcohol oxidase and alcohol dehydrogenase. The alcohol dehydrogenase is a NADH depending enzyme and the biosensors for ethanol based on this enzyme require the co-immobilization of both enzyme and co-enzyme. In addition this co-enzyme requires an overpotential of abut 1 V for oxidation and at this potential a number of other substances present in food samples, are also oxidized and can interfere in the

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measurement. Different chemical mediators could be used successfully to decrease the overpotential as well as to prevent the electrode pasivation.

Among the mediators taken into consideration when designing biosensors, Meldola Blue counts as a well studied, extensively used compound, being most famous for facilitating NADH oxidation. This mediator allows to achieve high sensitivity for the amperometric determination of NADH and to detect as low as 2×10^{-6} mol·L^{-1} [3], 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. The formal potential of Meldola Blue, assimilated with the arithmetic mean of cathodic and anodic peak potential, ranges from –0.051 to –0.175 V vs. SCE, depending on the sensor material and the method used for mediator entrapment [4÷7].

In recent years the number of reports concerning biosensors based on screen-printed electrodes was continuously increasing and Meldola Blue was often a preferred modifier when designing NADH detectors [8÷11].

In this paper we have chosen as electron transfer mediator, Meldola Blue (MB), a phenoxazine dye, having a fast rate of electron transfer with NADH. This approach allows not only to shift the equilibrium of the enzymatic reaction to the product side but also to reduce the overpotential for the oxidation of NADH.

This work presents the modification of a screen printed carbon electrode by including Meldola's Blue in the graphite [12].

The sensor was characterised and further developed to produce a low cost biosensor for ethanol based on the association of screen-printing technology and chronoamperometry.

As the construction of reusable biosensors by immobilising the low-weight cofactor was shown to be a very difficult task [13], the problems related to NAD were overcome in this work by the simple design of the sensors: disposable biosensors for ethanol were obtained by direct deposition of small amounts of alcohol dehydrogenase and NAD on the planar surface of a mediator-incorporating carbon electrode.

The final goal was to apply the biosensor for the detection of ethanol in beverages, the operating conditions being optimised.

Experimental

The enzyme ADH (EC 1.1.1.1.) from baker's yeast 264 U/mg solid and *β*-nicotinamide adenine dinucleotide, in its oxidized form of NAD^+ and reduced form NADH were purchased from Sigma Chemical Co. MB was from Aldrich. All the solutions were prepared in phosphate buffer *p*H 7. In amperometry and cronoamperometry 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. The screen printing electrodes with MB 2% were kindly provided by Prof. Jean-Louis Marty from University of Perpignan. They are prepared according to the procedure described elsewhere [12]. The disposable biosensors for ethanol were prepared by simply depositing on the working area of Melodola Blue-modified electrodes 2.5 μL of a mixture

containing 8 IU ADH and 1.3×10^{-3}mol/L NAD^+ (optimum amounts). The biosensors were left to dry overnight at 4°C. All the measurements were performed at 25°C.

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.

Results and Discussion

The electrochemical behaviour of Meldola Blue incorporated in the graphite ink of the screen-printed electrodes was studied. The modified electrodes were used in cyclic voltammetry experiments in which the potential was scanned between –0.4 V and +0,4 V *vs* Ag/AgCl at 0.1 V/s in 0.1 mol/L phosphate buffer *p*H 7. These studies revealed the catalytic properties of MB towards NADH oxidation. As we can observe in Fig. 1 in the presence of 5×10^{-3} mol/L NADH the magnitude of the oxidation peak for MB increase, whereas that of the reduction peak decreases. This is a typical feature of catalytic processes which follow an E-C mechanism:

$$\mathrm{MBH} \xrightarrow{E>E^0} \mathrm{MB^+ + H^+ + 2e^-} \quad (1)$$

$$\mathrm{NADH + MB^+} \longrightarrow \mathrm{NAD^+ + MBH} \quad (2)$$

$$\mathrm{Alcohol + NAD^+} \xrightarrow{\mathrm{ADH}} \mathrm{Acetaldehyde + NADH + H^+} \quad (3)$$

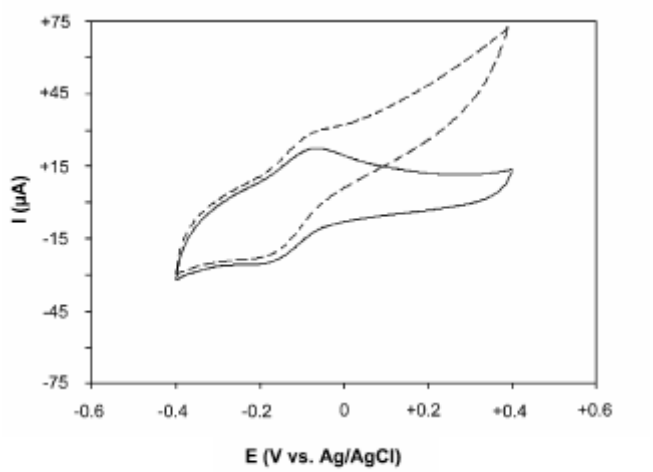

Fig. 1. *Voltammograms recorded in 0.1 mol/L phosphate buffer pH 7 with MB-incorporating screen-printing electrodes in the absence (——) and the presence (----) of $5x10^{-3}$ mol/L NADH.*

Therefore the changes in anodic current produced by these biosensors are proportional to alcohol concentration changes in the sample. The sensitive detection of the NADH were performed in this manner at +50 mV *vs* Ag/AgCl, in the potential range were the

electrochemical interferences are reduced to a minimum. In order to optimize the mediator content in the screen-printed ink we have tested a series of electrodes containing 2÷6% Meldola Blue (w/w graphite). Taking into account the sensitivity of the sensors towards NADH detection, the noise level and the value of residual current, we found an optimum of 2% MB (w/w graphite). The analytical performances of 2% MB modified screen printed electrodes towards NADH are presented in Table 1.

Table 1. Analytical performance of 2% MB modified screen printed electrodes.

Limit of detection (mol/L x 10^{-6})	Linear range (mmol/L)	Equation
0.01	0.02 – 0.2	I (nA) = 75 + 2786 C_{NADH}

The procedure used for the biosensor preparation, with the enzyme deposited on the surface of the electrode, produces single-use biosensors, ideal for the analysis of small amount of liquid (30÷50μL) by chronoamperometry. Taking into account the difficulty of immobilizing the enzymatic cofactor NAD^+, this configuration was found must appropriate for fabrication of disposable biosensors based n the NAD – dependent dehydrogenase.

The response of ethanol biosensor was recorded by chronoamperometry at 30, 60, and 120s after applying the potential +50mV vs Ag/AgCl, for different concentration of substrate in the range 0.1÷20 mmol/L ethanol. We choose as optimum time for determination, 60s, and we did not observe significant differences compare with the biosensor response for 120s (Fig. 2). The coefficient of variation was 8.9% for n = 5 electrodes, which is acceptable for screen-printed electrodes and considering that the deposition of biocatalytic layer was performed manually.

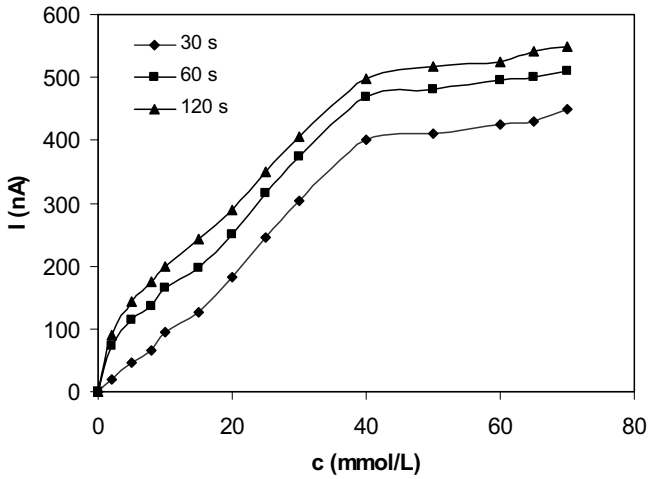

Fig. 2. *Response curves foe ethanol biosensors, recorded by chronoamperometry:* 30s, 60s, 120s after applying the potential.

The performances of the disposable biosensors were tested by applying it to the determination of the ethanol concentration of different beverages: whisky, gin and white wine. In order to fit the analyse concentration within the linear range of the calibration

curve, the sample required an appropriate dilution with phosphate buffer, 1:20000 for the wine and 1:50000 for the others beverages. The results obtained with the disposable biosensor were compared with Official Method of the European Community consisting of a distillation (Table 2). As can be seen, the results show excellent agreement with the results obtained by the Official Methods and with the label of the beverage.

Table 2. Ethanol determination in beverages.

Beverage	Biosensor determination (%) n = 5	Official Methods (%) n = 5	Label indicated alcohol content (%)
Whisky	38 ± 0.5	39.5 ± 0.2	40
Gin	37.3 ± 0.4	38.9 ± 0.2	40
Wine	11.7 ± 0.3	10.8 ± 0.1	11

Conclusions

The paper describes disposable biosensors for ethanol determination based on aldehyde dehydrogenase and screen-printing electrodes. The main advantages of this biosensor are the sample preparation and the short time of analysis compare with Official Methods. The application developed by our group highlight the flexibility which results from coupling screen-printing technology with the use of graphite inks for the construction of biosensors. Work is in progress to improve the stability and reproducibility of the sensors.

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