

**BIOSENSORS BASED ON CARBON PASTE  
ELECTRODES USING IMMOBILISED  
DEHYDROGENASE ENZYMES.  
AN OVERVIEW AND TRENDS**

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*Amperometric biosensors based on two different NADH catalytic systems are presented. Common to both types is the design strategy which consists in the direct mixing of the NAD<sup>+</sup>-dependent dehydrogenase enzymes and other modifying molecules with the carbon paste combined with physical barriers like dialysis membranes or conducting polymers. One type is based on the use of a redox mediator (the phenothiazine dye Toluidine Blue O) to make possible the electrocatalytic oxidation of the enzymatically produced NADH at potentials close to 0 V. The second type is based on the use of the electrochemically generated conducting polymers poly(o-aminophenol) (PAP) and poly(o-phenylenediamine) (PPD), which allow not only the immobilization of enzyme and cofactor at the electrode surface but also the amperometric detection of*

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*NADH at potentials as low as 0 V without other immobilized electron transfer mediators. An ethanol biosensor based on the first catalytic system and ethanol, lactate and glutamate sensors based on the second one are presented.*

## Introduction

It is well known that Biosensors research has received a growing attention in the last few decades justified by its important applications, not only in medicine and veterinary science, where efficient access to biochemical information has always been very important, but also in additional areas so important as environmental monitoring and control, food processing, bioprocessing, agriculture, pharmaceuticals etc. [1]

Among the electrochemical biosensors, amperometric devices offer real advantages over some of other transduction technologies as is shown by their leading position between the systems presently available [2]. The design strategy that we have selected to develop different enzymatic amperometric sensors is the employment of carbon paste as the electrode material, using as an immobilization approach the direct mixing of the enzymes and other modifying molecules with the carbon paste, combined with physical barriers like dialysis membranes or conducting polymers. It is well known that, in spite of its organic character, the mixture of electrically conducting graphite powder and an organic pasting liquid (such as paraffin oil or silicon) can be used to immobilize enzymes while retaining their catalytic activity and since the late 1980s when Matuszewsky et al. [3] used GOD directly blended into the carbon paste this approach has become the focus of intense interest in several laboratories [4,5].

Such strategy offers the following advantages:

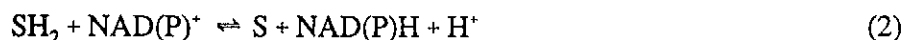
1. The procedure for the electrode fabrication is very simple, relatively inexpensive (requesting no special equipment) and no time consuming.
2. It offers a wide flexibility for the incorporation of numerous modifiers (enzymes, cofactors and mediators).
3. Moreover, the amount of modifier can easily be controlled and the electrode surface can easily be renewed.

In spite of this important advantages, there are some drawbacks associated with this kind of electrode material:

1. There is a limited amount of enzymes available for the substrate conversion because only the protein molecules on the electrode /solution interface are accessible for the substrate.
2. The modified carbon paste is prepared by hand mixing and it is important to be sure that a homogeneous paste is obtained. If not, irreproducibility could be obtained in the electrode response.

Nevertheless, the advantages of using carbon paste as a matrix for the amperometric biosensor design could exceed its limitations whenever the sensors can be made in a reproducible way (necessary condition for the mass production of any kind of biosensors) [6].

Among the different enzymes that can be used as biocomponents for the construction of amperometric sensors oxidoreductases or "redox enzymes" are particularly interesting for coupling to this kind of transducers since an electron transfer reaction is involved in the natural cycle of the enzymes. Two of the major groups of oxidoreductases are oxidase and NAD(P)<sup>+</sup>-dependent dehydrogenase enzymes which catalyzes reactions of the following general type, respectively



where SH<sub>2</sub> represents the substrate and S the product. Of these two groups, oxidase enzymes are the most commonly used biocomponents in biosensor design, in spite of the fact that the use of dehydrogenase enzymes offers some important advantages [7,8]:

1. Oxygen is not involved in the enzymatic reaction and therefore it does not interfere in the detection step.
2. Moreover, the dehydrogenase group is the largest one among the oxido-reductases, there are more than 400 NAD or NADP<sup>+</sup>-dependent enzymes. Therefore, if an electrodic system for the sensible and sensitive detection of the NADH enzymatically produced could be available, we could apply a single biosensor design for the determination of a great variety of substrates, among them the most important analytes in biological systems.

Despite these advantages, the making of biosensors for dehydrogenase substrates comes up against some important problems:

1. Major difficulties are associated with the regeneration of the oxidized coenzyme. The electrochemical oxidation of NADH on bare electrodes needs high applied overpotentials (1.1 V at carbon and 1.3 V at platinum electrodes [9,10]). At these potentials many interfering reactions arising from other oxidizable species can also occur. In addition, under these conditions the NADH oxidation is complicated by some radical side reactions. These unwanted reactions irreversibly consume the cofactor and can produce electrode fouling [11].
2. Another important difficulty associated with the biosensor design is

the need of a soluble cofactor ( $\text{NAD(P)}^+$ ) which must be effectively immobilized on the electrode surface.

It is the aim of this paper to show two different catalytic systems for NADH oxidation, and how these systems can be coupled to dehydrogenase enzymes in order to develop amperometric biosensors.

### Electrocatalytic Oxidation of NADH by an Electron Transfer Mediator

A way to decrease the high overpotentials required for NADH oxidation at solid electrodes is to directly mix into the carbon paste a soluble electron transfer mediator [7,8]. In this context, we have developed an ethanol sensor based on the use of the phenothiazine dye Toluidine Blue O (TBO) as the electron transfer mediator for the oxidation of the NADH enzymatically produced [12, 13]. Thus, the enzyme Alcohol dehydrogenase (ADH), the cofactor  $\text{NAD}^+$  and the mediator (TBO) were dispersed into a carbon paste matrix. The electrode surface was then protected by covering with a dialysis membrane (12000 Dalton cut-off), to prevent aqueous soluble species in the paste from dissolving into the background electrolyte. The reaction sequence which takes place at the electrode surface is outlined in Fig. 1. When ethanol is added to the background solution it passes through the membrane to the electrode surface where it is enzymatically oxidized in the presence of  $\text{NAD}^+$  to form acetaldehyde and NADH. The NADH formed is reoxidized by reaction with the oxidized mediator. The reduced form of TBO obtained is in turn electrochemically reoxidized at the working potential (+0.05 V vs. Ag/AgCl), obtaining a current directly related with the ethanol concentration in solution. As it can be observed with this configuration it is possible to oxidize NADH at a substantially decreased overvoltage, overcoming some of the limitations associated with the direct NADH oxidation at bare electrodes.

The proposed biosensor responds linearly to ethanol concentrations be-

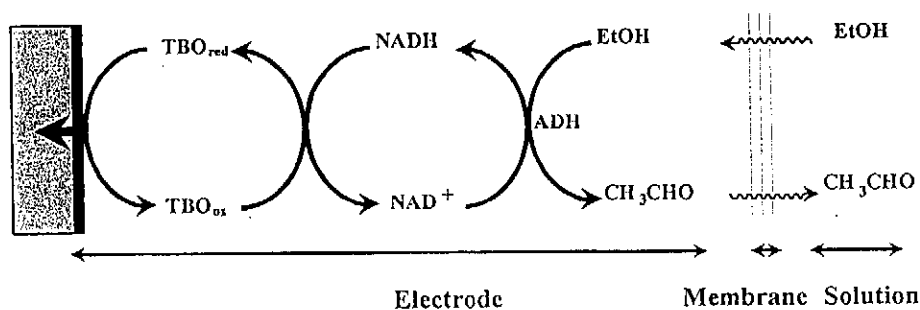


Fig. 1 Reaction scheme for the determination of ethanol with a TBO- $\text{NAD}^+$ -ADH modified carbon paste electrode covered by a dialysis membrane

tween  $2 \times 10^{-5}$  M and  $2 \times 10^{-4}$  M with a sensitivity of  $3.4 \times 10^5$  nA M<sup>-1</sup> and a relatively short response time (at 68 s 95% of steady state current is attained). Other features of this enzymatic sensor are its low cost and fast preparation.

With respect to the sensor stability, the response to ethanol is constant for one working day. However, after storing the sensor in the background solution overnight, this became slightly colored and the amperometric response to ethanol is practically null. This lack of stability is probably due to the slow desorption of the immobilized reagents, mainly the mediator which may pass through the membrane.

## Electrocatalytic Detection of Nicotinamide Coenzymes by Conducting Polymers

With the aim of searching other systems for the effective immobilization of enzymes and mainly the cofactor, we have checked a new modification scheme. This new approach involves coating the electrode surface with a thin layer of an electroactive and electronically conductive polymer. As a consequence, we have found that poly(*o*-aminophenol) (PAP) and poly(*o*-phenylenediamine) (PPD) films allow not only the immobilization of the enzyme and the cofactor at the electrode surface but also the amperometric detection of NADH at potentials as low as 0 V (vs. Ag/AgCl) without other immobilized electron transfer mediators [14].

The two kinds of conducting polymers assayed (PPD and PAP films) were electrochemically grown on carbon paste electrodes by potential-sweep electrolysis in aqueous solutions. Both monomers are oxidized irreversibly, producing a peak at ca. 0.5 V. After the first reverse scan, the electrochemical responses of the coupling products formed on the electrode surface were observed at more negative potentials. These oxidation-reduction peaks increased with successive potential scans, indicating that increasing amounts of the electroactive polymeric products (PAP or PPD) are deposited on the electrode surface. Voltamperograms obtained in the electropolymerization step are analogous to those previously described for the same polymers at another electrode surface [15].

The proposed structures for the electrogenerated conducting polymers [16, 17] are shown in Fig. 2. It is well known that one of the best catalytic structures for NADH oxidation is the charged *p*-phenylenediimine group. Thus much of the reported compounds which can shuttle electrons from NADH to an electrode at a substantially decreased overvoltage are compounds with this basic structural element in their molecules, for example phenoxazines such as Meldola Blue or phenothiazines such as Toluidine Blue O [7,8]. As it can be seen, because the polymer structure has the same basic structural element, it seems not strange that this kind of polymers can catalyze NADH oxidation.

The electrocatalytic activity of the PPD-modified carbon paste electrodes

for the oxidation of NADH and NADPH is demonstrated in Fig. 3. The uncatalyzed oxidation of both NADH and NADPH at a bare carbon paste electrode in pH 7.0 phosphate buffer yields a single irreversible peak in cyclic voltammetry at potentials of 0.5 V, close to the potentials previously reported for the coenzyme oxidation at other carbon electrodes. Otherwise, cyclic voltamperograms of NADH (Fig. 3) and NADPH (not shown) measured with a PPD-modified carbon paste electrode show an anodic current starting at potentials below 0 V, giving rise to a prewave with a half-wave potential of 60 mV for NADH and 50 mV for NADPH. This means that PPD-modified carbon paste electrodes decrease the NADH oxidation overpotentials for more than 400 mV. Analogous results were obtained for the PAP-modified carbon paste electrode, although with this polymer the cofactor oxidation prewave has a half-

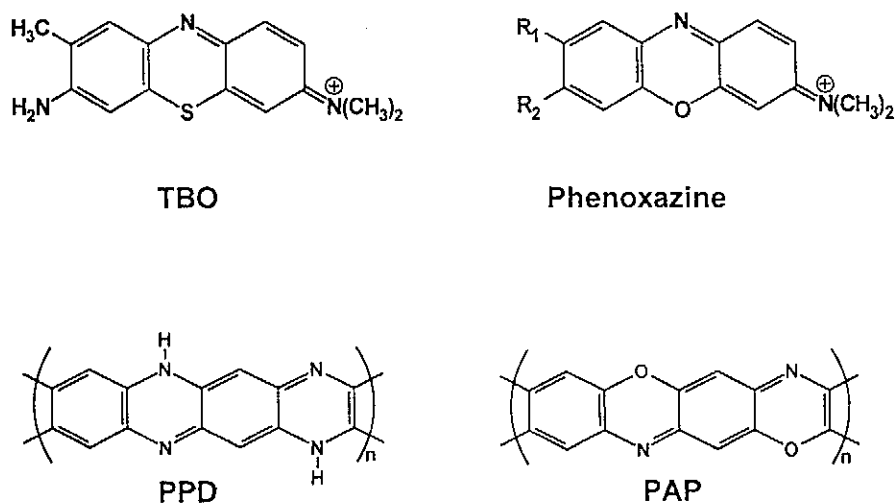


Fig. 2 Structures of typical NADH electrocatalysts and those proposed in literature for the conducting polymers PAP and PPD

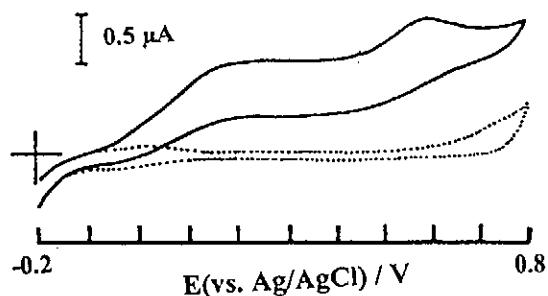


Fig. 3 Cyclic voltammograms of NADH (solid line) and background electrolyte (deaerated 0.1 M phosphate buffer pH 7.0, dashed line) recorded at a PPD-modified carbon paste electrode. NADH concentration  $10^{-4}$  M, potential scan rate  $20 \text{ mV s}^{-1}$

-wave potential of 200 mV for NADH and 300 mV for NADPH. The second anodic process observed in the voltamperogram corresponds to the direct, unmediated oxidation of NADH, as suggested by its peak potential close to that corresponding to the uncatalyzed NADH oxidation at bare carbon paste electrode.

It is important to point out that the catalytic oxidation of NADH on PPD- and PAP-modified carbon paste electrodes depends strongly on the pH of the electropolymerization medium. Thus, the catalytic amperometric response to NADH of polymer films synthesized at different pH values was evaluated, and it was observed that the electrocatalytic activity on NADH oxidation decreases when the pH of the electropolymerization solution increases showing a tendency similar to that presented by the conductivity of this kind of polymers. Although the catalytic current is higher for the films prepared at pH 1 and 3, a pH 5.0 acetate buffer was preferred because a significant loss of enzymatic activity occurred when more acidic solutions were used for the preparation of enzyme-modified electrodes.

The film modified electrodes were used for the amperometric detection of NADH and NADPH at applied potentials of 0.15 V, although amperometric signals useful for coenzyme detection at trace level can be obtained even at applied potentials as low as 0 V. Calibration characteristics are summarized in Table I. Limits of detection in the range of  $10^{-9}$  M were obtained when PPD films were used. Moreover, the PPD-modified electrode can be applied to practical analytical measurements over a period of more than 15 days, and the time required to obtain a steady-state response was less than 15 seconds in all the cases. As it can be observed, in the linear range PPD-modified electrodes allow a more sensitive NADH and NADPH detection. So this modified electrode was selected for further applications.

Table I Calibration characteristics for PPD- and PAP- modified carbon paste electrodes.

	Sensitivity nA M <sup>-1</sup>	L. O. D. M	Linear range M
PPD / NADPH	$4.9 \times 10^6$	$3 \times 10^{-9}$	$10^{-8} - 2 \times 10^{-7}$
PPD / NADH	$7.3 \times 10^6$	$10^{-9}$	$10^{-8} - 2 \times 10^{-7}$
PAP / NADH	$1.5 \times 10^6$	$3 \times 10^{-8}$	$10^{-7} - 9 \times 10^{-6}$

Nevertheless, when calibration over the whole concentration range examined ( $10^{-8} - 10^{-3}$  M) was presented, it was observed that at high NADH concentrations ( $5 \times 10^{-4}$  M) the same currents were reached using PAP- or PPD-modified electrodes. This dependence is typical of a substance which adsorbs according a Langmuir isotherm and apparently reflects the gradual coverage of the modified electrode surface by an adsorbed species, probably NADH. In order to elucidate the nature of the catalytic step, the effect of the potential scan rate

on the prewave limiting current and the uncatalyzed oxidation peak current of NADH was examined at PAP- and PPD-modified carbon paste electrodes. Whereas the catalytic prewave current does not depend on the potential scan rate, the peak current is linearly related to the square root of the potential scan rate. These observations, together with the fact that the limiting catalytic current was found to be independent of temperature in the range of 5–50 °C (opposite effect of temperature on the rate of the preceding chemical reaction and the surface excess of the adsorbed reactant) allow us to propose that the catalytic current is controlled by the rate of a chemical reaction in which adsorbed NADH is involved.

### Amperometric Biosensors Based on PPD Films

According to the results presented we have selected PPD-modified electrodes for the design of amperometric biosensors using NAD(P)<sup>+</sup>-dependent biocatalysts. The design strategy used was similar to that previously pointed out. The enzyme and the cofactor were mixed in the paste and covered with a PPD film electrogenerated in an aqueous monomer solution at pH 5. When the coenzyme is present, the enzymatic reaction leads to the formation of NADH, which is detected amperometrically through its electrochemical oxidation catalyzed by the PPD film. The concentration of the substrate (analyte) is reflected in the concentration of NADH generated and the resulting oxidation current. Figure 4 shows the reaction path for a general dehydrogenase enzyme- NAD<sup>+</sup>-modified carbon paste electrode covered by an electrogenerated electrocatalytic PPD film. Thus the polymer film acts both as a protective membrane and as a catalyst, and no other immobilized mediators are needed. Following this approach, the enzymes alcohol dehydrogenase (ADH), Glutamate dehydrogenase (GluDH) and Lactate dehydrogenase (LDH) were coupled to the PPD-modified carbon paste electrodes to obtain ethanol [18], glutamate [19] and lactate sensors [20], respectively. In Fig. 5 typical amperometric responses of these biosensors to increasing concentrations of ethanol lactate and glutamate are presented.

In general, reactions catalyzed by NAD(P)<sup>+</sup> dehydrogenase enzymes have an equilibrium constant which highly favours the substrate side. A displacement of the equilibrium can be achieved to some extent by coupling the enzymatic reaction with the electrocatalytic oxidation of NADH, increasing the NAD<sup>+</sup> concentration and raising the pH of the reaction mixture, as it was obtained in the ethanol and glutamate sensors. In the case of the lactate dehydrogenase the catalyzed reaction has an equilibrium highly unfavourable and, in order to force this enzymatic reaction to the product side, the enzyme glutamic pyruvic transaminase (GPT) was coimmobilized with LDH and NAD<sup>+</sup> and the reaction sequence in this case is summarized in Fig. 6. Glutamate is added to the background solution.



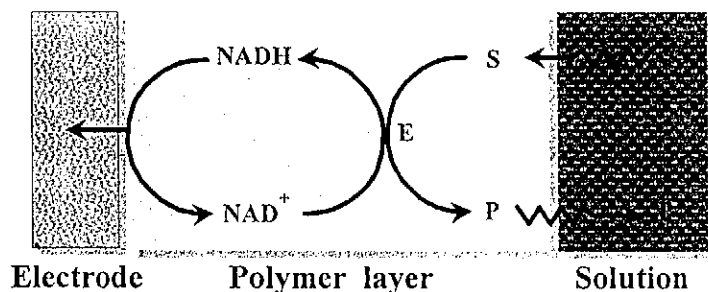


Fig. 4 Reaction scheme for a general amperometric biosensor based on PPD-carbon paste electrodes modified with NAD<sup>+</sup> and a dehydrogenase enzyme

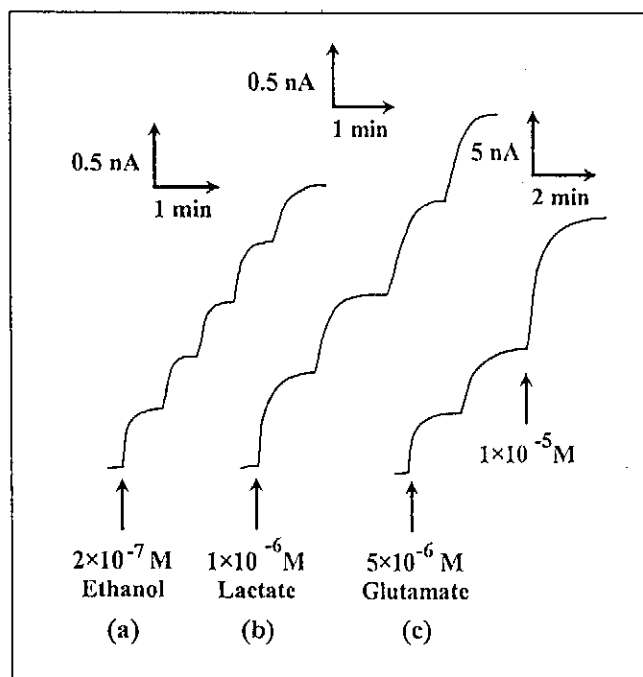


Fig. 5 Typical amperometric responses to successive injections of the corresponding substrates of (a) ethanol, (b) lactate and (c) glutamate biosensors. Applied potential 0.15 V

The analytical characteristics of the three PPD-modified carbon paste biosensors under optimum conditions are shown in Table II. These sensors showed very good sensitivity towards the corresponding substrates with a relatively wide linear range and limits of detection of  $2 \times 10^{-8} \text{ M}$  for ethanol,  $3 \times 10^{-6} \text{ M}$  for glutamate, and  $6 \times 10^{-7} \text{ M}$  for lactate. The reproducibility between

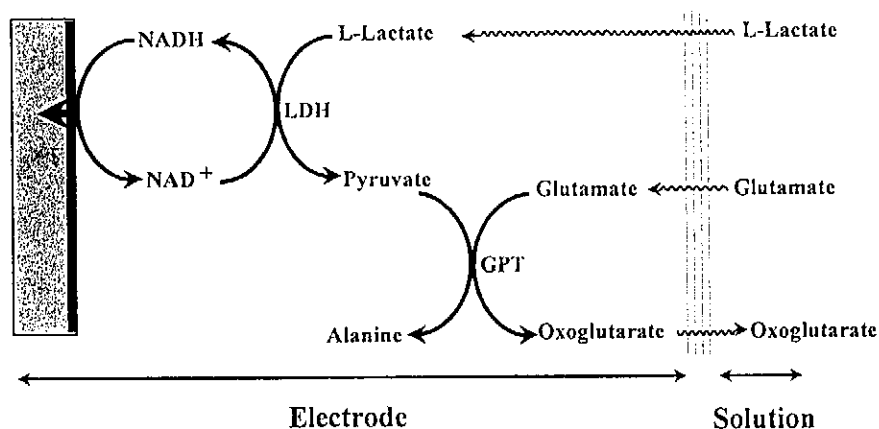


Fig. 6 Reaction scheme for the lactate biosensor

Table II Analytical characteristics of the designed PPD-modified carbon paste biosensors. Lactate biosensor has a second polymeric layer of non-conducting PAP

Analyte	Sensitivity $\text{nA M}^{-1}$	Linear range M	L. O. D. M	R. S. D. %	$t_R$ s
Ethanol	$3.1 \times 10^6$	$3 \times 10^{-8} - 3 \times 10^{-6}$	$2 \times 10^{-8}$	3.4	20
Glutamate	$4.6 \times 10^5$	$5 \times 10^{-6} - 8 \times 10^{-5}$	$4 \times 10^{-6}$	4.8	120
Lactate	$5.6 \times 10^5$	$5 \times 10^{-7} - 8 \times 10^{-5}$	$6 \times 10^{-7}$	4.1	80

different electrodes is good in all the cases (relative standard deviation less than 5%) and the sensors respond rapidly to changes in the substrate concentration producing steady-state currents within 20 s, 120 s, and 80 s, respectively.

The selectivity depends on both enzyme and electrochemical detection. The dehydrogenase enzymes used are relatively selective for the corresponding substrates. Thus the ethanol biosensor does not respond to methanol, ethylene glycol and glycerol although interferences from propanol and butanol were observed. With respect to lactate biosensor no response was obtained for malonic, glycolic, quinic and isocitric acids. Only malic acid shows a low response, about 1% of the response to lactate at the same concentration level. Glutamate dehydrogenase is highly selective for L-glutamate and no enzymatic interferences were found.

On the other hand, interference by direct electrochemical oxidation at the detection potential of easily oxidizable substances, such as uric acid, ascorbic acid, L-cysteine, glutathione and paracetamol, was observed. Nevertheless, in the lactate biosensor design we have developed a new approach in order to minimize such interferences [20], i.e. covering PPD-modified carbon paste

electrodes with a second electrochemically synthesized, non-conducting poly(*o*-aminophenol) film. The double polymer layer-modified carbon paste electrode at an applied potential of 0 V improves considerably the selectivity of the device. In Table III sensitivity of the proposed lactate biosensor to the different interferences is presented.

Table III Influence of the interfering substances on the response of the PPD-PAP-modified lactate biosensor at an applied potential of 0 V

Compound	Sensitivity nA M <sup>-1</sup>
Lactate	5.6×10 <sup>5</sup>
Ascorbate	6.6×10 <sup>5</sup>
Urate	8.6×10 <sup>3</sup>
Cysteine	2.9×10 <sup>5</sup>
Glutathione	4.4×10 <sup>4</sup>
Paracetamol	-

The biosensors designed were used for the determination of ethanol in alcoholic beverages (cider, wine, whisky), lactate in cider, and glutamate in chicken bouillon cubes.

In all the cases the diluted samples yielded well-defined amperometric responses similar to those of the corresponding standards. The results obtained in every case are shown in Table IV. An enzymatic spectrophotometric method based on the detection of NADH at 340 nm was used as a standard method. As it can be observed, the results obtained with the corresponding biosensor compared favourably with the standard spectrophotometric method for the different assayed samples.

Table IV Comparison of the ethanol (% v/v), glutamate (% w/w) and lactate (g l<sup>-1</sup>) concentrations obtained with the proposed biosensors with those from the standard spectrophotometric method

Analyte	Sample	Biosensor	Spectrophotometric
Ethanol	cider	5.68 ± 0.14	5.57
Ethanol	wine	12.52 ± 0.18	12.42
Ethanol	whisky	40.09 ± 0.07	39.96
Lactate	cider	12.6 ± 0.3	12.3
Glutamate	chicken bouillon cubes	2.57 ± 0.05	2.58

## Further Developments

The work summarized until now clearly indicates that carbon paste electrodes can be widely applied in the design of biosensors for substrates of  $\text{NAD}^+$ -dependent dehydrogenase enzymes. Recently, we have found a new NADH electrocatalytic system based on carbon paste electrodes modified with  $\text{NAD}^+$ .

Despite the fact that the electrochemical oxidation of adenine at carbon electrodes has been extensively studied [21], not much work about oxidation of adenine nucleotides, and still less about the oxidation of  $\text{NAD}^+$  adenine moiety, has been reported so far. We have found that products associated with the oxidation of  $\text{NAD}^+$  or in general adenine nucleotides present catalytic activity for NADH oxidation, decreasing the overpotential for more than 500 mV. Work is in progress to characterize these electrocatalysts and develop biosensors for different dehydrogenase enzyme substrates based on the new catalytic system.

## Conclusion

The research described here shows that carbon paste electrodes can widely be applied to the amperometric biosensor design, particularly for substrates of NAD-dependent dehydrogenase enzymes. All the sensors described allow the rapid measurement of the corresponding substrate combined with fast preparation and low cost.

A new approach to develop biosensors for this kind of substrates, which clearly simplifies the design and construction, is described. This approach implies the use of conducting polymer films not only for the immobilization of enzyme and cofactor but also for electrocatalyzing the oxidation of NADH enzymatically produced at applied potentials as low as 0 V. We have developed biosensors based on PPD films but there are a great variety of polymer layers that can be assayed. This means that in our opinion this approach will continue to be a useful source of many future studies.

Moreover, the versatility and easy of operation of the sensors based on this new approach are demonstrated. The great number and diversity of dehydrogenase enzymes known today makes this approach adequate for the monitoring of a wide variety of bioanalytes. This could be another topic for further research.

## Acknowledgements

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