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Enzymatic immobilisation in kappa-carrageenan gel suitable for organic phase enzyme electrode (OPEE) assembly

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Abstract

Organic phase enzyme electrodes (OPEEs) are a new class of biosensors that have extended the field of application of biosensors. The use of biosensors in organic solvent actually makes it possible to analyse insoluble or scarcely soluble substrates in aqueous medium. The best approach for obtaining a new OPEE starts from the optimisation of the experimental conditions in order to obtain the maximum activity and stability of the enzyme to be coupled to the transducer. In a previous paper we therefore optimised the choice of the solvent, while in this paper a new enzyme immobilisation method in kappa-carrageenan gel and its application to the construction of four different mono- or bienzymatic OPEEs are described and evaluated.

Keywords: Enzyme immobilisation; Organic solvent; OPEE

1. Introduction

Relatively recent research [1–5] (especially those performed by Klibanov and coworkers [6–9]) has produced the remarkable finding that many enzymes can function in organic solvents (whether or not the latter contain small amounts of added water [10,11]).

As a consequence, the use of enzymatic biocatalysts also capable of being employed in nonaqueous solvents presents yet another possible opportunity for biosensor techniques traditionally applied to the aqueous environment [12].

The attempts currently being made in this direction are justified by:

–the possible application of organic phase enzyme electrodes (OPEEs) to challenging new environments or additional hydrophobic substrates,

–the possible determination of compounds that are insoluble in water, but soluble in nonaqueous solvents,

–the possible extension of the useful analytical range of biosensors, whenever the compounds are partially soluble in water but very soluble in nonaqueous solvents,

–the increased operational stability of the sensor due to the increased thermal stability of some enzymes in organic solvents,

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–the decreased interference of hydrophilic ionic species,
–the decrease of microbial contamination, which can improve the operational lifetime of the biosensor,
–the simplified immobilisation technique (namely, the direct adsorption of enzymes onto inert supporting materials, or directly onto the surface of the electrode, is facilitated and the enzyme loss generally hindered by the organic phase).

In fact, owing to the insolubility of the enzymes in hydrophobic organic solvents, simple adsorption onto a solid support is often sufficient when working in nonaqueous solvent. The adsorption of enzymes onto solid phases can be achieved by using finely divided particles or macroporous structures with large surface areas. There is a wide availability of good, ready-made support materials, such as alumina, graphite foil, carbon fibres and, especially, porous glass beads. The bound enzyme will have no propensity to desorb from the surface and immobilisation is easily achieved by impregnating the support with an aqueous solution of the enzyme, without any need for covalent bound formation, or the entrapment procedures generally required in conventional aqueous-based systems. Moreover, organic solvents may attack the covalent binding of enzymes to the support.

However, in some hydrophilic solvents, or in solvent–water media, the enzyme adsorbed on the electrode surface may gradually be lost. To overcome this problem, some researchers introduced bifunctional cross-linking agents (glutaraldehyde) in sensor construction [13]. Biocomposite sensors have also been used in organic phase media; carbon paste in particular has reportedly been used for enzyme immobilisation in graphite–Teflon composite electrodes [14,15]. Another class of biocomposite electrodes involve the complexation of long polycationic redox conducting polymers with the surface (lysine) residues of redox enzymes [16,17]. Also, entrapment in gel or polymeric membranes is a very popular immobilisation method both in aqueous and organic phase biosensing. A polymeric material that has found wide use in organic phase biosensor design is the poly(ester sulphonic acid) anionomer produced by Eastman Kodak. On several occasions Wang and Reviejo used this polymer as an immobilisation matrix for OPEEs [18]. The enzyme entrapped in poly(aniline) films containing sodium polyvinylsulphonate as dopant was recently proposed by Iwuoha et al. [19].

In recent works [20–22] we have used a typical immobilisation method involving the entrapment of the enzyme in polymeric gel. In this case a kappa-carrageenan gel was used which proved very suitable for the immobilisation of several types of enzymes, working both in polar (e.g., chloroform) and nonpolar (e.g., *n*-hexane) organic solvents. This paper describes the new method developed by us and its use to fabricate four different mono- or bienzymatic OPEEs.

2. Experimental

2.1. Apparatus and materials

Tyrosinase (EC 1.14.18.1) from mushroom (2400 U/mg), catalase (EC 1.11.1.6) from bovine liver (11000 U/mg), butyrylcholinesterase from horse serum (500 U per mg of solid), choline oxidase from Alcaligenes species (500 U per mg of solid), L-α-phosphatidylcholine, butyrylcholine chloride and dialysis membrane (D-9777) were supplied by Sigma, St. Louis, Mo, USA. The phenol and kappa-carrageenan were purchased from Fluka, Buchs, Switzerland. Hydrogen peroxide 30% (m/v) from Merck (Darmstadt, Germany), and Phospholipase D from *Streptomyces chromofuscus* (100 U per mg of solid) was supplied by Boehringer-Mannheim (Milan, Italy).

The *n*-hexane was supplied by Carlo Erba, Milan, Italy, while chloroform (Chromasolv stabilised with amylene) was supplied by Riedel de Haen, Seelze-Hannover, Germany. The oxygen electrode was a mod. 4000-1 purchased from

Universal Sensors, New Orleans, USA, the original membrane of which was replaced by a Teflon membrane and the O-ring by one also made of Teflon, as well as the external body of the electrode, also especially made of Teflon and suitably constructed.

Measurements were performed using a digital multimeter Mitek MK5001, or a Digital pH meter multimeter apparatus, model 200, supplied by Bell Engineering, coupled with an Amel (Italy) model 868 recorder. A Metrohm (Switzerland) 641 VA Detector was used as potentiostatic power supply and to transform the 'current' signal into a 'tension' signal. Temperature was maintained at a constant value by a Julabo model VC 20B thermostat (Germany). The 25-ml thermostatted cell glass used for measurements was obtained from Marbaglass (Italy).

The small percentage of water in the dried organic solvents or in water-saturated solvents was determined using a Mettler (Switzerland) mod. DL18 Karl-Fischer automatic titrator. Using this method the water content of the anhydrous chloroform was found to be 0.030% by weight, the anhydrous *n*-hexane 0.010% by weight, while the water saturated chloroform and *n*-hexane contained 0.090% and 0.014% by weight of water, respectively.

3. Methods

3.1. OPEE assembly

All the OPEEs described in the present paper were fabricated using one or two immobilised enzymes coupled to a commercial-type gas diffusion amperometric electrode for oxygen; we had to replace the original plastic cap and O-ring of the electrode by others made of Teflon in order to avoid organic solvent attack. In the first approach, the enzyme (or enzymes) dissolved in a few microliters of a suitable buffer solution was simply immobilised between the gas permeable membrane of the electrode and a dialysis membrane; in the second approach the enzyme was first immobilised in kappa-carrageenan gel and the latter sandwiched as in the first case. Briefly, the kappa-carrageenan gel (one or two layers) entrapping the enzyme, prepared as described below, was placed on the head of an amperometric gas diffusion electrode for oxygen, between the gas permeable membrane of the electrode and a dialysis membrane; the whole system was then fixed to the Teflon cap by means of a Teflon O-ring.

3.2. Enzyme immobilisation in kappa-carrageenan gel

3.2.1. Basic method

A 2% (w/w) solution of kappa-carrageenan was prepared by dissolving 0.2 g of the polysaccharide in 10 ml of distilled water, gradually heating (40°–50°C) the solution and maintaining it under constant stirring. The warm solution was placed on a Petri dish and, after cooling, some disks (≈ 0.5 cm diameter) were cut from the gel obtained. After drying at room temperature, the kappa-carrageenan gel disks can be stored as long as needed. To obtain the enzyme membrane, a suitable enzymatic solution of one or two enzymes was added to a disk that was then stored overnight at 5°C and rehydrated with the suitable buffer just before using.

Some modification of this basic procedure was introduced according to how each of the OPEEs studied was arranged.

3.2.2. Immobilisation of tyrosinase for monoenzymatic OPEE assembly

One hundred microliters of the warm (≈ 40°–50°C) kappa-carrageenan solution were placed dropwise on a Petri dish before it set completely at room temperature. Twenty-five microliters of the buffer enzyme solution were then injected into each gel drop, after which the gel drop was layered to obtain a disk. Storage

and assembly before use were as in the basic method. The enzyme solution used consisted of a 200 μl phosphate buffer (1/15 mol/l, pH 6.5) containing 5.0 mg (2400 U/mg) of tyrosinase.

3.2.3. Immobilisation of catalase for monoenzymatic OPEE assembly

The jelly-like kappa-carrageenan disk obtained as in the previous method was dipped in a test tube containing the buffer enzymatic solu-

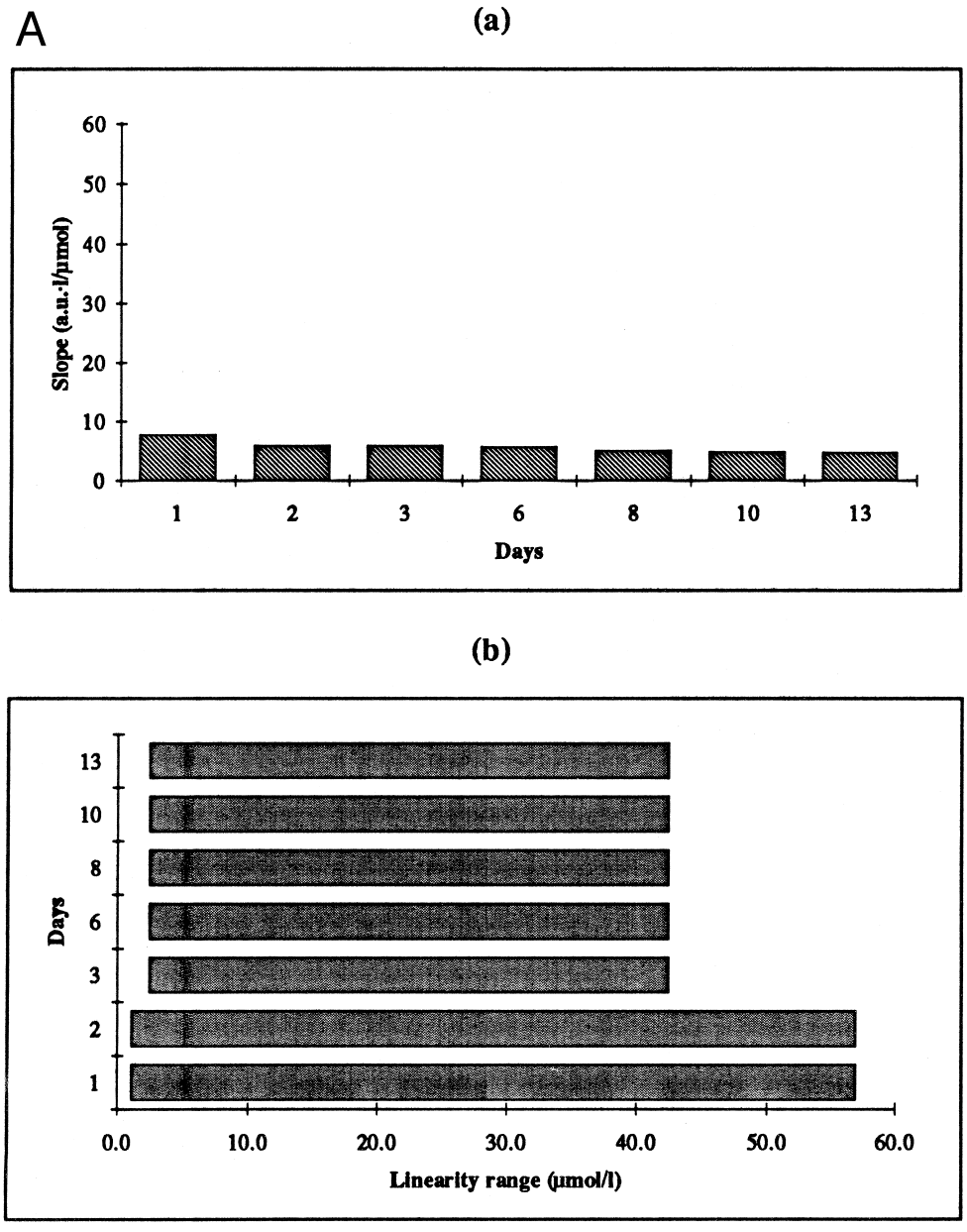

Fig. 1. (A) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the tyrosinase OPEE. Enzyme immobilized in dialysis membrane, *n*-hexane as medium. (B) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the tyrosinase OPEE. Enzyme immobilized in kappa-carrageenan gel, *n*-hexane as medium. Arrows indicate the replacement of a dialysis membrane with a new one.

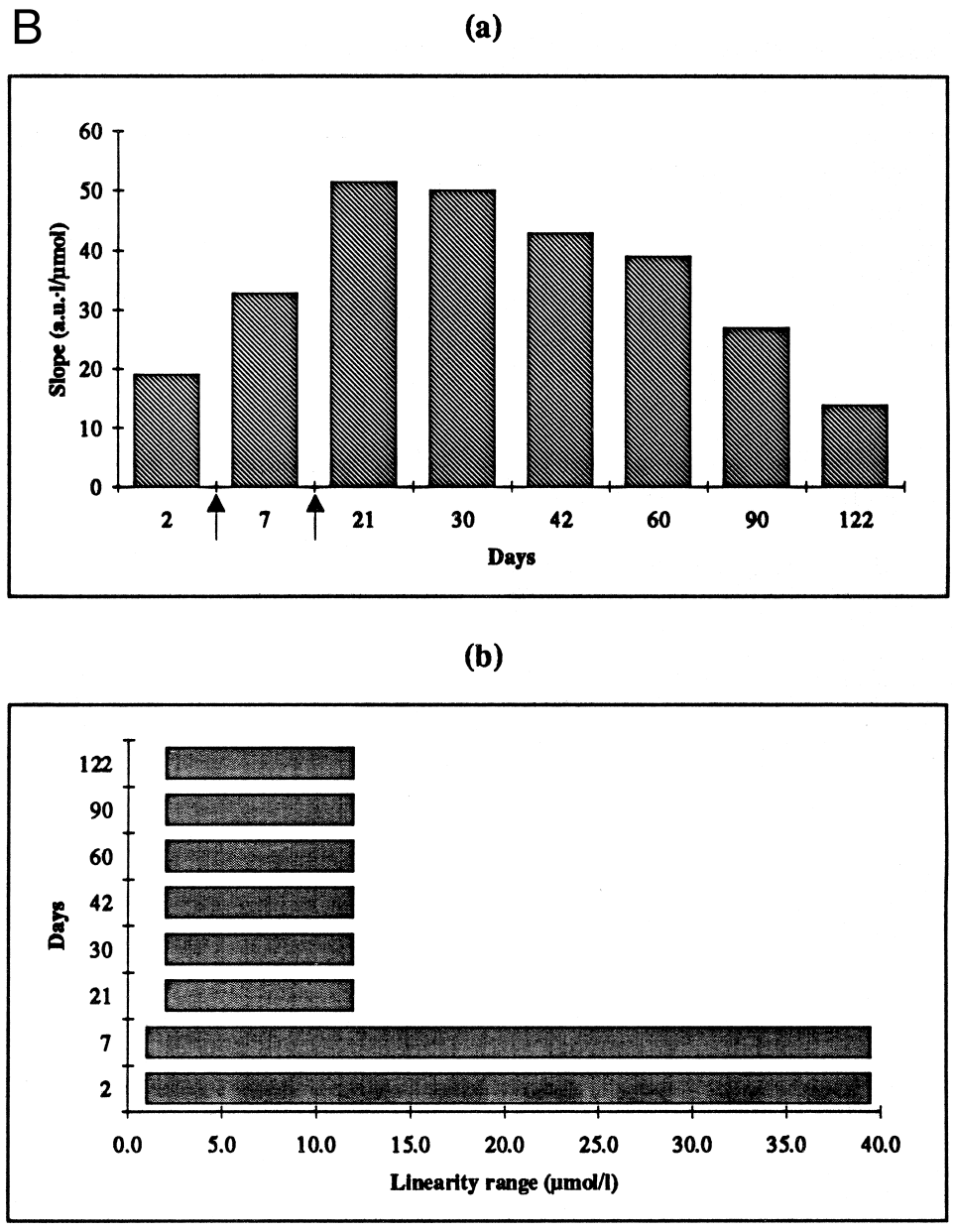

Fig. 1 (continued).

tion. After sealing the tube was allowed to stand for 6–7 h at 5°C. The disk was then removed and placed on a glass dish. Storage and assembly before use were as in the basic method. The enzyme solution used consisted of 25 µl of a phosphate buffer (1/15 mol/l, pH = 7) containing 5.0 mg (11,000 U/mg) of catalase.

3.2.4. Immobilisation of choline oxidase and phospholipase D, or butyrylcholinesterase, for bienzymatic OPEE assembly

The basic procedure was followed as far as the addition of two enzymes buffer solution to a kappa-carrageenan gel disk, and then another gel disk was placed on top of the first and both

disks pressed together carefully. Storage and assembly before use were as in the basic method.

The enzyme solution used consisted of 25 μl of glycine buffer 0.1 mol/l, pH 8.0 or 8.5, containing 500 U of butyrylcholinesterase and 500 U of choline oxidase, or 100 U of phospholipase D and 500 U of choline oxidase, respectively.

Lastly, in the case of the bienzymatic immobilisation of choline oxidase and phospholipase

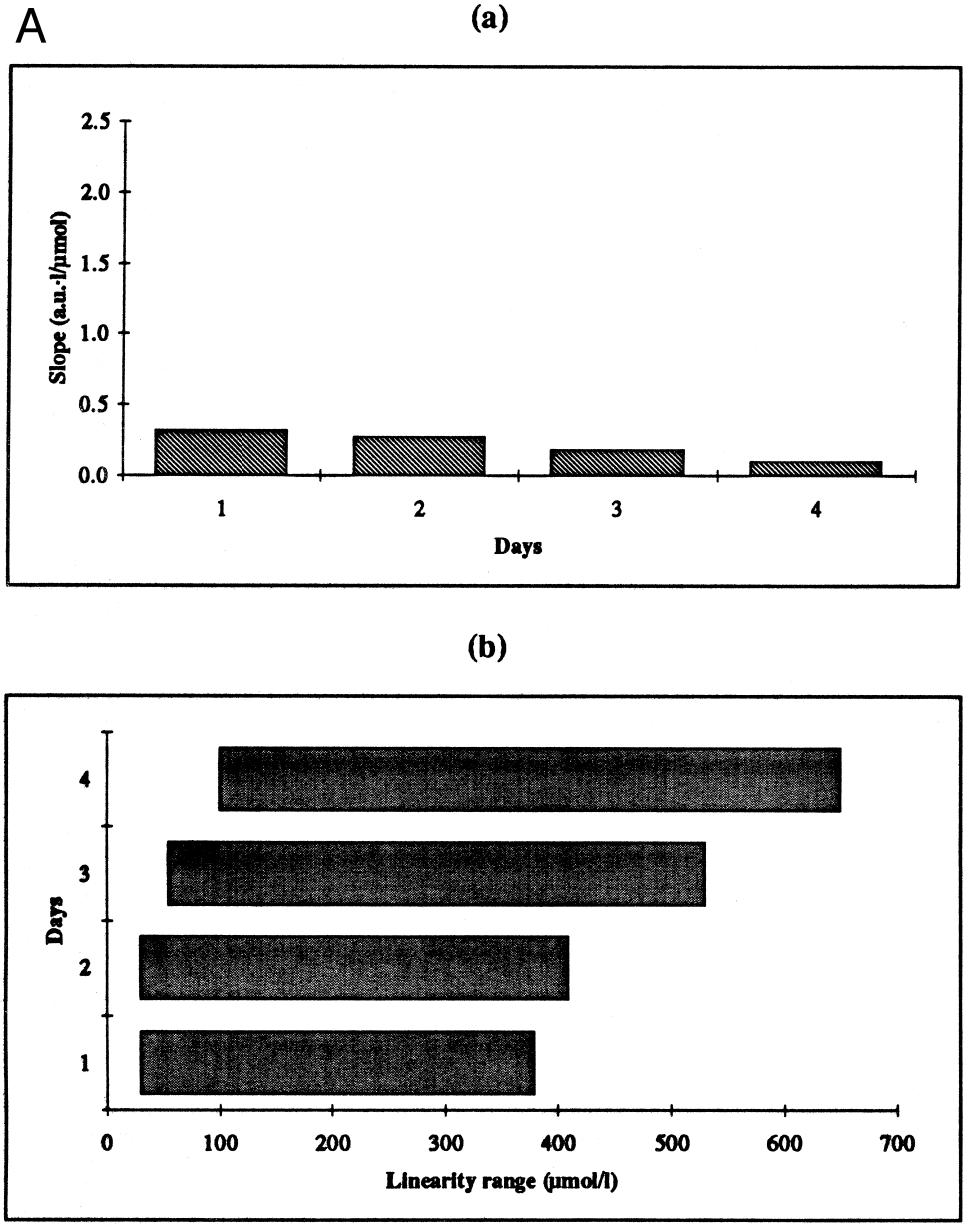

Fig. 2. (A) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the catalase OPEE. Enzyme immobilized in dialysis membrane, water saturated chloroform as medium. (B) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the catalase OPEE. Enzyme immobilized in kappa-carrageenan gel, water-saturated chloroform as medium.

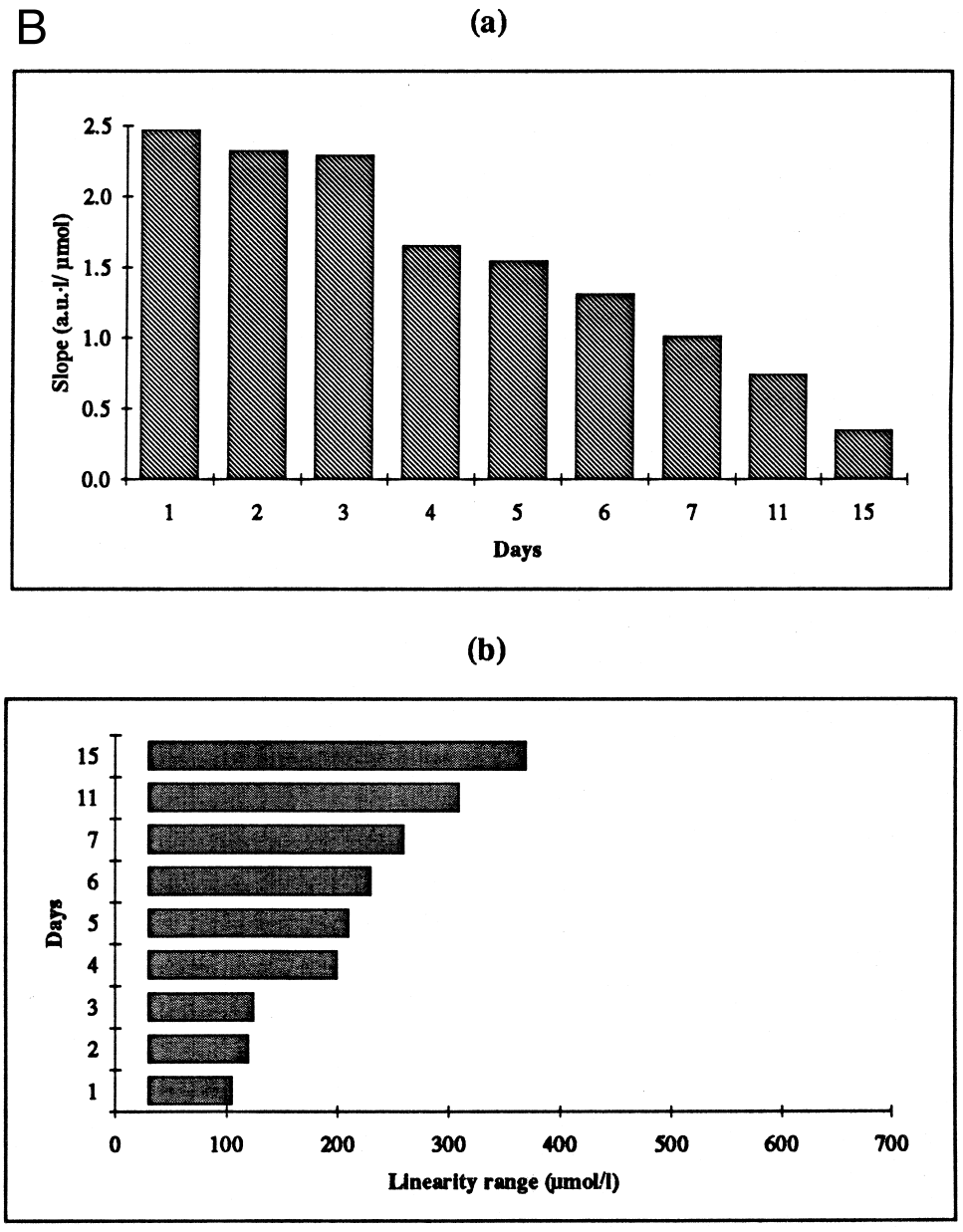

Fig. 2 (continued).

D, the following more recent procedure yields very good results: a kappa-carrageenan gel disk is moistened with 25 µl of glycine buffer (0.1 mol/l, pH 8.5) and fitted to the electrode cap; then 1.0 mg of lyophilised choline oxidase (12 U/mg) and 1.0 mg of lyophilised phospholipase D (45 U/mg) are successively added. A dialysis membrane is then placed on top and after 10 min the electrode is used for the measurement.

4. Results and discussion

We have developed four mono-, or bienzyme electrodes capable being used in nonaqueous

solvents—namely *n*-hexane, chloroform, or mixtures thereof, according to the enzymes employed. We initially constructed enzymatic sensors of relatively simple conception using enzymes immobilised on the head of an amperometric gas diffusion oxygen electrode by means of a dialysis membrane, which performed well in investigating the behaviour of the electroen-

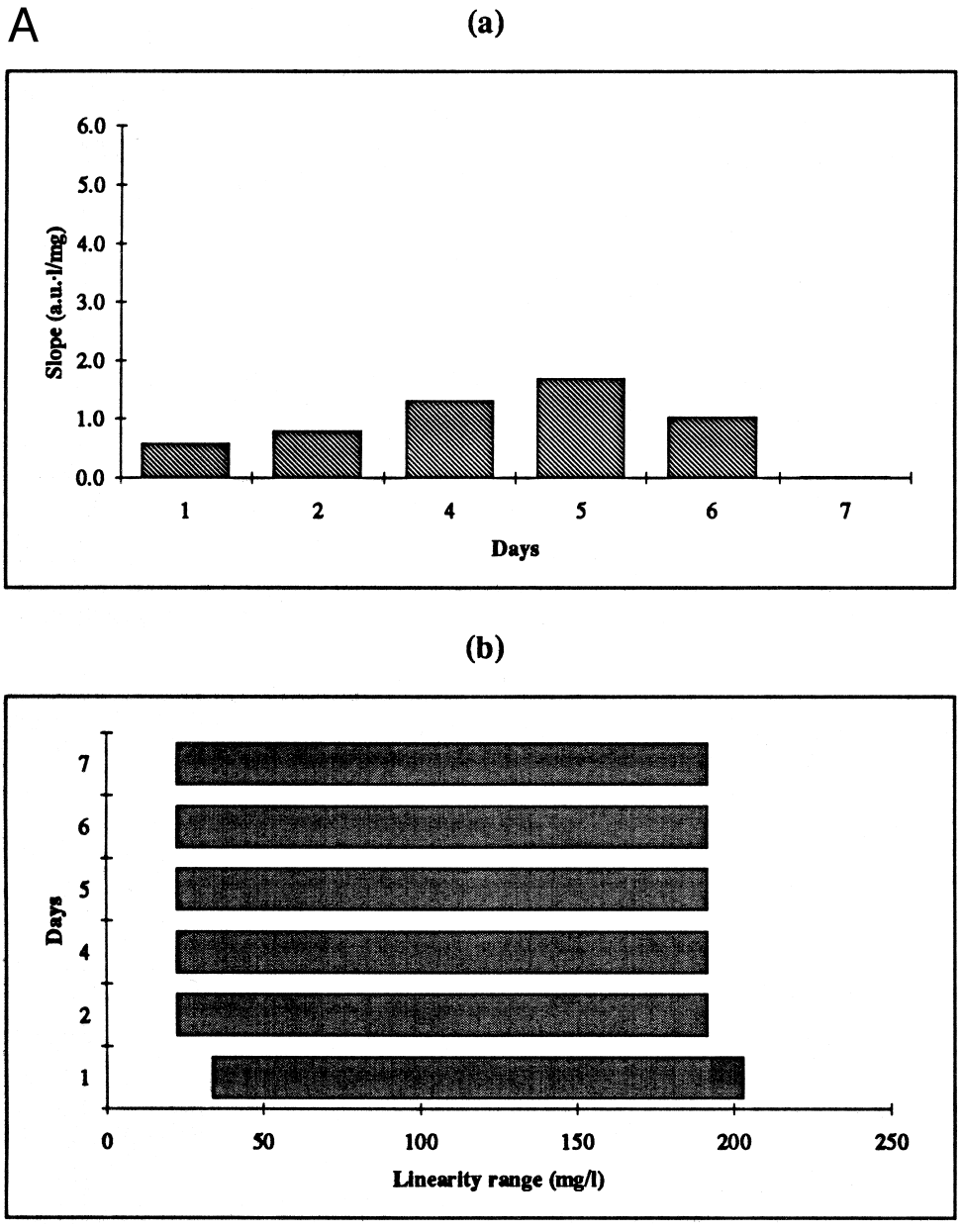

Fig. 3. (A) Behaviour of (a) the slope and linear range of the mean calibration graphs obtained throughout the 'lifetime' of the biosensor based on phospholipase D and choline oxidase. Both enzymes immobilised in dialysis membrane, water-saturated mixture of chloroform and hexane (50% v/v) as medium. (B) Behaviour of (a) the slope and (b) linear range of the mean calibration graphs obtained throughout the 'lifetime' of the biosensor based on phospholipase D and choline oxidase. Both enzymes immobilised in kappa-carrageenan gel, water-saturated mixture of chloroform and hexane (50% v/v) as medium.

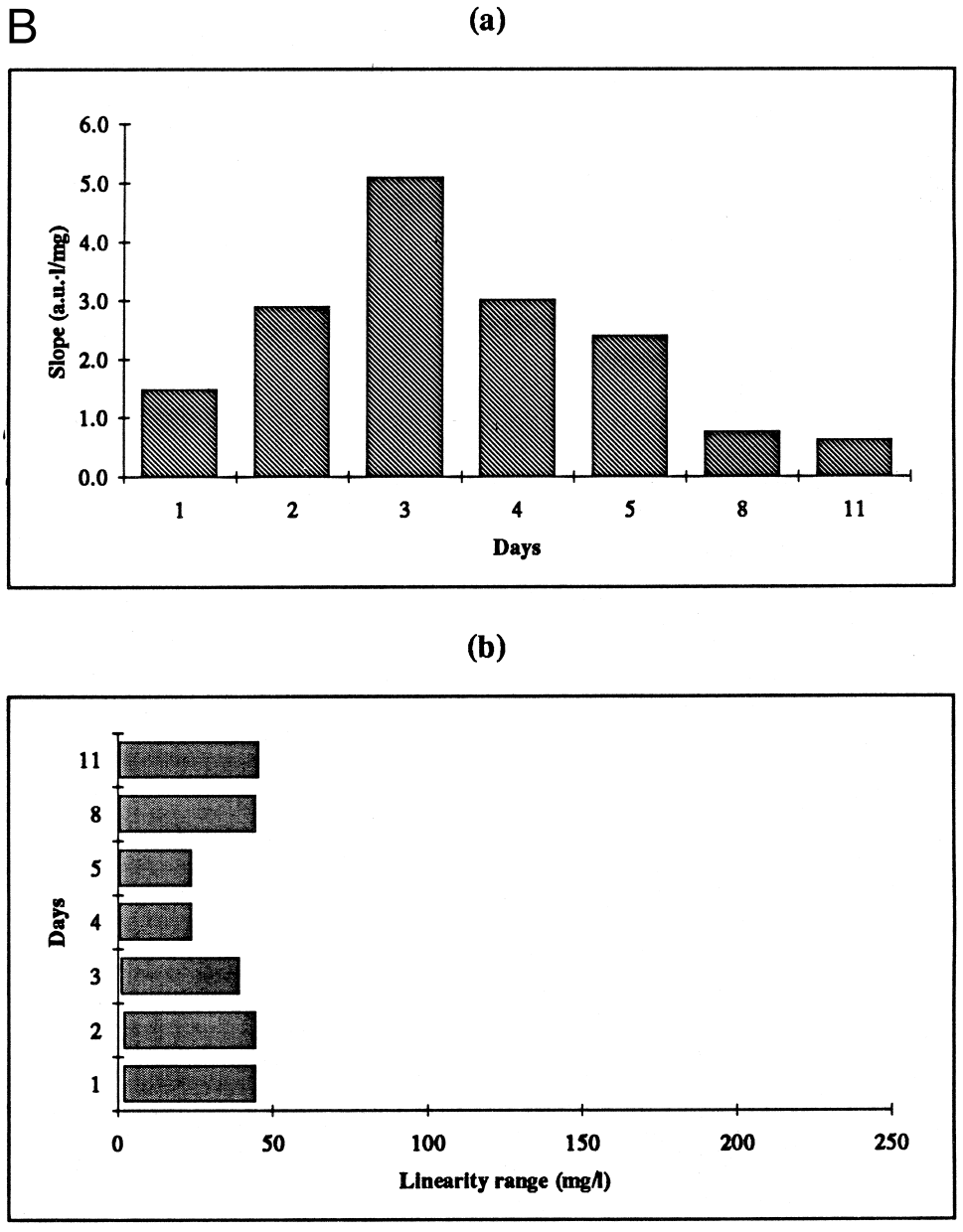

Fig. 3 (continued).

zymatic system. We then described a new method for immobilising enzymes, namely entrapment in kappa-carrageenan gel, which has proved useful in solving the question of the true lifetime of enzymes when operating in non-aqueous media. The biosensors obtained were thus more efficient.

Figs. 1–4 show the experimental results in the form of histograms showing the sensitivity and linear range behaviour throughout the lifetime of the four OPEEs studied, using both the immobilisation methods described above. When the experiments were carried out in the operating conditions in which the enzyme was immo-

bilised solely by means of a dialysis membrane, during the tests performed in nonaqueous solvents, the sensor's working life was found to depend essentially on the progressive wear and tear on, and on the ultimate breakdown of, the dialysis membrane. This occurs very rapidly in chloroform, very slowly in *n*-hexane and more slowly in a mixture of the two. We studied this point using two different experimental conditions, i.e., enzyme directly immobilised in the

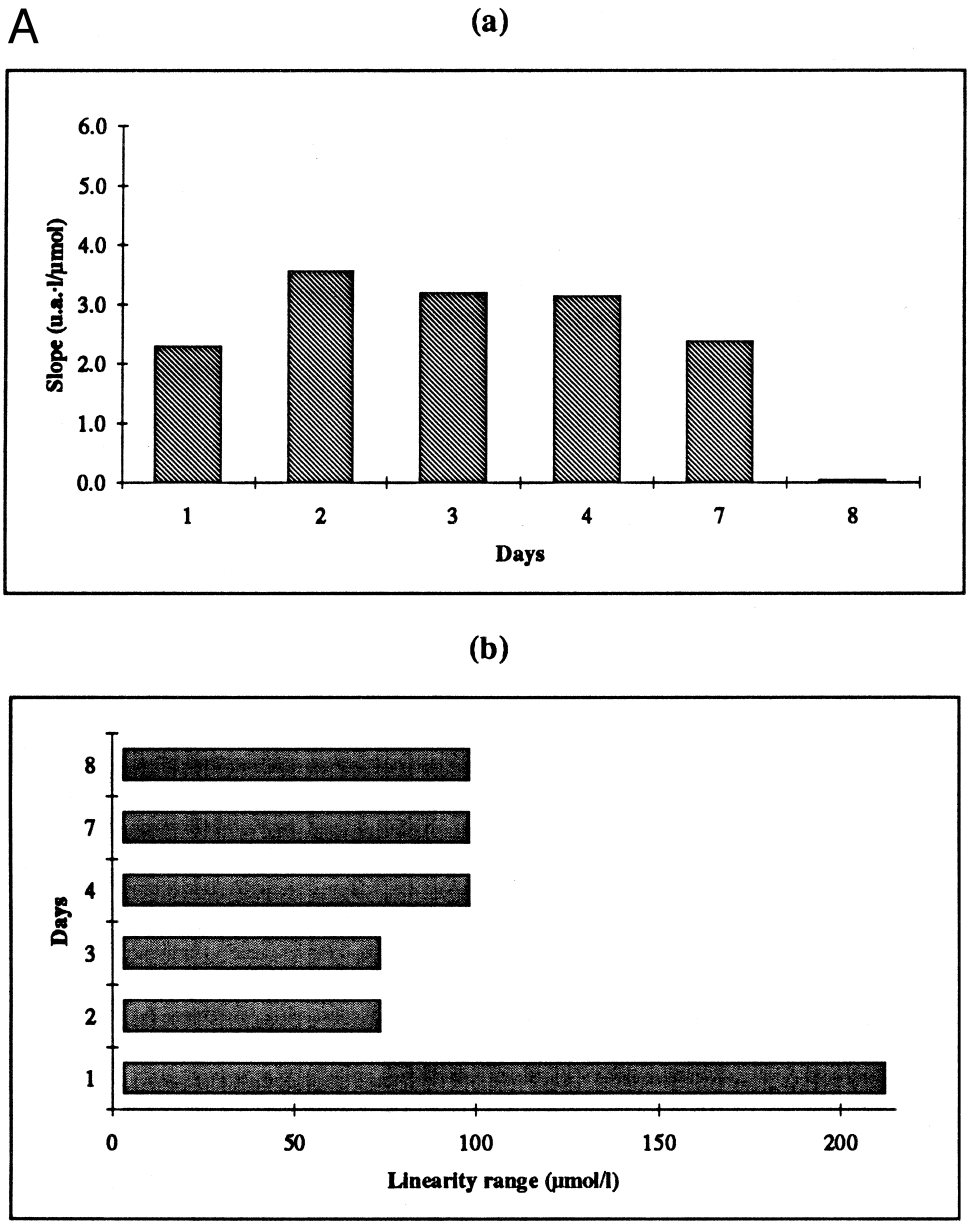

Fig. 4. (A) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the biosensor based on butyrylcholinesterase and choline oxidase. Both enzymes immobilized in dialysis membrane, water-saturated chloroform and hexane (50% v/v) mixture as medium. (B) Behaviour of (a) the slope and (b) the linear range of the mean calibration graph obtained daily throughout the lifetime of the biosensor based on butyrylcholinesterase and choline oxidase. Both enzymes immobilized in kappa-carrageenan gel, water-saturated chloroform and hexane (50% v/v) mixture as medium.

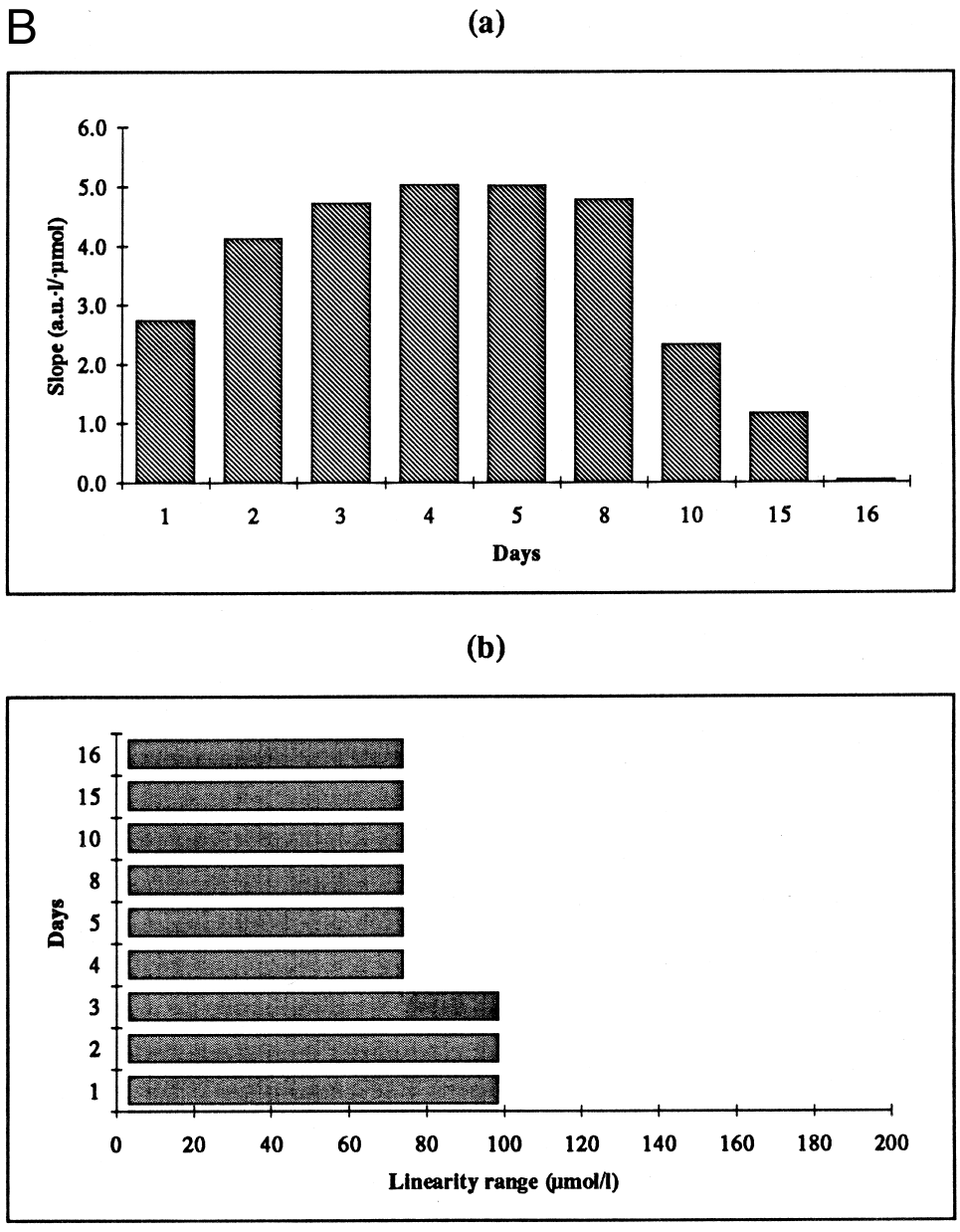

Fig. 4 (continued).

dialysis membrane, or enzyme entrapped in kappa-carrageenan gel and the gel fixed to the oxygen sensor by means of a dialysis membrane. This comparison was performed using four different kinds of OPEEs, two monoenzymatic (one with tyrosinase and one with catalase) and two bienzymatic (one with butyrylcholinesterase + choline oxidase and one with phospholipase D + choline oxidase) so as to obtain more systematic data and to distinguish whether enzymatic activity loss is due prevalently to the membrane breaking, or to deactivation caused by the organic solvent.

Results show that, within certain limits, the immobilisation in kappa-carrageenan ensures a longer biosensor lifetime than simple immobili-

sation in a dialysis membrane. In this way it is possible to compare the lifetimes of the catalase sensor using two different immobilisation methods (Fig. 2A and B) or those of bienzymatic biosensors (Figs. 3 and 4A and B); in this case, the dialysis membranes were actually never changed for the duration of the respective lifetimes.

However, the very long lifetime values found in some experiments using the immobilisation method in kappa-carrageenan (see, for instance, the results for the tyrosinase OPEE in Fig. 1) are to be attributed above all to the possibility provided by the new technique of immobilisation in kappa-carrageenan of easily replacing the dialysis membrane whenever its immersion in organic solvent causes it to break down. In fact, as it is entrapped in kappa-carrageenan gel, the enzyme is not immediately dispersed in solution, as in the case of the dialysis membrane immobilisation method, each time a sudden breakdown occurs in the dialysis membrane. On the contrary, after replacement of the dialysis membrane and a partial rehydration of the enzymatic gel using buffer solution, a good preservation of the enzymatic activity and an increase of the biosensor sensitivity are usually observed if the enzyme is entrapped in kappa-carrageenan. It should be pointed out, however, that also under these working conditions there is in any case some loss of enzyme activity over time, as indicated by the progressive decrease in the sensitivity of the biosensor in the last few days of its lifetime. In all cases, an increase of the sensitivity during the first days of the lifetime was also observed. This seems to be attributable overall to the varying imprinting conditions [23], probably due to the variation of the configuration of the aqueous microenvironment surrounding the enzyme molecules.

As far as the practical utilisation of the fabricated biosensors is concerned, by using the new method of enzymatic immobilisation in kappa-carrageenan gel it may be claimed that the increased sensitivity observed when the enzyme is immobilised in kappa-carrageenan instead of using a simple dialysis membrane is probably not always exceptional but much more significant, as it is linked to an increase also in the working lifetime, even though the latter of course varies according to the solvent used. Of course, an appreciable increase in sensitivity generally corresponds to a narrower linear range (see Figs. 1–4).

Moreover at present the four OPEEs developed using the kappa-carrageenan immobilisation method yield good results when applied to the determination of the substrate concentration of the respective enzymes, namely for phenols [20], hydrogen peroxide [22], butyrylcholine [24] and lecithin [25], respectively. Tests are being run in which the substrates analysed are contained both in standard solutions and in real matrices, always operating in *n*-hexane, or chloroform, or in a 50% by volume mixture of chloroform and *n*-hexane, respectively.

5. Conclusions

The OPEEs presented here have proved suitable for the analysis of the respective substrates and the new immobilisation method in kappa-carrageenan gel ensured a good activity and stability of the enzymes when working in non-aqueous solvent. In particular, the method ensures that the biosensors fabricated in this way show satisfying lifetimes and a good sensitivity. The result is comparable (or better) performances compared to those obtained by means of OPEEs [26–30] developed using other immobilisation methods reported in literature and cited in the introduction of this paper [13–19]. For each of the OPEEs, the small modification to the basic immobilisation method described probably leads to better enzyme loading and the damping of the enzyme with a suitable buffer, in each case, allows the ‘imprinting effect’ to be exploited.

The organic solvent choice is not discussed herein, as it was reported in previously published papers [20,21,31], or in the course of

publication [32]. We merely mention that in each case we had to take into account both OPEE performance and the solubility of the substrates. Indeed, in all cases this choice was made in order to perform applications, e.g., the analysis of real matrices, obtaining good analytical results.

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