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Development and Applications of a Bienzymatic Amperometric Glycerol Biosensor Based on a Poly(Neutral Red) Modified Carbon Film Electrode

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Abstract: A bienzymatic biosensor for glycerol has been designed, based on the co-immobilization of two enzymes, glycerol kinase and glycerol-3-phosphate oxidase, the glycerol kinase phosphorylating glycerol to glycerol-3-phosphate. The enzymes were immobilized by cross-linking with glutaraldehyde on carbon film electrodes with poly(neutral red) as mediator of the enzymatic reaction. The polymer film was made on the carbon film substrate by potential cycling from a solution containing 1 mM neutral red. Glycerol, as well as glycerol-3-phosphate, was determined in amperometric mode at −350 mV vs. saturated calomel electrode. Several buffers and pH values were tested for the immobilized enzymes' response and the optimum experimental conditions were found to be in phosphate buffer at pH 8.0. The linear response range to glycerol was directly dependent on the concentration of adenosine-5′-triphosphate (ATP). With 3 mM ATP, glycerol was determined in the range 5–147 μM. A monoenzymatic glycerol-3-phosphate biosensor was also evaluated and found to have a working range from 20–700 μM. Application of the

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bienzymatic biosensor to natural samples was tested by glycerol determination in dry white and red wines and the method was validated by spectrophotometric enzyme assay.

Keywords: Bienzymatic biosensor, glycerol, glycerol kinase, glycerol-3-phosphate oxidase, carbon film, poly(neutral red)

1. INTRODUCTION

Chemical analysis of complex samples such as wine and other beverages is of great interest in achieving an adequate quality of production. Wine is a complex mixture of several hundred compounds present at different concentrations. Among these the dominant ones are: water, ethanol, glycerol, sugars, organic acids, and various ions (Niculescu et al. 2003). Glycerol is the most important secondary product of alcoholic fermentation in wine, contributing to the sensory properties, whilst it is added to beer and other alcoholic beverages in order to improve their taste. The amount of glycerol formed during the fermentation process is about one tenth of the alcohol formed, with final concentrations varying from 1 to $10\,g\,L^{-1}$ (Compagnone et al. 1998). Deviations from this value might indicate technological alterations during the process or deterioration of the harvested grapes (Amerine and Ough 1980). Therefore, a rapid, sensitive, accurate and, at the same time, economic method is desirable for glycerol determination in foods and beverages.

The Association of Analytical Communities (AOAC) official methods of analysis are gravimetry for determining glycerol in both dry and sweet wines and liquid chromatography for determination of glycerol in wines and grape juice. These methods are either very time consuming and tedious, expensive, or are not suitable for routine analysis.

The use of biosensors and bioreactors for the determination of glycerol was investigated by different authors. A glycerol dehydrogenase–based reactor with spectrophotometric detection of nicotinamide adenine dinucleotide plus hydrogen (NADH) has been used for the measurement of glycerol in human serum and wine by using immobilization on nylon tubing (Hinsch and Sundaram 1980) or on controlled pore glass beads (Cañizares and Luque de Castro 1995). A glycerokinase thermistor was also used to monitor glycerol during industrial fermentation (Rank et al. 1995). Glycerol in wine and human serum was determined spectrophotometrically with glycerokinase and glycerol-3-phosphate oxidase immobilized on nonporous glass beads (Kiranas et al. 1997). Another method for glycerol determination in wine is based on immobilized glycerol dehydrogenase and NADH oxidase with chemiluminescent detection (Kondruweit et al. 1994).

Electrochemical detection still represents the most successful approach for glycerol determination because of its sensitivity, reliability, and cost

effectiveness. Moreover, electrochemical biosensors can be easily configured for flow-through or flow-injection analysis (FIA) allowing real-time monitoring in process control. One of the first attempts at this was a potentiometric biosensor involving bound glycerol dehydrogenase, NAD^+, and hexacyanoferrate (III) (Chen et al. 1982). Other possibilities studied used either bound galactose oxidase (Johnson et al. 1982) or co-immobilised glycerol kinase and glycerol-3-phosphate oxidase (Merchie et al. 1992) with amperometric detection of H_2O_2 and O_2, respectively. Analysis of glycerol in human plasma with electrochemical detection has been attempted using glycerol kinase and glycerol-3-phosphate oxidase enzymes physically retained at the electrode with a microdialysis probe (Murphy and Galley 1994).

The present work focuses on the design and development of a bienzymatic biosensor for the amperometric detection of glycerol in wines. The biosensor is based on the association of carbon film electrodes and the enzymes glycerol kinase (GK) and glycerol-3-phosphate oxidase (GPO), immobilized by cross-linking with glutaraldehyde. Poly(neutral red) (PNR) was used as a redox mediator, which has been recently developed by us for use with carbon film electrodes (Ghica and Brett 2006). These carbon film electrodes are highly promising for application as short-term-use or as disposable sensors and biosensors, e.g., (Florescu and Brett 2005, Pauliukaite and Brett 2005, Paulikaite et al. 2005, Ghica and Brett 2005, de Luca et al. 2005).

The enzymatic reactions exploited are:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerolkinase, } MgCl_2} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + PNR_{ox} \xrightarrow{\text{glycerolphosphateoxidase}} \text{Glycerone-3-phosphate} + PNR_{red}$$

the PNR_{red} being reoxidized at the electrode surface, as will be discussed later.

Studies concerning the optimization of the experimental conditions, and of the stability and selectivity of the biosensor, were carried out. Finally, the optimized biosensor was successfully used to determine glycerol in wine samples and the results were compared with those obtained by spectrophotometric enzyme assay (Eggstein and Kuhlmann 1974).

2. EXPERIMENTAL

2.1 Reagents and Buffers

Glycerol kinase (GK, E.C. 2.7.1.30, $19\,U\,mg^{-1}$) as lyophilized powder from *Bacillus stearothermophilus*, bovine serum albumin (BSA), adenosine 5′-triphosphate disodium salt (ATP), DEAE-dextran hydrochloride and D-lactitol monohydrate were purchased from Sigma Chemical Co., St. Louis, MO, USA. Glycerol-3-phosphate oxidase (GPO, E.C. 1.1.3.21, $40\,U\,mg^{-1}$) as

lyophilized powder from *Aerococcus viridans*, DL-α-glycerol phosphate disodium salt hexahydrate, anhydrous glycerol, and glutaraldehyde (GA) (70% v/v) were purchased from Fluka Chemie AG, Buchs, Switzerland. Magnesium chloride hexahydrate was from Aldrich Chemie GmbH&Co KG, Steinheim, Germany.

Other reagents used were: α-D(+)-glucose, D(+) fructose, DL-lactic acid, L-ascorbic acid, urea (Sigma Chemical Co. St. Louis, MO, USA); tartaric acid (PAHI, Lisbon, Portugal), citric acid, DL-malic acid, and ethanol (Merck, Darmstadt, Germany), DL-*iso*-leucine (BDH, Poole, England), and β-alanine and KCl (Fluka Chemie AG, Buchs, Switzerland).

For electrochemical experiments, the supporting electrolytes were 0.1 M sodium phosphate buffer (NaPB) (pH 6.0–8.0), 0.1 M trizma-HCl buffer (pH 7.0–9.0), and 0.1 M borate buffer (pH 8.0–10.0) with 0.1 M KCl.

Solutions were prepared with ultrapure water obtained from a Millipore Milli-Q purification system (resistivity $> 18\,\mathrm{M}\Omega\,\mathrm{cm}$) and all reagents were analytical reagent grade, unless specified.

Stock solutions of 100 mM glycerol ($\geq$99.5%) and 100 mM glycerol-3-phosphate were prepared and kept in the refrigerator at 4°C.

2.2 Instrumentation

Electrochemical polymerization of neutral red to form poly(neutral red), (PNR), as well as amperometric measurements, was carried out using a Bioanalytical Systems (BAS, West Lafayette, IN, USA) CV-50W electrochemical analyzer. The electrochemical three-compartment cell employed in these experiments consisted of poly(neutral red)/enzyme modified carbon film electrode as the working electrode, a platinum wire auxiliary electrode, and a saturated calomel electrode (SCE) as reference.

The pH measurements were made with a CRISON 2001 micro pH-meter at room temperature.

2.3 Electrode Preparation and Enzyme Immobilization

Electrodes were made from carbon film resistors (2 Ω nominal resistance) 6 mm in length and 1.5 mm in diameter. The resistors were fabricated from ceramic cylinders by pyrolytic deposition of carbon from methane in a nitrogen atmosphere (Brett et al. 2001). One of the two tight-fitting metal caps, linked to external contact wires, was removed and the other was covered in plastic and protected by normal epoxy resin. The cylinder was then covered in epoxy resin except on the bottom surface. In this way the working electrode was a disc electrode of area ca. $0.020\,\mathrm{cm}^2$ (Gouveia-Caridade and Brett 2005).

Poly(neutral red) films were formed on the carbon film electrode substrate by electropolymerization (Ghica and Brett 2006). Prior to polymerization, electrodes were cycled in 0.025 M KPB + 0.1 KNO_3 (pH 6.0) in the potential range −1.0 V to +1.0 V at a sweep rate of 100 mV s^{-1} for approximately 3 minutes, when a reproducible voltammogram of the carbon film electrode was obtained. Electropolymerization of neutral red, from a solution containing 1 mM neutral red in 0.025 M KPB + 0.1 M KNO_3 (pH 6.0), was carried out by potential cycling from −1.0 V to +1.0 V at a sweep rate of 50 mV s^{-1} for 20 cycles.

For the glycerol-3-phosphate biosensor, glycerol-3-phosphate oxidase (GPO) was immobilized onto the surface of the PNR-modified carbon film electrode by cross-linking with glutaraldehyde (GA) and using bovine serum albumin (BSA) as protein carrier. A mixture of 2 μL GA (2.5% in water), 2 μL BSA (1% in water), and 4 μL GPO (20 mg mL^{-1}) was prepared. From this mixture 3 μL was placed onto the surface of the PNR-modified carbon film electrode and allowed to dry at room temperature for at least 1 hour. The biosensors were then stored for 1 day at 4°C before use.

For the glycerol biosensor, GPO and GK were immobilized together by cross-linking with glutaraldehyde. A mixture of 1 μL GA (2.5% in water), 1 μL GPO (20 mg mL^{-1}), and 4 μL GK (24 mg mL^{-1}) was prepared. From this mixture 2 μL was placed onto the surface of the PNR-modified carbon film electrode and allowed to dry at room temperature for at least one hour. These biosensors were stored for 2 days at 4°C before use.

When not in use, both types of biosensor were kept at 4°C in 0.1 M sodium phosphate buffer solution (pH 8.0) or in 0.1 M sodium phosphate buffer with 1% DEAE dextran + 5% lactitol stabilizers to investigate which was better.

2.4 Analysis of Wine Samples

For analysis of glycerol in wine, 10 μL aliquots of the wine were added to 10 mL of 0.1 M NaPB + 0.1 M KCl (pH 8.0), corresponding to 1000-fold dilution, and the standard addition method was used to determine the glycerol concentrations.

Independent analysis of glycerol concentrations was done using the spectrophotometric enzyme assay kit (Eggstein and Kuhlmann 1974) (Cat 10 148 270 035, Boehringer, Mannheim, Germany). This involves conversion of glycerol to glycerol-3-phosphate, the adenosine diphosphate (ADP) produced being converted to ATP and pyruvate by reaction with phosphoenolpyruvate, catalyzed by pyruvate kinase. The pyruvate is then reduced to L-lactate by NADH in the presence of L-lactate dehydrogenase. The NADH consumption is measured spectrophotometrically at 340 nm. Measurements were done using a Speccord S100 spectrophotometer (Carl Zeiss, Jena, Germany).

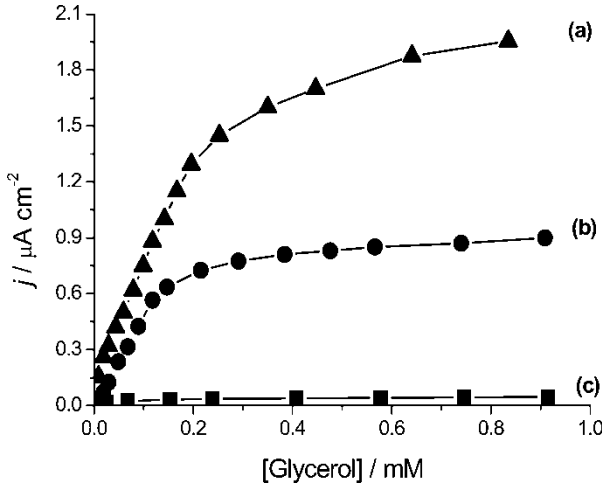

Figure 1. Calibration curves for glycerol obtained with different GPO:GK ratios (a) 1:4; (b) 1:5; and (c) 1:1 in 0.1 M trizma-HCl (pH 8.5) + 3 mM $MgCl_2$ and 3 mM ATP.

3. RESULTS AND DISCUSSION

3.1 Optimization of the Experimental Conditions

3.1.1 Choice of the Enzyme Ratio

Glycerol-3-phosphate oxidase and glycerol kinase were immobilized by cross-linking with glutaraldehyde as described in the experimental section, and the performance of the glycerol biosensor was tested using different GPO and GK enzyme ratios of 1:1, 1:4, and 1:5 (Figure 1). Table 1 summarizes the results. It can be seen that the 1:1 ratio gives a

Table 1. Characteristics of the glycerol biosensor for different GPO:GK ratios in 0.1 M trizma-HCl buffer + 0.1 M KCl (pH 8.5) solution

GPO:GK	Linear range/μM	Sensitivity/ $\mu A\,mM^{-1}cm^{-2}$	LOD/μM	K_m/μM
1:1	10–68	0.29	15.3	67
1:4	10–142	6.37	3.45	217
1:5	10–118	4.97	7.12	252

much lower current response and smaller linear range than do the other two ratios and has a smaller Michaelis-Menten constant. Although the 1:4 and 1:5 ratios give similar responses, the highest current and best performance characteristics were obtained using a 1:4 GPO:GK ratio. In this case, the biosensor exhibited linearity from 10 to 142 μmol L^{-1} with a detection limit ($S/N = 3$) of 3 μmol L^{-1} of glycerol.

3.1.2 Influence of Buffer and pH

Glycerol kinase is stable over the pH range 6.0–9.8, exhibiting maximum activity at pH 9.8 (Hayashi and Lin 1967), while glycerol phosphate oxidase exhibits broad optimum activity over the pH range 6.5–9.0 (Esders and Michrina 1979).

The pH dependence of the amperometric response was studied separately for the immobilized GPO enzyme biosensor and for the GPO/GK bienzyme biosensor in three different 0.1 M buffer electrolyte solutions to which 0.1 M KCl was added: trizma-HCl (pH 7.0–9.0), sodium phosphate (pH 6.0–8.0), and borate (pH 8.0–10.0). In Fig. 2A the response of the GPO biosensor to 0.50 mmol L^{-1} glycerol-3-phosphate is presented and, as seen, the response in phosphate buffer solution is higher than in the other two buffers by up to approximately 50% over the values of pH tested, maximum response being obtained for pH 8.0. The pH dependence of the bienzyme biosensor response to 0.10 mmol L^{-1} glycerol (unsaturated response) is shown in Fig. 2B. As for the glycerol-3-phosphate biosensor, the phosphate buffer leads to the highest signal with a slow increase with increasing pH, the highest value being at pH 8.0. The response in trizma-HCl buffer was approximately 55% of that obtained in phosphate buffer; the same being observed with borate buffer.

These results led to the selection of 0.1 M NaPB + 0.1 M KCl, pH 8.0, as buffer electrolyte solution in further studies.

3.1.3 Influence of Co-substrates

Glycerol kinase catalyzes the phosphorylation of glycerol using ATP as co-substrate. The catalytic efficiency of the reaction is improved in the presence of Mg^{2+} because the enzyme is more active toward the MgATP complex; it was found that the best response was achieved with 3 mM Mg^{2+} and 3 mM ATP (Compagnone et al. 1998). The response of the glycerol biosensor with equal concentrations of the cofactors was studied in the range from 0 to 4 mM and is shown in Fig. 3 for a 0.166 mmol L^{-1} glycerol solution. The highest biosensor signal was obtained using 3 mmol L^{-1} ATP and $MgCl_2$, so this was selected for further work.

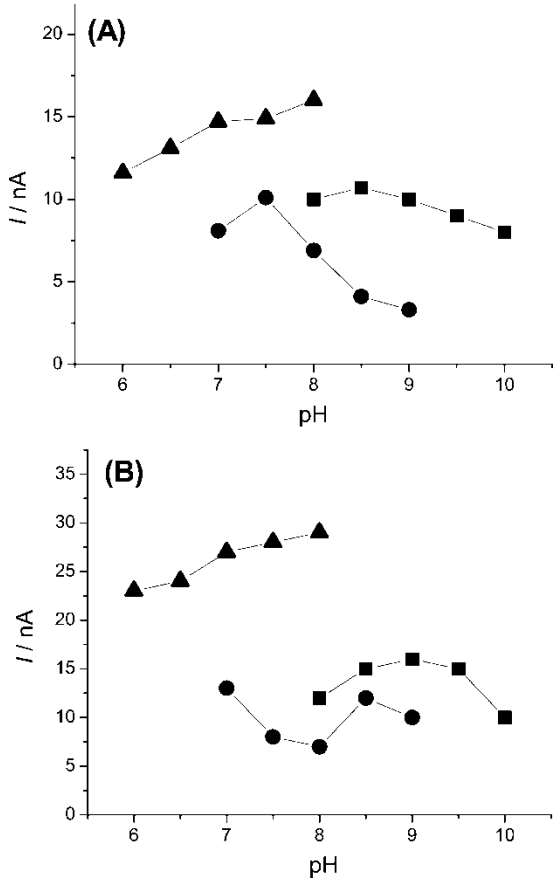

Figure 2. pH dependence of the response to (A) 0.50 mmol L^{-1} glycerol-3-phosphate and (B) 0.10 mmol L^{-1} glycerol in 0.1 M KCl + 0.1 M buffers: (▲) NaPB; (■) borate; and (●) trizma-HCl.

3.2 Glycerol-3-phosphate and Glycerol Biosensors

3.2.1 Electrochemical Behavior and Effect of Applied Potential

The PNR-modified carbon film electrodes with immobilized GPO or with immobilized GPO/GK were characterized by cyclic voltammetry. Figure 4 shows a typical cyclic voltammogram obtained with the PNR/GPO/GK biosensor in 0.1 M NaPB + 0.1 M KCl (pH 8.0) with and without addition of 0.10 mmol L^{-1} glycerol. In the absence of substrate, the bienzyme biosensor displayed the typical electrochemical behavior of the PNR mediator in cyclic voltammetry. An increase in the oxidation peak current at −500 mV vs. SCE was observed when glycerol was added to the

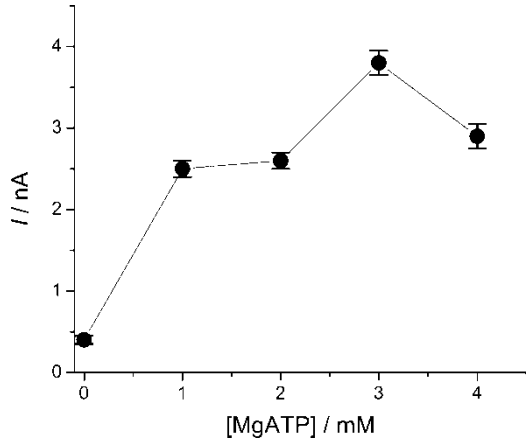

Figure 3. Response of glycerol biosensor to 0.166 mmol L^{-1} glycerol for different $MgCl_2$ and ATP concentrations.

solution—this by itself is an indication that PNR can act as redox mediator for the enzymatic reaction of glycerol. The same behavior was exhibited by the PNR/GPO biosensor when glycerol-3-phosphate was added (data not shown).

The effect of applied potential on the response of the monoenzyme biosensor in the presence of 5 mM glycerol-3-phosphate is presented in Fig. 5. In the potential range from −600 to −200 mV vs. SCE the current increases almost linearly with increasing potential and then remains almost constant from −200 to +200 mV. The bienzyme biosensor for glycerol showed a similar behavior with respect to applied potential.

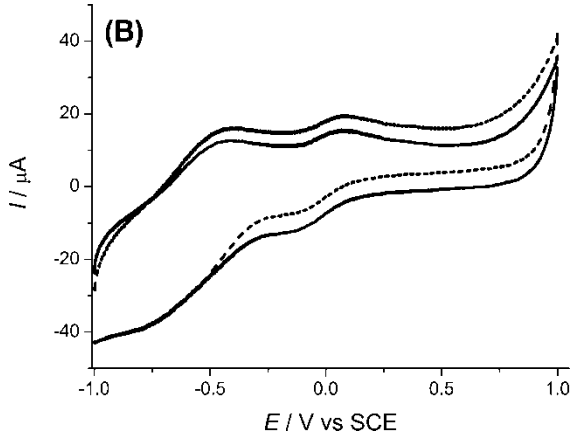

Figure 4. Cyclic voltammogram of PNR/GPO/GK modified electrode in 0.1 M NaPB + 0.1 M KCl without (—) and with (– – –) addition of 0.10 mmol L^{-1} glycerol.

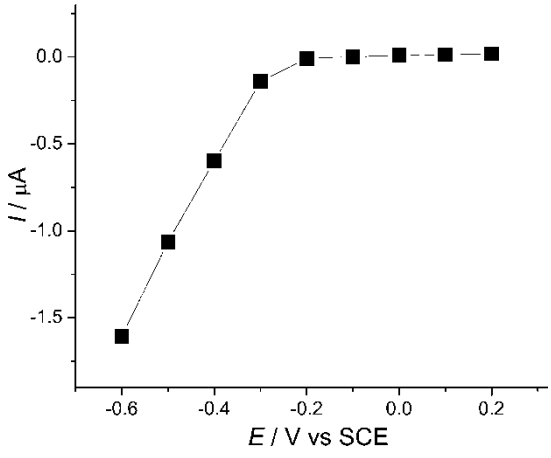

Figure 5. Effect of applied potential on the current response of the GPO-based biosensor in the presence of 5.0 mmol L^{-1} glycerol-3-phosphate.

As a compromise between the sensitivity of the biosensor and the reduction of possible electrochemical interferences, a working potential of -350 mV vs. SCE was chosen for amperometric measurements for both the monoenzymatic and for the bienzymatic biosensors. This represents an improvement with respect to other biosensors for the determination of glycerol using the bienzymatic system: GPO/GK: $+650$ mV vs. Ag/AgCl using a platinum electrode (Merchie et al. 1992; Compagnone et al. 1998), $+600$ mV vs. SCE using a dialysis electrode (Albery et al. 1993) or $+650$ mV vs. Ag/AgCl using a microdialysis electrode (Murphy and Galley 1994).

Experiments with hydrogen peroxide at the PNR-coated electrode at applied potentials of -0.35 V (not shown), led to cathodic currents confirming that hydrogen peroxide is reduced to water. The biosensor response to glycerol or glycerol-3-phosphate addition is a change to a more anodic current.

The mechanism is, therefore, proposed to be as follows. Glycerol kinase catalyzes the phosphorylation of glycerol to glycerol-3-phosphate in the presence of ATP and $MgCl_2$.

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerolkinase, } MgCl_2} \text{Glycerol-3-phosphate} + \text{ADP}$$

Glycerol-3-phosphate oxidase then catalyzes the oxidation of glycerol-3-phosphate to glycerone-3-phosphate according to:

$$\text{Glycerol-3-phosphate} + PNR_{ox} \xrightarrow{\text{glycerolphosphateoxidase}} \text{Glycerone-3-phosphate} + PNR_{red}$$

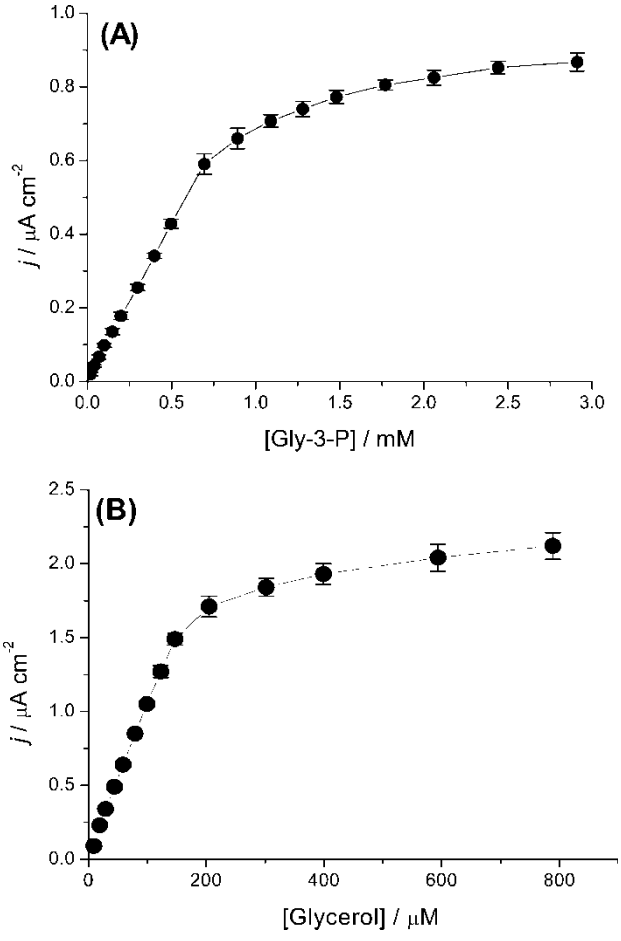

Figure 6. Calibration curves for (A) glycerol-3-phosphate obtained with the GPO biosensor and (B) glycerol obtained with the GPO/GK biosensor in 0.1 M NaPB + 0.1 M KCl (pH 8.0) + 3 mM $MgCl_2$ + 3 mM ATP.

The reduced mediator is oxidised electrochemically at the carbon film electrode.

3.2.2 Biosensor Response to Substrate

The PNR/GPO/GK biosensor was applied to the amperometric determination of glycerol under stirred conditions at −350 mV vs. SCE. After stabilization of the baseline current, glycerol was injected into the buffer solution, containing ATP and $MgCl_2$ and the response was an increase in current.

Table 2. Biosensor characteristics in 0.1 M NaPB + 0.1 M KCl (pH 8.0) solution

Type of biosensor	Linear range/μM	Sensitivity/ $\mu A\,mM^{-1}cm^{-2}$	LOD/μM
Glycerol	10–147	10.1 ± 0.05	4
Glycerol-3-phosphate	20–700	0.79 ± 0.06	15

It should be noted that the measurement of only glycerol-3-phosphate in a sample containing both glycerol and glycerol-3-phosphate can be performed with the monoenzymatic biosensor PNR/GPO or with the bienzymatic biosensor PNR/GPO/GK under the same conditions as for glycerol, but without addition of ATP-$MgCl_2$.

Calibration curves for glycerol-3-phosphate and glycerol are shown in Fig. 6 and the behaviors were typically those of Michaelis-Menten kinetics. The main characteristics of the optimized biosensors are presented in Table 2. Good linear ranges are observed with a sensitivity for glycerol of $10.1 \pm 0.05\,\mu A\,mM^{-1}\,cm^{-2}$, much higher than that for glycerol-3-phosphate of $0.79 \pm 0.06\,\mu A\,mM^{-1}\,cm^{-2}$ and the detection limit goes down to 4 μM. The apparent Michaelis-Menten constants were determined from the Lineweaver-Burk plots, see example in Fig. 7: for GPO it was 0.78 ± 0.06 mM ($n = 3$) and for the GPO/GK combination it was lower, as would be expected, at 0.24 ± 0.02 mM ($n = 5$).

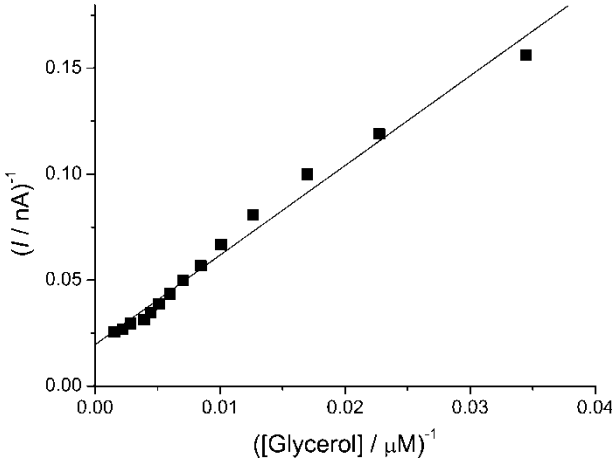

Figure 7. Lineweaver-Burk plot from a calibration curve for the glycerol biosensor; experimental conditions as in Fig. 6B.

3.2.3 Storage and Operational Lifetime

One of the major concerns in the development of biosensors is the lifetime of the immobilized enzyme in storage and operational conditions.

The operational stability of the bienzyme electrode was tested during 8 days performing one calibration curve per day. After this time there was a decrease of 28% in glycerol response compared with the first day. The mono-enzyme electrode response to glycerol-3-phosphate decreased 13% compared to the initial value.

The long-term stability of the bienzyme electrode was also examined following different periods of storage at 4°C in buffer electrolyte to which was added 1% DEAE dextran and 5% lactitol. This mixture has been reported to stabilize GPO in solution (Gibson et al. 1992a) as well as immobilized enzymes (Gibson et al. 1992b). The results demonstrated that the decrease in sensitivity did not exceed 35% after 37 days of storage. The long-term stability without DEAE dextran and lactitol was also tested. After the same period of 37 days the decrease in sensitivity was of only 21% compared with freshly prepared electrodes, so in our case the use of stabilizers for storage at 4°C decreased biosensor stability.

3.2.4 Biosensor Selectivity

The behavior of commonly considered interferents, normally present in wine, was investigated. All the tested compounds (sugars, amino acids, and organic acids) were present at concentrations of 2×10^{-4} mol L^{-1} and the glycerol concentration was 4×10^{-5} mol L^{-1}. The results in Table 3 show that

Table 3. Interference effect of various compounds on the assay of glycerol

Interferent	Relative response of glycerol for 1:5 ratio of glycerol to interferent (%)
—	100
Glucose	100
Fructose	100
Ethanol	95
Ascorbic acid	390
Citric acid	105
Lactic acid	105
Malic acid	95
Tartaric acid	98
Alanine	102
Leucine	105
Urea	102

Table 4. Determination of glycerol in wine samples

Type of wine	Glycerol/g L^{-1} (amperometry)	Glycerol/g L^{-1} (spectrophotometry)
Red wine 1	2.22 ± 0.13	2.32 ± 0.07
Red wine 2	3.24 ± 0.13	3.28 ± 0.07
White wine 1	1.81 ± 0.09	1.94 ± 0.03
White wine 2	2.86 ± 0.06	2.60 ± 0.10

interference is small except for ascorbic acid, which leads to considerable positive interference. However, ascorbic acid in wine is found in much lower concentrations than glycerol so this should not be a problem for the determination of glycerol in wine samples.

3.3 Glycerol Determination in Wines

With the optimized bienzyme electrode, four Portuguese dry wines (two red and two white) were analyzed using the standard addition method. Taking into account the fact that the concentration of glycerol in wine is far higher than the working range of the biosensors, dilution of the samples was necessary. For comparison purposes the different wine samples were validated using a spectrophotometric enzyme assay (Eggstein and Kuhlmann 1974). Concentrations calculated for the four wines are summarized in Table 4 as the mean value of three determinations. The results are in very good agreement except for one of the white wines, where the spectrophotometric results are slightly lower. Nevertheless, even in this case the biosensor can certainly be applied with an error of less than 10% and remains a valuable tool for monitoring glycerol.

4. CONCLUSIONS

It has been demonstrated that immobilization of glycerol kinase and glycerol-3-phosphate oxidase at poly(neutral red) modified carbon film electrodes results in a successful biosensor for glycerol that can also be used for glycerol-3-phosphate. After optimization of the experimental parameters, this bienzyme electrode has a detection limit of 4 μmol L^{-1} for glycerol and 15 μmol L^{-1} for glycerol-3-phosphate and a linear range large enough to enable glycerol determination in natural samples, such as wine.

Bienzymatic electrodes using poly(neutral red) as mediator have good selectivity and good operational and storage stability without the necessity of additional stabilizers to improve lifetime.

The features of the proposed biosensor make it a good and more rapid alternative for the determination of glycerol in wines: the results are in good agreement with those obtained by spectrophotometric enzyme assay and the required sample treatment—only dilution by a factor of a thousand—is minimum.

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