ANALYTICA CHIMICA ACTA 609 (2008) 201–209

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

Integrated multienzyme electrochemical biosensors for the determination of glycerol in wines

*M. Gamella, S. Campuzano, A.J. Reviejo, J.M. Pingarrón**

Dpto. Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 18 October 2007
Received in revised form
20 December 2007
Accepted 24 December 2007
Published on line 8 January 2008

Keywords:
Self-assembled monolayers
Enzyme biosensors
Glycerol
Wines

ABSTRACT

The construction and performance of integrated amperometric biosensors for the determination of glycerol are reported. Two different biosensor configurations have been evaluated: one based on the glycerol dehydrogenase/diaphorase (GDH/DP) bienzyme system, and another using glycerol kinase/glycerol-3-phosphate oxidase/peroxidase (GK/GPOx/HRP). Both enzyme systems were immobilized together with the mediator tetrathiafulvalene (TTF) on a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold electrode by using a dialysis membrane. The electrochemical oxidation of TTF at +150 mV (vs. Ag/AgCl), and the reduction of TTF^+ at 0 mV were used for the monitoring of the enzyme reactions for the bienzyme and trienzyme configurations, respectively. Experimental variables concerning both the biosensors composition and the working conditions were optimized for each configuration. A good repeatability of the measurements with no need of cleaning or pretreatment of the biosensors was obtained in both cases. After 51 days of use, the GDH/DP biosensor still exhibited 87% of the original sensitivity, while the GK/GPOx/HRP biosensor yielded a 46% of the original response after 8 days. Calibration graphs for glycerol with linear ranges of 1.0×10^{-6} to 2.0×10^{-5} or 1.0×10^{-6} to 1.0×10^{-5} M glycerol and sensitivities of 1214 ± 21 or 1460 ± 34 μA M^{-1} were obtained with GDH/DP and GK/GPOx/HRP biosensors, respectively. The calculated detection limits were 4.0×10^{-7} and 3.1×10^{-7} M, respectively. The biosensors exhibited a great sensitivity with no significant interferences in the analysis of wines. The biosensors were applied to the determination of glycerol in 12 different wines and the results advantageously compared with those provided by a commercial enzyme kit.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Glycerol is a natural chemical constituent of many foodstuffs being currently measured in fruit juice, wine, vegetable oil, beer, tobacco, honey, etc. The glycerol content is increasingly considered as a useful quality indicator for these food products. For example, its determination is of interest as a fermentation indicator and its production is related to the presence of microorganisms in food products [1].

Apart from ethanol and carbon dioxide, glycerol is the most abundant product resulting from the fermentation process involving *Saccharomyces cerevisae*, thus being present in almost all alcoholic beverages and spirits. The amount of glycerol formed during fermentation (about 1:10 of the alcohol formed) is influenced by several factors, such as grape variety, degree of ripeness, fermentation temperature, SO_2 concentration, pH of grape must, nitrogen composition, aeration, yeast strain and inoculation level [2]. Glycerol is typically found at con-

* *Corresponding author*. Tel.: +34 913944315; fax: +34 913944329.
E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).
0003-2670/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.12.036

centrations of 4–10 g L^{-1} in dry wine and in the case of the botrysized late harvest wines, levels in excess of 20 g L^{-1} are not uncommon [3]. In this latter case, grape berries infected by *Botrytis cinerea* already contain significant amounts of glycerol as a result of the fungus metabolism, which explains the high glycerol levels found in this wine style. Glycerol contributes to the taste properties and smoothness of wine as well as to the sensory characteristics in beers, and it can be a carbon source for spoilage bacteria, originating highly volatile acidity and aroma compounds that confer unacceptable flavor to beers. Hence, the determination of glycerol is important for industrial quality control, requiring rapid, sensitive, reliable and at the same time economical methods for routine analysis [4]. Furthermore, the content of glycerol is also of interest to detect possible adulteration of wine with added glycerol [2].

According to the literature, several analytical methods can be employed for glycerol determination.

The official methods for determining glycerol are that recommended by the AOAC involving gas and liquid chromatography [5,6]. Both methods are time-consuming and tedious or expensive and not suited to routine analysis [7].

Furthermore, enzymatic methods combining the selectivity of enzymes involved with different detection systems have been used [1,8–11]. However, these methods imply the use of more than one enzymatic pathway and require tedious sample pretreatment and the preparation of many reagents. Biosensors, and in particular amperometric biosensors can be employed for glycerol determination, and they can be envisaged as serious competitors for conventional techniques [12,13].

Recently, we have demonstrated that it is possible to construct robust integrated amperometric biosensors by co-immobilizing the biomolecules with the mediator tetrathiafulvalene. This was accomplished by cross-linking atop a 3-mercaptopropionic acid self-assembled monolayer-modified gold disk electrode [14,15]. Therefore, a related approach for the construction of integrated amperometric biosensors for the determination of glycerol is reported in this article. Two different enzyme systems have been checked involving glycerol dehydrogenase and diaphorase on the one hand, and glycerol kinase, glycerol-3-phosphate oxidase and peroxidase co-immobilization, on the other hand. The enzyme systems were immobilized together with TTF on the MPA-modified gold electrode by using a dialysis membrane. The analytical performance of the biosensors derived from both enzyme systems has been evaluated, and there were successfully applied to the determination of glycerol in wine samples.

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were performed on a BAS LC-4C amperometric detector connected to a Linseis L6512 recorder. A P-Selecta ultrasonic bath, and a P-Selecta Agimatic magnetic stirrer were also used. A BAS gold disk electrode (Ø 3 mm) was used as electrode substrate to be coated with the MPA-SAM. A BAS MF-2063 Ag/AgCl/KCl 3 M reference electrode and a Pt wire counter electrode, were also employed. A 10 mL glass electrochemical cell was used.

2.2. Reagents and solutions

Buffer solutions were prepared daily. A 5.0 mM NAD^+ (Sigma) in 0.05 M phosphate buffer of pH 9.0 was employed with the GDH/DP biosensor, while a 3.0 mM ATP (Aldrich) and 3.0 mM $MgCl_2$ (Scharlau) in 0.05 M phosphate buffer of pH 9.0 solution was used in the case of GK/GPOx/HRP biosensor.

Stock 0.01 M glycerol (Scharlau) solutions were prepared in the corresponding buffer solution specified above. More dilute standards were prepared by suitable dilution with the same buffer solution.

A 40 mM mercaptopropionic acid (Research Chemicals Ltd.) solution, prepared in a 75/25% (v/v) ethanol/water mixture, was employed for the formation of the monolayers. For the preparation of the GDH/DP biosensors, a 2.5 U μL^{-1} glycerol dehydrogenase solution (from *Cellulomonas* sp., EC 1.1.1.6, Sigma) and a 0.54 U μL^{-1} diaphorase solution in phosphate buffer of pH 7.4 (from *Clostridium kluyveri*, EC 1.8.1.4, Sigma) were used. In the case of GK/GPOx/HRP biosensors, a 3.95 U μL^{-1} glycerokinase solution (from *Cellulomonas* sp., EC 2.7.1.30, Sigma), a 1.0 U μL^{-1} glycerol-3-phosphate oxidase solution (from *Pediococcus* sp., EC 1.1.3.21, Sigma) and a 12.3 U μL^{-1} horseradish peroxidase solution (Type II from *Horseradish*, EC 1.11.1.7, Sigma) were employed. A 0.5 M tetrathiafulvalene (Aldrich) solution in acetone was used in both configurations. Dialysis membranes (10 K MWCO) were purchased from Cultek®.

Other solutions employed were as follows: a 2 M KOH (Panreac) aqueous solution; 0.5 M stock solutions of ascorbic acid (Fluka), citric acid (Merck), tartaric acid (Merck), L-malic acid (Merck), D-glucose (Panreac), D-fructose and D-galactose (Sigma), and L-arabinose (BDH) prepared in the corresponding buffer solution. All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system.

Moreover, a D-glycerol kit (10148270035 (R-Biopharm®)) was used in order to compare the results obtained for the determination of glycerol in wines using the developed biosensors.

2.3. Procedures

Before SAM deposition, the gold disk electrode was pretreated as described previously [14]. MPA-SAMs were formed by immersion of the clean AuE in a 40 mM MPA solution in EtOH/H_2O_2 (75/25, v/v) for at least 15 h. Then, the modified electrode was rinsed with deionized water and dried with an argon stream.

Co-immobilization of the enzyme and the mediator was carried out as follows:

(a) *Biosensor GDH/DP*: A 3 μL aliquot of the 0.5 M TTF solution was deposited on the SAM-modified AuE. Once the electrode surface had dried at ambient temperature, a 3 μL aliquot of the 0.54 U μL^{-1} DP solution was deposited on and let to dry again. Finally 3 μL of the 2.5 U μL^{-1} GDH solution were spread on the modified electrode surface

(b) *Biosensor GK/GPOx/HRP*: A 3 μL aliquot of the 0.5 M TTF solution was deposited on the SAM-modified AuE. Once the electrode surface had dried at ambient temperature, a 2 μL aliquot of a 12.3 U μL^{-1} HRP solution was deposited on and let to dry again. Next, 3 μL of the 1.0 U μL^{-1} GPOx solution were placed on the modified electrode surface and dried at ambient temperature. Finally, 3 μL of the 3.95 U μL^{-1} GK solution was spread on the modified electrode surface.

After deposition of the mediator and the enzymes, a 1.5 cm^2 piece of the dialysis membrane was fixed on top of the electrode surface and secured with an O-ring.

Amperometric measurements were performed by applying a potential of +150 mV (vs. Ag/AgCl) when working with GDH/DP biosensors, and of 0 mV in the case of GK/GPOx/HRP biosensors.

2.4. *Determination of glycerol in wines*

As it will be commented below, no matrix effect was observed and, therefore, the glycerol concentration was calculated by interpolation of the amperometric signal from the sample solutions into a calibration graph constructed with standard glycerol solutions in the 4.0×10^{-6} to 1.0×10^{-5} M (0.37–0.92 mg L^{-1}) concentration range.

The sample treatment consisted only of an appropriate dilution in order to adjust the concentration of glycerol in wine to the dynamic concentration range of the sensors. So a 50–200 times previous dilution of the samples with the corresponding buffer solution was carried out prior the analysis. Next, 40 μL of the diluted sample were added to the electrochemical cell containing 5.0 mL of the corresponding buffer solution which was used as the supporting electrolyte, in each case, and the amperometric measurements were carried out by applying the desired potential and allowing the steady-state current to be reached.

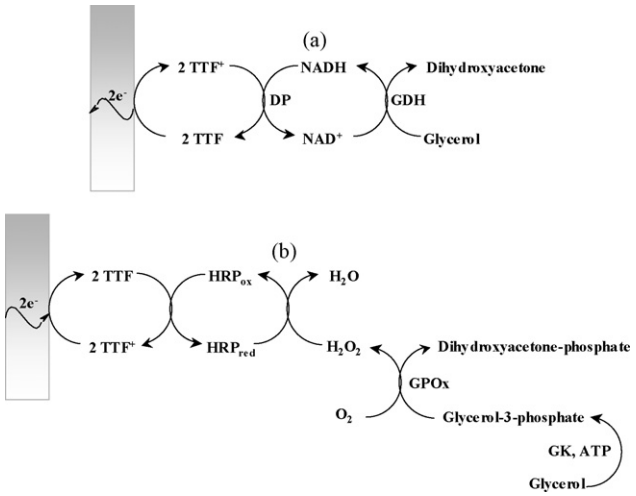
In all cases, the results were compared with those obtained with a commercial enzyme kit from R-Biopharm® using spectrophotometric detection.

3. Results and discussion

The biocatalytic schemes on which the determination of glycerol is based with both biosensing approaches are displayed in Scheme 1.

The enzyme reactions involved with the GDH/DP biosensor imply oxidation of glycerol to dihydroxyacetone catalyzed by GDH, together with simultaneous reduction of cofactor NAD^+ to NADH. The NADH is then re-oxidized by TTF^+, reaction catalyzed by the enzyme diaphorase [12]. The generated TTF is amperometrically re-oxidized at the modified electrode, with the resulting current being dependent on the glycerol concentration. Tetrathiafulvalene is well known as an appropriate mediator for the NAD^+/NADH system [16], exhibiting suitable electrochemical properties.

On the other hand, the biosensing scheme for the GK/GPOx/HRP bioelectrode shows as glycerol is converted to 1-glycerophosphate in the presence of GK and ATP as its cofactor. This product can then be oxidized by oxygen in the presence of glycerol phosphate oxidase to produce dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide formed is reduced in the presence of HRP, and regeneration of its reduced form is mediated by TTF. Therefore, the TTF^+ generated is electrochemically reduced when the applied potential is more negative than the formal potential of the TTF/TTF^+ redox couple [17].



Scheme 1 – Schematic diagram displaying the enzyme and electrode reactions involved in the glycerol determination at a GDH/DP biosensor (a) and a GK/GPOx/HRP biosensor (b).

3.1. *Optimization of variables*

Optimization of experimental variables concerning the behavior of both biosensors was accomplished in all cases by amperometry in stirred solutions.

3.1.1. *GDH/DP biosensor*

The TTF loading used was the same that the one optimized in previous works [14,15]. Consequently, only the influence of the enzymes loadings was checked concerning the biosensor composition. As expected, the amperometric response of the biosensor, measured at +0.15 V, increased notably with GDH loading up to a value of 7.5 U (Fig. 1). Higher GDH loadings produced a dramatic current decrease which is likely due to the blocking of the electrode surface by the huge amount of immobilized enzyme.

Biosensors prepared without co-immobilized DP did not show significant amperometric responses at the applied potential. The presence of DP on the electrode surface significantly accelerated the NADH re-oxidation reaction with TTF^+ until a value of 1.5 U [12]. Consequently, the selected composition of the bioelectrode for further work was 7.5 U GDH/1.5 U DP/1.5 μmol TTF.

Once the composition of the biosensor was established, the effect of the applied potential on the glycerol amperometric response was tested. Fig. 2 shows the results obtained for a glycerol concentration of 2.0×10^{-5} M. As expected considering the use of TTF as mediator, the current increased in the potential range between 0.00 and +0.15 V. The observed decrease of the amperometric response above +0.15 V is in

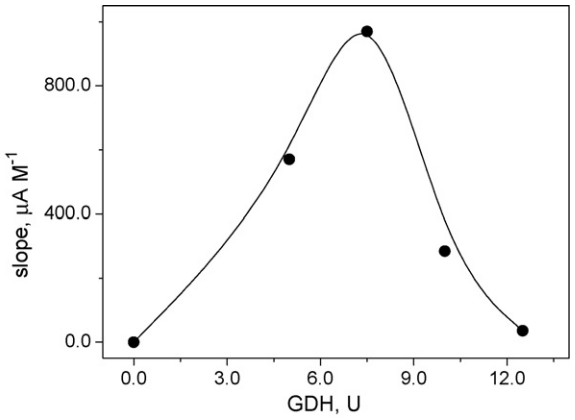


Fig. 1 – Effect of the GDH loading immobilized atop the GDH/DP biosensor (using constant DP and TTF loadings of 1.5 U and 1.5 μmol, respectively, on the slope of glycerol calibration graphs in the 4.0×10^{-6} to 1.0×10^{-5} M concentration range. Supporting electrolyte: 0.05 M phosphate buffer (pH 9.0) containing 3 mM NAD^+. E_{app} = +0.15 V.

agreement with similar behaviors reported for biosensors using TTF as mediator [14,15], and can be attributed to a leakage of TTF from the electrode surface at these potentials, induced after irreversible oxidation of TTF^+ to TTF^{2+}, which is soluble in aqueous solutions and decomposes. Moreover, no amperometric signal was observed in the 0.00–0.25 V potential range with biosensors constructed in absence of GDH or DP. Also no response for glycerol was obtained at a GDH–DP–MPA–AuE, thus indicating that no direct oxidation of NADH occurred with this biosensor design and that the transport of electrons was made from the enzyme to the electrode surface through the mediator. According to the obtained results, an applied potential of +0.15 V was selected for further work.

Experiments with various NAD^+ cofactor concentrations were carried out in the range of 0–10 mM. The sensitivity increased with the concentration of the cofactor until 0.5 mM

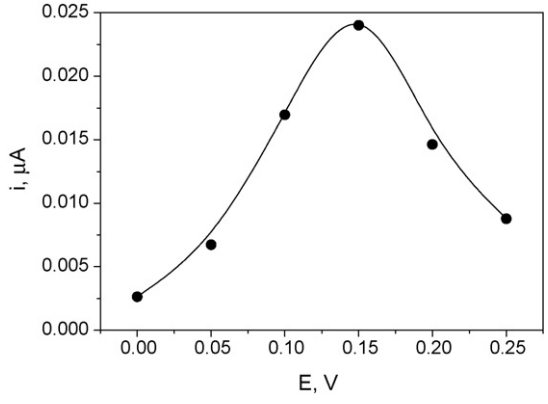


Fig. 2 – Effect of the applied potential at a GDH/DP biosensor on the amperometric signal of 2.0×10^{-5} M glycerol in a 0.05 M phosphate buffer solution (pH 9.0) containing 5 mM NAD^+.

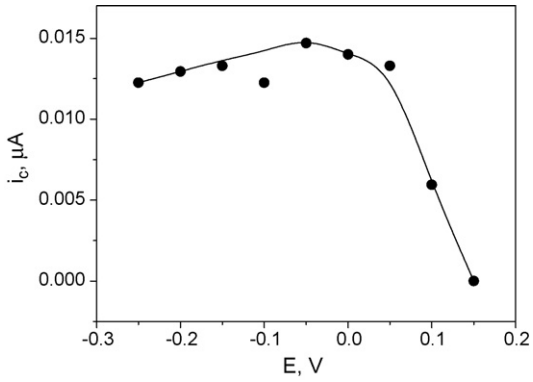


Fig. 3 – Effect of the applied potential at a GK/GPOx/HRP biosensor on the amperometric signal of 1.0×10^{-5} M glycerol in a 0.05 M phosphate buffer solution (pH 9.0) containing 3 mM ATP and 3 mM $MgCl_2$.

and then stabilised. Since the equilibrium of the employed reaction favours glycerol breakdown only in the case of NAD^+ being in large excess [18], a final concentration of 5.0 mM was chosen for the experiments.

The optimum pH for this bienzyme electrode was evaluated in the 6.0–11.0 range (data not shown). The biosensor displayed an optimum activity towards glycerol at pH values comprised between 8.5 and 10.0, which is close to the optimum pH value for the activation of GDH previously reported 9.0 and 8–10 [19,20]. According to this, a 0.05 M phosphate buffer solution of pH 9.0 (containing 5.0 mM NAD^+) was chosen for further work.

3.1.2. *GK/GPOx/HRP biosensor*

Regarding biosensor preparation, only the influence of the GK and GPOx loadings were checked, whereas both HRP and TTF loadings were those optimized previously for a hydrogen peroxide biosensor [17]. According to the results obtained in these studies (data not shown), the composition of the trienzyme electrode was 11.8 U GK/3.0 U GPOx/24.6 U HRP/1.0 μmol TTF.

The influence of the applied potential on the biosensor response to 1.0×10^{-5} M glycerol was examined over the +0.15 to −0.25 V range. Fig. 3 shows as the reduction current increased rapidly as the applied potential varied from +0.15 to 0.00 V, then almost leveling off in the potential range from 0.00 to −0.20 V. Since no significant differences in sensitivity were observed when applying potential values of 0.00 and −0.10 V, a working potential of 0.00 V was selected for future work in order to achieve a sensitive detection and also to minimize the number of potential interferents able to be reduced at the electrode. Moreover, as expected and accordingly with the involved enzyme reactions, no amperometric signals were found in the whole potential range for bioelectrodes constructed with no GK, or GPOx, or HRP or TTF (TTF/GPOx/HRP, TTF/GK/HRP, TTF/GK/GPOx, and GK/GPOx/HRP).

Similarly to most of the kinase enzymes, GK catalyzes the phosphorylation of glycerol using ATP as cosubstrate. Furthermore, the catalytic efficiency of the reaction is improved in the presence of Mg^{2+} because the enzyme is more active toward the ATP(Mg^{2+}) complex. Also Mg^{2+} ions have an activating

Table 1 – Repeatability and reproducibility of the amperometric measurements obtained with the integrated biosensors

	Biosensor	
	GDH/DP	GK/GPOx/HRP
R.S.D. (%) for calibration graph slopes ($n = 10$)	7.5 (4.0×10^{-6} to 2.0×10^{-5} M)	7.0 (2.0×10^{-6} to 1.0×10^{-5} M)
R.S.D. (%) for repetitive measurements ($n = 10$)	3.2 (2.0×10^{-5} M)	4.9 (1.0×10^{-5} M)
R.S.D. (%) for calibration graph slopes with different biosensors ($n = 5$)	11.7 (4.0×10^{-6} to 2.0×10^{-5} M)	10.1 (2.0×10^{-6} to 1.0×10^{-5} M)

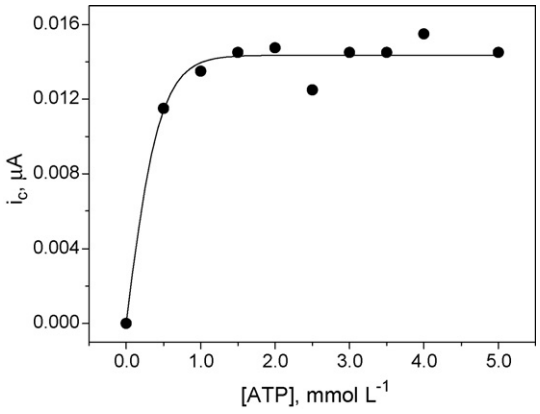


Fig. 4 – Effect of the ATP concentration in the buffer solution (containing 3 mM $MgCl_2$) on the amperometric signal of 1.0×10^{-5} M glycerol at GK/GPOx/RP biosensor. E_{app} = 0.00 V.

effect on GPOx activity [21]. The effect of ATP concentration on the biosensor response is shown in Fig. 4 for 1.0×10^{-5} M glycerol. The highest current was observed from ca. 1.5 mM ATP. In order to ensure that an excess of cofactor is present a 3.0 mM ATP concentration was selected for further work. Similar experiments concerning Mg^{2+} concentration led to choose also a 3.0 mM Mg^{2+} concentration for subsequent work.

Finally, the effect of the pH on the amperometric response was evaluated over the 5.5–12.0 range, for a glycerol concentration of 5.0×10^{-6} M. The current response increased between pH values of 8.0 and 9.5, and decreased at pHs higher than 9.5. According to this, a 0.05 M phosphate buffer solution of pH 9.0 (containing the appropriate cofactors) was chosen.

3.2. *Stability of the glycerol amperometric biosensors*

The stability of the biosensor response is one of the most critical factors for assessing the possibilities of a biosensor to be applied in control processes and routine monitoring. Different aspects regarding the stability of the biosensors were considered:

The repeatability of the measurements was evaluated by constructing 10 successive calibration plots for glycerol in the concentration range specified in Table 1 with the same biosensor. As can be seen relative standard deviation (R.S.D.) values of 7.5% and 7.0% were obtained for the slope values of such calibration plots for GDH/DP and GK/GPOx/HRP electrodes, respectively, indicating a good repeatability of the measurements with no need of cleaning or pretreatment of the biosensors. Moreover, R.S.D.s of 3.2% and 4.9%, respectively, were obtained for the steady-state current corresponding to 10 repetitive measurements of glycerol (see Table 1).

The reproducibility of the responses obtained with different biosensors was also checked. Results obtained with five different GDH/DP or GK/GPOx/HRP bioelectrodes yielded R.S.D. values for the slope of the corresponding calibration plots of 11.7% and 10.1%. This showed that the fabrication procedure of both biosensors was reliable, thus allowing acceptably reproducible electroanalytical responses to be obtained with different biosensors constructed in the same manner.

Finally, the useful lifetime of one single biosensor was checked by performing daily calibration graphs for glycerol in the above-mentioned concentration range. After use, the biosensor was stored in its corresponding buffer solution at 4 °C. After 51 days of continuous use, the GDH/DP biosensor still exhibited 87% of the original sensitivity. The operational stability of GK-based biosensor was not so good as for the GDH-based biosensor. After 8 days, the biosensor yielded a 46% of the original response, which can be attributed to the denaturation of the immobilized enzymes.

Concerning the robustness of the biosensors, we verified that their storage in buffer at room temperature instead of at 4 °C did not affect the stability.

3.3. *Analytical characteristics*

Under the optimized working conditions, typical calibration curves for enzyme systems were obtained with both biosensors. Table 2 summarizes the analytical characteristics of the corresponding calibration graphs. The limits of detection were calculated according to the $3s_b/m$ criterion, where m is the slope of the linear calibration plot, and s_b was estimated as

Table 2 – Analytical characteristics of the calibration plots for glycerol obtained with GDH/DP and GK/GPOx/HRP biosensors

Biosensor	Linear range	Slope (μA M^{-1})	r	LOD ($\times 10^{-7}$ M)
GDH/DP	1.0×10^{-6} to 2.0×10^{-5} M (0.092–1.84 mg L^{-1})	1214 ± 21	0.999	4.0
GK/GPOx/HRP	4.0×10^{-7} to 1.0×10^{-5} M (0.037–0.92 mg L^{-1})	1460 ± 34	0.999	3.1

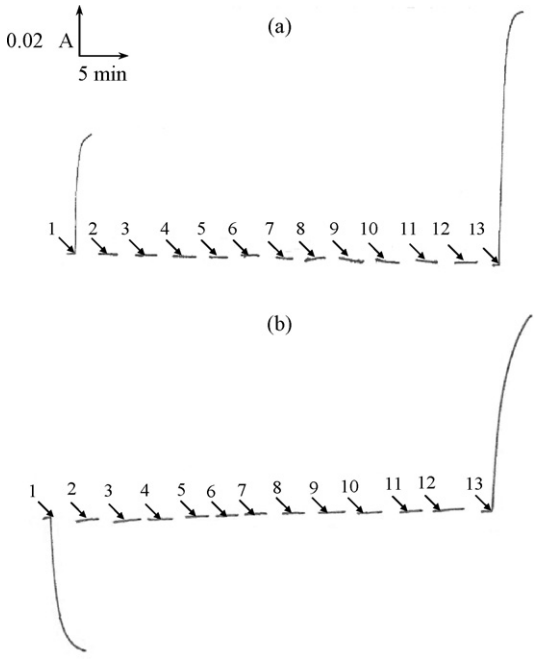


Fig. 5 – Amperometric responses obtained after additions of 20 μL of a 0.01 M glycerol solution (1) and of 0.01 M solutions of different potential interferents (ethanol (2), glucose (3) fructose (4), arabinose (5), galactose (6), lactic acid (7), malic acid (8), gluconic acid (9), acetic acid (10) tartaric acid (11), citric acid (12) and ascorbic acid (13)) to the corresponding buffer solution, obtained at a GDH/DP biosensor (a) and a GK/GPOx/HRP biosensor (b). Other conditions as in Figs. 1 and 4, respectively.

the standard deviation ($n = 10$) of the amperometric signals from different solutions of glycerol at a concentration level of 1.0×10^{-6} M.

3.4. *Interference study*

Wines contain various kinds of components that may affect the biosensor amperometric response. In particular, ethanol (10–14%, v/v), some sugars (glucose: 0.01–0.95 g L^{-1}; fructose: 0.01–0.83 g L^{-1}; galactose: 0.00–0.13 g L^{-1}; arabinose: 0.01–0.30 g L^{-1}) and some organic acids (tartaric acid: 1.2–4.8 g L^{-1}; malic acid: 0.16–5.20 g L^{-1}; citric acid: 0.12–0.88 g L^{-1}; L(+)-lactic acid: 0.044–4.2 g L^{-1}; gluconic acid: 0.001–2.8 g L^{-1}; acetic acid: 0.15–0.90 g L^{-1}; ascorbic acid: 0.005–0.012 g L^{-1}), may be considered as sources of potential interferences [22]. Therefore, the influence of these compounds on the quantification of glycerol was investigated under the experimental conditions specified above. The substances tested were glucose, fructose, galactose, arabinose and ascorbic, citric, malic and tartaric acids. As can be seen in Fig. 5, among all of these compounds, only ascorbic acid yielded an amperometric response under the working conditions with both biosensors, which is due to the electrochemical oxidation of this compound at the applied potential to the bioelectrode. Although the presence of the SAM inhibits in a high extent this oxidation processes, as it was demonstrated by recording cyclic voltammograms at bare gold and MPA-modified gold electrodes, it has been reported that the presence of TTF catalyses the ascorbic acid oxidation [23], and therefore a significant interference was produced. Nevertheless, it is important to remark that the content of glycerol in the samples to be analyzed is remarkably higher than the possible content of ascorbic acid. Consequently, no significant interference should be expected in the analysis of wine samples.

3.5. *Determination of glycerol in wines*

Both types of developed biosensors were applied to the determination of glycerol in several types of wine. As indicated in the experimental section, no matrix effect was observed for any of these samples, and therefore, the determination was accomplished by interpolation of the corresponding amperometric signals into appropriate calibration plots. Dilutions of 1:50 to 1:200 are necessary, depending on the glycerol concentration of the sample. No changes were observed in the slope and intercept values of the glycerol calibrations plots for at least 1 week.

Glycerol determination were carried out in 12 different types of wine (Table 3) and three replicates were made for each sample with each biosensor. The confidence intervals were calculated for a significance level of 0.05, and R.S.D. values were in all cases <10% (Table 3). As can be seen, red wines usually have higher glycerol contents than white ones, which is partially because a higher fermentation temperature is used in the production of red wines [2]. An important aspect to be remarked is that the overall time needed for the analysis of a wine sample is no longer than 3 min. The results obtained with the biosensors were compared with those provided by applying the R-Biopharm® enzyme kit for glycerol (Fig. 6). The characteristic parameters of the corresponding linear least squares regression curves are summarized in Table 4. It is clear that the estimated slopes and intercepts do not differ significantly from the values 1 and 0, respectively [24]. Thus, there is no evidence for systematic differences between the three sets of results obtained by using the developed biosensors and the enzymatic kit. However, the methodologies involved with the integrated multienzyme biosensors developed show advantages in operation simplicity (only and appropriate dilution of the sample is needed), rapidity (3 min against 30 min) and lower cost when they are compared with the use of the enzyme kit.

3.6. *Comparison with other glycerol biosensors reported in the literature*

Finally, we have compared the analytical performance of the developed biosensors with that of other glycerol electrochemical biosensors reported in the literature. Characteristics such as the type of electrode and enzyme immobilization, the redox mediator if used, working potential, type of sample analyzed, range of linearity of the corresponding calibration graph, sensitivity, limit of detection achieved and useful lifetime are listed for all of them in Table 5.

Concerning the applied potential, the biosensors used in this work employed some of the less extreme detec-

Table 3 – Determination of glycerol in wines using GDH/DP and GK/GPOx/HRP biosensors as well as by using a R-Biopharm® enzyme kit

Sample	GDH/DP		GK/GPOx/HRP		Enzyme kit
	$g\,L^{-1}$	R.S.D.$_{n=3}$ (%)	$g\,L^{-1}$	R.S.D.$_{n=3}$ (%)	
Red wines					
1	8.5 ± 0.8	3.8	8.38 ± 0.01	0.5	8.1
2	12 ± 1	4.7	11 ± 1	3.9	11.3
3	18 ± 5	9.9	18 ± 3	6.4	18.4
4	9 ± 2	9.6	9 ± 2	6.9	9.5
5	8 ± 2	8.0	8 ± 2	8.8	7.5
White wines					
6	5.25 ± 0.06	0.5	5.3 ± 0.4	3.2	5.6
7	6.7 ± 0.3	2.0	6.6 ± 0.6	3.5	7.0
8	4.4 ± 0.7	6.1	4.0 ± 0.1	1.0	4.2
9	6.3 ± 0.5	3.0	7.0 ± 0.8	4.6	6.6
10	5.6 ± 0.7	5.0	5.5 ± 0.3	2.3	5.9
11	6.5 ± 0.9	5.4	6.8 ± 0.6	3.6	6.4
12	4.5 ± 0.4	3.2	6.0 ± 0.3	2.1	5.4

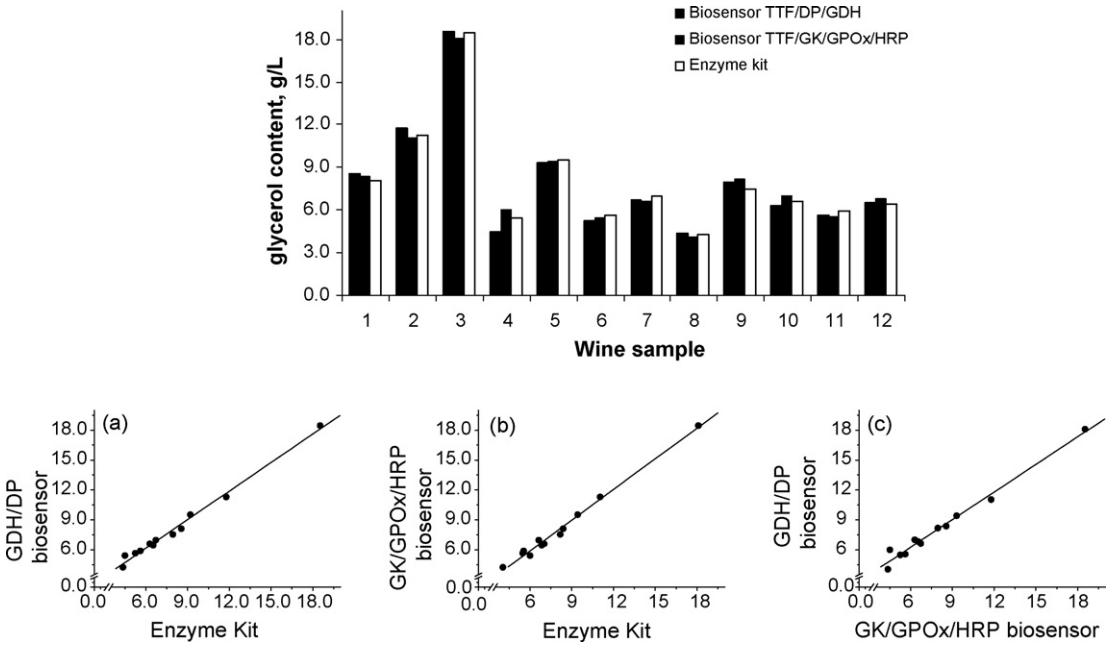


Fig. 6 – Comparison of the results obtained for the determination of glycerol in wines using GDH/DH and GK/GPOx/HRP biosensors and the R-Biopharm® enzyme.

Table 4 – Characteristic parameters of the linear least squares regression curves displayed in Fig. 6

Representation	Slope	Intercept	r
GDH/DP biosensor vs. enzymatic kit ($n = 12$) (Fig. 6a)	0.95 ± 0.07	0.4 ± 0.6	0.995
GK/GPOx/HRP biosensor vs. enzymatic kit ($n = 12$) (Fig. 6b)	1.02 ± 0.07	−0.2 ± 0.6	0.995
GDH/DP biosensor vs. GK/GPOx/HRP biosensor ($n = 12$) (Fig. 6c)	0.92 ± 0.09	0.7 ± 0.8	0.991

tion potentials values, which implies a better selectivity towards potential interferents present in the wine samples. Regarding sensitivity, this is as good as the best ones reported previously, with lower limits of detection achieved. Probably the most remarkable advantage is related to the high stability achieved with the biosensor designs, especially with the GDH/DP bioelectrode, showing their suitability for monitoring of fermentation process and long-term storage.

Therefore, in general, it can be said that the developed biosensors exhibit a good analytical performance in terms of sensitivity, time of response, stability and reproducibility when compared with other biosensors reported in the literature.

Table 5 – Amperometric biosensors reported in the literature for the determination of glycerol

Electrode	Enzyme/s	Immobilization	Mediator	Potential	Sample	L.R.	Sensitivity	L.D.	Stability	Ref.
Pt	GK/GPOx	Immobilization of GK in a glass vedas reactor coupled with GPOx on a preactivated Immobilon AV membrane kept at the electrode surface	–	+650 mV vs. ?	Grapes must	2.0×10^{-6} to 1.0×10^{-3} M[a]	–	5.0×10^{-7} M[a]	GPOx membrane up to 1 month	[21]
Aluminium substrate electrode	GDH	Co-immobilization of GDH/NAD/$K_4Fe(CN)_6$ into a polyaniline film	$K_4Fe(CN)_6$	+400 mV vs. SCE	Grape juice	5.0×10^{-6} to 2.0×10^{-3} M[b]	–	1.0×10^{-6} M[b]	–	[25]
Rods of spectroscopic graphite	GDH	GDH has been integrated in redox hydrogels using an Os-complex-modified non-conducting polymer and poly(ethyleneglycol)-diglycidyl ether	Os-complex	+200 mV vs. Ag/AgCl	Wine	1–200 μM[a]	32 mA M^{-1} cm^{-2}[a]	1 μM[a]	Maintain only 80% of their initial signal after 20 h of continuous substrate injection	[26]
SPE	pGlyDH	Co-immobilization of the enzyme and mediator by cross-linking with glutaraldehyde. Finally the electrode was covered with semi-permeable terylene film	N-(4-Hydroxybenzylidene)-4-ferrocenylaniline	+400 mV vs. Ag/AgCl	Wine	–	–	–	Enzyme activity decreases rapidly	[27]
GCE	(a) GDH/DP; (b) GK/GPOx/HRP	Enzymes were immobilized on interchangeable membranes by polycarbamoyl sulfonate-prepolymer technique	(a) Ferricyanide; (b) ferrocyanide	(a) +350 mV vs. Ag/AgCl; (b) −300 mV vs. Ag/AgCl	From anaerobic fermentation process	(a) 0.01–1.0 mM[a]; (b) 0.01–1.5 mM[a]	(a) 6.02 mA M^{-1} cm^{-2}[a]; (b) 1.42 mA M^{-1} cm^{-2}[a]	–	Signal loss after continuous use: (a) 40% after 90 h; (b) 30% after 16 h	[12]
bMBZrPRs-SPEs	GDH	Cross-linking with glutaraldehyde and nafion	MB	+100 mV vs. Ag/AgCl pseudoreference electrode	Taken during fermentation of chemically defined grape juice medium	1.0×10^{-5} to 1.0×10^{-4} M[b]	–	9.4×10^{-6} M[b]	–	[28]
bPB-SPEs	GDH/NADH oxidase from *Thermus thermophilus*	Cross-linking with glutaraldehyde and nafion	PB	−50 mV vs. Ag/AgCl pseudoreference electrode	–	0.01–1.0 mM[a]	–	2.2×10^{-6} M[a]	–	[29]
CPE	GDH	GDH and NAD^+ were co-immobilized using an electropolymerized layer of non-conducting poly(*o*-phenylenediamine)	Oxidation products of NAD^+	+150 mV vs. Ag/AgCl	Plant-extract syrup	9.97×10^{-7} to 1.0×10^{-4} M[b]	1.62×10^5 nA M^{-1}	4.3×10^{-7} M[b]	The third day a decrease of about 20% of the initial response is observed	[30]
Carbon film	(a) GPOx; (b) GK/GPOx	Cross-linking with glutaraldehyde	Poly(neutral red)	−350 mV vs. SCE	Wines	(a) 10–147 μM[b]; (b) 20–700 μM[b]	(a) 10.1 μA mM^{-1} cm^{-2}[b]; (b) 0.79 μA mM^{-1} cm^{-2}[b]	(a) 4 μM[b]; (b) 15 μM[b]	Signal loss after continuous use: (a) 13% after 8 days; (b) 28% after 8 days	[13]
AuE	(a) GDH/DP; (b) GK/GPOx/HRP	Co-immobilization of the corresponding enzymes and the mediator onto the modified MPA–AuE with a dialysis membrane	TTF	(a) +150 mV vs. Ag/AgCl; (b) 0 mV vs. Ag/AgCl	Wines	(a) 1.0×10^{-6} to 2.0×10^{-5} M[b]; (b) 4.0×10^{-7} to 1.0×10^{-5} M[b]	(a) 1214 μA M^{-1}[b] (20.7 mA M^{-1} cm^{-2}[b]); (b) 1460 μA M^{-1}[b] (25.0 mA M^{-1} cm^{-2}[b])	(a) 4.0×10^{-7} M[b]; (b) 3.1×10^{-7} M[b]	Signal loss after continuous use: (a) 13% after 51 days; (b) 54% after 8 days	This work

bMBZrPRs-SPEs: bulk screen-printed electrodes modified with zirconium phosphate and meldola blue and by electrochemical deposition of a Reineckate film; bPB-SPEs: Prussian blue bulk modified screen-printed electrodes; CPE: carbon paste electrode; DP: diaphorase; GCE: glassy carbon electrode; GK: glycerokinase; GPOx: glycerol-3-Phosphate oxidase; HRP: horseradish peroxidase; MB: meldola blue; MPA: 3-mercaptopropionic acid; PB: Prussian blue; SCE: saturated calomel electrode; SPE: screen-printed electrodes; TTF: tetrathiafulvalene.

[a] FIA mode.

[b] Batch mode.

4. Conclusions

The results described above demonstrate fairly well that the use of integrated TTF/GDH/DP and TTF/GK/GPOx/HRP amperometric biosensors accomplishes the requirements of precision, rapidity, sensitivity, simplicity and low cost required to be considered as useful analytical tools for the wine industry, providing rapid and reliable analytical methodologies for the quantification of glycerol, which are suitable to be employed for rapid judging the quality of wines. The comparison of the analytical performance achieved with both biosensors shows that a better operational stability and an easier and shorter construction procedure were found for the GDH biosensor. Therefore, this configuration is recommended for the monitoring of glycerol in wine samples.

Acknowledgements

The financial support of the projects PETRI nos. PTR95-0893.0.1.02.OP and CTQ2006-02743BQU are gratefully acknowledged. María Gamella acknowledges a pre-PhD fellowship of the Comunidad Autónoma de Madrid.

REFERENCES

[1] C. Cordella, J.-F. Antinelli, D. Cabrol-Bass, Trends Anal. Chem. 22 (2003) 115.
[2] Đ. Šehović, V. Petravić, V. Marić, Kem. Ind. 53 (2004) 505.
[3] P. Ribereau-Gayon, D. Dubordieu, B. Doneche, A. Lonvaud, Les bacteries lactiques, in: Dunod (Ed.), Traité d'enologie. Microbiologie du vin. Vinifications, La Vigne, Paris, 1998, pp. 225–236.
[4] H.M. Oliveira, M.A. Segundo, J.L.F.C. Lima, V. Grassi, E.A.G. Zagatto, J. Agric. Food Chem. 54 (2006) 4136.
[5] AOAC Official Methods of Analysis 968.09, 972.10, 17th ed.
[6] M. Esti, G. Volpe, D. Compagnone, G. Mariotti, D. Moscone, G. Palleschi, Am. J. Enol. Vitic. 54 (2003) 39.
[7] M.I. Prodomidis, C.D. Stalikas, S.M. Tzouwara-Karayanni, M.I. Karayannis, Talanta 43 (1996) 27.
[8] B. Merchie, A. Girard, B. Maisterrena, P. Michalon, R. Couturier, Anal. Chim. Acta 263 (1992) 85.
[9] M. Kiyoshi, M. Hiroaki, H. Masashi, D. Toyohiko, O. Yutaka, Agric. Biol. Chem. 55 (1991) 1055.
[10] I.L. Mattos, J.M.F. Romero, M.D.L. de Castro, M. Valcárcel, Analyst 120 (1995) 179.
[11] L.J. Murphy, P.T. Galley, Anal. Chem. 66 (1994) 4345.
[12] J. Katrlík, V. Mastihuba, I. Voštiar, J. Šefčovičová, V. Štefuca, P. Gemeiner, Anal. Chim. Acta 566 (2006) 11.
[13] M.E. Ghica, C.M.A. Brett, Anal. Lett. 39 (2006) 1527.
[14] S. Campuzano, R. Gálvez, M. Pedrero, F. Javier Manuel de Villena, J.M. Pingarrón, J. Electroanal. Chem. 526 (2002) 92.
[15] S. Campuzano, R. Gálvez, M. Pedrero, F. Javier Manuel de Villena, J.M. Pingarrón, Anal. Bioanal. Chem. 377 (2003) 600.
[16] P.C. Pandey, S. Upadhyay, B.C. Upadhyay, H.C. Pathak, Anal. Biochem. 260 (1998) 195.
[17] S. Campuzano, M. Pedrero, J.M. Pingarrón, Talanta 66 (2005) 1310.
[18] M. Burton, in: S.P. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, vol. I, Academic Press, New York, 1955, p. 397.
[19] L.A. Decker (Ed.), Glycerol dehydrogenase. In Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, New Jersey, 1977, p. 17.
[20] J.E. Strickland, D.N. Millen, Biochim. Biophys. Acta 159 (1968) 221.
[21] D. Compagnone, M. Esti, M.C. Messia, E. Peluso, G. Palleschi, Biosens. Bioelectron. 13 (1998) 875.
[22] Web page: www.enoforum.com.
[23] A.S.N. Murthy, Anita, Biosens. Bioelectron. 11 (1996) 191.
[24] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 5th ed., Pearson Education, Harlow, UK, 2005, p. 126.
[25] A. Eftekhari, Sens. Actuators B 80 (2001) 283.
[26] M. Niculescu, R. Mieliauskiene, V. Laurinavicius, E. Csöregi, Food Chem. 82 (2003) 481.
[27] I. Lapenaite, B. Kurtinaitiene, J. Razumiene, V. Laurinavicius, L. Marcinkeviciene, I. Bachmatova, R. Meskys Niculescu, A. Ramanavicius, Anal. Chim. Acta 549 (2005) 140.
[28] A. Radoi, D. Compagnone, M. Batič, J. Klinčar, L. Gorton, G. Palleschi, Anal. Bioanal. Chem. 387 (2007) 1049.
[29] A. Radoi, D. Compagnone, E. Devic, G. Palleschi, Sens. Actuators B 121 (2007) 501.
[30] M.I. Álvarez Gómez, S.B. Saidman, M.J. Lobo-Castañón, A.J. Miranda-Ordieres, P. Tuñón-Blanco, Anal. Chem. 72 (2000) 520.