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# Simultaneous detection of ethanol, glucose and glycerol in wines using pyrroloquinoline quinone-dependent dehydrogenases based biosensors

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**Abstract**

Amperometric biosensors based on corresponding dehydrogenases have been developed for the determination of ethanol, glucose and glycerol. The enzymes have been integrated in redox hydrogels using an Os complex-modified non-conducting polymer employed as the electrochemical mediator and poly(ethyleneglycol)-diglycidyl ether (PEGDGE) as the cross-linking agent. The developed biosensors showed a sensitive response to ethanol, glucose and glycerol within the concentration range 2.5–250, 20–800, and 1–200 μM, detection limits of 1.2, 9 and 1 μM, and sensitivities of 220, 87 and 32 mA $M^{-1}$ $cm^{-2}$, respectively. The ethanol, glucose and glycerol content of several types of wine was determined with these biosensors, and the results were compared with those obtained by spectrophotometric methods. The developed biosensors have been successfully employed for simultaneous determination of all these substrates at the required sensitivity.


*Keywords:* Ethanol; Glucose; Glycerol; Biosensor; Wine analysis

## 1. Introduction

Chemical analysis of complex samples as wine and other beverages is becoming of great importance due to the general effort of achieving an adequate quality of their production. Particularly for wines, differentiation among variety of vine, geographical origin, and production year is very important in authenticity studies. Wine is a complex mixture of several hundred compounds present simultaneously at different concentrations. The dominants ones are water, ethanol, glycerol, sugars, organic acids, and various ions. Except ethanol and glycerol, other aliphatic and aromatic alcohols, amino acids and phenolic compounds are present at much lower concentrations.

Determination of *glucose* concentration, which often is not only the carbon source for the fermentation yeasts, but also the growth-limiting substrate, is very important during the fermentation process and for the final quality control. *Ethanol* is not only the main product of alcoholic fermentation, but it also has important effects on the yeast growth (Kitagawa et al., 1991). The ethanol content produced by fermentation, ranges in concentration from a few percent up to about 14%. Above this concentration, ethanol destroys the enzymes involved in fermentation, and the process stops. Continuous measurement and control of the ethanol concentration during fermentation is necessary for process optimization and as an alarm for process failure.

*Glycerol* is a secondary fermentation product of alcoholic fermentation, contributing to the viscosity and smoothness of a wine, with a favourable effect on the taste. The amount of glycerol formed during the fermentation process is about 1:10 of the alcohol formed, with final concentrations varying from 1 to 10 g/l (Compagnone, Esti, Messia, Peluso, & Palleschi, 1998). Deviations from this value might indicate technological alterations during the process or deterioration of the harvested grape (Amerine & Ough, 1980).

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Beverage industry may only fulfil the prerequisites for this quality assessment when highly sophisticated analytical methods are introduced into the technological process (Amerine & Ough, 1980). A number of compounds have to be simultaneously and selectively analysed, a task, which is presently achieved only by using very expensive, laborious and time consuming methods like gas chromatography eventually coupled with mass spectrometry (Ng, 2002), liquid chromatography coupled with absorbance or fluorescence detection (Vitrac, Monti, Vercauteren, Deffieux, & Mérillon, 2002), spectrometry (Lázaro, Luque de Castro, & Valcárcel, 1987; Segundo & Rangel, 2002) vibrational spectrometry (Chalmers & Griffiths, 2002) or enzymatic test-kits. In addition, these methods often require a large amount of chemicals, some of which are of major concern with respect to the protection of the environment.

On the other hand, research focused on fast-responding, sensitive and selective biosensors showed their potential applicability for food quality monitoring, including the control of raw materials and final products, and in industrial technologies (Luong, Groom, & Male, 1991). Different scientific groups have focused on the development of biosensors for the determination of glycerol, ethanol and glucose. Compagnone et al. (1998) and Merchie, Girard, Maisterrena, Michalon, and Couturier (1992) developed enzyme sensors for glycerol using covalently immobilized glycerokinase and glycerol-3-phosphate oxidase in conjunction with a platinum-based hydrogen peroxide probe (Compagnone et al., 1998; Merchie et al., 1992). Another possibility was explored involving glycerol dehydrogenase, $NAD^+$ and hexacyanoferrate (III), the enzymatically generated signal being detected by a potentiometric method (Chen & Starzman, 1982). Also, an amperometric method involving glycerol dehydrogenase and ferrocene as electron mediator has been studied (Davis & Hill, 1984). Most of these electrodes have been operated at high over-potentials, and/or required the addition of a soluble cofactor, an obvious drawback, when monitoring in such complex samples as wine.

The construction of ethanol or glucose biosensors has been achieved using either the corresponding oxidases (alcohol—Marko-Varga, Johansson, & Gorton, 1994; Ohara, Vreeke, Battaglini, & Heller, 1993; Vijayakumar, Csöregi, Heller, & Gorton, 1996) or glucose oxidase (Garjonyte & Malinauskas, 1999; Tian & Zhu, 2002) or $NAD^+$-dependent dehydrogenases (alcohol—Cal, Xue, Zhou, & Yang, 1997; Dennison, Hall, & Turner, 1996; Tobalina, Pariente, Hernandez, Abruna, & Lorenzo, 1999) or glucose dehydrogenase (Skoog & Johansson, 1991) by immobilizing these enzymes on suitable transducers. Such biosensors display some basic drawbacks: the oxidase-based ones are dependent on the oxygen content (Garjonyte & Malinauskas, 1999) or are often based on diffusion-type mediators such as ferrocene derivatives (Kutner, Wu, & Kadish, 1994), while the $NAD^+$-dehydrogenases based biosensors require the addition of the soluble cofactor, and often a mediator as well, which complicates their construction, decreases their long-term stability and increases the overall cost for manufacturing. In addition, the electrochemical detection of the reduced form NADH is associated with high over-potentials.

The earlier-mentioned drawbacks have highly motivated research targeting the isolation and purification of new enzymes, among which the oxygen-independent pyrroloquinoline quinone (PQQ)-enzymes received great interest (Laurinavicius et al., 2002). PQQ-enzymes are attractive not only due to their oxygen independence and no cofactor requirement, but also, because some were reported to display a direct electron transfer between their active centre and suitable electrodes, thus making the construction and application of biosensors based on such enzymes more simple.

The present work focuses on the design and development of recently isolated and purified PQQ-dependent dehydrogenase-based electrodes for monitoring of ethanol, glucose and glycerol. The obtained biosensors were found to be a versatile tool for a fast and sufficiently accurate method to simultaneously determine the earlier-mentioned analytes in model and real samples.

## 2. Experimental

### 2.1. Enzymes, chemicals and materials

Glucose dehydrogenase from *Erwinia* sp. *34-1* (PQQ-GDH, 8.3 U/mg protein), alcohol dehydrogenase from *Gluconobacter* sp. *3-3* (PQQ-ADH, 57 U/mg protein), and glycerol dehydrogenase from *Gluconobacter* sp. *3-3* (PQQ-GlyDH, 2.5 U/mg protein) were isolated and purified as described previously (Marcinkeviciene et al., 2000, 1999a, 1999b).

Glycerol dehydrogenase–$NAD^+$ dependent from *Cellulomonas* sp. (Cat. No. G 3512) was from Sigma (Steinheim, Germany) and glucose dehydrogenase-$NAD^+$ dependent from *Bacillus megaterium* (Cat. No. 113732) was from Merck (Darmstadt, Germany).

D (+) Glucose anhydrous (Cat. No. 152527) was from ICN Biomedicals Inc. (Aurora, OH, USA), ethanol 99.7% from Kemetyl (Stockholm, Sweden), glycerol (Cat. No. G-6279) and β-nicotinamide adenine dinucleotide (Cat. No. N-1511) from Sigma. Poly(vinylimidazole) complexed with Os(4,4′-dimethylbipyridine)$_2$Cl ($PVI_{13}$dmeOs) was synthesized according to a previously published procedure (Ohara, Rajagopalan, & Heller, 1994). Poly(ethylene glycol) diglycidyl ether (PEGDGE) (Cat. No. 08210) was from Polysciences Inc. (Warrington, PA, USA).

All reagents used for the preparation of supporting electrolytes were of analytical grade. Sodium acetate anhydrous GC (Cat. No. 1.06268), acetic acid 100% (Cat. No. 1.00063), and calcium chloride dihydrate (Cat. No. 1.02382) were supplied from Merck (Darmstadt, Germany). These reagents were used for the preparation of 100 mM sodium acetate buffer pH 6.0 containing 1 mM calcium chloride. All solutions were prepared using bidistilled water purified in a Milli-Q system (Millipore, MA, USA).

### 2.2. Biosensor preparation

Enzyme modified graphite electrodes (type RW001, 3.05 mm diameter, Ringsdorff Werke GmbH. Bonn, Germany) were prepared as follows: first, rods of spectroscopic graphite were cut, and polished on fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI, USA) to produce a flat surface, followed by rinsing the electrode surface with water and drying at room temperature before coating with enzyme. Next, a drop of 6 μl of the mixture containing the enzyme, the redox polymer and the cross-linking agent, was added on the electrode surface, and the hydrogel was allowed to condition for 20 h at 4 °C. Stock solutions of PQQ–GDH (5.2 mg/ml), PQQ–GlyDH (5 mg/ml), PQQ–ADH (3 mg/ml), and $PVI_{13}$dmeOs (5 mg/ml) were made in buffer and used for the preparation of working solutions in all experiments. The cross-linking agent was freshly prepared in water and used within 15 min.

The optimal composition of the biocatalytic film for the three different biosensors was as follows:

1. PQQ–GDH–$PVI_{13}$dmeOs–PEGDGE biosensor: enzyme/redox polymer/cross-linker ratio is 4:4:1 (w/w/w).
2. PQQ–GlyDH–$PVI_{13}$dmeOs–PEGDGE modified electrode: enzyme/redox polymer/ cross-linker ratio is 4:4:1 (w/w/w).
3. PQQ–ADH–$PVI_{13}$dmeOs–PEGDGE modified electrode: enzyme/ redox polymer/ cross-linker ratio is 3:4:1 (w/w/w).

### 2.3. Spectrophotometric experiments

The method used for the validation of the obtained results was based on the following reactions:

$$\text{Glucose} + \text{NAD}^+ \xrightarrow{\text{GDH}} \text{Gluconic acid} + \text{NADH} + \text{H}^+$$

$$\text{Glycerol} + \text{NAD}^+ \xrightarrow{\text{GlyDH}} \text{Dihydroxyacetone} + \text{NADH} + \text{H}^+$$

where GDH and GlyDH indicate glucose and glycerol dehydrogenases–$NAD^+$ dependent, respectively. The absorbance change caused by the produced NADH permitted the spectrophotometric quantification of the analytes at 340 nm using an Ultraspec 1000, UV/visible Spectrophotometer (Pharmacia Biotech, Boule Nordic AB, Huddinge, Sweden).

### 2.4. Instrumentation

All electrochemical experiments were carried out in a three-electrode electrochemical cell, using the modified graphite electrode as the working, an Ag/AgCl (0.1 M KCl) as the reference, and a platinum wire as the counter electrode, respectively.

The cyclic voltammetry and chronoamperometric experiments were controlled by a BAS 50 W electrochemical analyser (Bioanalytical System Inc., West Lafayette, IN, USA) connected to a computer.

In-flow amperometric measurements were performed in a single channel flow injection system containing a manual sample injection valve (Valco Instruments Co. Inc., Houston, TX, USA) with a 50 μl injection loop, and a peristaltic pump (Alitea AB, Stockholm, Sweden) which pumped the carrier solution through teflon tubings (0.5 mm i.d.) to a flow-through wall jet electrochemical flow cell. The response of the prepared biosensors to different concentrations of substrates was checked at +200 mV vs. Ag/AgCl, potential controlled by a potentiostat (Zäta-Elektronik, Höör, Sweden). The signal was recorded on a single channel strip-chart recorder (model BD 111, Kipp & Zonen, Delft, The Netherlands). The selectivity studies were performed in a similar electrochemical system containing a specially designed flow injection cell, with two working electrodes.

The supporting electrolyte for all experiments was 100 mM acetate buffer pH 6.0, containing 1 mM $CaCl_2$.

### 2.5. Real sample

Commercial wine samples were analysed by flow injection using the developed biosensors. Taking into account that the concentration of the compounds of interest in wine is far outside the working range of the sensors, a dilution of the sample was necessary prior to analysis to adjust the sample concentration to the linear range of the developed biosensors.

## 3. Results and discussion

The measurement of the key compounds (i.e. ethanol, glucose, glycerol) plays an important role in the quality control during the wine fermentation process and of the final product. During this work, biosensors based on PQQ-dependent dehydrogenases for the earlier-mentioned substrates have been developed and used for their simultaneous analysis, enabling a rapid and cheap

measurement in real samples of wine without the need of separation.

The PQQ–ADH used in this work contains three subunits: the first one contains PQQ and heme c groups, the second two heme c, and the third with no redox groups (Razumiene, Niculescu, Ramanavicius, Laurinavicius, & Csöregi, 2002). The other two enzymes, glucose dehydrogenase and glycerol dehydrogenase, are also PQQ-dependent, but they do not contain heme as cofactor. Due to the structural similarities of the studied enzymes, a common sensing mechanism could be proposed for the three biosensors based on the earlier-mentioned enzymes. Thus, the main enzyme substrate (glucose for PQQ–GDH, glycerol for PQQ–GlyDH and ethanol for PQQ–ADH) is first oxidized while the enzyme's cofactor is simultaneously reduced. The active form of the enzyme is regenerated via the interaction with the electrochemical mediator (Os-modified redox polymer), which is maintained in its oxidized form by the positive potential applied at the electrode (see Fig. 1).

The electrochemical behaviour of the modified electrodes was studied in detail. In the absence of the substrate (ethanol, glucose or glycerol), the enzyme (PQQ–ADH, PQQ–GDH or PQQ–GlyDH)-based biosensor displayed the typical electrochemical behaviour of the Os-mediator in cyclic voltammetry. When the substrate was present, a catalytical oxidation current appears and rises from about +50 mV vs. Ag/AgCl. As a compromise between the sensitivity of the biosensor and the reduction of electrochemical interference, a working potential of +200 mV vs. Ag/AgCl was considered for further amperometric measurements (see Fig. 2).

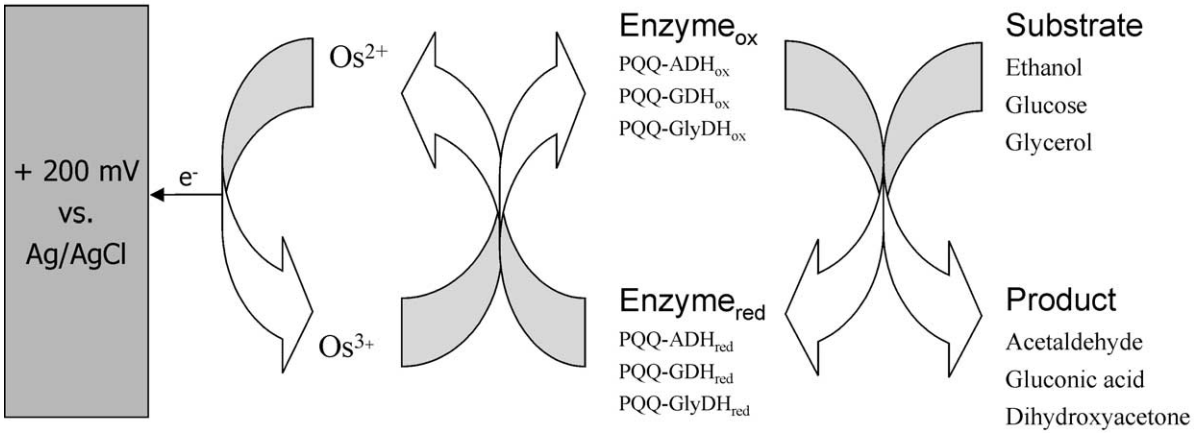


Fig. 1. Proposed mechanism of the bioelectroconversion of the substrates for redox hydrogel integrated PQQ–dehydrogenase modified electrodes.

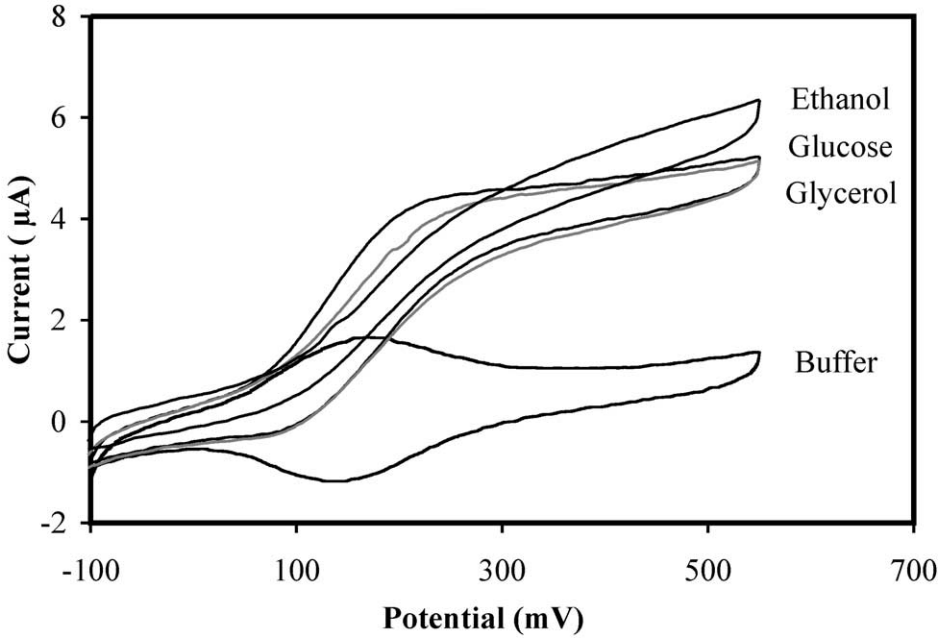


Fig. 2. Cyclic voltammograms obtained in 100 mM sodium acetate buffer pH 6.0 containing 1 mM calcium chloride and in 5 mM substrate (ethanol, glucose or glycerol) solution at a sweep rate of 10 mV/s for graphite electrodes modified with enzyme (PQQ–ADH, PQQ–GDH or PQQ–GlyDH), $PVI_{13}$dmeOs and PEGDGE.

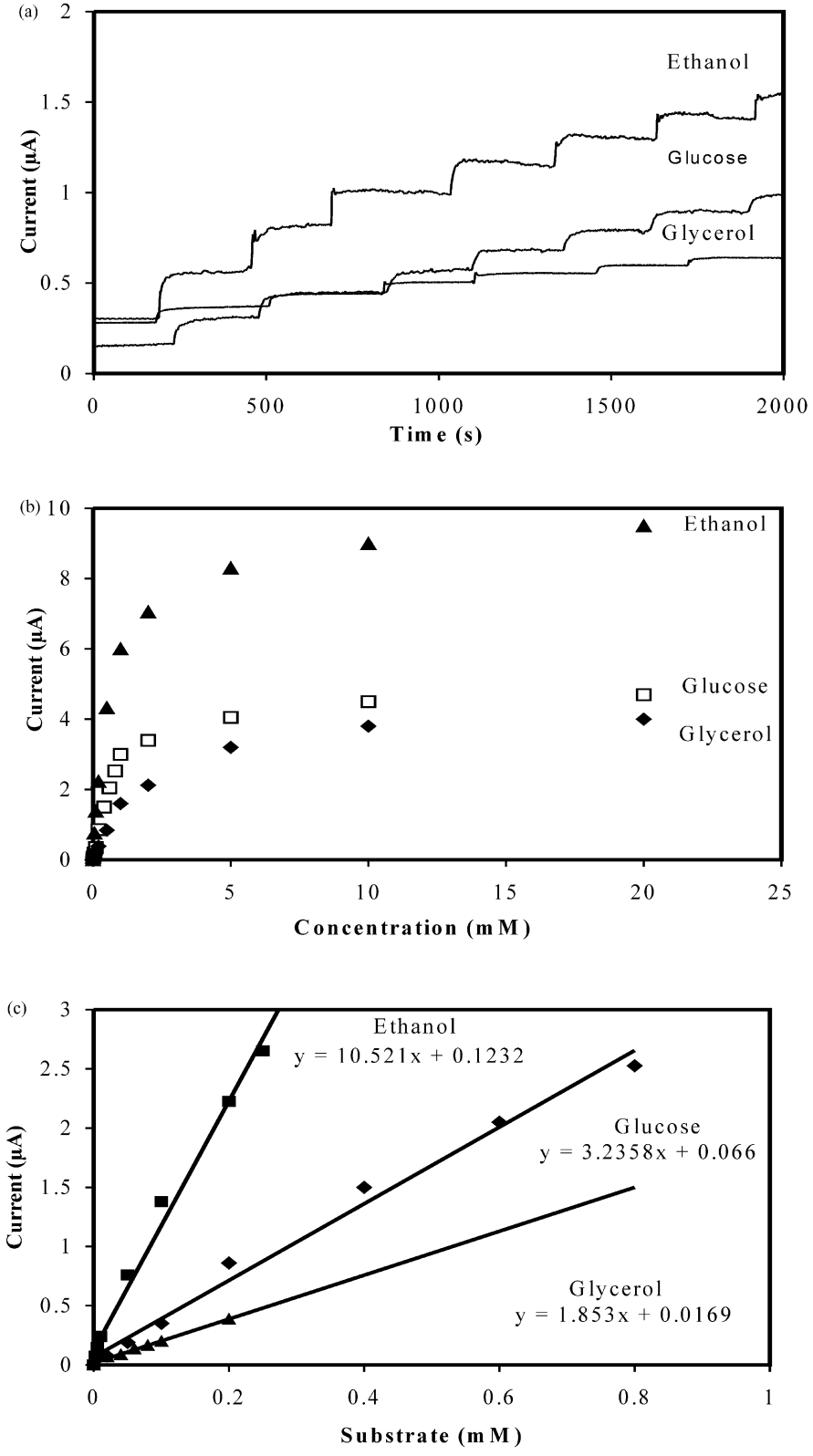


Fig. 3. (a) Current-time plots obtained for successive additions of 100 mM substrate (10 μl) in the amperometric cell, using the biosensors as the working electrodes. Experimental conditions: 5 ml of 100 mM sodium acetate buffer pH 6.0 containing 1 mM calcium chloride, applied potential 200 mV (vs. Ag/AgCl). (b) Calibration curves for glucose, ethanol, and glycerol recorded in a flow injection system with the corresponding substrate-specific biosensor used as the working electrode. Experimental conditions: working potential 200 mV (vs. Ag/AgCl), flow rate 0.5 ml/min, injection volume 50 μl, carrier buffer 100 mM sodium acetate pH 6.0 containing 1 mM calcium chloride. (c) Linear ranges for the calibration curves presented at point b. Conditions as at point b.

First, the biosensors were tested in a batch system. Fig. 3a shows current-time recordings obtained at PQQ–dehydrogenase modified graphite for successive additions of substrate solution (i.e. ethanol for PQQ–ADH sensor, glucose for PQQ–GDH sensor, and glycerol for PQQ–GlyDH sensor). Then, the developed biosensors were integrated in a single-manifold flow injection line and samples containing the analytes were manually injected into the carrier flow and transported to the electrochemical cell equipped with the working, reference, and auxiliary electrodes. The enzyme modified electrodes were optimized with regard to their composition (e.g. enzyme/polymer/crosslinker ratio), flow rate, and pH and were evaluated using standard solutions. The main characteristics of the optimized biosensors are presented in Table 1. The response for glucose on the PQQ–GDH biosensor showed a typical Michaelis kinetics. The detection limit was 9 μM, while the dynamic range of the sensor was between 20 and 800 μM glucose. The dynamic range of the ethanol sensor was between 2.5 and 250 μM, which implies that the wine sample must be considerably diluted (i.e. between 4000 and 400 000 times) before the analysis is performed. The dynamic range of the biosensor for glycerol is also much below the concentration required to measure in wine samples. This is of considerable importance since by diluting the real sample, the concentration of the interfering species is also considerably reduced.

The calibration curves of the optimized biosensors together with their linear range are presented in Fig. 3b and c, respectively.

The operational and storage stability of the three biosensors was also tested. While about 90% response of the ethanol sensor was kept after continuous operation for 100 h, the other two electrodes were less stable, maintaining only 80% (glycerol sensor) and 60% (glu-

Table 1
Biosensors characteristics

| Type of sensor | $K_m$ (mM) | $I_{max}$ (μA) | S (mA/M cm$^2$) | DL (μM) | LR (μM) | $LR_{wine}$ (M) |
|---|---|---|---|---|---|---|
| Ethanol | 0.62±0.02 | 9.56±0.09 | 220.27 | 1.2 | 2.5–250 | 1–3.5 |
| Glucose | 0.80±0.05 | 4.85±0.09 | 86.60 | 9.0 | 20–800 | 0.002–1 |
| Glycerol | 2.01±0.09 | 4.46±0.06 | 31.69 | 1.0 | 1–200 | 0.01–01 |

DL, detection limit (calculated as three times the signal-to-noise ratio); LR, $LR_{wine}$, linear range; $I_{max}$ and $K_m^{app}$ were evaluated from Michaelis–Menten equation: $I = (I_{max} \times [A])/(K_m^{app} + [A])$; $[A]$ analyte concentration; S, sensitivity (calculated as $I_{max}/K_m^{app} \times$ Electrode surface).

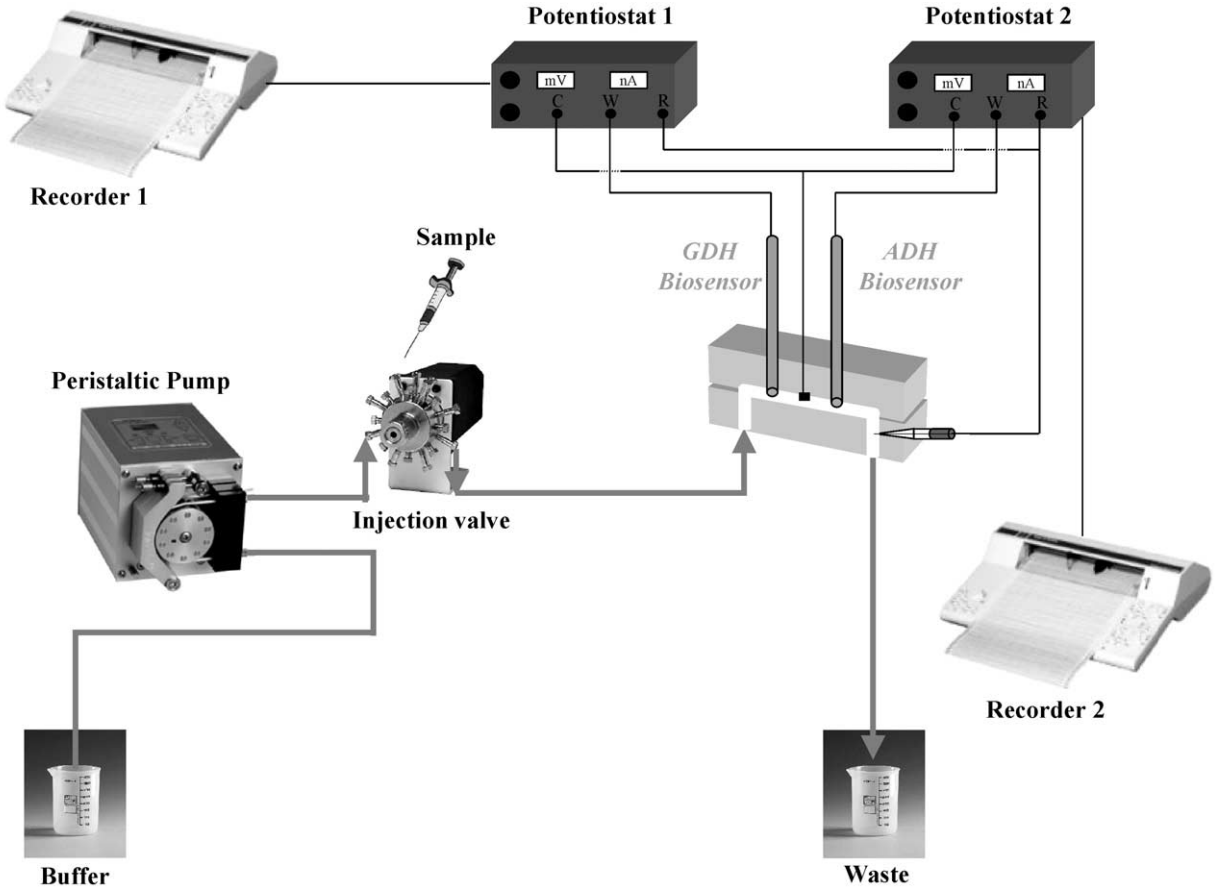


Fig. 4. Schematic presentation of the flow injection system with the specially designed dual cell with two working electrodes used for selective detection of different substrates.

cose sensor) of their initial signal after 20 h of continuous substrate injection. The storage stability was good for all three biosensors, less than 20% of their initial response being lost after 1 month of storage at 4° C (results not shown).

The selectivity of the PQQ–dehydrogenases and PQQ–dehydrogenase-based biosensors was previously investigated (Laurinavicius et al., 2002; Marcinkeviciene et al., 1999a, 1999b, 2000), but this was performed only for substances from the same class of compounds (different aliphatic alcohols for ethanol and glycerol sensors and saccharides for glucose sensor). In this work, selectivity studies were performed in order to examine whether the individual analytes (i.e. glucose, ethanol, glycerol) could be measured simultaneously. A specially constructed flow injection cell, with two working electrodes, was employed in this context (see Fig. 4).

When using a PQQ–GDH-biosensor as the first working electrode and a PQQ–ADH-biosensor as the second one (see Fig. 5), upon injecting glucose no

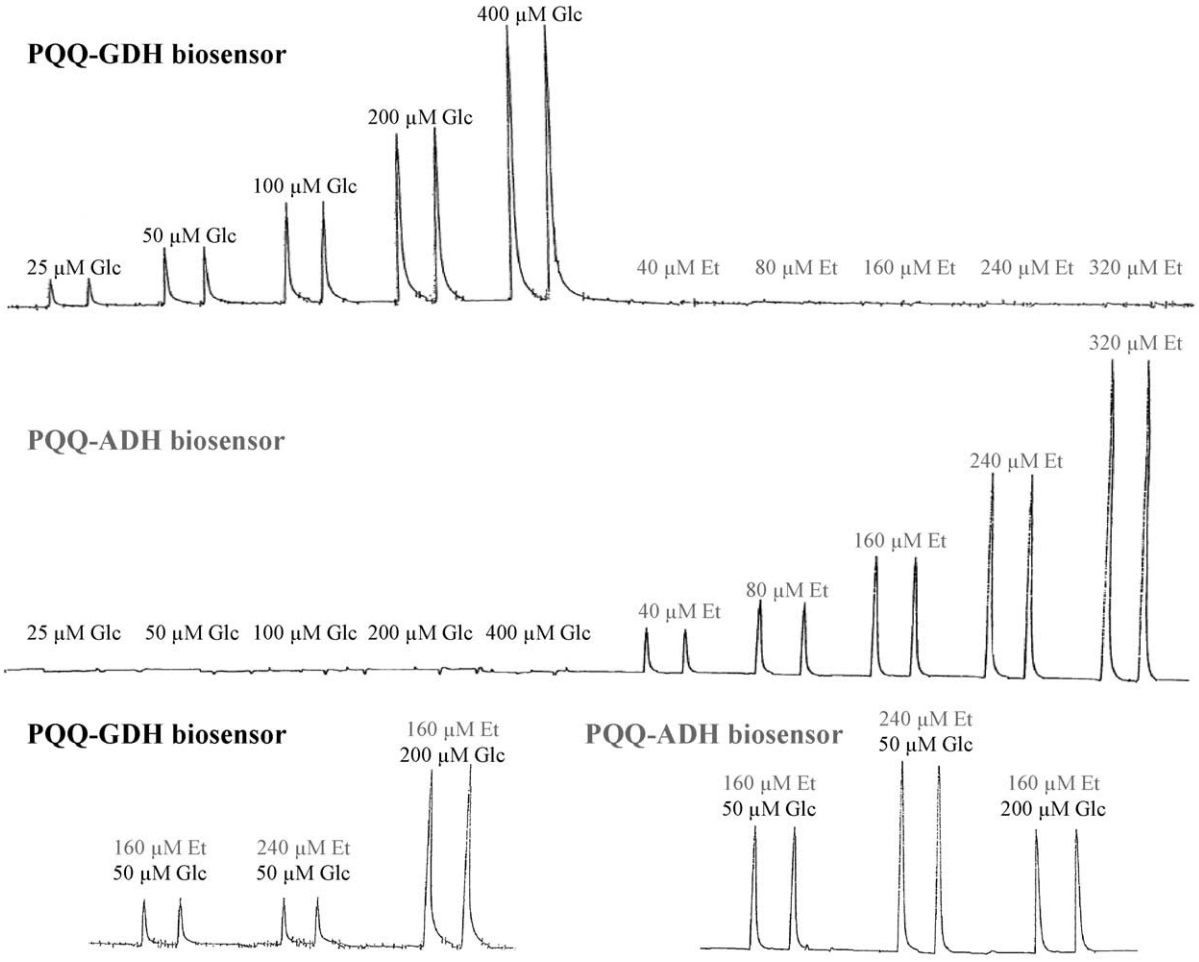


Fig. 5. Signal profiles of glucose and ethanol biosensors using a model solution in a flow injection system equipped with two working electrodes. Experimental conditions: flow rate 0.5 ml/min, applied potential 200 mV (vs. Ag/AgCl). Symbols: Glc, glucose; Et, ethanol.

Table 2
Comparison of results obtained with different methods for glucose, ethanol and glycerol determination in wine

| Type of wine | Glucose | | Ethanol | | Glycerol | |
|---|---|---|---|---|---|---|
| | Biosensor analysis, g/l | Spectrometric analysis, g/l | Biosensor analysis,% | Declared by producer,% | Biosensor analysis, g/l | Spectrometric analysis, g/l |
| Ungarischer Rotwein, 1998, Balaton, (Hungary) | 8.50±0.283 | 8.44±0.063 | 12.05±1.20 | 12 | 10.20±1.69 | 9.80±1.13 |
| Bleriot, Trocken, (France) | 3.05±0.049 | 2.65±0.071 | 9.70±0.424 | 11.5 | 7.00±1.69 | 6.60±1.21 |
| Valpolicella, 1998, Negrar, (Italy) | 2.20±0.141 | 2.28±0.182 | 12.50±2.12 | 12 | 7.10±1.56 | 7.00±1.68 |

response could be seen at the ethanol biosensor, while the glucose electrode accurately followed the substrate addition, and vice versa. When a mixture of the two analytes was injected, the signal for glucose and for ethanol could be differentiated on the two biosensors. Similar experiments have been performed for the simultaneous analysis of glucose and glycerol, and ethanol and glycerol, respectively obtaining similar results (results not shown), namely the electrodes responded selectively only for their analytes except for the glucose–glycerol system. In this case a high response for glucose (i.e. 140% vs. the sensitivity for glycerol) was obtained on the PQQ–GlyDH biosensor. Current experiments are targeting the elucidation of the observed phenomenon and the modification of the design of the sensor and/or analysis system, accordingly. However, the present sensors still can be used for simultaneous monitoring, but a separate measurement of glucose on the PQQ–GDH electrode in real wine samples is necessary for a correct quantification of glycerol.

Initial experiments on real wine samples were performed in the earlier-described flow injection line by injecting the samples diluted with carrier buffer to the range where the recorded signal could be interpolated in the dynamic range of each sensor (see Table 2).

As seen, the results obtained by the developed biosensors showed a good agreement with the values obtained by corresponding spectrophotometric methods (glucose and glycerol) or declared by the producer (ethanol), and the applicability for the determination of these key compounds in wine samples could be successfully demonstrated.

## 4. Conclusion

The characteristics of the developed biosensors recommend the optimized electrodes as a good alternative for the simultaneous determination of glucose, ethanol and glycerol in wines: the results are comparable to the alternative procedures, the repeatability is good, and the required sample treatment is minimum (i.e. dilution). The fact that all three biosensors could be operated in similar conditions regarding carrier buffer (pH and ionic strength) and applied potential is of considerable importance for the simultaneous, on-line measurement of the three analytes in real samples.

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