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In memoriam prof. dr. Liviu Oniciu

AMPEROMETRIC BIOSENSORS FOR GLUCOSE AND ETHANOL DETERMINATION IN WINE USING FLOW INJECTION ANALYSIS

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ABSTRACT. Reagentless amperometric biosensors for glucose and ethanol were developed and successfully applied for monitoring glucose and ethanol concentrations in wine during the fermentation process. The glucose biosensor was based on commercially available glucose oxidase and horseradish peroxidase co-immobilized on solid graphite using Os(II)-redox hydrogel (RH) [1]. In the case of ethanol biosensor, the quinohemoprotein dependent alcohol dehydrogenase was immobilized on the graphite electrode surface using the same RH [2]. Both biosensors were operated at low applied potentials (-50 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$ for glucose biosensor, and +250 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$ for ethanol biosensor), where biases from interferences are minimal. The bioelectroanalytical parameters, estimated from flow injection analysis measurements, were found as follows: sensitivity, 0.73 ± 0.01 μA mM^{-1} for glucose and 0.45 ± 0.01 μA mM^{-1} for ethanol; linear range up to 1 mM in both cases; detection limit, 7.0 μM for glucose and 8.9 μM for ethanol. The results for real samples were found in good agreement with those reported by Barsan et al. [3].

Keywords: *amperometric biosensors; ethanol; flow injection analysis; glucose; wine.*

INTRODUCTION

The measurement of ethanol and glucose plays an important role in the control of wine fermentation process and for assesing the quality of the final product. Methods commonly used for their determination like chromatography [4], spectrophotometry [5] or enzymatic test-kits [6] require long analysis times, complex instrumentation, high costs or tedious sample treatment. These drawbacks can be avoided using amperometric biosensors due to their characteristics as high selectivity, low cost, relative simple preparation and good stability [7].

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PQQ-dependent dehydrogenases are attractive due to their oxygen independence and to the fact that display a direct electron transfer between their active center and certain electrodes [8, 9]. Quinohemoprotein dependent alcohol dehydrogenase (PQQ-ADH)-based biosensors were previously reported for the detection of ethanol in alcoholic beverages [2, 10].

Oxidases are usually more stable than dehydrogenases and their use imply the monitoring of hydrogen peroxide produced by the enzymatic reaction at applied potential higher than 500 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$. The use of redox mediators immobilized on the electrode surface [11] is beneficial in order to overcome the use of such high applied potentials.

The properties of osmium redox polymers allow besides co-immobilization with the enzyme on the electrode surface, the use of low applied potentials for biosensor operation [12]. Thus, such biosensors are reagentless and less prone to interferences.

The aim of this work was to develop simple and low cost reagentless enzyme biosensors based on PQQ-ADH and glucose oxidase (GOx), respectively, for monitoring of key analytes in wine during the fermentation process. Os(II)-redox hydrogel was used for “wiring” the enzymes and the electrode and the whole mixture was cross-linked with poly(ethylene glycol) diglycidyl ether (PEGDGE) [13]. The biosensors present good reproducibility and their use has the advantage of requiring minimum sample treatment (dilution).

This work represents a part of a training session taking place at Fattoria dei Barbi, Montalcino, Italy, where several analytes of interest such as glucose and ethanol were analyzed by alternative techniques.

RESULTS AND DISCUSSIONS

I. Electrochemical behavior of the modified electrodes

The detection principle of glucose and ethanol biosensors is presented in figure 1.

The bienzyme system, presented in figure 1A, on one hand ensures a high selectivity of the measurements because at the low applied potential (-50 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$) biases from interferences are minimal, and on the other hand, offers an increased sensitivity due to the presence of RH which mediates the electron transfer between HRP and the graphite electrode.

The monoenzyme system used for ethanol detection (figure 1B) is based on the electrical connection of PQQ-ADH to graphite electrode, via RH. The bioelectrocatalytic cycle is closed by electrochemical oxidation of Os(II) to Os(III) at a low applied positive potential (+250 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$).

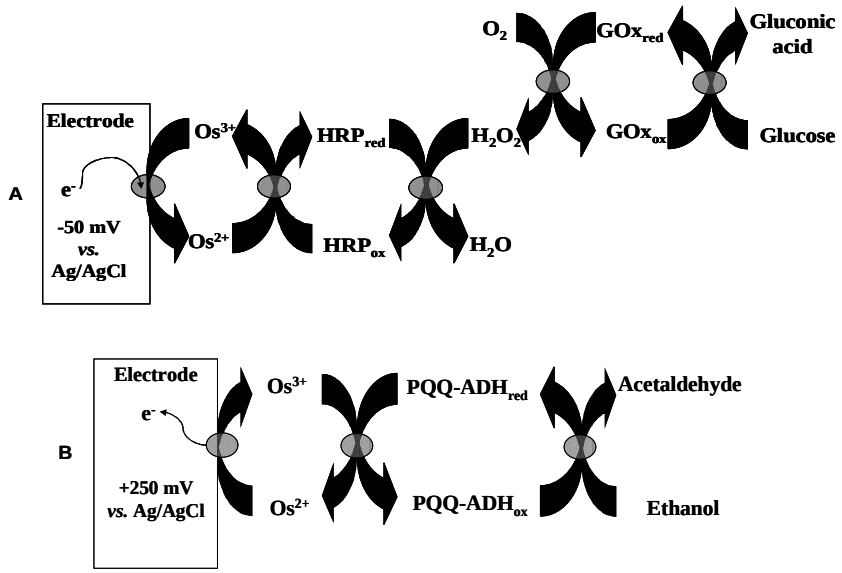

Figure 1. Detection principle for **(A)** glucose and **(B)** ethanol biosensors.

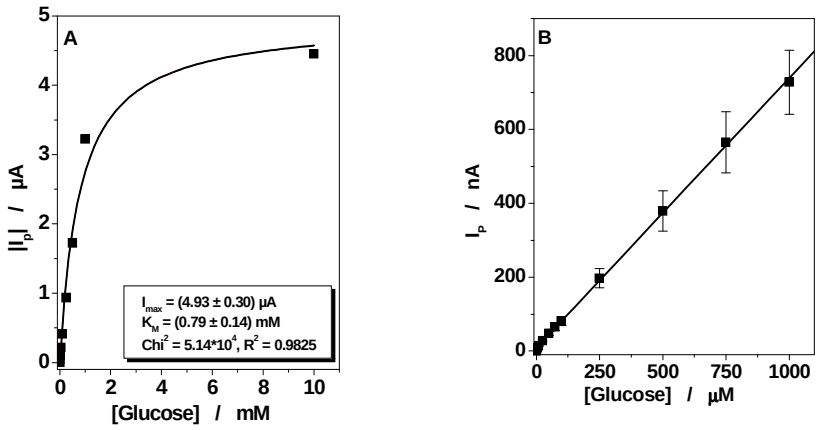

Figure 2. (A) Calibration curve and **(B)** linear range for glucose biosensor. *Experimental conditions*: flow rate, 0.5 ml min^{-1}; supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl, pH 7.2; applied potential, –50 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$.

In order to investigate their electrochemical behavior, the prepared biosensors were integrated in a FIA system. Amperometric measurements were performed by injecting constant volumes of increasing substrate concentrations and recording the corresponding peak currents. As expected, both enzymes presented the Michaelis-Menten behavior. A calibration curve for glucose is presented in figure 2A. The kinetic parameters were found as: I_{max} = (4.93 ± 0.30) μA and K_M = (0.79 ± 0.14) mM. The linear range of the calibration curve is presented in figure 2B. The error bars stand for standard deviation, estimated for 6 enzyme electrodes.

The corresponding bioelectroanalytical parameters are synthesized in table 1.

The same procedure was followed for the ethanol biosensors and the calibration curve is presented in figure 3. The kinetic parameters estimated from the calibration curve were: I_{max} = (2.25 ± 0.01) μA and K_M = (4.05 ± 0.05) mM. As can be seen from figure 3B, small error bars indicate a quite good reproducibility of the results (standard deviation calculated as the mean of three independent measurements). The ethanol biosensor characteristics are summarized in table 1.

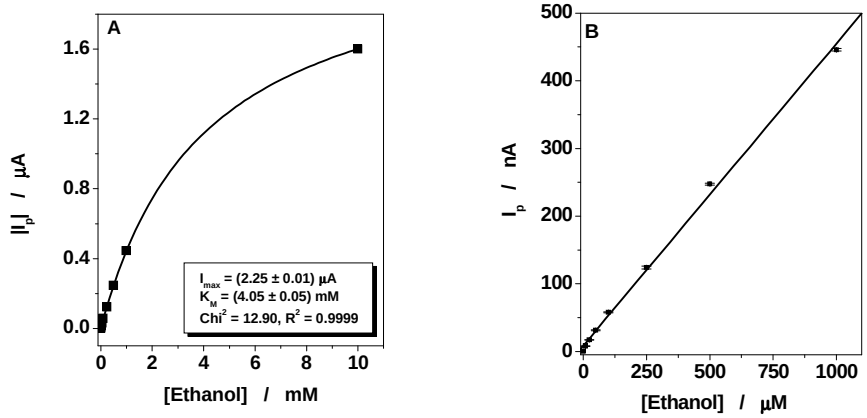

Figure 3. (A) Calibration curve and **(B)** linear range for ethanol biosensor. *Experimental conditions*: flow rate, 0.5 ml min^{-1}; supporting electrolyte, 0.1 M acetate buffer containing 1 mM $CaCl_2$, pH 6.2; applied potential, +250 mV *vs.* Ag/AgCl, KCl $_{0.1\,M}$.

The results are within the limits reported in the literature for different kind of glucose and ethanol biosensors. Good reproducibility, large linear range and relatively low detection limits make the developed biosensors suitable for applications in real samples.

Table 1.

Bioelectroanalytical parameters for glucose and ethanol biosensors.
Experimental conditions: see figures 2 and 3.

Biosensor	Sensitivity** (μA mM^{-1})	Linear range (μM)	Detection limit*** (μM)	R / N
Glucose	0.73 ± 0.01*	up to 1000	7.0	0.9997 / 11
Ethanol	0.45 ± 0.01*	up to 1000	8.9	0.9986 / 8

*standard deviation for 6 (glucose) or 3 (ethanol) measurements.
**calculated as the slope of the linear range.
***estimated for signal / noise ratio equal to 3.

II. Real samples measurements

After the biosensors were calibrated, measurements were performed in wine samples, collected at different fermentation times from a winery. Thus, the real samples were diluted (1:10000 for glucose and 1:400 for ethanol determination) in the corresponding buffers in order to get the response in the linear range of the calibration curves, and injected in the flow line. The results were expressed in g l^{-1} and represented as function of time elapsed from the beginning of sampling. As observed in figure 4, in 27 h from the beginning of the sampling (51 h from the beginning of the fermentation process), the glucose concentration decreased with 32.9 %, while ethanol concentration increased with 4.5 %.

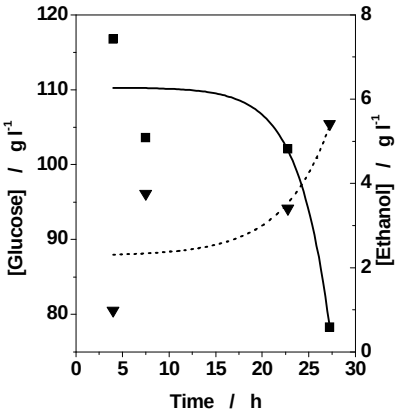

Figure 4. Variation in the glucose and ethanol concentrations during the alcoholic fermentation. Symbols: ■ glucose; ▼ ethanol. *Experimental conditions*: see figures 2 and 3. Obs: The first sample was collected after 24 h from the beginning of the fermentation process.

The results were similar to those obtained with a biosensor based on GOx-poly(neutral) red-BSA-GA adsorbed on carbon-film electrode as well as with those found by HPLC on analysis of the same samples [3]. The

observed differences are not significant and can be due to the different conservation conditions of the samples before being analyzed.

CONCLUSIONS

Bi- and *mono*enzyme biosensors for glucose and ethanol detection, based on GOx-HRP and PQQ-ADH, respectively, were developed and used in an off-line FIA system for wine fermentation monitoring.

The analytical parameters for both types of sensors were estimated from amperometric calibrations in flow injection mode. The biosensors presented good reproducibility and were successfully applied for analysis of glucose and ethanol during fermentation of wine.

EXPERIMENTAL SECTION

Reagents and solutions

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4.), PQQ dependent alcohol dehydrogenase from *Gluconobacter sp. 3.3* and horseradish peroxidase (HRP) (EC 1.11.1.7) were purchased from Sigma-Aldrich (Poole, UK), while poly(ethylene glycol) diglycidyl ether was supplied from Polysciences (Warrington, PA, USA).

Poly(1-vinylimidazole) complexed with Os (4,4'-dimethylbipyridine)$_2$Cl (PVI_{10}dmeOs) (figure 5) was prepared accordingly to a previously published procedure [12].

D(+) glucose anhydrous from Sigma-Aldrich (Poole, UK) and absolute ethanol 99.7% from Solveco Chemicals AB (Sweden) were used to prepare the standard solutions necessary for the sensor calibrations.

Acetic acid glacial 99% from Sigma-Aldrich (Poole, UK), sodium acetate dehydrate and calcium chloride dehydrate from Merck (Darmstadt, Germany) were used to prepare 0.1 M acetate buffer containing 1 mM $CaCl_2$ (pH 6.2). Disodium hydrogen phosphate dehydrate, sodium dihydrogen phosphate and potassium chloride purchased from Merck (Darmstadt, Germany), were utilized to prepare the 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.2). All reagents were of analytical grade and used as received. If not otherwise indicated, the solutions were prepared in purified water obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Biosensors preparation

Considering the good properties reported for the redox hydrogel-based biosensors, amperometric biosensors for the detection of glucose and ethanol were developed accordingly to a previously described method [2, 10].

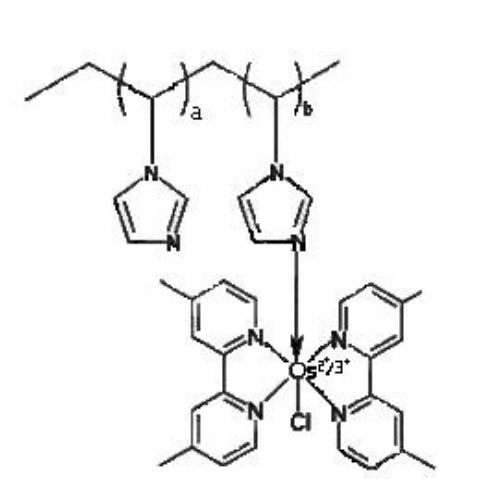

Figure 5. Structure of the Os(II)-redox hydrogel.

Prior to the modification, rods of spectroscopic graphite (Ringsdorff-Werke GmbH, Bonn-Bad, Germany, type RW001, 3.05 mm diameter) were mechanically polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI). The electrodes were rinsed with distilled water before coating them with 5 µL of enzyme mixtures, prepared as described below.

A mixture containing 2.9 mg ml^{-1} GOx, 0.7 mg ml^{-1} HRP, 1.15 mg ml^{-1} PVI_{10}–dmeOs, and 0.3 mg ml^{-1} PEGDGE (freshly prepared aqueous solution and used within 15 min) was used to prepare the glucose biosensors.

PQQ-ADH from *Gluconobacter* was previously reported as bioselective receptor for ethanol biosensor [9]. A mixture containing 1.7 mg ml^{-1} PQQ-ADH, 2.2 mg ml^{-1} PVI_{10}–dmeOs, and 0.55 mg ml^{-1} PEGDGE (freshly prepared aqueous solution and used within 15 min) was used for the preparation of ethanol biosensors. The composition of the modified electrodes is given in table 2.

Table 2.

The composition of enzyme matrix.

Type of biosensor	Composition	(%)
Glucose	GOx-HRP-PVI_{10}dmeOs-PEGDGE	57 : 14.2 : 22.8 : 6
Ethanol	PQQ-ADH-PVI_{10}dmeOs-PEGDGE	37.5 : 50 : 12.5

The electrodes were left to dry at room temperature and kept at + 4 ^{0}C until tested.

If not otherwise indicated, the presented results are average values of three equally prepared electrodes.

Real samples preparation

Must during fermentation (Fattoria dei Barbi, Montalcino, Italy, 2005) was monitored during a period of 27 hours, by analyzing the glucose and

ethanol concentrations with the developed biosensors in an off-line flow injection analysis (FIA) system. Taking into account that the concentration of the measured compounds is outside the working range of the sensors, dilution of the samples was necessary before injecting them into the FIA system. The samples were diluted 1:10000 (v/v) for glucose determination and 1:400 (v/v) for ethanol determination with the corresponding buffer solutions.

Electrochemical measurements

A mono-line FIA set-up consisting of a manual injection valve (Valco Instruments Co. Inc., Houston, TX, USA) with an injection loop of 100 µL, a peristaltic pump (Alitea AB, Stockholm, Sweden), a wall-jet electrochemical cell, a potentiostat (Zäta-Elektronik, Höör, Sweden) and a single channel recorder (Model BD 111, Kipp & Zonen, Delft, The Netherlands) was employed to operate the amperometric biosensors. The working electrodes were the enzyme-modified graphite electrodes, the reference electrode a Ag/AgCl, $KCl_{0.1\ M}$ and the counter electrode a Pt wire. The system was operated at a constant potential of -50 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$ in the case of glucose biosensor and +250 mV *vs.* Ag/AgCl, $KCl_{0.1\ M}$ in the case of ethanol biosensor.

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REFERENCES

1. L. Gorton, G. Bremle, E. Csöregi, G. Jönsson-Pettersson, B. Persson, *Analytica Chimica Acta*, **1991**, *249*, 43.
2. M. Niculescu, R. Mieliauskiene, V. Laurinavicius, E. Csöregi, *Food Chemistry*, **2003**, *82*, 481.
3. M. M. Bârsan, J. Klincar, M. Batic, C. M. A. Brett, *Talanta*, **2007**, *71*, 1893.
4. R. Vonach, B. Lendl, R. Kellner, *Journal of Chromatography A*, **1998**, *824*, 159.
5. N. Choengchan, T. Mantima, P. Wilairat, P. K. Dasgupta, S. Motomizu, D. Nacapricha, *Analytica Chimica Acta*, **2006**, *579*, 33.
6. A. K. Sarker, H. Ukeda, D. Kawana, M. Sawamura, *Food Research International*, **2001**, *34*, 393.
7. M. I. Prodromidis, M. I. Karayannis, *Electroanalysis*, **2002**, *14*, 241.

8. A. Ramanavicius, K. Habermuller, E. Csöregi, V. Laurinavicius, W. Schuhmann, *Analytical Chemistry*, **1999**, *71*, 3581.
9. J. Razumiene, M. Niculescu, A. Ramanavicius, V. Laurinavicius, E. Csöregi, *Electroanalysis*, **2002**, *14*, 43.
10. M. Niculescu, T. Erichsen, V. Sukharev, Z. Kerenyi, E. Csöregi, W. Schuhmann, *Analytica Chimica Acta*, **2002**, *463*, 39.
11. L. Gorton, E. Dominguez, *Reviews in Molecular Biotechnology*, **2002**, *82*, 371.
12. T. J. Ohara, R. Rajagopalan, A. Heller, *Analytical Chemistry*, **1994**, *66*, 2451.
13. R. Antiochia, L. Gorton, *Biosensors & Bioelectronics*, **2007**, *22*, 2611.