



A new amperometric bienzymatic biosensor based on biocomposites for the determination of gluconic acid in wines

Xavier Cetó^a, Francisco Céspedes^a, Josefina Capdevila^b, Manel del Valle^{a,*}

^a Sensors and Biosensors Group, Department of Chemistry, Universitat Autònoma de Barcelona, Edifici Cn, 08193 Bellaterra, Barcelona, Spain

^b INCAVI – Station of Viticulture and Enology from Vilafranca, Pça. Àgora, 2, 08720 Vilafranca del Penedès, Barcelona, Spain

ARTICLE INFO

Article history:

Received 22 December 2010

Received in revised form 16 May 2011

Accepted 24 May 2011

Available online 30 May 2011

Keywords:

Gluconic acid

Amperometric biosensor

Wine

Bienzymatic

Gluconokinase

ABSTRACT

A new amperometric bienzymatic biosensor for gluconic acid based on the coimmobilization of gluconokinase (EC 2.7.1.12) and phosphogluconate dehydrogenase (EC 1.1.1.44) by polysulfone membrane entrapment onto the surface of a graphite-epoxy composite is reported. This biosensor represents an alternative to gluconate dehydrogenase (EC 1.1.99.3) based methods, which is no longer commercially available. Measurements were done at an applied potential of +0.800 V, room temperature and phosphate buffer pH 7.50; obtaining a linear response range for gluconic acid extended from 7.0×10^{-6} to 2.5×10^{-4} M. Constructed biosensors showed good reproducibility for calibrations using different electrodes (RSD of 1.74%). Finally, biosensor was applied to real wine samples, and the results obtained were validated by comparison with those provided by a reference laboratory. Good correlation was found when the biosensor results were plotted vs. the reference values (slope = 1.03 ± 0.04 , intercept = 0.01 ± 0.02 , $r^2 = 0.995$).

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

High concentrations of gluconic acid in wine are caused by the proliferation of a fungus (*Botrytis cinerea*) which is able, via the enzyme glucose oxidase, to generate gluconic acid from glucose (via glucono delta-lactone); this grapevines disease is called common rot or grey rot [1,2]. It develops during growth of the grape berry and is influenced by weather factors, such as moisture and rainfall, or by physiological factors, such as grape variety and bunch shape. Changes that are caused in white and red wines include alterations in colour due to a high activity of oxidases; an increase in volatile acidity, ash contents and dry extract contents through the formation of glycerol, polysaccharides, uronic acids and aldonic acids (essentially gluconic acid); and a decrease in titratable acidity [3]. Significantly, sensory properties of wine are altered by the presence of gluconic acid, which additionally renders it microbiologically unstable and results in long-term storage problems that can be solved only by reducing its content in wine.

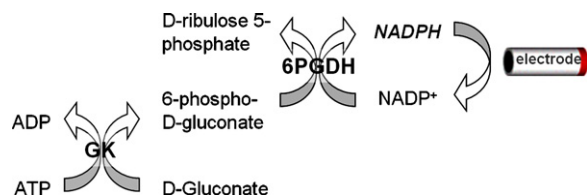
Therefore, gluconic acid concentration is an important analytical parameter used by oenologist to quantify the rottenness degree and assess quality of wine. Taking into account that basal amounts will always be present, *Organization Internationale du Vin* (OIV) recommends levels lower than 200–300 mg/l [4], whereas levels up to

1.0 g/l indicate an initial stage of the fungus infection and higher levels (up to 2–3 g/l) might indicate an activity of acetobacter bacteria. Nevertheless, we have to distinguish noble rot wines, obtained in very special and controlled conditions (which have to be proved), in which cases gluconic acid is a virtue instead of a defect.

D-Gluconate (the base form of gluconic acid) has been usually determined by enzymatic assay employing gluconate dehydrogenase (GADH) (EC 1.1.99.3) which catalyzes the oxidation of gluconic acid through an acceptor reduction (FAD, TTF, NAD⁺, etc.); depending on the acceptor, we can use different techniques such as spectrophotometry [5] or electrochemistry [6–8]. In this case, biosensors have been developed employing: glassy carbon electrodes with GADH immobilized on its surface [6,8], gold electrodes and immobilization through different membranes either with direct cofactor detection [9] or with TTF acting as a mediator [7]. Unfortunately, an inconvenient of these methodologies is that GADH enzyme is not longer commercially available, and in order to continue using the above principles, the only option would be to produce and isolate the enzyme from microbiological cultures [10]. There are also some commercial enzymatic kits for spectrophotometric determination and batch measurements that employ other enzyme systems [11]. Apart of the enzymatic assay, alternative analytical techniques also employed are high performance liquid chromatography [12,13], gas chromatography [14], capillary electrophoresis [15,16] or near-infrared spectroscopy [17]; but these methods are time consuming and require advanced laboratory facilities, while we report a more simple on-field biosensor system based on composites electrodes.

* Corresponding author. Tel.: +34 93 5811017; fax: +34 93 5812379.

E-mail address: manel.delvalle@uab.es (M. del Valle).



Scheme 1. Schematic diagram of the biosensor reactions involved for gluconic acid determination.

The development of composites based on a conductive phase dispersed in a polymeric matrix, has led to important advances in the analytical electrochemistry field, particularly in sensor devices. These materials combine the electrical properties of graphite with the ease of processing of plastics (i.e. epoxy, methacrylate, Teflon) and show attractive electrochemical, physical, mechanical and economical features compared to other noncombined conductive materials (i.e. gold, platinum, graphite). The electrochemical characteristics of composites have been widely studied in previous works [18]. Composites can also incorporate modifiers when they are in solid state, but if we want to incorporate a liquid phase (like an enzyme suspension) we have to use a membrane immobilization protocol like the use of polysulfone.

Polysulfone is a porous polymer widely used as support material for composite membranes and as ultrafiltration membrane. It displays very good chemical and thermal stability and excellent film-forming ability. Moreover, polysulfone films are hydrophobic hence providing the enzyme with an *in vivo*-like environment. Polysulfone is therefore an attractive structural material and has been studied as support for the immobilization of enzymes [19,20].

The aim of this paper is to propose a biosensor alternative to GADH based methods, as a simple tool able to satisfy industry requirements which demands sensor-based system to replace heavy laboratory equipment. For this purpose, we have developed an electrochemical composite biosensor based on the use of two commercial enzymes (gluconate kinase and 6-phospho-D-gluconate dehydrogenase). With these, an amperometric bienzymatic system for the determination of gluconic acid based on the phosphorylation by gluconate kinase and oxidation of the resulting product by 6-phospho-D-gluconate dehydrogenase and accompanied with NADP reduction is reported (Scheme 1). The developed biosensor was tested for real wine sample determination and their results compared and validated with those obtained by a reference laboratory.

2. Materials and methods

2.1. Apparatus

Amperometric measurements were done using a LC-4C amperimeter (BAS Inc., West Lafayette, IN, USA) connected to a personal computer by a data acquisition system ADC-42 PicoTechnology (Pico Technology Limited, St. Neots, Cambridgeshire, UK) for data recording and visualization. Electroanalytical experiments were carried out at room temperature (25 °C) using a three electrode configuration: a double junction electrode Ag/AgCl Orion 900200 (Thermo Electron Corporation, Beverly, MA, USA) was used as the reference electrode, a platinum-based electrode (Crison 52-67, Barcelona, Spain) was used as the auxiliary one and the biosensor was used as the working electrode. A magnetic stirrer provided the convective transport during the amperometric measurements.

2.2. Reagents and solutions

All solutions were prepared using deionised water from a Milli-Q system (Millipore, Billerica, MA, USA). Potassium D-gluconate (99%), adenosine 5'-triphosphate disodium salt (ATP) (99%), adenosine 5'-diphosphate sodium salt (ADP) (95%), polysulfone (Ps) (average $M_n \sim 22,000$ by Membrane Osmometry) and N,N-dimethylformamide (DMF) (99.5%, over molecular sieve) were purchased from Sigma-Aldrich (St. Louis, MO, USA). β -Nicotinamide-adenine dinucleotide phosphate ($NADP^+$) (95%), β -nicotinamide-adenine dinucleotide phosphate (reduced form) ($NADPH$) (93%) and magnesium chloride hexahydrate (99%) were purchased from Merck KGaA (Darmstadt, Germany). Gluconate kinase (GK) (EC 2.7.1.12, 1500 U/ml) and 6-phospho-D-gluconate dehydrogenase (6PGDH) (EC 1.1.1.44, 150 U/ml) were purchased from CPC Biotech (Napoli, Italy).

The solutions were freshly prepared using the buffer solution. $NADP^+$ and ATP- $MgCl_2 \cdot 6H_2O$ solutions were prepared before each experiment; also gluconate 0.1 M solution was prepared and used as stock, and then 10^{-3} M and 10^{-2} M gluconate solutions were prepared from the stock, to reduce the increment of volume after additions in batch measurements. Gluconate salt is used as stock instead of gluconic acid; given that gluconic acid dissociates in water at pH 7.00 to form the gluconate anion ($pK_a = 3.7$), its anion form will be considered along all the text.

2.3. Electrode fabrication

Working electrodes were prepared following the conventional methodology in our laboratories [18]. A resin EpoTek H77 (Epoxy Technology, Billerica, MA, USA) and its corresponding hardener compound were mixed in the ratio 20:3 (w/w). The composite was prepared adding a 15% of graphite (w/w) into the epoxy resin before hardening [21], then it was homogenised for 60 min and finally the composite paste electrode was allowed to harden during 72 h at 80 °C. Electrode surface was then polished with different sandpapers of decreasing grain size; final electrode area 28 mm².

2.4. Membrane preparation

The polysulfone composite membranes were prepared following the methodology previously established [22]. First of all we have to prepare the membrane suspension, which process could be summarized in these two steps. Polysulfone (Ps) was dissolved in DMF, obtaining a Ps solution in DMF and afterwards it was mixed with graphite; the resulting suspension was mixed for 10 min under continuous stirring. The proportions employed for the membrane preparation, once optimized, were 10:2:88 for Ps, DMF and graphite, respectively.

A thin film of this mixture was manually deposited onto the epoxy-graphite electrode surface. Immediately after depositing the Ps solution onto the electrode surface, it was precipitated by causing a phase inversion that was achieved by immersing the electrode in cold water (approximately 4 °C) [23] where the non-solvent (H_2O) displaced the solvent (DMF) and made Ps insoluble. This process led to a controlled phase change of the Ps cast on the electrode from liquid to solid. Taking advantage of the phase inversion process, the enzyme was incorporated into the membrane substituting the cold water for an aqueous solution of the enzyme. Electrodes in this way prepared, with Ps composite films on their surface, were rinsed thoroughly with doubly distilled water prior to use. The modified electrodes with films incorporating the enzyme were stored in phosphate buffer solution pH 7.50 at 4 °C.

2.5. Procedures

Amperometric measurements were made in a 0.1 M phosphate buffer with 0.1 M potassium chloride at pH 7.50. Cyclic voltammograms were made under quiescent condition and amperometric detection was made under forced convection by stirring the solution using a magnetic stirrer.

The amperometric detection was carried out in steady-state conditions, once the current intensity became stable, after addition of different microvolumes of a gluconate solution. Results were obtained as relative responses where the residual current intensity was taken as zero. All the experiments were done in triplicate, and the results given are the averages of three measurements with their corresponding relative standard deviations (RSD). The working potential was selected from preliminary cyclic voltammograms.

The bienzymatic scheme required the addition of two cofactors which were used as reaction starters after adjusting the zero of current value, so all the possible electroactive compounds that could affect the signal are neglected. For the measurements a final concentration of 0.9 mM of NADP⁺, 7.0 mM of ATP and 7.0 mM of MgCl₂·6H₂O were used [24].

Magnesium was added because it is known to act as an activator of the gluconokinase enzyme [25], but at higher concentrations it could also act as an inhibitor [26]. It was also checked and tested that its presence could increase the signal in a factor between 3 and 6 times; therefore its role is important and was added to any further experiment.

2.6. Determination of gluconic acid in wine samples

Given that analysis of gluconic acid in wines did not require any sample pretreatment; the batch mode was employed for the amperometric measurements: only the dilution of the sample by an appropriate factor was made to adequate the gluconic acid wine concentration to the linear zone of the calibration curve and to eliminate possible matrix effects. In this way, 200 µl of sample were added to the electrochemical cell containing 10 ml of buffer, and the amperometric measurements performed. Afterwards, amperometric measurement was not started until the baseline signal was stabilized; at this time the zero of current value (counterbalancing the residual current) was adjusted. Then the cofactors were added and the enzymatic reactions started, obtaining the amperometric signal (incremental signal). Finally this value was interpolated on the calibration graph constructed with gluconic acid standards. The obtained results were compared to those obtained by the reference laboratory (INCAVI) using the reference method.

3. Results and discussion

3.1. Enzymatic scheme

3.1.1. Gluconate kinase

Firstly a simpler scheme based only on GK enzymatic reaction was tested, so as in the bienzymatic system gluconate was phosphorylated reducing ATP concentration. For its monitoring, ATP was electrochemically oxidized through a modified electrode with copper nanoparticles [27]. In this case, a decrease in the signal proportional to gluconate concentration was observed due to ATP consumption via GK enzyme. This methodology was rejected when we observed that ADP reagent was also electrochemically oxidized; this is caused by the oxidation of adenine moiety and the response difference is caused by the R group [28,29]. Its response (slope of the calibration graph) was approximately a half of ATP response, making its contribution hardly distinguishable. Due to the difficulty of having an electrode with a selective response of ATP towards

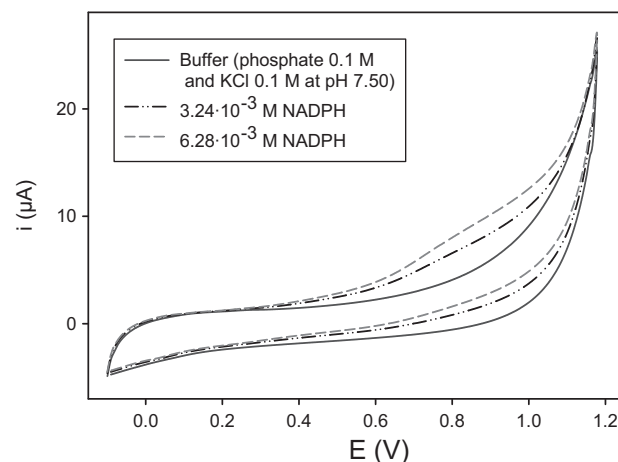


Fig. 1. Cyclic voltammogram for buffer, a 3.24×10^{-3} M and a 6.28×10^{-3} M NADPH solution.

ADP, this system was rejected and the bienzymatic alternative was attempted.

3.1.2. Gluconate kinase and 6-phospho-D-gluconate dehydrogenase

The biocatalytic scheme used for the determination of gluconic acid is displayed in Scheme 1. The determination involves two enzymatic reactions, in the first step gluconate is phosphorylated by GK in the presence of ATP producing 6-phospho-D-gluconate which is oxidized with NADP⁺ to D-ribulose 5-phosphate by 6PGDH, generating NADPH. The next, the NADPH formed in the second enzymatic reaction is electrochemically oxidized at the electrode surface, making its signal proportional to gluconic acid concentration on the sample.

Given that the response of the system depends of NADPH generated in the second enzymatic reaction, and to guarantee that our signal is kinetically related to the substrate we want to determine (gluconic acid), we must have the system controlled by the first reaction employing the substrate. Therefore, as we were following NADPH formed during the second reaction (with 6PGDH), a higher proportion (ca. 40% more) in terms of activity of this enzyme was added. This is to make sure that in case of enzyme saturation, all the gluconate that reacts will be detected.

3.2. Bienzymatic system

3.2.1. Selection of the optimal potential

The first step was to check the NADPH response vs. the graphite-epoxy composite, so a cyclic voltammogram was performed to choose the optimum potential. A cyclic scan was carried between -0.1 V and 1.2 V and with a scan rate of 90 mV s^{-1} . Three measurements were taken: buffer, a 3.2 mM and a 6.3 mM solution of NADPH, confirming that the observed signal is originated from NADPH oxidation (Fig. 1). The three voltammograms were compared and a maximum was found at approximately 800 mV vs. SCE for NADPH oxidation, selecting this potential as the working potential. This optimal potential was confirmed by carrying out a voltammogram in the presence of gluconic acid using the biosensor as working electrode, with which the same maximum for the oxidation of NADPH was found.

Furthermore, analogous voltammograms were done for the other reaction products and substrates, like ATP or ADP, to confirm that no other interfering effect was present in the signal. No response was found along all the scan range, so all the signal in

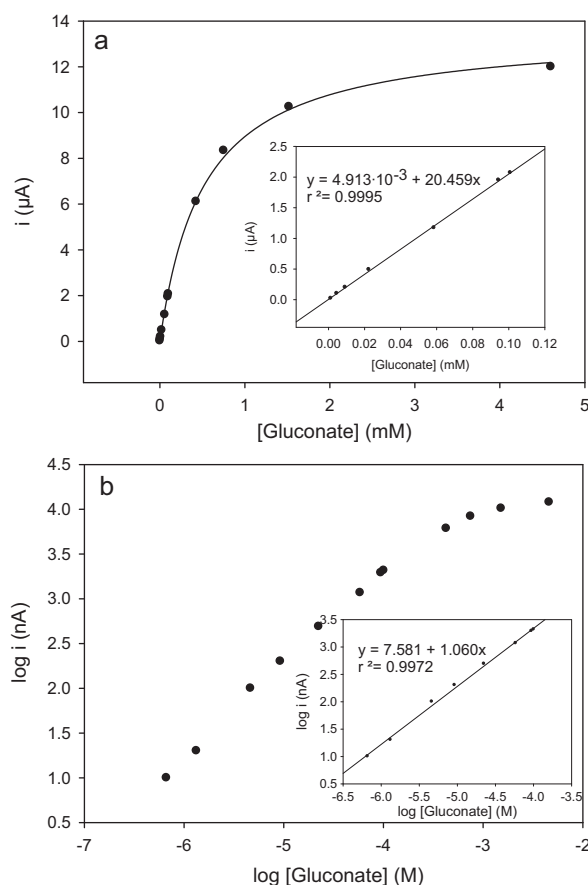


Fig. 2. Characterization of the response of the bienzymatic system in phosphate buffer pH 7.50 with an applied potential of +0.800 V vs. SCE, where (a) corresponds to the linear and (b) to the logarithmic representation.

our measurements was originated from the oxidation of NADPH formed by the bienzymatic system.

3.2.2. Bienzymatic response

The next step was to confirm that the bienzymatic system worked correctly and that NADPH formed in the second reaction could be monitored amperometrically. In this way, a first determination employing the graphite-epoxy composite was performed to confirm we had enough signal for the determinations and to estimate the required amount of enzyme needed for the immobilization step.

Under stirring conditions and with all the reagents in solution, buffer solution containing 7.5 U of GK and 10.5 U of 6PGDH, a kinetic determination of gluconate was carried out. Given a clear Michaelis–Menten behaviour was obtained (Fig. 2), data was adjusted to the non-linear model obtaining a good correlation coefficient ($r=0.9994$) and the apparent constants $K_{M,app} = 0.52 \pm 0.05$ mM and $v_{max,app} = 13.5 \pm 0.5$ μA, with a pseudo-first order zone between 1.5×10^{-6} and 1.0×10^{-4} M, $r^2 = 0.9996$ and a slope of 20.48 ± 0.15 mA M⁻¹. Once confirmed the correct system response and that the availability of enough sensitivity for our purposes, we proceed to the immobilization of the enzymes.

Given that the enzymes used were available in aqueous solution instead of the lyophilized form, we had to change the typical immobilization protocol used with graphite-epoxy biocomposites [18] based on entrapment through physical incorporation of the solid into the composite matrix.

Then, we decided to use a polysulfone (Ps) membrane strategy which was successfully used before [22]. Membrane composition

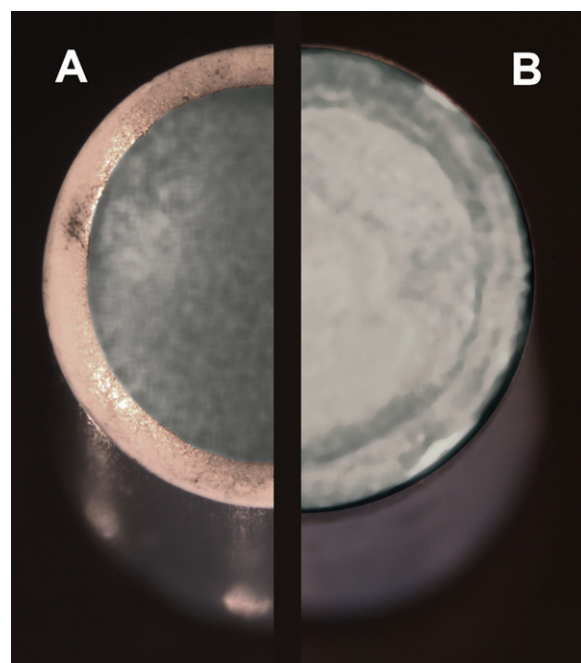


Fig. 3. Epoxy-graphite composite electrode image before (A) and after (B) Ps membrane deposition.

is based in three components, which proportions were changed to obtain the better responses for our case. Firstly, the Ps:DMF ratio was studied, this ratio affects membrane viscosity and the adherence of the resultant membrane with the graphite-epoxy electrode surface. The second parameter was graphite incorporation; given Ps is not a conductive material, its presence increased the signal reducing the influence of membrane diffusion. Departing from the conditions reported in previous work, membrane composition was changed and its response was studied. For the optimization process Umetrics software (MODDE 8) was followed; the optimization process used surface response models, taking membrane composition as variables and its electrochemical behaviour (electroanalytical response evaluated by cyclic voltammetry measurements with ferrocyanide/ferricyanide, gluconate calibration parameters and electrochemical impedance characteristics) as system responses. With this procedure the optimum composition response was experimentally tested and confirmed as the working composition (see section 2.4). The appearance of the obtained membrane, and its comparison before Ps deposition on the electrode surface, could be observed in Fig. 3.

3.3. Biosensor response

Following the procedure described in Section 2, an enzyme mixture containing (15 U of GK and 21 U of 6PGDH) was used to cause Ps precipitation, trapping enzyme in the porous membrane and obtaining our biosensor. Electrodes were rinsed thoroughly with double-distilled water and stored in buffer at 4 °C when not in use. Three replicates were done each time to compare biosensor response and to evaluate its reproducibility.

Amperometric determinations in batch conditions were carried out by systematic additions of different volumes of gluconic acid standard solutions at the selected potential ($E_{app} = 800$ mV vs. SCE). Although immobilization, Michaelis–Menten behaviour was still observed, but with a loss of signal due to the decrease of enzyme units contained in the Ps matrix. Also a good correlation was obtained when adjusting the non-linear model ($r=0.9996$), with apparent constants: $K_{M,app} = 0.52 \pm 0.05$ mM and

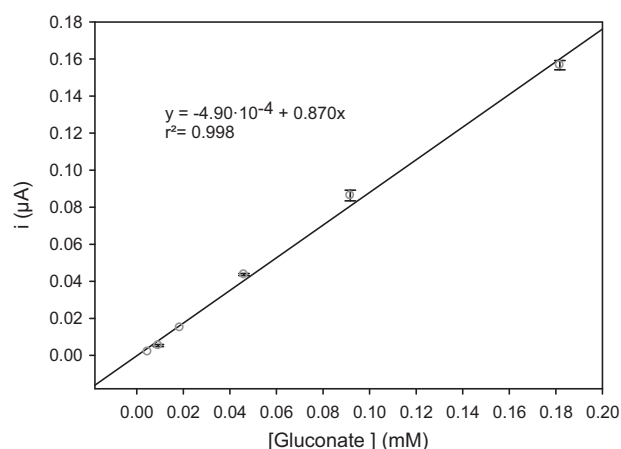


Fig. 4. Amperometric calibration curves obtained with different biosensors for different amounts of gluconate standards, between 4.5×10^{-6} M and 2.0×10^{-4} M. Error bars correspond to experiments carried with 3 different electrodes.

$\nu_{\max,app} = 0.614 \pm 0.020 \mu\text{A}$. If we compare the $K_{M,app}$ value obtained for the enzymes in solution and the one obtained for the immobilized system, there are no significant differences detected; this means that the affinity of the enzyme to the substrate was not affected by the immobilization procedure and that the enzymes preserved their activity. Satisfactory response was obtained for the biosensor and also a good reproducibility for the calibrations between different electrodes was available (Fig. 4); the obtained linear range (pseudo-first order zone) for gluconic acid was between 7×10^{-6} and 2.5×10^{-4} M (slope = $870 \pm 20 \mu\text{A M}^{-1}$, intercept = $-4.9 \times 10^{-4} \pm 1.69 \times 10^{-3} \mu\text{A}$ and $r^2 = 0.998$) and a RSD of 1.74% between the slope of different electrodes ($n = 3$). Although the lifetime is not very long (up to 5–7 calibrations; with a decrease in slope lower than 10%), due to the low RSD between different electrodes demonstrates a disposable scheme of use, without the need to perform calibration for each electrode.

In Table 1 biosensor response parameters are compared with the ones reported in the literature for gluconic acid determination. Comparing to the previous configurations, the first consideration that must be taken into account is that GADH is unfortunately no longer commercially available and that we are comparing a bienzymatic system with a single reaction system; also Michaelis–Menten constant refer to different systems. Applied potential is slightly higher in our case, given it is a different enzyme system and that no mediator is employed and it could be reduced with the use of Meldola Blue for example [22], but it has been demonstrated that this does not represent a problem when carrying real sample determination. Sensitivity is of the same order and linear range is wider satisfying food industry requirements. Finally reproducibility between different electrodes is very good and slightly better than that reported by the other authors.

The linear range of the biosensor was sensitive enough to allow a high dilution factor (approximately 1/50, reducing possible matrix effects) for the application in real wine samples; with these conditions, the determination could satisfy food industry requirements (5.0×10^{-4} to 2.5×10^{-3} M of gluconic acid in wine samples). There-

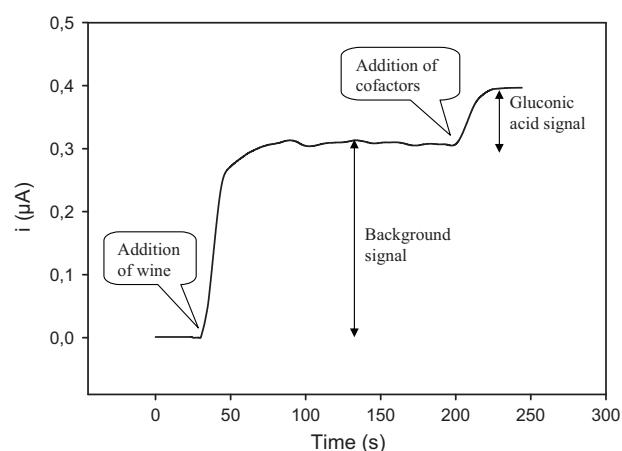


Fig. 5. Current–time recordings obtained from amperometric experiments. First increase of signal corresponds to wine addition, second increase corresponds to addition of cofactors and the start of the enzymatic reactions (incremental signal). Working potential: +0.800 V vs. SCE in 0.1 M phosphate buffer pH 7.50.

fore, the next step was to test the biosensor in the determination of gluconic acid in wine samples and to evaluate its results.

3.4. Application to real samples

The developed biosensor was used for the analysis of gluconic acid in wine samples under batch conditions as described above. Firstly we checked that no matrix effect was observed, so we compared the slope of a calibration graph obtained with the addition of gluconic acid in absence (typical calibration graph) and presence of wine sample (addition standard method). No significant differences were found in the slopes of the two calibration graphs, indicating that no matrix effect is present.

Thanks to this, real sample concentration could be simply calculated by interpolation of the corresponding amperometric signals into the calibration plot prepared by addition of different amounts of gluconic acid standards. The amperometric signal (incremental signal) is recorded as described in Section 2 in two steps: first the wine signal is recorded and afterwards cofactors are added generating an increased current due to gluconic acid present in wine; recorded signal is schematized in Fig. 5.

In these conditions, 9 white wine samples were analyzed taking three replicates for each sample. Results obtained with the biosensor were plotted vs. those obtained by the reference laboratory (INCAVI) in order to compare both methodologies and to validate biosensor results; in addition this comparison would be also useful to detect any interference effect if there were observed some lack of correlation between the two methodologies. Linear least-squares regression was fitted and the characteristic parameters were calculated, also theoretical line was plotted (Fig. 6). A satisfactory correlation is shown ($r^2 = 0.995$) with slope value near one (1.03 ± 0.04) and intercept near zero (0.01 ± 0.02), containing both theoretical 1.0 and 0.0 values in the confidence interval. These parameters show that there are no significant differences between the results obtained with both methodologies and validate the applicability of the developed biosensor for gluconic determination in wine samples; furthermore it also demonstrates the selectivity

Table 1
A comparison of analytical characteristics towards gluconic acid of amperometric biosensors.

Electrode	Enzyme	E_{det} vs. SCE (V)	$K_{M,app}$ (mM)	Sensitivity ($\mu\text{A M}^{-1}$)	Linear range (M)	RSD (%)	Ref.
TTF-GADH-MPA-AuE	GADH	+0.15	0.21	1995	6×10^{-7} to 2×10^{-5}	3.4	[7]
Film coated GADH-BQ CPE	GADH	+0.4	0.88	1170	1×10^{-4} to 4×10^{-3}	4.8	[6,8]
Epoxy-graphite-Ps	GK + 6PGDH	+0.8	0.52	870	7×10^{-6} to 2.5×10^{-4}	1.7	This work

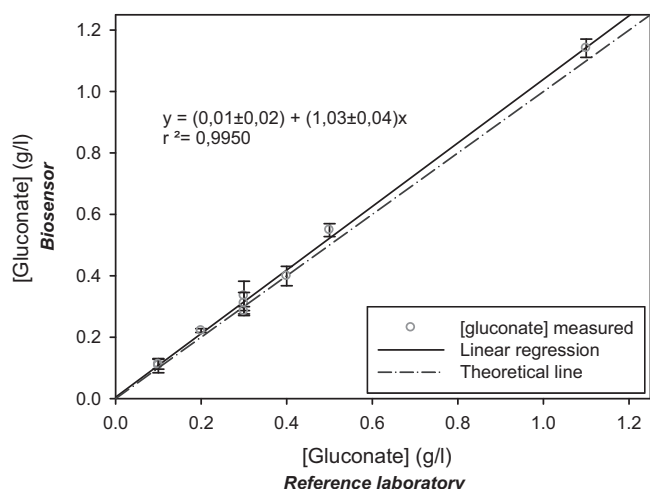


Fig. 6. Comparison of the results obtained with the biosensor (y axis) and the ones obtained by the reference laboratory (x axis). Error bars correspond to biosensor replicate analysis.

of the employed methodology towards gluconic acid given that despite all the possible interferents found in wines no interference effects were detected. Apart from reported wines, some red wines were also assayed, although they were not included here because they provided values too low for the reference methodology employed in the reference laboratory; but for the low values found, it seems they were not origin of any interference effect.

4. Conclusions

An alternative bienzymatic biosensor to quantify gluconic acid based on commercially available reagents is reported. It was successfully applied to quantify gluconic acid in real wine samples, validating biosensor performance through comparison of the results obtained with those obtained by a reference laboratory. Its performance characteristics may satisfy food industry requirements of precision, rapidity, sensitivity, simplicity and low cost required to be considered as a useful analytical tool. Hence, the biosensor-based methodology developed for the quantification of D-gluconate is suitable to be employed for rapid assessment of the quality of wines.

Further improvements may be to incorporate mediators into the biocomposite materials, or to reduce the working potential and analysis time, if a faster residual current stabilization might be attained. Also biosensors as described could be prepared using screen printed technology (SPE) and finally used in a disposable manner.

Acknowledgments

This work was partly supported by MICINN projects TEC2007-68012-C03-02 and CTQ2010-17099 and by program ICREA Academia. X. Cetó thanks the support of Dept. d'Innovació, Universitats i Empresa de la Generalitat de Catalunya for the predoctoral grant.

References

- [1] C. Mathias, F. Elisabeth, K. Caroline, L. Caroline, P. Jean-Marc, S. Adeline, V. Muriel, *FEMS Microbiol. Lett.* 277 (2007) 1–10.
- [2] R.A. Peinado, J.C. Mauricio, J.M. Ortega, M. Medina, J. Moreno, *Biotechnol. Lett.* 25 (2003) 1887–1891.
- [3] P. Ribéreau-Gayon, D. Dubourdieu, B. Donèche, A. Lonvaud, *Handbook of Enology, Volume 1, The Microbiology of Wine and Vinifications*, 2nd ed., Wiley-Blackwell, London, 2000.
- [4] OIV, *Recueil des méthodes internationales d'analyse des vins et des moûts*, Paris, 2005.
- [5] T. Tsukatani, K. Matsumoto, *Anal. Chim. Acta* 530 (2005) 221–225.
- [6] T. Ikeda, K. Miki, F. Fushimi, M. Senda, *Agric. Biol. Chem. Tokyo* 52 (1988) 1557–1563.
- [7] S. Campuzano, M. Gamella, B. Serra, A.J. Reviejo, J.M. Pingarrón, *J. Agric. Food Chem.* 55 (2007) 2109–2114.
- [8] T. Ikeda, S. Miyaoka, F. Matsushita, D. Kobayashi, M. Senda, *Chem. Lett.* (1992) 847–850.
- [9] M. Darder, E. Casero, F. Pariente, E. Lorenzo, *Anal. Chem.* 72 (2000) 3784–3792.
- [10] E. Shinagawa, K. Matsushita, O. Adachi, M. Ameyama, *Agric. Biol. Chem. Tokyo* 45 (1981) 1079–1085.
- [11] I. Mato, J.F. Huidobro, M.P. Sanchez, S. Muniategui, M.A. FernandezMuino, M.T. Sancho, *J. Agric. Food Chem.* 45 (1997) 3550–3553.
- [12] R. Larcher, G. Nicolini, T.R. Villegas, D. Bertoldi, C. Puecher, *Vitis* 48 (2009) 201–204.
- [13] A. Cherchi, L. Spanedda, C. Tuberioso, P. Cabras, *J. Chromatogr. A* 669 (1994) 59–64.
- [14] M. Cocchi, P. Lambertini, D. Manzini, A. Marchetti, A. Ulrici, *J. Agric. Food Chem.* 50 (2002) 5255–5261.
- [15] M.D.G. Moreno, R.C. Mejias, R.N. Marin, C.G. Barroso, *Eur. Food Res. Technol.* 215 (2002) 255–259.
- [16] I. Mato, J.F. Huidobro, J. Simal-Lozano, M.T. Sancho, *Anal. Chim. Acta* 565 (2006) 190–197.
- [17] M. Urbano-Cuadrado, M.D. Luque de Castro, P.M. Pérez-Juan, J. García-Olmo, M.A. Gómez-Nieto, *Anal. Chim. Acta* 527 (2004) 81–88.
- [18] F. Céspedes, E. Martínez-Fàbregas, S. Alegret, *TrAC-Trends Anal. Chem.* 15 (1996) 296–304.
- [19] J. Bryjak, M. Bryjak, A. Noworyta, *Enzyme Microb. Technol.* 19 (1996) 196–201.
- [20] L. Giorno, E. Drioli, G. Carvoli, A. Cassano, L. Donato, *Biotechnol. Bioeng.* 72 (2001) 77–84.
- [21] R. Olivé-Monllau, M. Baeza, J. Bartroli, F. Céspedes, *Electroanalysis* 21 (2009) 931–938.
- [22] B. Prieto-Simón, E. Fàbregas, *Biosens. Bioelectron.* 22 (2006) 131–137.
- [23] C.A. Smolders, A.J. Reuvers, R.M. Boom, I.M. Wienk, *J. Membr. Biol.* 73 (1992) 259–275.
- [24] H. Izu, O. Adachi, M. Yamada, *FEBS Lett.* 394 (1996) 14–16.
- [25] H.Z. Sable, A.J. Guarino, *J. Biol. Chem.* 196 (1952) 395–402.
- [26] C.J. Coffee, A.S.L. Hu, *Arch. Biochem. Biophys.* 149 (1972) 549–559.
- [27] J.M. Gutiérrez, L. Moreno-Barón, M.I. Pividori, S. Alegret, M. del Valle, *Microchim. Acta* 169 (2010) 261–268.
- [28] N. de los Santos Álvarez, P.M. Ortea, A.M. Pañeda, M.J.L. Castañón, A.J.M. Ordieres, P.T. Blanco, *J. Electroanal. Chem.* 502 (2001) 109–117.
- [29] C.G. Fu, L.N. Song, Y.Z. Fang, *Anal. Chim. Acta* 399 (1999) 259–263.