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Integrated Electrochemical Gluconic Acid Biosensor Based on Self-Assembled Monolayer-Modified Gold Electrodes. Application to the Analysis of Gluconic Acid in Musts and Wines

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An integrated amperometric gluconic acid biosensor constructed using a gold electrode (AuE) modified with a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA) on which gluconate dehydrogenase (GADH, 0.84 U) and the mediator tetrathiafulvalene (TTF, 1.5 μmol) were coimmobilized by covering the electrode surface with a dialysis membrane is reported. The working conditions selected were $E_{app} = +0.15$ V and 25 ± 1 °C. The useful lifetime of one single TTF-GADH-MPA-AuE was surprisingly long. After 53 days of continuous use, the biosensor exhibited 86% of the original sensitivity. A linear calibration plot was obtained for gluconic acid over the 6.0×10^{-7} to 2.0×10^{-5} M concentration range, with a limit of detection of 1.9×10^{-7} M. The effect of potential interferents (glucose, fructose, galactose, arabinose, and tartaric, citric, malic, ascorbic, gallic, and caffeic acids) on the biosensor response was evaluated. The behavior of the biosensor in a flow-injection system in connection with amperometric detection was tested. The analytical usefulness of the biosensor was evaluated by determining gluconic acid in wine and must samples, and the results obtained were validated by comparison with those provided by using a commercial enzyme test kit.

KEYWORDS: Self-assembled monolayers; enzyme biosensors; gluconic acid; wines

INTRODUCTION

Gluconic acid is one of the predominant organic acids found in honey, vinegars, and noble rot wine, which are produced from grapes infected with the fungi *Botrytis cinerea* (*1*). This infection, called gray rot disease, produces skin contraction and dehydration of grape berry, with the subsequent increase in polysaccharides concentration (sugar amounts up to 30−40° Brix are attained). *B. cinerea* produces fissures in the berry allowing proliferation of acetic acid bacteria (*Acetobacter* and *Gluconobacter*), which are fed by the sweet juice that escapes from the berry, thus increasing the gluconic acid concentration above 3 g/L and the volatile acidity of musts (*2*). Moreover, the contents of tartaric acid, malic acid, and available nitrogen decrease, and the concentration of glycerol increases in grapes affected by *B. cinerea* (*2*).

The gray rot disease develops during growth of grape berry and is dependent on meteorological factors, such as moisture and rainfall, and on physiological factors, such as grape variety, bunch shape, and viticultural practices (*3*). This disease causes substantial losses to wine farmers, as it decreases the quality and size of crops, and to winemakers, as a result of microbiologically induced changes in grape composition.

The rot extent affecting grapes is usually related to the gluconic acid concentration in the resulting must. Thus, concentrations above ∼1 g/L reflect a substantial proportion of rotten grapes. Recently, Couto et al. (*4*) have evidenced that levels of gluconic acid up to 1 g/L indicate an initial stage of grape infection by fungi, whereas higher levels (up to 2−3 g/L) might be taken as indication of the activity of acetic acid bacteria.

D-Gluconate has been usually determined by high performance liquid chromatography (*5*−*7*), gas chromatography (*8*), capillary electrophoresis (*9*), near-infrared reflectance spectroscopy (*10*), and enzyme assays with spectrophotometric (*11*) or fluorimetric determination (*1*), but these methods are time-consuming. Although some enzyme electrodes using D-gluconate dehydrogenase have been developed, they were not applied to determine gluconic acid in foodstuffs (*12*−*14*).

Recently, we have reported on the construction and performance of robust integrated amperometric enzyme biosensors in which the biomolecules were coimmobilized, together with the mediator tetrathiafulvalene (TTF), by cross-linking with glutaraldehyde atop 3-mercaptopropionic acid (MPA) self-assembled monolayers (SAMs) on gold disk electrodes (AuEs) (*15*, *16*).

Therefore, following a somewhat similar approach, we report, in this Article, the construction and performance of an integrated

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amperometric biosensor for gluconic acid. The design of the bioelectrode is based on a MPA-modified AuE on which TTF and gluconate dehydrogenase (GADH) were coimmobilized, in this case by covering the electrode surface with a dialysis membrane.

EXPERIMENTAL PROCEDURES

Apparatus and Electrodes. Amperometric measurements were performed on a Metrohm 641VA potentiostat connected to a Linseis L6521B recorder. A P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. Flow-injection (FI) experiments were carried out using a Gilson Minipuls-2 peristaltic pump and a Rheodyne model 5020 injection valve with variable injection volumes.

A BAS gold disk electrode (3-mm ϕ) was used as electrode substrate to be coated with the modified 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 in batch experiments, and a large volume (50 mL) home-made glass wall-jet cell was employed for flow-injection measurements.

Reagents and Solutions. Stock 0.1 M sodium gluconate (Sigma) solutions were prepared in 0.05 M phosphate buffer of pH 6.0 (*17*). More dilute standards were prepared by suitable dilution with the same phosphate buffer solution, which was also used as the supporting electrolyte both in batch and in flow-injection measurements.

A 40 mM mercaptopropionic acid (MPA) (Research Chemicals Ltd.) solution, prepared in a 75/25% v/v ethanol/water mixture, was employed for the formation of the monolayers. A 0.28 U μL^{-1} GADH solution (from *Pseudomonas sp.* Sigma, NAD(P) independent, EC 1.1.99.3) was used for the enzyme immobilization, and a 0.5 M tetrathiafulvalene (TTF, Aldrich) solution in acetone was used for mediator immobilization. Dialysis membranes (10 K MWCO) were purchased from Cultek.

Other solutions employed for the interference study were as follows: a 2 M KOH (Panreac) aqueous solution, and 0.5 M stock solutions of ascorbic acid (Fluka), citric acid (Merck), tartaric acid (Merck), L-malic acid (Merck), D-glucose (Panreac), D-fructose, D-galactose, gallic and caffeic acids (Sigma), and L-arabinose (BDH) prepared in 0.05 M phosphate buffer of pH 6.0.

All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system.

Moreover, a D-gluconic acid/D-glucono-δ-lactone test combination kit (10428191035 (R-Biopharm)) was used to compare the results obtained in the analysis of gluconic acid in real samples.

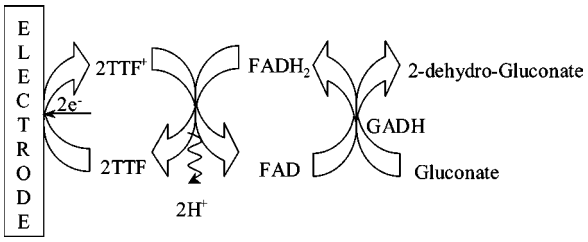
Procedures. Before the SAM deposition, the gold disk electrode was pretreated as described previously (*15*). MPA-SAMs were formed by immersion of the clean AuE in a 40 mmol L^{-1} MPA solution in EtOH/H_2O (75/25, v/v) for at least 15 h (*15*). Next, the modified electrode was rinsed with deionized water and dried with an argon stream.

Coimmobilization of the enzyme and the mediator was carried out as follows: 3 μL of a 0.28 U μL^{-1} GADH solution was deposited on the SAM-modified AuE. Once the electrode surface had dried at ambient temperature, a 3-μL aliquot of the 0.5 M TTF solution was deposited onto it and allowed to dry again. Next, a 1.5-cm^2 piece of a dialysis membrane was fixed on top of the electrode surface and secured with an O-ring. The use of the dialysis membrane gave rise to a more stable coimmobilization of the enzyme and the mediator on the modified AuE than that obtained using other immobilization approaches such as cross-linking with glutaraldehyde. In this later case, significant loss of enzyme was observed after immersion of the biosensor in the solutions.

Amperometric measurements were performed by applying in all cases a potential of +150 mV (vs Ag/AgCl). The carrier stream for FI experiments was a 0.05 M phosphate buffer of pH 6.0, with a flow rate of 0.2 mL min^{-1}.

Sample Treatment. Analysis of gluconic acid in wines and musts required no sample treatment when the amperometric batch mode was employed. So, 10 μL of sample was added to the electrochemical cell containing 5.0 mL of supporting electrolyte, and the amperometric measurements were carried out. When the FI methodology was employed, only an appropriate dilution with the supporting electrolyte was needed, and 150 μL of sample solutions previously diluted 100 times were injected in the carrier solution.

Scheme 1. Schematic Diagram Displaying the Enzyme and Electrode Reactions Involved in the Gluconic Acid Determination at a TTF-GADH-MPA-AuE



As no matrix effect was observed for the samples analyzed, the gluconic acid concentration was calculated by interpolation of the amperometric signal from the sample solutions into a calibration graph constructed with standard gluconic acid solutions in the 2.0×10^{-6} to 1.0×10^{-5} M concentration range when amperometry in stirred solution was used, and in the 2.0×10^{-5} to 1.0×10^{-4} M range when the FI system was employed.

In all cases, the obtained results were compared to those obtained with a commercial enzyme kit from R-Biopharm using spectrophotometric detection. This method is based on the phosphorylation of gluconate by gluconate kinase in the presence of ATP; then the 6-phospho-D-gluconate produced was oxidized with $NADP^+$ by 6-phosphogluconate dehydrogenase. The reduced form of NADP, NADPH, formed in this oxidation reaction is measured spectrophotometrically at 340 nm. The results were also compared to those obtained using the "LISA 200 Wine Analyzer System", a fully automatic analyzer that performs both colorimetric and enzymatic determinations, including acids (acetic, malic, lactic, citric, gluconic), sugars (glucose, fructose, saccharose), and other constituents.

RESULTS AND DISCUSSION

The biocatalytic scheme used for the determination of gluconic acid is displayed in **Scheme 1**. This scheme involved the oxidation of gluconic acid to 2-dehydro-gluconate catalyzed by GADH, which was simultaneously reduced to the reduced form GADH-$FADH_2$. The GADH-$FADH_2$ is reoxidized by TTF^+, and the generated TTF is amperometrically oxidized at the modified electrode, with the resulting current being dependent on the gluconic acid concentration.

Optimization of Variables. The optimization of experimental variables concerning the behavior of the biosensor was accomplished by amperometry in stirred solutions.

Considering that the TTF loading was optimized in previous works (*15*, *16*), only the influence of the GADH loading was checked concerning the biosensor composition. As it can be seen in **Figure 1**, the amperometric response of the biosensor, measured at +0.15 V, increased notably with GADH loading up to a value of 0.84 U, showing even a slight decrease for higher loadings. Consequently, the selected composition of the bioelectrode for further work was 0.84 U of GADH and 1.5 μmol of TTF.

Once the composition of the biosensor was chosen, the influence of the applied potential on the gluconic acid amperometric response was tested. **Figure 2** shows the results obtained for a gluconic acid concentration of 2.0×10^{-5} M. A catalytic effect could be observed at the TTF-GADH-MPA-AuE in the potential range between 0.00 and +0.50 V, with a maximum limiting current at +0.15 V. Moreover, no amperometric signal was found in the whole potential range when no enzyme was immobilized on the electrode, and also no response for gluconic acid was obtained at a GADH-MPA-AuE, thus indicating that

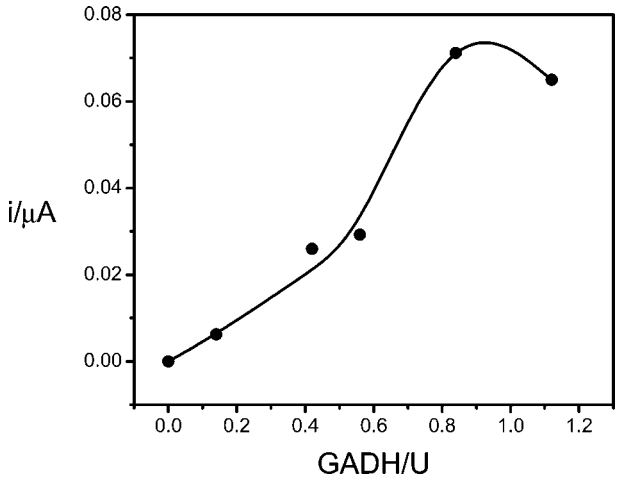


Figure 1. Effect of the GADH loading immobilized atop the MPA-AuE (using a constant TTF amount of 1.5 μmol) on the amperometric signal obtained for a 4.0×10^{-5} M gluconic acid solution. Supporting electrolyte: 0.05 M phosphate buffer (pH 6.0). $E_{app} = +0.15$ V.

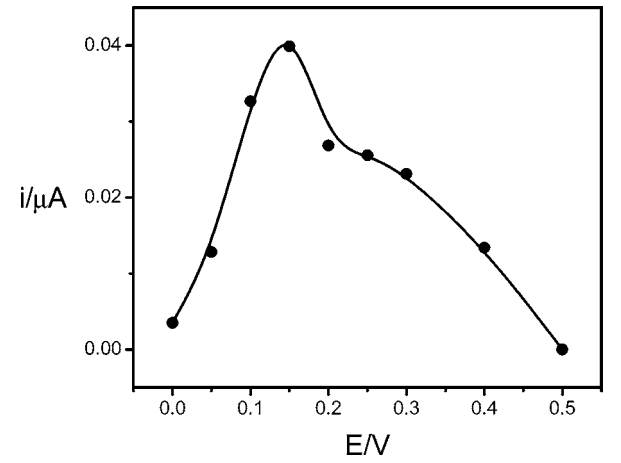


Figure 2. Effect of the applied potential on the amperometric signal of 2.0×10^{-5} M gluconic acid in a 0.05 M phosphate buffer solution (pH 6.0), at a TTF-GADH-MPA-AuE.

no direct oxidation of $FADH_2$ occurred with this biosensor design and that the transport of electrons was made from the enzyme to the electrode surface through the mediator. The decrease of the amperometric response above +0.15 V is in agreement with similar behaviors reported for biosensors using TTF as mediator (*15*−*19*) and can be attributed to a leakage of TTF from the electrode surface at more positive potentials, induced after irreversible oxidation of TTF^+ to TTF^{2+}, which is soluble in aqueous solutions and descomposes. An applied potential of +0.15 V was selected for further work to obtain the highest sensitivity.

Stability of the Gluconic Acid Amperometric Biosensor. 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 biosensor were considered.

First, the repeatability of the measurements was evaluated by constructing 10 successive calibration plots for gluconic acid in the 2.0×10^{-6} to 1.0×10^{-5} M concentration range with the same biosensor. A relative standard deviation (RSD) value of 3.4% was obtained for the slope values of such calibration plots, indicating a good repeatability of the measurements with no need of cleaning or pretreatment procedure to the biosensor. Moreover, a RSD of 4.2% was obtained for the steady-state current corresponding to 10 repetitive measurements of 5.0×10^{-6} M gluconic acid.

Second, the reproducibility of the responses obtained with different TTF-GADH-MPA-AuE was checked. Results for five different bioelectrodes yielded a RSD of 7.4% for the slope values of the corresponding calibration plots for gluconic acid in the 2.0×10^{-6} to 1.0×10^{-5} M concentration range. This clearly demonstrated that the fabrication procedure of the TTF-GADH-MPA-AuE was reliable, thus allowing reproducible electroanalytical responses to be obtained with different biosensors constructed in the same manner.

Finally, the useful lifetime of one single TTF-GADH-MPA-AuE was checked by performing daily calibration graphs for gluconate in the above-mentioned concentration range. After use, the biosensor was stored in phosphate buffer of pH 6.0 at 4 °C. After 53 days of continuous use, the biosensor still exhibited 86% of the original sensitivity. However, a 38% decrease in sensitivity was observed on the 66th day. This decrease can be attributed to the denaturation of some of the immobilized enzyme molecules.

Kinetic Constants and Analytical Characteristics. The enzyme reaction for gluconic acid at the TTF-GADH-MPA-AuE obeyed a Michaelis−Menten behavior. Thus, a linear log-$[(i_{max}/i) - 1]$ versus log[gluconic acid] graph was obtained with a slope value close to 1 (0.97 ± 0.03), indicating that the immobilization procedure did not alter the Michaelis−Menten-type kinetics. Next, calculation of the apparent Michaelis−Menten constant and the maximum rate of the reaction was accomplished from the Lineweaver−Burk plot. Values of K_M^{app} $(2.1 \pm 0.1) \times 10^{-4}$ M and $V_{max} = 0.57 \pm 0.01$ μA were obtained, with the confidence intervals calculated for a significance level of 0.05 ($n = 3$). The K_M^{app} value for the TTF-GADH-MPA-AuE was even smaller than that reported for the GADH reaction in solution (0.8−2.3 mM) (*14*), which indicates that the affinity of the enzyme to the substrate is practically non-affected by the immobilization procedure used (*20*).

Under the optimized working conditions, a typical calibration curve for an enzyme system was obtained for gluconic acid. A linear calibration graph was obtained over the 6.0×10^{-7} to 2.0×10^{-5} M concentration range ($r = 0.999$, slope $= 1995 \pm 31$ μA M^{-1}, intercept $= (7 \pm 1) \times 10^{-4}$ μA). The limits of detection and determination were calculated, according to the $3s_b/m$ and 10 s criteria, respectively, where m is the slope of the linear calibration plot and s_b was estimated as the standard deviation ($n = 10$) of the amperometric signals from 2.0×10^{-7} M gluconic acid. These values were 1.9×10^{-7} and 6.4×10^{-7} M, respectively.

Interference Study. Musts and wines contain various kinds of components that may affect the biosensor response. In particular, a high concentration of sugars, and of some organic acids (tartaric (2−6 g/L), malic (1−6.5 g/L), citric (0.1−1 g/L), and ascorbic acids) in musts, may be considered as sources of potential interferences. Furthermore, catechol- and galloyl-containing polyphenols are also potential interferents in wines. Therefore, the influence of some of these compounds on the quantification of D-gluconate was investigated under the experimental conditions specified above. The substances tested were glucose, fructose, galactose, arabinose, and ascorbic, citric, malic, and tartaric acids, as well as caffeic acid, to represent the hydroxycinnamates in white wines, and gallic acid, to represent galloyl-containing polyphenols. **Figure 3** compares the amperograms obtained for the addition of 20 μL of a 0.01 M gluconic acid solution to the electrochemical cell with those obtained after adding the different potential interferents. Caffeic

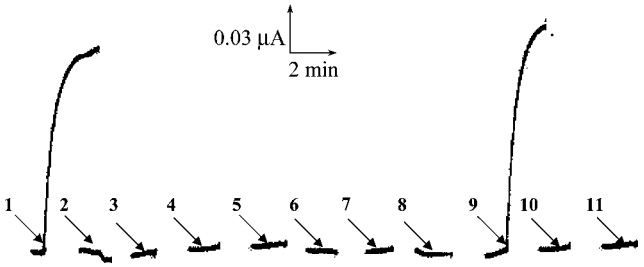


Figure 3. Amperograms obtained after additions of 20 μL of a 0.01 M gluconic acid solution (1) and of 0.01 M solutions of glucose (2), fructose (3), arabinose (4), galactose (5), tartaric acid (6), citric acid (7), malic acid (8), and ascorbic acid (9), as well as after additions of 20 μL of a 13 mg/L caffeic acid solution (10) and 126 mg/L gallic acid solution (11), in 0.05 M phosphate buffer solution of pH 6.0; $E_{app} = +0.15$ V.

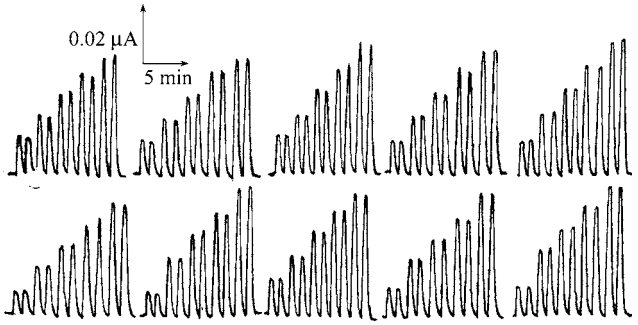


Figure 4. Amperometric responses at the TTF-GADH-MPA-AuE from 10 successive calibration plots for gluconic acid in the 2.0×10^{-5} to 1.0×10^{-4} M concentration range under flow-injection conditions. Carrier solution, 0.05 M phosphate buffer of pH 6.0; flow rate, 0.2 mL min^{-1}; $V_i = 150$ μL; $E_{app} = +0.15$ V.

and gallic acids were added at the highest concentrations found in wines for these compounds (13 and 126 mg/L, respectively) (*21*). As can be observed, among all of these compounds, only ascorbic acid yielded a significant amperometric response under the working conditions, 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 process, as it was demonstrated by recording cyclic voltammograms at Au and MPA-Au electrodes, it has been reported that the presence of TTF catalyzes the ascorbic acid oxidation (*22*), and therefore a significant interference was produced. Nevertheless, it is important to remark that the content of gluconic acid 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 the proposed real samples.

Flow-Injection with Amperometric Detection at the TTF-GADH-MPA-AuE. The good performance exhibited by the SAM-based gluconic acid biosensor in the batch mode led us to evaluate its behavior in flow-injection systems in connection with amperometric detection. The carrier solution employed was the 0.05 M phosphate buffer of pH 6.0, used in the batch measurements as supporting electrolyte.

Characteristic flow-injection parameters, such as flow rate and sample volume injected, were first optimized using a detection potential of +0.15 V. Regarding the flow rate effect, the usual behavior for enzyme-based FIA was found, with a decrease of the flow-injection peak height for relatively high flow rates, which is expected if the response time of the biosensor is not very short and the sample plug passes faster (*23*). A higher $i_p/W_{1/2}$ ratio, where i_p is the FI peak height and $W_{1/2}$ is the peak width at half-height, was obtained for a flow rate of 0.2 mL min^{-1} and an injection volume of 150 μL.

Selection of the detection potential was carried out by checking the i_p values over the 0.00 to +0.50 V range. A behavior similar to that depicted in **Figure 2** for batch conditions was obtained, and, therefore, we decided to use the same detection potential, +0.15 V, for further work.

Different aspects concerning the stability of the TTF-GADH-MPA-AuE under these flow conditions were also tested. The repeatability of the FI amperometric measurements was evaluated by constructing 10 successive calibration plots for gluconic acid in the 2.0×10^{-5} to 1.0×10^{-4} M concentration range, with the same electrode (**Figure 4**). A RSD value of 5.4% was obtained for the slopes of these calibration plots. Furthermore, a series of 20 repetitive injections of a 5.0×10^{-5} M gluconic acid solution yielded a RSD value for i_p of 2.0%. These results demonstrated a good stability of both the enzyme and the mediator on the SAM-modified electrode despite the hydrodynamic conditions. According to this diagram, when the TTF-GADH-MPA-AuE is employed as an amperometric detector in a FIA system, a sampling frequency of 15 samples/h is possible.

A linear calibration plot was obtained over the 7.0×10^{-7} to 1.0×10^{-4} M concentration range ($r = 0.998$, slope 400 ± 9 μA M^{-1}, intercept 1.1 ± 0.4 μA). The limits of detection and determination, calculated according to the same criteria mentioned above, were 6.3×10^{-7} and 2.1×10^{-6} M, respectively.

Analysis of Real Samples. The developed biosensor was used for the analysis of gluconic acid in must and wine samples by applying both the batch amperometry and the flow-injection with amperometric detection methodologies.

As indicated in the experimental section, no matrix effect was observed for any of these samples. This was confirmed by comparing statistically (by applying Student's *t*-test) the slope values of the calibration graphs obtained by application of the standard additions methods to must and wine samples (1130 ± 75 and 1255 ± 65 μA M^{-1}, respectively) with the slope value of the calibration graph prepared with gluconid acid standards (1195 ± 31 μA M^{-1}). Therefore, the sample analysis was accomplished by interpolation of the corresponding amperometric signals into the appropriate calibration plots.

The batch amperometric method in stirred solutions was applied to the determination of gluconic acid in 44 different types of wine and 98 musts samples. Some of the wine samples contained sulfite added as stabilizer. The results obtained in this study were compared to those obtained by applying both the R-Biopharm test kit and the "Lisa 200" wine analyzer (**Figure 5**). This comparison was made by plotting the biosensor results versus those obtained for 83 of the 98 must samples, and for 30 of the 44 wine samples, with the LISA 200 analyzer (**Figure 5a** and **c**, respectively), as well as versus those obtained for 15 must and 14 wine samples when the enzymatic kit was employed (**Figure 5b** and **d**, respectively). Furthermore, a plot of all of the biosensor results versus both reference methods was also constructed. The characteristic parameters of the corresponding linear least-squares regression curves are summarized in **Table 1**. As can be seen in all cases, the correlation between the results is highly satisfactory, and the values found fall within the ranges reported as usual for musts and wines.

Furthermore, the FI methodology was also applied for the analysis of some of the same samples analyzed by the batch mode (6 must samples and 2 wine samples). The results obtained by FIA agreed fairly well with those achieved by applying the batch mode. The parameters of the correlation plot obtained

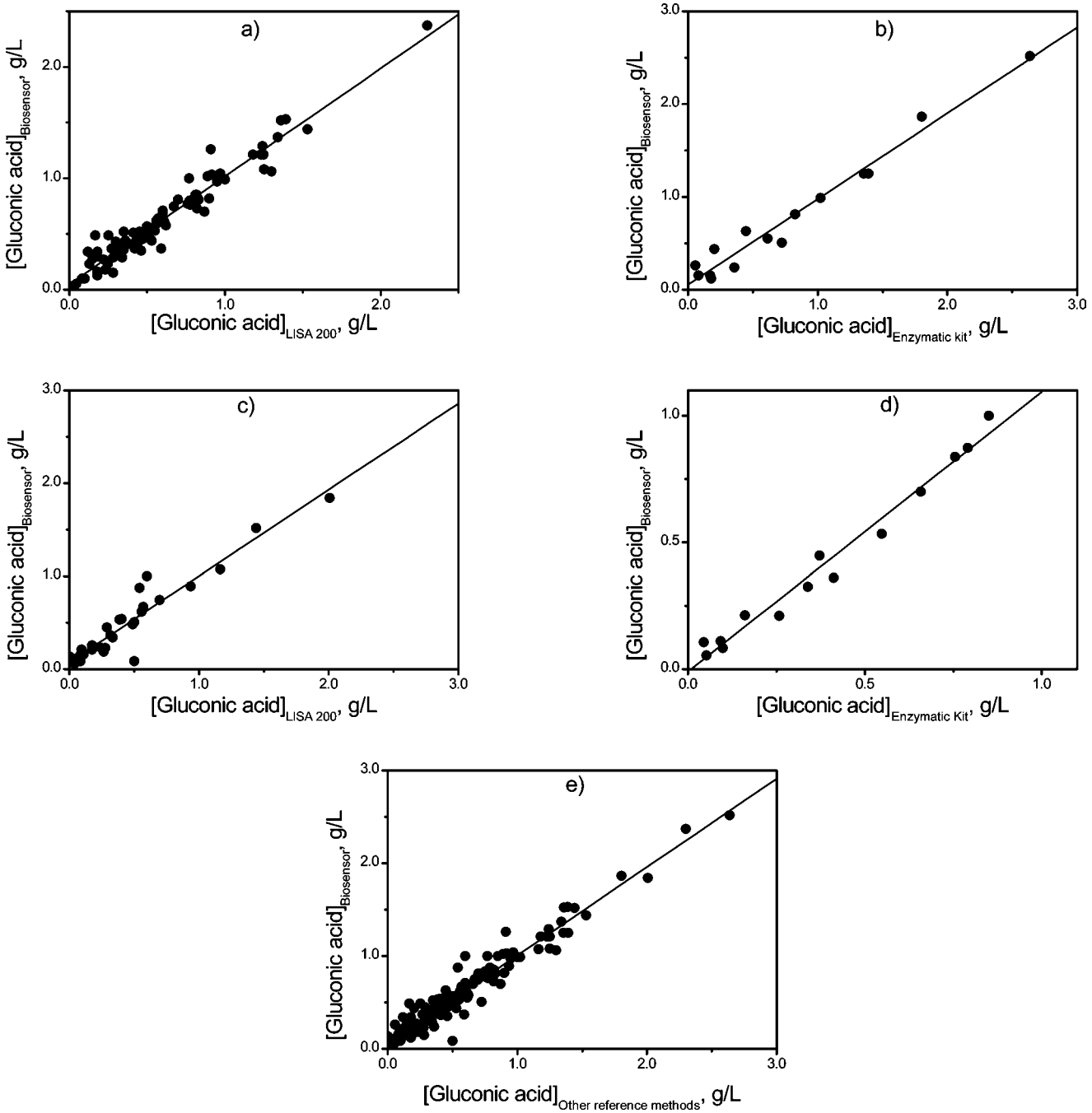


Figure 5. Comparison of the results obtained with the biosensor using the batch amperometric mode and those obtained with (**a**) "LISA 200" wine analyzer for musts samples; (**b**) the commercial R-Biopharm test kit for must samples; (**c**) "LISA 200" wine analyzer for wine samples; (**d**) R-Biopharm test kit for wine samples; and (**e**) applying both "LISA 200" wine analyzer and R-Biopharm test kit to all of the samples analyzed.

for all of the tested samples were slope = 1.0 ± 0.2, intercept = −0.0 ± 0.2, and r = 0.986. Another correlation plot was constructed against data obtained by using the R-Biopharm test kit, and the parameters found were slope = 1.0 ± 0.1, intercept = −0.0 ± 0.1, and r = 0.994. These parameters show clearly that there were no significant differences between the results obtained by applying the different methodologies used.

In conclusion, it can be said that the results described above demonstrate fairly well that the use of an integrated gluconate dehydrogenase-tetrathiafulvalene amperometric biosensor accomplishes the requirements of precision, rapidity, sensitivity, simplicity, and low cost required to be considered as a useful analytical tool for the food industry, and the methodologies

Table 1. Characteristic Parameters of the Linear Least-Squares Regression Curves Displayed in **Figure 5**

representation	slope	intercept	r
musts: biosensor vs LISA 200 (**Figure 5a**) (n = 83)	0.97 ± 0.06	0.05 ± 0.04	0.969
musts: biosensor vs enzymatic kit (**Figure 5b**) (n = 15)	0.9 ± 0.1	0.1 ± 0.1	0.984
wines: biosensor vs LISA 200 (**Figure 5c**) (n = 30)	0.9 ± 0.1	0.07 ± 0.08	0.949
wines: biosensor vs enzymatic kit (**Figure 5d**) (n = 14)	1.1 ± 0.1	–0.01 ± 0.04	0.988
all samples: biosensor vs both reference methods (**Figure 5e**) (n = 142)	0.95 ± 0.04	0.06 ± 0.03	0.970

developed for the quantification of D-gluconate are suitable to be employed for rapid judging of the quality of musts and wines.

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