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Integrated multienzyme electrochemical biosensors for monitoring malolactic fermentation in wines

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ABSTRACT

Integrated amperometric biosensors for the determination of L-malic and L-lactic acids were developed by coimmobilization of the enzymes L-malate dehydrogenase (MDH) and diaphorase (DP), or L-lactate oxidase (LOX) and horseradish peroxidase (HRP), respectively, together with the redox mediator tetrathiafulvalene (TTF), on a 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold electrode by using a dialysis membrane. The electrochemical oxidation of TTF at +100 mV (vs. Ag/AgCl), and the reduction of TTF^+ at −50 mV were used for the monitoring of the enzyme reactions involved in L-malic and L-lactic acid determinations, respectively. Experimental variables concerning the biosensors composition and the detection conditions were optimized for each biosensor. Good relative standard deviation values were obtained in both cases for the measurements carried out with the same biosensor, with no need of cleaning or pretreatment of the bioelectrodes surface, and with different biosensors constructed in the same manner. After 7 days of continuous use, the MDH/DP biosensor still exhibited 90% of the original sensitivity, while the LOX/HRP biosensor yielded a 91% of the original response after 5 days. Calibration graphs for L-malic and L-lactic were obtained with linear ranges of 5.2×10^{-7} to 2.0×10^{-5} and 4.2×10^{-7} to 2.0×10^{-5} M, respectively. The calculated detection limits were 5.2×10^{-7} and 4.2×10^{-7} M, respectively. The biosensors exhibited a high selectivity with no significant interferences. They were applied to monitor malolactic fermentation (MLF) induced by inoculation of *Lactobacillus plantarum* CECT 748^T into a synthetic wine. Samples collected during MLF were assayed for L-malic and L-lactic acids, and the results obtained with the biosensors exhibited a very good correlation when plotted against those obtained by using commercial enzymatic kits.

1. Introduction

From earliest development, wine has a special place in our customs, diet and social gatherings. The understanding of the complex transformation of the grape must into wine allows the producers to monitor and control the different steps of this process in order to obtain more refined products. The winemaking process includes alcoholic fermentation conducted by yeast and a secondary fermentation performed by lactic acid bacteria, called malolactic fermentation (MLF) [1,2]. During this latter process, which most commonly occurs after completion of alcoholic fermentation, L-malic acid is converted into L-lactic acid and CO_2 (so hence the term, fermentation) [3,4]. The transformation from a diacid (malic acid) to a monoacid (lactic acid) influences the quality and taste of wines [5]. In addition to the deacidification, this second fermentation is considered to contribute to the complexity of the flavour, to influence on the final taste, and to confer a degree of microbiological stability to the wine [4]. Depending on the wine type, the climatic zone of production or the requirements for commercialization [6], this process should be either avoided, controlled or even encouraged [2,7]. For these reasons, the determination of L-malic and/or L-lactic acids in wines and during the MLF is of great interest and can be considered as a real "quality test" [6], necessary to allow the winemaker to take the proper decisions. These determinations are frequently performed in oenological laboratories [2,8,9]. MLF is conducted by lactic acid bacteria (LAB), mainly *Oenococcus oeni*, and some of the *Lactobacillus* (*L. plantarum*, *L. brevis*, *L. hilgardii*, *L. buchneri*, etc.), *Pediococcus* (*P. pentosaceus*), and *Leuconostoc* genera.

In recent years, starter culture technologies involving the inoculation of lactic bacteria into wine have been developed for managing the MLF [4]. However, failures usually occur because of the lack of adaptation of the cultures to wine, or because of cellular damage during storage of the commercial malolactic bacteria. Thus, it is desirable to supply winemakers with new simple, rapid and low cost analytical systems to monitor MLF and to establish the best strategy for its management [4].

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Monitoring of MLF is usually carried out by measuring the total acidity and the volatile acidity of wine [10], by means of chromatography methods [11] or by electrophoresis [12]. These methods are not adapted to the competences and financial constrains of small winemakers [2].

The most common methods for the determination of malic and lactic acids are based on the use of enzymes [13–20], including the official methods based on the reaction of lactic and malic acid with nicotinamide adenine dinucleotide coenzyme (β-NAD^+), catalyzed by malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) [9,21,22]. Enzymatic methods imply the use of more than one enzymatic pathway and require tedious sample treatment and the preparation and consumption of many reagents. Biosensors, in particular amperometric biosensors offer fast, cheap and smart easy-to-handle devices able to detect selectively and quantify L-malic and L-lactic acids in real time and in situ. They can be envisaged as serious competitors for conventional techniques, representing an attractive alternative for small industries [2,5,6,23].

In our group, we demonstrated that it is possible to construct robust integrated amperometric biosensors by co-immobilizing the biomolecules with the mediator TTF on 3-mercaptopropionic acid (MPA) self-assembled monolayer modified gold electrodes [24–26]. Therefore, a related approach for the construction of integrated amperometric biosensors for the determination of L-malic and L-lactic acids is reported in this article. In the case of lactate bioelectrode, the device implies coimmobilization of L-lactate oxidase (LOX) and horseradish peroxidase (HRP). The malate bioelectrode is based on the coupling of L-malate dehydrogenase (MDH) and diaphorase (DP). These enzymes were co-immobilized together with TTF on MPA-modified gold electrodes by using a dialysis membrane. The analytical performance of the developed biosensors was evaluated, and they were successfully applied to the quantification of both analytes during MLF induced by inoculation of *L. plantarum* in synthetic wine samples prepared in our laboratory, demonstrating their potential usefulness for oenological measurements.

2. Experimental

2.1. Apparatus and electrodes

Amperometric measurements were performed on a BAS LC-4C amperometric detector connected to a Linseis L6512 recorder. A Varian Cary-3 Bio UV-visible absorption spectrophotometer, a HONO stove, a Trade Raypa AES-75 autoclave, a P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used.

XBAS-NS-AU gold disk electrodes ($\phi \sim 3$ mm) were used as electrode substrates to be modified. A BAS MF-2052 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 the experiments.

2.2. Reagents and solutions

Buffer solutions were prepared daily. A 5.0 mM NAD^+ (Sigma) in 0.05 M phosphate buffer of pH 7.0 was employed with the MDH/DP biosensor, while pH 6.5 0.05 M phosphate buffer solution was used with the LOX/HRP biosensor. Stock 0.01 M L-malic (Sigma) or L-lactic (Scharlau) acid solutions were prepared in the corresponding buffer solution mentioned above. More dilute standards were prepared by suitable dilution with the same buffer.

A 40 mM mercaptopropionic acid (Research Chemicals Ltd.) solution, prepared in a 75/25% (v/v) ethanol/water mixture, was employed for the formation of the monolayers.

For the preparation of the MDH/DP biosensors, a 2.0 U μL^{-1} MDH solution (from *Thermus flavus*, EC 1.1.1.37, Sigma) and a 0.54 U μL^{-1} DP solution (from *Clostridium kluyveri*, EC 1.8.1.4, Sigma) prepared in phosphate buffer of pH 7.0 were used. In the case of LOX/HRP biosensors, a 1.0 U μL^{-1} LOX solution (from *Pediococcus* sp., EC 1.1.3.2, Sigma) and a 12.1 U μL^{-1} HRP solution (Type II from *Horseradish*, EC 1.11.1.7, Sigma) prepared in phosphate buffer of pH 6.5 were employed. A 0.5 M TTF (Aldrich) solution in acetone was used with both biosensors. Dialysis membranes (10K MWCO) were purchased from Cultek®.

Other solutions employed were: a 2 M KOH (Panreac) aqueous solution; 0.01 M stock solutions of sodium gluconate (Sigma), ethanol and glycerol (Scharlab), D-lactic acid (Sigma), ascorbic acid (Fluka), citric acid and acetic acid (Sigma), tartaric acid (Fluka), D-glucose (Panreac), D-fructose and D-galactose (Sigma), and L-arabinose (BDH) prepared in the corresponding buffer solution.

All chemicals used were of analytical-reagent grade, and water was obtained from a Millipore Milli-Q purification system. Moreover, L-malic and L-lactic acid spectrophotometric-enzymatic kits (Enzyplus®) were used in order to compare the results obtained for the determination of both analytes with the developed biosensors.

Other reagents used were: sodium chloride (Sigma), ammonium sulphate (Panreac), magnesium sulphate (Sigma), manganese sulphate heptahydrate (Sigma), disodium phosphate (Sigma) and yeast extract (Scharlau).

L. plantarum CECT 748^T was purchased from the Spanish Type Culture Collection (CECT). The Man Rogosa Sharpe (MRS) medium (Scharlau) was used for *L. plantarum* growth.

2.3. Procedures

Before carrying out the deposition of the MPA monolayer, the gold disk electrodes (AuE) were pretreated as described previously [24]. MPA SAMs were formed by immersion of the clean AuE in a 40 mM MPA solution in $EtOH/H_2O$ (75/25, v/v) for at least 15 h. Then, the modified electrode was rinsed with deionized water and dried with an argon stream. The enzymes and the mediator were immobilized onto the MPA-modified AuE as follows:

(a) *MDH/DP biosensor*: A 3-μL aliquot of the 0.5 M TTF solution was dropped on the modified electrode surface and let to dry at room temperature. Then, a 3-μL aliquot of the 0.54 U μL^{-1} DP solution, and 2 μL of the 2.0 U μL^{-1} MDH solution were sequentially casted on the electrode surface, allowing drying in between.

(b) *LOX/HRP biosensor*: A 2-μL aliquot of the 0.5 M TTF solution was deposited on the SAM-modified AuE. Once the electrode surface had dried at room temperature, a 2-μL aliquot of the 12.1 U μL^{-1} HRP solution was deposited on and let to dry again. Finally, 2 μL of the 1.0 U μL^{-1} LOX solution were dropped on the modified electrode surface and dried at room temperature.

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

Amperometric measurements were performed by applying a potential of +100 mV (vs. Ag/AgCl) when working with MDH/DP biosensors, and of −50 mV in the case of LOX/HRP biosensors. The working media consisted of a 0.05 M phosphate buffer solution of pH 7.0 containing 5 mM NAD^+ for malic acid determination, and a 0.05 M phosphate buffer solution of pH 6.5 for lactic acid measurements.

2.4. MLF

2.4.1. Synthetic wine samples preparation

The synthetic wine contained: tartaric acid (5 g L^{-1}), L-malic acid (3.5 g L^{-1}), glucose (2.0 g L^{-1}), fructose (2.0 g L^{-1}),

sodium chloride (0.2 g L^{-1}), ammonium sulphate (1.0 g L^{-1}), disodium phosphate (2.0 g L^{-1}), magnesium sulphate heptahydrate (0.2 g L^{-1}), manganese sulphate (0.05 g L^{-1}), ethanol (0.05 g L^{-1} <> 6.3%), acetic acid (0.05 g L^{-1}) and yeast extract (2.0 g L^{-1}) [27].

To prepare it, the amount needed for each compound was weighed and dissolved in deionized water, the pH was adjusted to 3.4 with KOH, and the mixture sterilized in an autoclave for 50 min at a pressure of 0.50 kg cm^{-2}. Once sterile, the mixture is cooled, the necessary volume of commercial ethanol and acetic acid (previously filtered with nylon filters of 0.20 μm) added and led to 1 L with sterile water.

2.4.2. Bacteria growth conditions

L. plantarum cultures were grown overnight in MRS broth at 30 °C, which allowed the growing stationary phase to be reached. Appropriated dilutions of the overnight culture were used to inoculate the synthetic wine to perform the MLF. Counting of colonies was carried out by the serial dilution method in MRS plates, which were incubated at 30 °C during 2 days.

2.4.3. Wine inoculation

MLF microvinifications were performed in our laboratory using the synthetic wine described above. This synthetic wine contained 3.5 g L^{-1} malic acid, 0.05 g L^{-1} ethanol and its pH value was 3.4. The MLF fermentation process was carried out by inoculating the wine with *L. plantarum* CECT 784^T.

To carry out the process, 200 mL of wine were transferred to 250 mL volumetric flasks and inoculated with two concentration levels of bacteria (1.0×10^5 and 1.0×10^6 cfu mL^{-1}) using an overnight *L. plantarum* culture.

The effect of temperature on the malolactic conversion was evaluated by testing two batches, one at cellar temperature, 18 °C, and another one at 30 °C. Each batch was run in duplicate. A batch of the wine without bacteria was also prepared at both temperatures as sample controls.

Aliquots (1 mL) of wine batches (with and without bacteria) were collected every day until the end of the MLF process. Samples collected prior to the inoculation of the wine were also taken. The collected samples were assayed for viable bacterial counts. Subsequently, they were centrifuged to remove the lactic acid bacteria, and were assayed for L-malic and L-lactic acids determination using both biosensor and spectrophotometric kits.

2.5. Wine analysis

As it will be commented below, no matrix effect was observed and, therefore, the L-malic and L-lactic acid concentrations were calculated by interpolation of the corresponding amperometric signals from the sample solutions into a calibration graph constructed with standard solutions of both acids in the 2.0×10^{-6} to 1.0×10^{-5} M concentration range.

The sample treatment consisted only of an appropriate dilution in order to fit the concentration of L-malic and L-lactic acids in the wine to the specified concentration range. A 1–100 times dilution with the corresponding buffer solution was then carried out prior the analysis. Next, 10–80 μL of the diluted sample were added to the electrochemical cell containing 5.0 mL of the corresponding buffer solution which was used as supporting electrolyte, and the amperometric measurements were carried out by applying the desired potential and allowing the steady-state current to be reached.

The obtained results were compared with those given using with commercial enzyme kits with spectrophotometric detection.

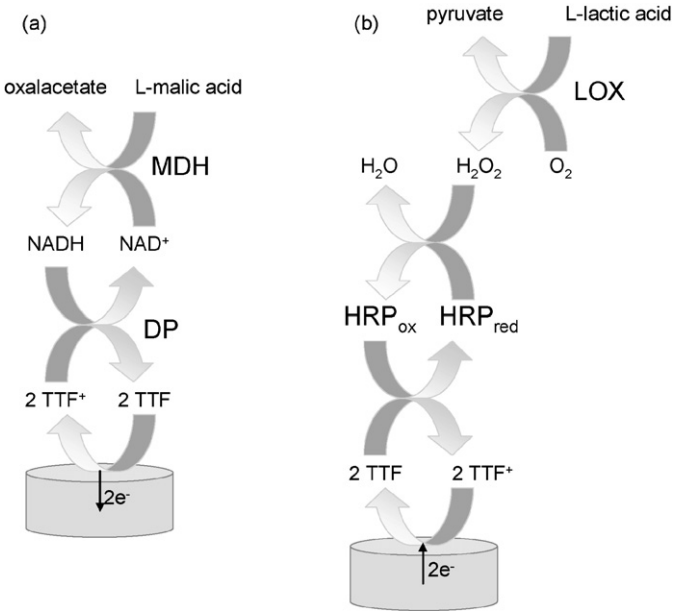

Fig. 1. Schematic diagram displaying the enzyme and electrode reactions involved in the L-malic and L-lactic acids determination with MDH/DP (a), and LOX/HRP (b) biosensors.

3. Results and discussion

The biocatalytic schemes depicting the functioning of the developed biosensor for the determinations of L-malic and L-lactic acids are displayed in Fig. 1.

The coupled enzyme reactions involved in the MDH/DP biosensor imply oxidation of L-malic acid to oxalacetate catalyzed by MDH, with the simultaneous reduction of cofactor NAD^+ to NADH. The NADH is then re-oxidized by TTF^+, this reaction being catalyzed by the enzyme diaphorase [28]. The generated TTF is amperometrically oxidized at the modified electrode surface, with the resulting current being dependent on the L-malic acid concentration. The redox mediator used, TTF, has been claimed previously as an appropriate mediator for the NAD^+/NADH system [29,30] exhibiting a suitable electrochemical performance.

On the other hand, the biosensing scheme for the LOX/HRP bioelectrode involves the oxidation of L-lactic acid by oxygen in the presence of LOX to produce piruvate and hydrogen peroxide. The hydrogen peroxide formed is reduced in the presence of HRP, and regeneration of the HRP reduced form is mediated by TTF. The electrode reaction implies the electrochemical reduction of the generated TTF^+ at an applied potential more negative than the formal potential of the TTF/TTF^+ redox couple [26].

The enzymes immobilization strategy employed in both cases involved the use of a MPA SAM and a dialysis membrane to entrap the enzymes. The role of MPA is preventing protein denaturation at the gold electrode surface and, therefore, improving the bioelectrodes stability. This advantageous characteristic of MPA-modified electrodes was previously demonstrated with other enzyme electrodes [24–26], where a much poorer stability of the biosensors constructed without MPA SAMs was observed.

Furthermore, the use of glutaraldehyde as cross-linking reagent to coimmobilize the enzymes and the mediator, similarly to the previously reported approach [24,25], was checked. However, in the case of malic and lactic acid biosensors this process did not provide acceptable results since the enzymes did not keep attached to the modified electrode surface. This fact was attributed to the relatively high amount of proteins deposited on the electrode. Accordingly,

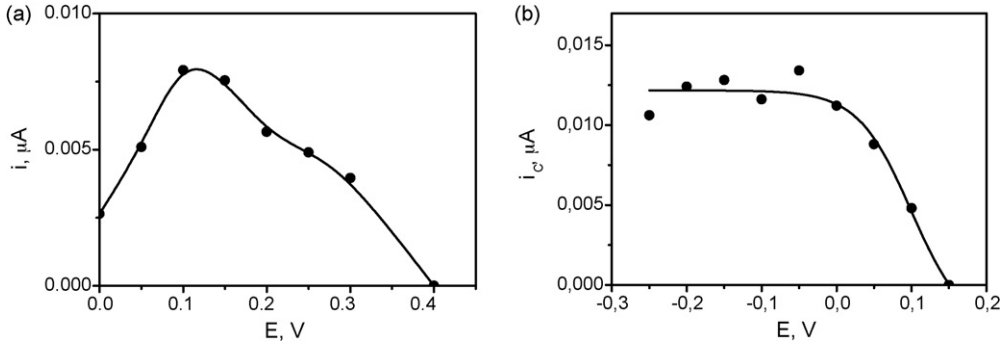

Fig. 2. Effect of the applied potential for MDH/DP (a) and LOX/HRP (b) biosensors on the amperometric signal for 5.0×10^{-6} M malic acid and lactic acid, respectively, in the appropriate buffer solution (MDH/DP biosensor: 0.05 M phosphate buffer, pH 7.0, containing 5 mM NAD^+ and LOX/HRP biosensor: 0.05 M phosphate buffer pH 6.5).

we decided to use a dialysis membrane to physically entrap the enzymes and mediator avoiding their leaking [30].

3.1. Optimization of the working variables

Optimization of experimental variables affecting the performance of both biosensors was accomplished by amperometry in stirred solutions.

3.1.1. MDH/DP biosensor

The TTF loading used was that optimized previously (1.5 μmol) [30]. Therefore, concerning the biosensor composition, only the influence of the enzymes loadings was evaluated. The slope value of the calibration graph constructed for L-malic acid in the 2.0×10^{-5} to 8.0×10^{-5} M concentration range, at an applied potential of +0.20 V, increased notably with MDH loading up to a value of 4.0 U (data not shown). Higher MDH loadings produced a dramatic current decrease which is likely due to the blocking of the electrode surface by the large amount of immobilized protein. Biosensors prepared without co-immobilized DP did not show significant amperometric responses at such applied potential. The presence of DP on the electrode surface significantly accelerated the NADH re-oxidation with TTF^+ up to a value of 1.5 U enzyme. Consequently, the selected composition of the bioelectrode for further work was 4.0 U MDH/1.5 U DP/1.5 μmol TTF.

Once the composition of the biosensor was established, the effect of the applied potential (in the 0.00 to +0.40 V range) on the L-malic acid amperometric response was tested (Fig. 2a). As expected using TTF as mediator, the current obtained for 5.0×10^{-6} M L-malic acid increased over the potential range between 0.00 and +0.10 V, and exhibited a sharp decrease above this value. This behaviour is in agreement with previous reports [24,25,30], and can be attributed to the TTF leakage from the electrode surface at these potentials, induced upon irreversible oxidation of TTF^+ to TTF^{2+}, which is soluble in aqueous solutions and decomposes. Also, we verified that no significant amperometric responses were observed in the potential range scanned when the biosensors were constructed without MDH or DP. Furthermore, no response for L-malic acid was obtained at a MDH/DP/MPA/AuE, thus indicating that no direct oxidation of NADH occurred with this biosensor design at the applied potential, and that the transport of electrons was accomplished from the enzyme to the electrode surface through the mediator. According to the obtained results, an applied potential of +0.10 V was selected for further work.

The most appropriate working pH for a biosensor depends on both the enzyme activity and the influence of pH on the electrochemical performance of the mediator. The optimum pH for this bienzyme electrode was evaluated in the 5.0–10.0 range (data not shown). The biosensor displayed an optimum activity at pH values comprised between 6.5 and 7.5. Accordingly, a 0.05 M phosphate buffer solution of pH 7.0 (containing 5.0 mM NAD^+) was chosen for further work.

Also, the concentration of NAD^+ in the electrochemical cell was optimized. The amperometric response increased significantly with NAD^+ up to a concentration of 5 mM, then levelling off for higher concentrations in the 0–20 mM range checked. Therefore, 5 mM NAD^+ was selected for further work.

3.1.2. LOX/HRP biosensor

Similarly to that commented for the MDH/DP biosensor, only the influence of the LOX loading was checked with respect to the LOX/HRP biosensor preparation, whereas both HRP and TTF loadings were those optimized previously for a hydrogen peroxide biosensor [26]. The steady-state current for 5.0×10^{-6} M lactic acid, measured at a potential of 0.00 V showed higher values for LOX loadings of 2.0 U (data not shown). Accordingly, the composition of the bienzyme electrode was 2.0 U LOX/24.6 U HRP/1.0 μmol TTF.

The influence of the applied potential on the biosensor response to 5.0×10^{-6} M L-lactic acid was examined over the +0.15 to −0.25 V range (Fig. 2b). The cathodic current increased rapidly when the applied potential was varied from +0.15 to −0.05 V, reaching a steady state for more negative values. An applied potential value of −0.05 V was chosen in order to accomplish a sensitive detection and also to minimize the number of potential interferents able to be reduced at the electrode surface. Moreover, as expected and accordingly with the involved enzyme reactions, no cathodic amperometric signals were found in the whole potential range for bioelectrodes constructed with no LOX, or HRP or TTF (HRP/TTF, LOX/TTF, and LOX/HRP MPA/AuEs).

Also, the effect of pH on the amperometric response was evaluated over the 4.0–9.0 range for a L-lactic acid concentration of 5.0×10^{-6} M. The current response increased between pH values of 4.0 and 6.5, and decreased at pHs higher than 6.5. According to this, a 0.05 M phosphate buffer solution of pH 6.5 was chosen as working pH.

3.2. Stability of the MDH/DP and LOX/HRP biosensors

Biosensors responses need to fulfill some requirements concerning their stability to be able to be applied in control processes and routine monitoring. Different aspects regarding the stability of the biosensors were considered.

The repeatability of the measurements was evaluated by constructing 10 different calibration plots with the same biosensor, for both MDH/DP and LOX/HRP biosensors, under the optimized conditions commented above and in the 2.0×10^{-6} to 1.0×10^{-5} M analyte concentration range. Relative standard deviation (RSD) values of 5.8% and 10.4% were obtained for the slope values of the 10 calibration plots for malic acid and lactic acid using MDH/DP and LOX/HRP biosensors, respectively. These values indicate an

acceptable repeatability for the measurements carried out with both biosensors with no need of cleaning or pretreatment for the bioelectrodes surface. Moreover, RSD values of 2.0% and 6.3% were obtained for the steady-state current corresponding to 10 repetitive measurements of 5.0×10^{-6} M malic and lactic acid, respectively.

The reproducibility of the responses obtained with different biosensors was also evaluated. Results for six different MDH/DP and six different LOX/HRP biosensors yielded RSD values for the slope of the corresponding calibration plots in the 2.0×10^{-6} to 1.0×10^{-5} M concentration range of 6.5% and 8.3%, respectively. These values demonstrated that the fabrication procedure of both biosensors was reliable, allowing reproducible amperometric responses to be obtained with different biosensors constructed in the same manner following the developed methodologies.

Also, the useful lifetime of one single biosensor was checked by performing daily calibration graphs for the corresponding substrate in the concentration range mentioned above. Once the measurements were carried out, the biosensors were stored in the corresponding buffer solution at 4 °C. After 7 days of continuous use, the MDH/DP biosensor still exhibited 90% of the original sensitivity, decreasing to 50% after 10 days of use. The operational stability of the LOX/HRP biosensor is a little worse, exhibiting a 91% of the initial sensitivity after 5 days and retaining only half of the initial sensitivity after a week of use, which can be attributed to the denaturation of the immobilized enzymes. Furthermore, we verified that storage of the biosensors between uses in the corresponding buffer solution at room temperature instead of at 4 °C did not affect their useful lifetime.

We also observed that storage of the biosensors in the appropriate buffer solution at 4 °C for at least 15 days after their construction and without using them, did not produce significant variations in the slope values of the corresponding calibration plots for malic and lactic acid.

3.3. Kinetic parameters and analytical characteristics of the MDH/DP and LOX/HRP biosensors

Saturation curves for both biosensors exhibited typical calibration curves for enzyme systems. Plots of $\log[(i_{max}/i - 1)]$ vs. log[malic acid] or log[lactic acid] yielded linear graphs with slope values of 0.99 ± 0.01 and 1.07 ± 0.09, respectively, thus suggesting a Michaelis–Menten type behaviour. Moreover, in order to elucidate the rate limiting step, and considering that the TTF^+/TTF electrochemical reaction is rapid [24], the rate constants were calculated from the slope values of the ln *i* vs. time plots [31] using L-malic acid or NADH as substrates. The calculated rate constants ($n = 3$) were $(2.1 \pm 0.8) \times 10^{-3}$ and $(2.4 \pm 0.5) \times 10^{-3}$ s^{-1}, respectively. Although these values are very similar, the malic acid oxidation step seems to be slightly more sluggish. From the Lineweaver–Burk plot, an apparent Michaelis–Menten constant value of 0.49 ± 0.09 mM was obtained.

Concerning LOX/HRP biosensor, a similar methodology yielded a rate constant of $(75 \pm 2) \times 10^{-3}$ s^{-1} using L-lactic acid as substrate, and a K_{app}^{M} value of 0.17 ± 0.09 mM, very similar to that of LOX in solution, 0.23 mM, which means that the immobilization method does not affect the enzyme reaction kinetics.

Table 1 summarizes the analytical characteristics of the corresponding calibration graphs, under the optimized working conditions. The limits of detection were calculated according to the $3s_b/m$ criterion, where *m* is the slope of the linear part of the calibration plot stated in Table 1, and s_b was estimated as the standard deviation of the amperometric signals from 10 different solutions of L-malic and L-lactic acids at a concentration level of 5.0×10^{-7} M.

Furthermore, the biosensor responses can be considered as rapid since the steady-state currents were reached in 167 and 32 s for L-malic and L-lactic acids, respectively.

Table 1
Analytical characteristics of the calibration plots for L-malic and L-lactic acids obtained with MDH/DP and LOX/HRP biosensors, respectively.

Biosensor	Linear range (M)	Slope ($\mu A\,M^{-1}$)	*r*	LOD ($\times 10^7$ M)
MDH/DP	5.2×10^{-7} to 2.0×10^{-5}	(1583 ± 75)	0.998	5.2
LOX/HRP	4.2×10^{-7} to 2.0×10^{-5}	(2711 ± 190)	0.998	4.2

3.4. Interference study

Several compounds were checked as potential intereferents for the biosensors amperometric responses. In particular, ethanol, glycerol, some sugars (glucose, fructose, galactose, arabinose) and

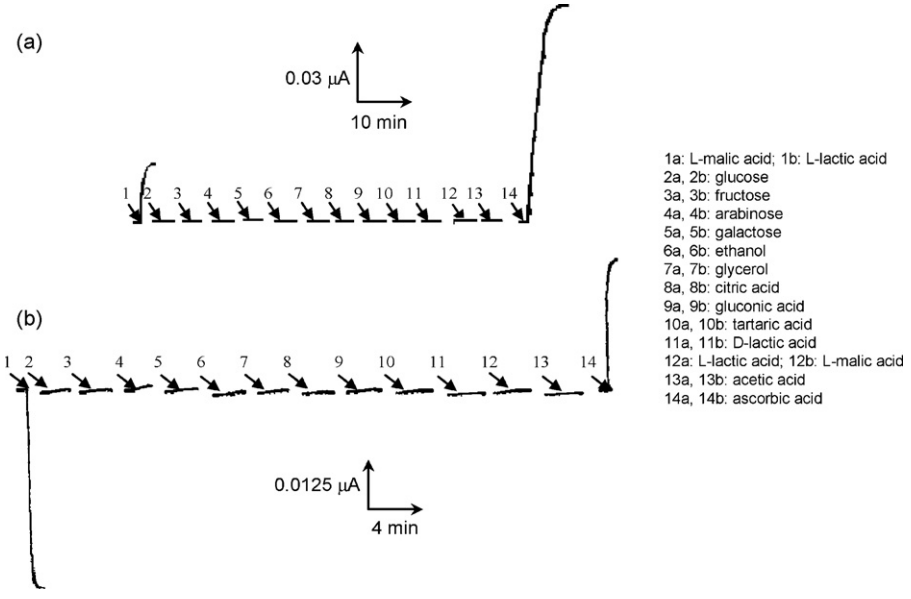

Fig. 3. Amperometric responses obtained after additions of 20 μL of a 0.01 M analyte solution (1), and of 0.01 M solutions of different potential interferents (2–14) to the corresponding buffer solution, at a MDH/DP biosensor (a) and a LOX/HRP biosensor (b). MDH/DP biosensor: supporting electrolyte: 0.05 M phosphate buffer (pH 7.0) containing 5 mM NAD^+; E_{app} = +0.10 V. LOX/HRP biosensor: supporting electrolyte: 0.05 M phosphate buffer pH 6.5; E_{app} = −0.05 V.

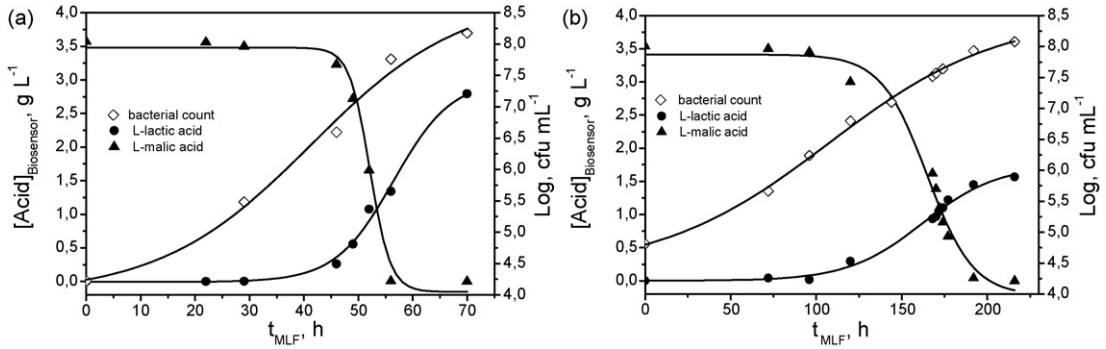

Fig. 4. Monitoring of MLF induced by *L. plantarum* CECT 748^T in a synthetic wine (see text for composition) at 30 °C (a) and at 18 °C (b). Other conditions as in Fig. 3.

some organic acids (tartaric acid, citric acid, gluconic acid, acetic acid, ascorbic acid) may be considered as sources of potential interferences [32]. Therefore, the influence of these compounds on the quantification of L-malic and L-lactic acids was investigated under the experimental conditions specified above. As can be seen in Fig. 3, among all of these compounds, only ascorbic acid produced an amperometric response under the working conditions with both biosensors, which is due to the electrochemical oxidation of this compound at the applied potential to the bioelectrode, and to the reported catalytic oxidation of ascorbic acid by TTF [33]. Nevertheless, taking into account that the expected content of L-malic and L-lactic acids in the samples to be analyzed (see below) is much higher than the possible content of ascorbic acid (0.005–0.012 g L^{-1} in wines), no practical drawbacks can be envisaged from the presence of ascorbic acid. These results clearly demonstrate the high selectivity of the developed biosensors for the determination of the analytes in samples containing other saccharides or organic acids.

3.5. Application of the biosensors to the monitoring of malolactic fermentation

The developed biosensors were used to monitor the MLF induced by *L. plantarum* CECT 748^T in the synthetic wine prepared as described in Section 2.4.1. The evolution of L-malic and L-lactic acid levels was monitored as well as the bacterial growth. The synthetic wine composition mimics well the real must fermentation process where the alcohol content is lower than that obtained for the final wine product. Furthermore, *L. plantarum* 747^T is adequate for working with these relatively low alcohol contents, but not for alcohol levels characteristics of wines. Moreover, parallel measure-

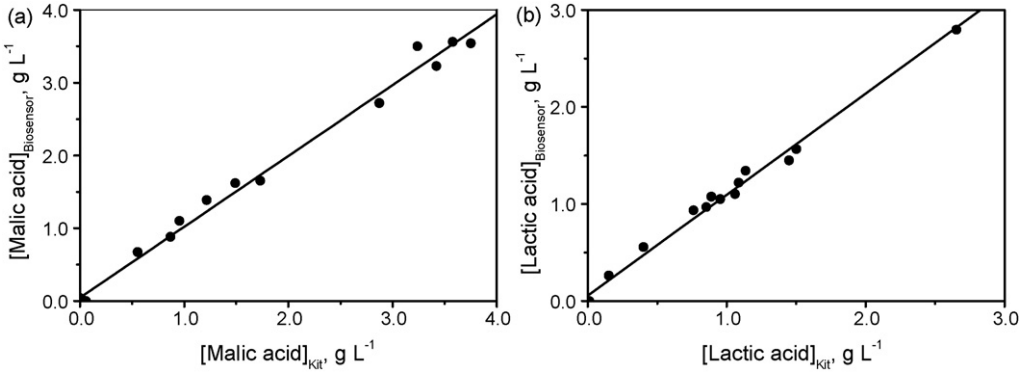

Fig. 5. Correlation between the results for the determination of L-malic (a) and L-lactic (b) acids during MLF obtained with MDH/DH and LOX/HRP biosensors and commercial enzymatic kits. Other conditions as in Fig. 3.

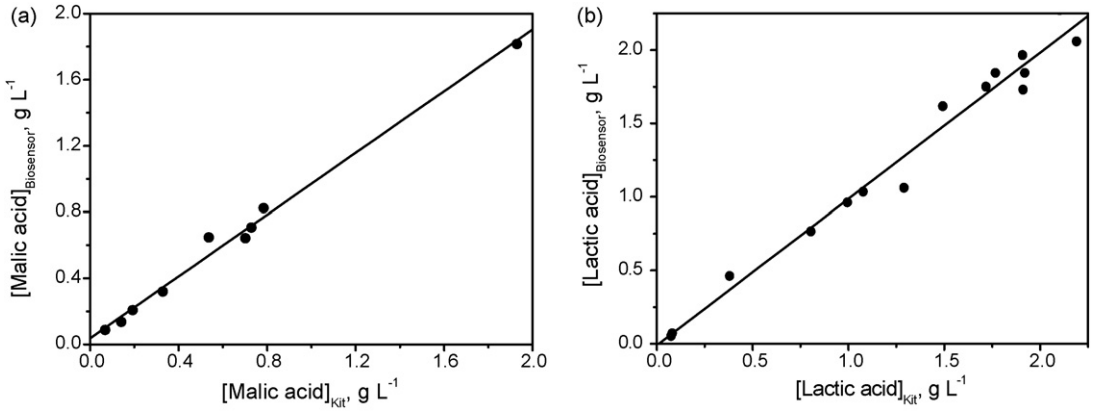

Fig. 6. Comparison of the results obtained for the determination of L-malic (a) and L-lactic (b) acids in commercial wines using MDH/DH and LOX/HRP biosensors and commercial enzymatic kits. Other conditions as in Fig. 3.

Table 2
L-Malic and L-lactic acids electrochemical biosensors reported in the literature.

Enzyme/s (mediator)	Electrode	Immobilization	E_{app} (V)	L.R.	Sensitivity	LOD	Stability	Ref.
MDH/SHL	Clark electrode	Entrapment in a dialysis membrane	+0.65 vs. Ag/AgCl	(0.01–1.2) mM	18.5 mA cm^{-2} M^{-1}	–	>30 days	[37]
(a) LDH/DP	CPE	Entrapment in the electrodic matrix	+0.20 vs. Ag/AgCl	(a) (0.011–1.5) mM	–	(a) 0.011 mM	Remaining sensitivity after 1 month storage:	[38]
(b) MDH/DP ($Fe(CN)_6^{3-}$ in both systems)				(b) (0.015–1.5) mM		(b) 0.015 mM	(a) 100% (b) 90%	
(a) LDH/DP	Graphite/NAD^+ composite	Entrapment in dialysis membranes	+0.30 vs. SCE	(a) (0.01–1.1) mM	–	(a) 11 μM	Remaining sensitivity after 5 months storage:	[39]
(b) MDH/DP ($Fe(CN)_6^{3-}$ in both systems)				(b) (0.01–1.3) mM		(b) 10 μM	(a) 90% (b) 100%	
LOX/HRP (ferrocene)	Graphite–Teflon composite	Entrapment in the electrodic matrix	0.00 vs. Ag/AgCl	(a) (5.0–100) μM	(a) 2980 μA M^{-1}	(a) 1.4 μM	1 week without surface regeneration	[35]
				(b) (2.5–1000) μM[a]	(b) 424 μA M^{-1} [a]	(b) 0.9 μM[a]		
(a) LOX (b) MDH (MB)	Graphite SPE	Entrapment in the sol–gel matrix	(a) +0.35 (b) −0.125 vs. Ag/AgCl	(a) (0–1) mM (b) (0–15) mM	(a) 2800 μA M^{-1} (b) 20 μA M^{-1}		(a) 2 weeks (without use) (b) –	[36]
L-LDH (MB)	Graphite SPE	Enzyme adsorption on the electrode surface	0.00 vs. Ag/AgCl	(20–200) μM	–	10 μM	Single use	[40]
(a) LOX	Pt	(a) Immobilization on nylon membranes	+0.65 vs. Ag/AgCl	(a) (0.05–1.0) × 10^{-3} M[a]	–	(a) 2 μM[a]	Initial response conserved after 150 sample injections:	[4]
(b) ME (PMS)		(b) Immobilization on aminopropyl-glass spheres		(b) (0.1–4.0) × 10^{-4} M[a]		(b) 3 μM[a]	(a) 65% (b) 90%	
ME (MB)	Carbon SPE	Cross-linking in a polietilenimina-GA membrane	+0.20 vs. Ag/AgCl	(0.01–1.0) × 10^{-3} M	–	10 μM	After 15–20 measurements, the initial response of the sensor is decreased by 25%	[41]
MQO (DPIP or PMS)	Graphite SPE	Entrapment in PVA-SbQ	DPIP: +0.05 vs. Ag/AgCl	DPIP: 5–250 μM	DPIP: 0.85 mA M^{-1}	DPIP: 5 μM	DPIP: 10 successive determinations	[2]
			PMS: −0.01 vs. Ag/AgCl	PMS: 5–150 μM	PMS: 1.7 mA M^{-1}	PMS: 5 μM		
LOX (hydroxymethyl ferrocene)	Gold disk	(a) Adsorption	+0.30 vs. SSCE	(a) to 0.3 mM	(a) 0.77 μA mM^{-1}	(a) 10 μM	In both cases the response decreases about 50% of its initial value after one assay	[23]
		(b) Covalent on a DTSP-SAM-modified gold disk electrode		(b) to 0.2 mM	(b) 0.69 μA mM^{-1}	(b) 40 μM		
(a) L-LOX, D-LDH, HRP	Clark	Immobilization on nylon membranes functionalized with carbonyl groups	−0.65 vs. Ag/AgCl	(a) (5–300) ppm	(a) 0.12 nA ppm^{-1} (11.5 μA M^{-1})	(a) 2.5 ppm (26 μM)	(a) 180–200 tests	[6]
(b) MDH, HRP				(b) (9–270) ppm	(b) 0.10 nA ppm^{-1} (13.4 μA M^{-1})	(b) 5 ppm (37 μM)	(b) 100–200 tests	
LOX	Pt	Adsorption	+0.30 vs. Ag/AgCl	(a) (0.004–0.5) mM	(a) 320 nA/mM	–	The response diminishes by 2.5% daily	[5]
(a) Resydrol or (b) poly(ethylene-3,4-dioxin-thiophene)				(b) (0.05–1.6) mM	(b) 60 nA/mM			
(a) MDH/DP	Gold disk	Coimmobilization of the enzymes and the mediator on a MPA SAM-modified gold disk electrode by using a dialysis membrane	(a) +0.10	(a) (0.52–20) μM	(a) 1583 μA M^{-1}	(a) 0.52 μM	Initial sensitivity conserved with continuous use	This work
(b) LOX/HRP (TTF in both systems)			(b) −0.05 vs. Ag/AgCl	(b) (0.42–20) μM	(b) 2711 μA M^{-1}	(b) 0.42 μM	(a) 90% after 7 days (b) 91% after 5 days	

CPE: carbon paste electrode; DP: diaphorase; DPIP: 2,6-dichlorophenol indophenol; DTSP: ditiobis-N-succinimidyl propionate; GA: glutaraldehyde; HRP: horseradish peroxidase; LDH: lactate dehydrogenase; LOX: lactate oxidase; MB: Meldola Blue; ME: malic enzyme; MDH: malate dehydrogenase; MPA: 3-mercaptopropionic acid; PMS: phenazine methosulphate; PVA-SbQ: photocrosslinkable polyvinyl alcohol containing stilbazolium groups; SAM: self-assembled monolayer; SCE: saturated calomel electrode; SSCE: sodium-saturated calomel electrode; SHL: salicylate hydroxylase; SPE: screen-printed electrode; TTF: tetrathiafuvalene.

[a] Flow injection analysis mode.

ments using commercial enzyme kits were also carried out in order to validate the results obtained with the biosensors. The kinetics of bacterial growth, and thus the degradation and production of L-malic acid and L-lactic acid, respectively, is known to be a function of the temperature. Therefore, MLF was monitored at two different temperatures, 30 and 18 °C. The obtained results are displayed in Fig. 4.

In both cases, the malic acid degradation began when the viable cell concentration was $\sim 10^6$ cfu mL^{-1}, in agreement with that reported in the literature [4,34], and the kinetics reached a maximum during the exponential cell growth phase for the bacteria. The different bacterial growth profiles at the two temperatures determined the course of the degradation and production of L-malic and L-lactic acid, respectively. At 30 °C, malic acid was consumed in less than 70 h, while fermentation was completed in 216 h at 18 °C.

At the end of fermentation carried out at 30 °C, the concentration of lactic acid in the synthetic wine was close to the level expected for complete conversion of malic acid (theoretical value of the lactic acid/malic acid ratio = 0.67).

As commented above, the obtained results were validated by comparison with those obtained with commercial enzymatic kits for L-malic and L-lactic acids. The measured concentrations for both acids using the biosensors were plotted against the data collected with the kits (Fig. 5). As it can be seen, linear least squares regression curves ($r = 0.996$) were obtained in both cases, with slope and intercept values of (0.97 ± 0.04) and (0.05 ± 0.10) g L^{-1} for malic acid, and (1.04 ± 0.04) and (0.05 ± 0.06) g L^{-1} for lactic acid. Obviously, the correlation between the results is highly satisfactory, thus demonstrating that the developed biosensors are useful analytical tools for real time on site monitoring of the MLF process.

Another important figure of merit is that we verified during the set of experiments performed to monitor MLF, that almost 500 measurements could be made using the same biosensor until a decrease in the analytical response was observed.

Moreover, we also carried out the analysis of commercial wine samples with the developed biosensors. Only an appropriate dilution of wine samples (1–100 times with the corresponding working buffer solution) was necessary to adjust the concentration of L-malic and L-lactic acids in wines to the linear range of the calibration plots. As indicated in Section 2, no matrix effect was observed for any of these samples and, therefore, the determination was accomplished by interpolation of the corresponding amperometric signals obtained after addition of the sample into calibration plots constructed with standards.

L-Malic and L-lactic acids determinations were carried out in 9 and 15 different types of wine, respectively, and three replicates were made for each sample with each biosensor. The results obtained were compared with those provided by using the commercial enzyme kits (Fig. 6). It can be seen that a very good agreement between the results obtained by the two methods was achieved. The slope values of the linear least squares regression plots were 0.93 ± 0.08 and 1.00 ± 0.09 for MDH/DP and LOX/HRP biosensor plots, respectively, with intercept values of 0.04 ± 0.06 and -0.01 ± 0.1 g L^{-1}, respectively. The correlation coefficients were 0.996 and 0.988. The confidence intervals for a significance level of 0.05 for the slope and intercept values included the unit and the zero values, respectively, indicating that the methods have no systematic errors and that the developed biosensors can be successfully used for the determination of malic and lactic acids in wines.

3.6. Analytical performance comparison with other L-malic and L-lactic acids electrochemical biosensors reported in the literature

The analytical performance of the developed biosensors was compared with that of other electrochemical biosensors reported in the literature which were applied to the analysis of wine samples. Characteristics such as the type of electrode and enzyme immobilization, the redox mediator (if used), working potential, range of linearity of the corresponding calibration graph, sensitivity, limit of detection achieved and useful lifetime are listed for all of them in Table 2.

Concerning the applied potentials, the biosensors developed in this work employed less extreme detection potentials than most of used with other biosensor designs, which implies a better selectivity against potential electroactive interferents. Furthermore, the sensitivity achieved in the determination of both acids is similar to the best ones reported previously for L-lactic acid [35,36] and L-malic acid [2] enzyme electrodes. The detection limits achieved with the biosensor designs reported in this work are lower than the best ones reported previously (1.4 μM for L-lactic acid [35] and 3.0 μM for L-malic acid [4]). A high sensitivity is needed for the determination of low levels of L-lactic and L-malic acids as occurs at the beginning or the end of MLF.

Concerning the useful lifetime, this can be considered as acceptable taking into account the simplicity of the biosensors preparation procedure and the demonstrated suitability for fermentation processes monitoring and long-term storage. Therefore, although the lack of data in the literature avoids a more extensive comparison, in general, it can be said that the developed biosensors exhibit a good analytical performance in terms of sensitivity, time of response, stability and reproducibility when compared with other biosensors reported in the literature. In particular, when the lactate biosensor performance is compared with the only one described in the literature using self-assembled monolayers on gold electrodes [23], it can be stated that the biosensor developed in this work exhibits a 10-fold lower detection limit, a 4 times higher sensitivity and a much higher stability. It is also important to remark that only one of the biosensors reported in the literature was applied to monitor MLF [4].

4. Conclusions

Integrated amperometric biosensors for the determination of L-malic and L-lactic acids were developed and applied for the monitoring of MLF carried out by *L. plantarum* CECT 748^T. These biosensors accomplish the requirements of precision, rapidity, sensitivity, simplicity and low cost required to be considered as useful analytical tools for the wine industry, providing rapid and reliable analytical methodologies for the quantification of both acids. The results obtained with the biosensors were in good agreement with data provided by commercial enzymatic kits, thus demonstrating that the bioelectrodes were suitable for monitoring and managing the MLF process. Considering their reduced costs of preparation, operation and maintenance and their very short response times (1–3 min), the developed biosensors appear to be particularly competitive with traditional spectrophotometric-enzymatic methodologies.

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