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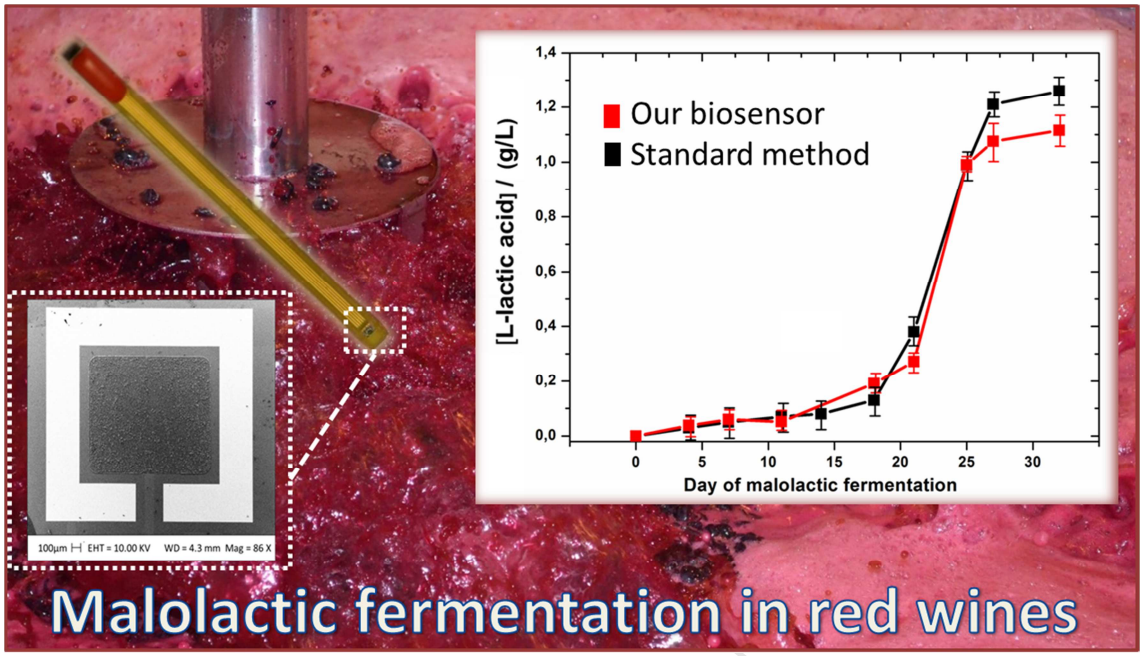
Graphical abstract



MONITORING OF MALOLACTIC FERMENTATION IN WINE USING AN ELECTROCHEMICAL BIENZYMATIC BIOSENSOR FOR L-LACTATE WITH LONG TERM STABILITY.

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Abstract

During malolactic fermentation process of wine, L-lactic acid is monitored and its evolution is strongly related with the quality of the final product. Nowadays the analysis of L-lactic acid is carried out off-line in a laboratory. However, there is a clear demand for analytical tools that enabled real-time monitoring of this process in field and biosensors have positioned as a feasible alternative in this regard. The development of an amperometric biosensor for L-lactate determination showing long-term stability is reported in this work. The biosensor architecture includes a thin-film gold electrochemical transducer selectively modified with an enzymatic membrane recognition element, based on a three-dimensional matrix of polypyrrole (PPy) entrapping lactate oxidase (LOX) and horseradish peroxidase (HRP) enzymes. The

experimental conditions of the biosensor fabrication regarding the pyrrole polymerization and the enzymes entrapment are optimized. The biosensor response to L-lactate is linear in a concentration range of 1×10^{-6} – 1×10^{-4} M, with a detection limit of 5.2×10^{-7} M and a sensitivity of - (13500 ± 600) μA M^{-1} cm^{-2}. The biosensor shows an excellent working stability, retaining more than 90 % of its original sensitivity after 40 days. This is the determining factor that allowed for the application of this biosensor to monitor the malolactic fermentation of three red wines, showing a good agreement with the standard colorimetric method.

Keywords: amperometric biosensor; bienzymatic membrane; electrosynthesized Polypyrrole; L-lactic acid analysis; wines; malolactic fermentation.

1. Introduction

Measuring and controlling L-lactic acid concentrations is required in fields as diverse as sport medicine [1], medical control [2] or food industry [3]. L-lactic acid is used as an indicator to monitor fermentation processes and its concentration is also strongly related to the flavor or the texture of the original product [4]. Also, L-lactic acid is applied as acidifier, preservative and pH regulator in confectionary industry [5], in fruit and vegetables industry or in the winemaking industry [6], among others. In the winemaking industry, the L-lactic acid concentration is related the quality of the final product [7]. It is produced mainly in the malolactic fermentation, in which the transformation of the malic acid into L-lactic acid and CO_2 takes place. Wine L-lactic acid concentrations can increase up to 3 g L^{-1} (0.028 M). In some elaboration processes, L-lactic acid is added as acidifier during the alcoholic fermentation in order to improve its clarification and guarantee the flavor during the aging of the wine. The presence of

L-lactic acid improves the sensorial qualities of wine, its freshness and contributes to the chemical and microbiological stability. In addition, it increases the total acidity and the buffer capacity of the wine. Therefore, the control of the L- lactic acid concentration can be used as a quality indicator of the final wine.

There are conventional analytical procedures for the determination of L-lactic acid in wines based on chromatography [8] and colorimetric methods [9]. In general, these methods require costly equipment and are time consuming, given that the analysis is carried out in an external laboratory. Therefore, in order to follow the fermentation process in real time, in field monitoring of L-lactic acid concentration would be desirable. In this context, biosensor devices emerge as a feasible alternative [10], and those based on electrochemical methods have shown to be very convenient. Electrochemical biosensor devices show several advantages, such as low detection limits, a wide linear response range or high selectivity and reproducibility [11]. Among them, amperometric sensors based on redox reactions catalyzed by oxidoreductase enzymes have been of widespread use [12]. These enzymes show additional advantages like their natural origin and no toxicity, high specificity and stability under moderate working conditions of pH and temperature. One of the most common strategies for the L-lactate determination makes use of L-lactate dehydrogenase (LDH) in presence of NAD^+/NADH as coenzyme [13]. However, the derived biosensor devices show several drawbacks related to the necessity of incorporating the NAD^+ cofactor, which in turn requires the implementation of a potential step once the sensor response is recorded in order to regenerate it. This step is carried out at relatively high potentials (above 300 mV), and this can cause interferences of other electroactive species present in the samples. Another alternative is the use of LOX as recognition element. This enzyme catalyzes the L-lactate oxidation to produce pyruvate and hydrogen peroxide in the

93 presence of dissolved oxygen. The hydrogen peroxide can then be reduced and the
94 resulting cathodic current is stoichiometrically related to the L-lactate concentration in
95 the sample. Here, the main drawback is that a high over-potential for the direct detection
96 of H_2O_2 is needed and this again can cause interferences of other oxidizable species
97 present in the samples. In order to circumvent this difficulty, a LOX based biosensor
98 comprising an electrochemical dissolved oxygen sensor was reported [14]. Other more
99 widespread strategies are based on the incorporation of a second enzyme, namely
100 horseradish peroxidase (HRP) that catalyzes the reduction of H_2O_2 to H_2O in the
101 presence of a suitable redox mediator that is concomitantly oxidized [15-17]. Detection
102 of these oxidized species takes place at low enough potentials to avoid any possible
103 interference from the sample. Moreover, the resulting biosensor shows enhanced
104 sensitivity thanks to the application of the LOX /HRP cascade enzyme reaction [18].

105 One of the main drawbacks of biosensor devices is the limited lifetime mainly
106 related to the biomolecules stability and the procedure applied for their incorporation
107 onto the transducer surface. Therefore, the chosen immobilization procedure has to be
108 studied in detail and optimized in order to maximize the working stability over time.
109 The enzyme entrapment in three-dimensional matrices [19, 20] proofed to be a good
110 alternative due to the simple fabrication and the not required modification of the
111 enzyme structure, which improves the lifetime of the biosensor. Besides, the enzyme
112 entrapment in a membrane of a conducting polymer by electropolymerization is a
113 common strategy in amperometric biosensors [21-23]. A one-step controlled process is
114 carried out under potentiostatic (set potential) or galvanostatic (set current) conditions in
115 a solution containing the monomer and the enzymes. Both approaches induce the
116 oxidation of the monomer and the formation of an electrogenerated polymer layer,
117 which physically entrap the enzymes, thus maintaining their original activity [24].

Conducting polymers, in particular PPy, have a stable electrical conductivity and can be electrogenerated under biocompatible conditions, in agreement with the enzyme requirements. The improved lifetime of biosensors fabricated using conducting polymers has been demonstrated [25]. Regarding to L-lactate biosensors, few works have been reported based on a two enzyme co-immobilization process onto the transducer surface using polymers. They make use of the polymer as a surface for the further enzyme physical adsorption [26] or covalent immobilization [27], but no paper has been reported describing the simultaneous one-step immobilization of LOX and HRP enzymes in an electrosynthesized PPy matrix.

In this work, the development and characterization of a new biosensor for L-lactate determination based on the co-immobilization of LOX and HRP in an electrosynthesized PPy film is described for first time. A thin-film gold microelectrode selected as the electrochemical transducer allows working with very small volumes and thus reducing reagent consumption during the polymer electrosynthesis. The fabrication of the enzymatic membrane has been optimized with respect to the electrosynthesis conditions and LOX:HRP ratio within the PPy membrane. L-lactate detection has been carried out by chronoamperometry in solutions containing potassium ferrocyanide as redox mediator. The analytical characteristics of the biosensor in terms of selectivity, sensitivity, linear range, limit of detection and working stability have been assessed and compared with other similar electrochemical biosensors. This sensor stands out for its extended lifetime of over 40 days and the successful application to the L-lactic acid monitoring during the malolactic fermentation process of three different red wines, obtaining concentration values in excellent agreement with those obtained using the standard colorimetric method.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of high purity, analytical grade or equivalent and were purchased from Sigma-Aldrich, unless stated otherwise. All solutions were prepared using de-ionized water. For the cleaning of the electrodes, ethanol 96% and 6 M sulfuric acid (H_2SO_4) were used. 5-µL 0.8 U μL^{-1} Lactate oxidase (LOX, from Pediococcus sp., lyophilized powder, ≥20 U mg^{-1} solid) aliquots were prepared and stored in a freezer at -20 ºC. Horseradish peroxidase (HRP, type VI-A, essentially salt-free, lyophilized powder, 250-330 U mg^{-1} solid) was stored in a refrigerator at 4 ºC and used as received. Pyrrole (reagent grade, 98%) was distilled every week and stored in a freezer at -20 ºC. A 0.05 M phosphate buffer solution (PB) prepared with potassium phosphate monobasic (KH_2PO_4) was used for all the optimization and electrochemical characterization experiments.

A set of PB solutions containing 2 mM of potassium ferrocyanide ($K_4[Fe(CN)_6]$), 2 mM of L-lactate (L-(+)-Lactic acid, ≥98%), 0.2 U LOX and a different HRP concentrations(0.2 U, 2.0 U, 4.0 U, 6.0 U, 8.0 U and 10.0 U), were prepared for the optimization of the LOX:HRP ratio. For the fabrication and optimization of the PPy film, several PB solutions containing different reagents and concentrations were used in order to find the best electrosynthesis conditions. The reagents were 0.1 M lithium perchlorate ($LiClO_4$), 0.1 M potassium chloride (KCl), 0.1 M potassium nitrate (KNO_3), pyrrole in a range of 0.2 M – 0.8 M, LOX from 2 U to 6 U and HRP from 40 U to 120 U. For the chronoamperometric L-lactate detection, a PB solution containing 0.1 M KCl, 2 mM of $K_4[Fe(CN)_6]$ and a L-lactate concentration from 1×10^{-7} M to 1×10^{-3} M was used. During the interference study, a PB solution with 0.1 M KCl, 2 mM $K_4[Fe(CN)_6]$ and 1×10^{-5} M of the analyzed interfering compounds was prepared. The

interferents considered were glycerol, glucose, ethanol, fructose, tartaric acid (all them purchased from Panreac, Spain), acetic acid, L-Malate (L-(−)-Malic acid, ≥99%), gluconic acid and ascorbic acid (Fluka, Spain).

2.2. Devices and equipment

The thin-film gold electrodes used as transducers were fabricated on silicon substrates at the Instituto de Microelectrónica de Barcelona (IMB-CNM) according to standard microelectronic technology [28]. The chip size was 3×3.5 mm^2 and included a cell with two electrodes, namely the working electrode with an area of 1.62 mm^2 and the counter electrode with an area of 2.27 mm^2 (Fig. S1a, in the Supporting Information (SI)). The chips were wire-bonded on a printed-circuit board (PCB) and packaged using Ebecryl photocurable polymer following a standardized photolithographic process developed at the IMB-CNM [29].

In order to ensure a low consumption of reagents during the polymer film fabrication, a 20 µL poly(methyl methacrylate) (PMMA) cell was designed and fabricated using a CO_2-laser system (Epilog Mini 24, Epilog Laser, United States). The cell was formed by two 3 mm x 30 mm x 20 mm PMMA layers. The bottom one was milled to host the transducer. The top layer was also machined to define the well of the electrochemical cell. Both parts were fixed with screws and an O-ring was used to avoid the fluid leakage. A 2-mm diameter stainless steel wire and a 0.5 mm diameter silver wire were used as counter and pseudo-reference electrode, respectively. A schematic picture of the assembled structure and an image of the actual configuration are showed in Fig. S1B and S1C, respectively, in the SI.

The electrochemical measurements were performed with an Autolab electrochemical workstation (PGSTAT-100 potentiostat – galvanostat, Ecochemie,

Uthecht, The Netherlands) controlled using a PC with GPES (General Purpose Electrochemical System) software. During the biosensor characterization, the three-electrode cell was completed with a Ag/AgCl (3.0 M KCl) double junction (Orion 92-02-00, Thermo Fisher Scientific Inc., Berverly, USA) as reference electrode, and the integrated on chip auxiliary electrode as counter. Polystyrene 96-well ELISA plates (Corning Incorporated, United States) and a Thermo Electron Multiskan EX plate reader (Thermo Fisher Scientific) were used for the optimization of the LOX:HRP concentration ratio off chip by recording the absorbance at 405 nm.For the morphological characterization of the polypyrrole films, an atomic force microscope (AFM, Nanoscope IV from Veeco, USA) operated in tapping mode, a scanning electron microscope (SEM, Auriga from Carl Zeiss, Spain) operated at 5–10 kV and a focused ion beam equipment (FIB, Crossbeam 1560 XB from Carl Zeiss) were used.

2.3. LOX:HRP ratio assessment

The optimization of the LOX:HRP ratio was carried out off-chip in solution, using a low-binding ELISA microtiter plate. 100-µL PB solutions containing 0.1 M KCl, 2 mM of potassium ferrocyanide, 2 mM of L-lactate and different amounts of the two enzymes were prepared in independent wells. The LOX activity was set to 0.2 U and six concentrations of HRP (0 U, 2 U, 4 U, 6 U, 8 U and 10 U) were tested. Therefore, the LOX:HRP ratios studied were 1:0, 1:10, 1:20, 1:30, 1:40. Triplicates of each solution were analyzed. Solutions were stirred for 45 s and then left for 10 min under quiescent solutions for the enzymatic reactions to take place. Then, absorbance values for each well were recorded at a wavelength of 405 nm. This value was chosen taking into account the potassium ferricyanide absorbance spectrum [30].

2.4. Enzymatic membrane fabrication

Before the electrosynthesis of the PPy layer, the gold electrodes were activated according to previous works by our group [28]. Firstly, they were carefully cleaned using 96 % ethanol, H_2SO_4 6 M and deionized water. Then, an electrochemical activation was performed in 0.1 M KNO_3 solution by applying 20 cyclic voltammetric scans in a potential range from +0.8 V to -2.2 V at 100 mV s^{-1}. The effectiveness of this activation process was verified in a 0.1 M KNO_3 solution containing 1 mM potassium ferrocyanide [31].

The PPy film electrodeposition process was optimized by studying the influence of several chemical parameters of the pre-polymerization solution such as nature of the required counter-ion (or dopant agent) to carry out the polymer electrosynthesis, the pyrrole concentration and the amount of enzymes on the resulting biosensor response. The nature of the counter-ion has a high relevance in the morphology of the electrogenerated PPy layer [32]. During the oxidation of the monomer, the generated radical cations react with other radical cations present in the sample for obtaining polymeric chains and their positive charge are compensated by the counter-ion. Then, three electrolytes ($LiClO_4$, KNO_3 and KCl) were tested in PB containing 0.5 M pyrrole, 4 U of LOX and 80 U of HRP. The counter-ion concentration was fixed to 0.1 M [25]. Then the effect of the monomer concentration and the concentration of both enzymes were sequentially studied in PB solutions. Four pyrrole concentrations from 0.2 M to 0.8 M, and three LOX:HRP concentrations (2:40, 4:80 and 6:120, in U) were selected. The LOX:HRP ratio was set following the results of the absorbance measurements carried out as described above. All measurements were done in triplicate.

Electrosynthesis of all the PPy films was carried out under potentiostatic conditions at a set potential of +0.85 V (vs. Ag pseudo-ref. electrode). In order to ensure the reproducibility of the PPy film thickness, the charge accumulated during the

electrogeneration was fixed to 500 mC cm^{-2} [33]. This charge guarantees an efficient enzyme entrapment inside the polymer matrix. Finally, the biosensors were rinsed with PB in order to remove the enzymes physically adsorbed on the PPy film and stored in PB at 4 ºC when not in use.

2.5. Electrochemical measurements

The process associated with the presented L-lactate biosensor is based on the oxidation of the L-lactate to pyruvate and hydrogen peroxide (H_2O_2) in presence of LOX (Fig. S2, in the SI). The hydrogen peroxide is then reduced in presence of HRP and the HRP is regenerated thanks to the potassium ferrocyanide mediator, which is oxidized to ferricyanide. This is again reduced back to ferrocyanide by applying an adequate potential and the recorded faradaic current is stoichiometrically related to the L-lactate concentration in the sample.

The biosensor response was evaluated by chronoamperometry setting an overpotential of +0.075 V (vs. Ag/AgCl, 3M), at which ferricyanide generated during the bienzymatic process is effectively reduced. The biosensor was calibrated in 10 mL PB solutions containing 2 mM ferrocyanide and L-lactate in a concentration range between 1×10^{-7} M and 1×10^{-3} M. Each L-lactate concentration was measured in triplicate.

The biosensor performance was assessed in terms of sensitivity, linear range and limit of detection to L-lactate. For the optimized biosensor, studies of repeatability, reproducibility and working stability were carried out using three biosensors fabricated under the same experimental conditions.

Selectivity studies were carried out in PB solutions containing 0.1 M KCl, 2 mM ferrocyanide and 1×10^{-5} M concentrations of glucose, glycerol, gluconic acid, L-malate, tartaric acid, fructose, acetic acid, ethanol or ascorbic acid interferences.

The working stability of the developed biosensor was tested by carrying out periodic calibrations in the L-lactate concentration range from 1×10^{-6} M to 1×10^{-4} M. The calibrations were carried out every 2 or 4 days during 52 days. The biosensor was stored in PB at 4 ºC between calibrations.

2.6. Analysis of real samples coming from wine malolactic fermentation processes

In order to evaluate the applicability of the developed biosensor to the malolactic fermentation monitoring, the analysis of real wine samples provided by the Catalan Institute of Vineyard and Wine (IRTA-INCAVI) was carried out. Three red wines based on Marcelanne, Petit Verdot and Syrah grape varieties, were tested. These wines were from the 2013 vintage and their vineyards were harvested in the region of Tarragona (Spain). When the alcoholic fermentation was completed, selected bacteria of strain of *Oenococcus Oeni* were inoculated to induce the malolactic fermentation. For each wine, samples collected along this fermentation process, with a concentration of L-lactic acid in a range of $0 - 2\times10^{-2}$ M ($0 - 1.7$ g L^{-1}), were analyzed. They were eleven samples for the "Wine 1" (Marcelanne) in an interval of 45 days, 5 samples for the "Wine 2" (Petit Verdot) in an interval of 16 days and 8 samples for the "Wine 3" (Syrah) in an interval of 30 days were analyzed with the proposed biosensor. For measuring, a 1:100 sample dilution was done in order to adjust the L-lactic acid concentration to the linear range of the amperometric biosensor. Results were compared with the standard enzymatic method used by the IRTA-INCAVI. This method is based on the enzymatically catalyzed reaction between the L-lactate and NAD^+ to produce NADH, whose concentration is stoichiometrically related to the L-lactic acid concentration in the

288 samples. The change of NADH concentration is measured spectrophotometrically at
289 340 nm [34].

290

291 **3. Results and discussion**

292 *3.1. Study of the LOX:HRP ratio in the cascade reaction*

293 In order to assess the optimal enzyme ratio whose coupled activity provided with
294 the best analytical signal, solutions containing different LOX:HRP proportions were
295 analyzed, as described in the experimental section. As it is shown in Fig. S3 in the SI,
296 the absorbance value increased with the HRP activity and levels off at concentration
297 values above 4 U. This HRP activity is related to the LOX:HRP ratio of 1:20, which
298 was chosen for the further biosensor fabrication.

299

300 *3.3. Optimization of the polymer electrogeneration conditions*

301 The methodology used for the PPy electrogeneration is critical for the biosensor
302 fabrication. In order to obtain a fast, easy and totally controlled method, potentiostatic
303 conditions were proposed. A preliminary study by cyclic voltammetry was done in
304 order to set the potential. First and second cyclic voltammograms during PPy
305 electrogeneration are showed and compared to the background solution cyclic
306 voltammogram in Fig. S4A (in the SI). The fast increase of current at potentials above
307 +0.60 V (vs Ag pseudo-ref. electrode) in the pyrrole containing solution was related to
308 the monomer oxidation-polymerization. A quasi-reversible signal at a half wave
309 potential of +0.23 V was also recorded, which can be ascribed to the polymer film
310 oxidation and reduction processes. From these results, the selected potential was set at
311 +0.85 V (vs. Ag pseudo-ref. electrode) and applied for 1 min. In order to ensure the

reproducibility of the PPy film thickness, the charge accumulated during the PPy formation was fixed to 500 mC cm^{-2}. An example of the chronoamperometric response obtained under these conditions is shown in Fig. S4B (in the SI).

The influence of three different counter ions (Cl^-, NO_3^- or ClO_4^-) on the PPy electropolymerization was assessed in terms of the sensitivity values achieved with the corresponding biosensors. Calibration curves were recorded in PB solutions containing 0.1 M KCl and L-lactate in a concentration range from 1×10^{-7} M to 1×10^{-3} M. The highest sensitivity was obtained when the PPy was generated in the presence of Cl^- ions ($-95x10^2$ µA M^{-1} cm^{-2}), while values of $-81x10^2$ µA M^{-1} cm^{-2} and $-69x10^2$ µA M^{-1} cm^{-2} were obtained with NO_3^- and ClO_4^- ions respectively. This lower sensitivity obtained with these ions may be related to their oxidative power that could affect the enzyme activity. Indeed, preliminary studies carried out with HRP entrapped in PPy films showed the significant decrease of the enzyme activity when ClO_4^- ions were used (data not shown).

Then, the effect of the monomer concentration on the biosensor response was studied in PB solutions containing 4 U LOX, 80 U HRP and 0.1 M KCl. Fig. S5A (in the SI) depicts the biosensor sensitivity related to the monomer concentration. It is shown that the highest sensitivity was achieved for a 0.4 M pyrrole concentration. At higher concentrations of monomer, the sensitivity and the linear range of the biosensor decreased. This could be related to the generation of a thicker polymer layer that negatively affected the diffusion of the redox mediator from the bulk of the solution to reach the electrode solution interface [35]. Also, at concentrations of pyrrole below 0.4 M no response of the biosensor was recorded and this could be related to the formation of a very thin polymer layer, which contained a low amount of enzymes entrapped in its structure.

Finally, the effect of the LOX and HRP concentrations was evaluated in PB containing 0.4 M pyrrole and 0.1 M KCl. Three LOX:HRP concentrations (2:40, 4:80 and 6:120, in U) were tested, keeping the LOX:HRP ratio to 1:20. As can be seen in Fig. S5B (in the SI), the highest sensitivity and widest linear range were achieved for the 4:80 U. Low enzyme concentrations resulted in a low amount of entrapped enzymes and a poor sensitivity. On the other hand, for high enzyme concentrations, the rate of the polypyrrole film formation decreased drastically (more than 30 minutes were required to reach the set charge of 500 mC cm^{-2}). This could be due to the transducer surface being blocked by the adsorption of the enzymes, which results in the recording of no-faradaic current and thus of no biosensor response, or to the high concentration of enzymes that could affect the polymerization rate.

As a summary, and based on the above described experiments, the optimum conditions for the electrosynthesis of the enzyme containing PPy film on the surface of the thin-film gold transducers were the following: potentiostatic conditions at +0.85 V (vs. Ag pseudo-ref. electrode) for 60 s and 500 mC cm^{-2} charge, in a PB solution containing 0.4 M pyrrole, 0.1 M KCl, 4 U of LOX and 80 U of HRP.

3.4. Morphological characterization of the PPY/LOX:HRP film

The thickness of the PPy film plays a significant role in the biosensor response. The thickness and the structure of the polymer matrix are related to the charge accumulated during the electrogeneration process and the nature and concentration of the substances involved in it (monomer, enzymes and counter-ion) [36]. A SEM image in Fig. 1A shows the typical surface morphology of these films [37]. The roughness, opening, porosity and globular morphology of the polypyrrole films allow for the adequate enzyme loading and improve the electronic conductivity of the film. Fig. 1B

depicts a SEM image of the silicon chip, with the PPy film selectively deposited on the working electrode. An AFM detailed image of the PPy surface shown in Fig. S6A in SI, gives more information about the surface morphology and roughness. A RMS roughness of 0.24 µm was obtained. The thickness of the polypyrrole film, measured by FIB, was between 1.30-1.40 µm (Fig. S6B, in SI).

3.5. Evaluation of the biosensor performance

Once the fabrication of the biosensor was optimized, chronoamperometric measurements were carried out at +0.075 V (vs Ag/AgCl) in PB containing 0.1 M KCl, 2 mM potassium ferrocyanide and increasing concentrations of L-lactate, in a range from 1×10^{-7} M to 1×10^{-3} M. The biosensor responses are shown in Fig. 2A. As expected a more negative current density for increasing L-lactate concentrations, was obtained. The current recorded in all cases increased and started to levels off at 100 s time after the potential application. This current was recorded for 200 s and the mean current value of the last 60 s (140-200 s) was used as the analytical signal. From this, it can be said that the sensor response was 100 s and the overall assay time is set to 200 s. The calibration curve is shown in Fig. 2B. A linear response was observed in a concentration range from 1×10^{-6} M to 1×10^{-4} M of L-lactate with a sensitivity of $-135 \pm 6\times10^{2}$ µA M^{-1} cm^{-2} (r = 0.998). A limit of detection (LOD) of $5 \pm 0.2\times10^{-7}$ M, calculated using the 3σ IUPAC criterion was obtained. For L-lactate concentration above $1x10^{-6}$M, the enzymatic biosensor is saturated, a according to the normal behavior of a Michaelis-Menten enzyme kinetics in biosensors. Regarding the reproducibility of the fabrication process, three different biosensors were calibrated on the same day, obtaining a relative standard deviation (R.S.D.) of the sensitivity lower than 4 %.

385 The biosensor response to possible interferences was assessed. For that, glycerol,
386 glucose, gluconic acid, fructose, acetic acid, citric acid, ethanol, L-malate, tartaric acid
387 and ascorbic acid were considered. The corresponding chronoamperometric responses
388 are shown in Fig. 3. Only a significant signal was obtained in the presence of ascorbic
389 acid, due to its electrochemical oxidation at the measurement potential of +0.075 V
390 [38]. However, red wines do not contain ascorbic acid [39] and it is not produced during
391 the malolactic fermentation. These results demonstrate the high selectivity of the
392 developed biosensor for the L-lactate determination.

393 The biosensor working stability with time was studied during 52 days. When not
394 in use, the biosensor was stored in the PB at 4 ºC. As can be seen in Fig. 4, a decrease of
395 the sensitivity around 5 % took place during the first five days, probably related to the
396 loss of the enzyme attached by physical absorption on the PPy film or the more weakly
397 entrapped in the PPy film (enzyme molecules closer to the outer surface of the
398 polymer). After this time, the biosensor response remained stable and kept over 90 % of
399 the initial sensitivity value for 42 days. From the day 45, there was a sudden decrease of
400 the response, which could be attributed to the denaturation of the immobilized enzymes.
401 However, this decrease represented only around 20 % of the initial sensitivity after 52
402 days of study.

403

404 *3.6. Application of the biosensor in real samples from malolactic fermentation of wines*

405 The proposed L-lactate biosensor was applied to the monitoring of the
406 malolactic fermentation in three different red wines. Samples collected different days
407 during the fermentation process were provided by the IRTA-INCAVI. The 1:100
408 diluted wine samples were analyzed in triplicate with the biosensor. In parallel, the

409 same samples were analyzed by the IRTA-INCAVI with the colorimetric standard
410 method. The results from both methods were compared by plotting the L-lactic acid
411 concentration (in g L^{-1}) versus the day of the malolactic fermentation process. As shown
412 in Fig. 5, there is an excellent agreement between the two values, with absolute errors
413 below 0.09 g L^{-1} in all the cases. The evolution of the lactic acid during the fermentation
414 processes was as expected. There was an initial slow formation, followed by an
415 exponential increase and a final stabilization of the L-lactic acid concentration, which
416 indicates the end of the malolactic fermentation. This behavior was particularly clear in
417 the monitoring with "Wine 3". However, for the "Wine 1" and "Wine 2", the
418 concentration of L-lactic acid was still increasing, which means that the process of
419 transformation of malic acid to L-lactic acid was still in progress.

420 During its application, the biosensor was calibrated four times within a range of 1×10^{-6}
421 M to 5×10^{-4} M of L-lactate. These calibrations were carried out at the beginning,
422 between the first and the second set of wines (30 analyses), between the second and the
423 third set of wines (15 analyses) and at the end of the experiment (24 analyses). The
424 results showed a stable sensor response, which retained over 90 % of the initial value
425 after completing all the analyses (see Table 1).

426 *3.7. Comparison with other L-lactate electrochemical biosensors*

427 The developed biosensor was compared with other electrochemical biosensors
428 for L-lactate determination reported in the literature. The main characteristics of these
429 devices are summarized in Table 2. Regarding the immobilization method, the proposed
430 biosensor is the only one constructed by simultaneously entrapping both LOX and HRP
431 in a PPy film, which simplifies the fabrication of the device. Also, biosensor production
432 time is very short and reagent consumption has been very low.

The approach presented in this work clearly outperforms other devices in terms of operational stability and in terms of biosensor performance. It is worth mentioning that even though some of them show the required sensitivity and linear range for the measurement of L-lactic acid during the malolactic fermentation, all of them lack the long-term stability required to monitor this process, which usually takes place for up to 40 days in most wines. The proposed PPY/LOX:HRP biosensor, with a lifetime of 42 days is the only one that could be applied to this monitoring process.

It should also be highlighted that even though some of those biosensor devices have been applied to the L-lactic acid determination in real samples, such as beers, wines or milk, the long-term stability of the proposed biosensor enable the real-time of the malolactic fermentation using the same biosensor during all the process.

4. Conclusions

A bienzymatic amperometric biosensor based on the co-immobilization of LOX and HRP entrapped in an electropolymerized PPy film was developed and characterized for the L-lactic acid analysis in wine samples collected during the malolactic fermentation. The biosensor fabrication was optimized in terms of electrogeneration conditions. The analytical performance was thoroughly assessed and demonstrated that the developed biosensor response presented a high sensitivity, a low LOD and a wide linear range, together with an excellent selectivity. Besides, the lifetime of the developed biosensor of more than 40 days is of key importance for monitoring L-lactate in a real scenario.

In order to demonstrate the potential application in real samples, the monitoring of the L-lactic acid concentration during the malolactic fermentation in red wines was

457 carried out and results were in excellent agreement with those obtained by the standard
458 colorimetric method.

459 Taking into account the simplicity of the biosensor fabrication, its compatibility
460 with the microelectronic technology, the long working stability and the characteristics
461 of the response, the developed PPY/LOX:HRP biosensor for the L-lactate acid
462 determination represents a competitive and feasible approach for the online monitoring
463 of malolactic fermentation in wineries.

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471

472 **Appendix A. Supplementary data**

473 Supplementary data associated with this article can be found in the online
474 version.

475

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Table 1. Data of calibration curves for L-lactate carried out during the analysis of the malolactic fermentation process of the three wines.

Calibration	Sensitivity ($\mu A\ M^{-1}\ cm^{-2)}$)	R^2
1st	-13465	0.995
2nd	-12782	0.993
3rd	-12998	0.994
4th	-14064	0.993

Table 2. L-lactate electrochemical biosensors described in the literature.

Enzymes	Electrode	Mediator	Immobilization	Sensitivity	Linear range (M)	LOD (M)	Stability	Samples	Reference
LOX/HRP	Carbon	Ferrocene	MWCNT/PS membrane	116880 µA M^{-1} cm^{-2}	1.1×10^{-6} - 5.6×10^{-5}	5.6×10^{-7}	40 % after 2 weeks	Wine Beer	[17]
LOX/HRP	Carbon		Screen printed	0.84 µA M^{-1} L (flow system)	1×10^{-5} - 2×10^{-4}	1×10^{-6}	90 % after 50 injections	Yoghurt Milk	[16]
LOX/HRP	Gold disk	TTF	MPA-SAM	2711 µA M^{-1}	4.2×10^{-7} - 2×10^{-5}	4.2×10^{-7}	91 % after 5 days	Synthetic wines	[15]
LDH	Glassy carbon		MWCNT-CHIT	8300 µA M^{-1} cm^{-2}	5×10^{-6} - 1.2×10^{-4}	7.6×10^{-7}	65 % after 7 days		[40]
LOD/LDH	ITO		PANI physical absorption	0.038 µA M^{-1}	1×10^{-4} - 1×10^{-3}	5×10^{-5}	3 weeks		[41]
LOD	Gold	PVI-Os	CNT-CHIT	0.0197 µA M^{-1} cm^{-2}	Up to 8×10^{-4}	5×10^{-6}			[42]
LOX/HRP	Gold	Ferrocyanide	PPy entrapment	14100 µA M^{-1} cm^{-2}	1×10^{-6} - 1×10^{-4}	5×10^{-7}	90 % after 42 days	Red wines	This work

***** *ITO: indium tin oxide; TTF: tetrathiafulvalene; PVI-Os: polyvinylimidazole-Os; MWCNT: Multiwalled carbon nanotubes; PS: polystyrene; MPA: 3-mercaptopropinic acid; SAM: self-assembled monolayer; CNT: carbon nanotubes; CHIT:* chitosan.

475 **Figure Captions**

476

477 **Fig. 1**. SEM images of, (A) the PPy/LOX:HRP film; (B) the silicon chip showing the
478 polymer film selectively deposited on the working electrode surface.

479 **Fig. 2** A) Chronoamperometric response of the PPY/LOX:HRP biosensor for L-lactate
480 concentrations from 5×10-7 M (a) to 1×10-3 M (g). B) Biosensor calibration curve.
481 Each point represents the mean current value of three replicates recorded consecutively
482 with the same biosensor, with the error bars being the corresponding standard deviation.

483 **Fig. 3.** Current density response of the biosensor in solutions containing possible
484 interferences. All of them are in a concentration of 1×10^{-5}M and were compared to the
485 signal recorded in the background electrolyte (no analyte).

486 **Fig.4**. Variation of the biosensor sensitivity along 50 days.

487 **Fig. 5**. Analysis of wine samples collected during the malolactic fermentation process
488 for three red wines. Dashed lines and filled circles show the values of L-lactic acid
489 obtained with the colorimetric standard method and filled lines and squares ones, those
490 measured with the proposed PPY/LOX:HRP biosensor. In the case of the biosensor, the
491 standard deviation values of three replicates are represented as error bars in the plot. In
492 the case of the colorimetric method, the error bars represent the uncertainty at 95 %.

Fig. 1

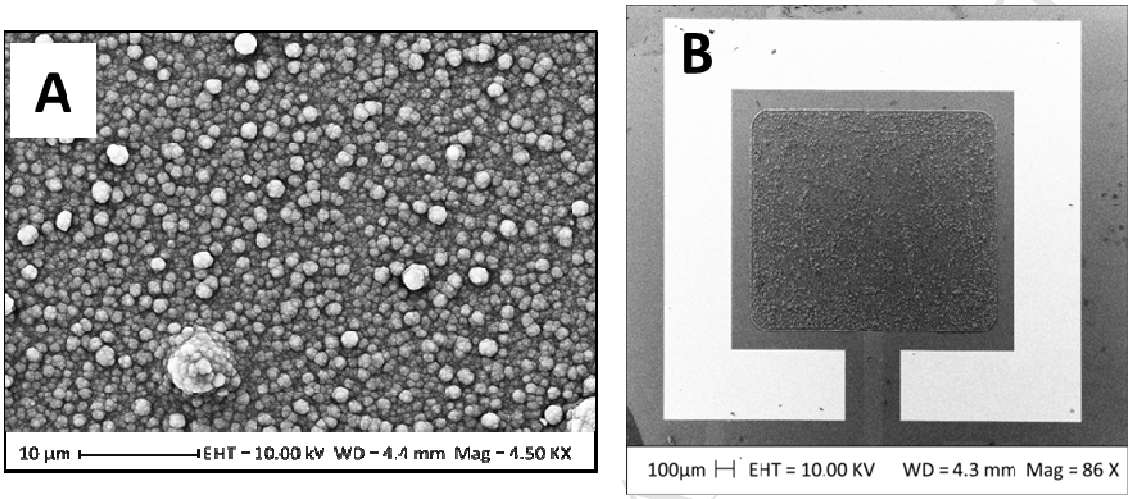


Fig. 2

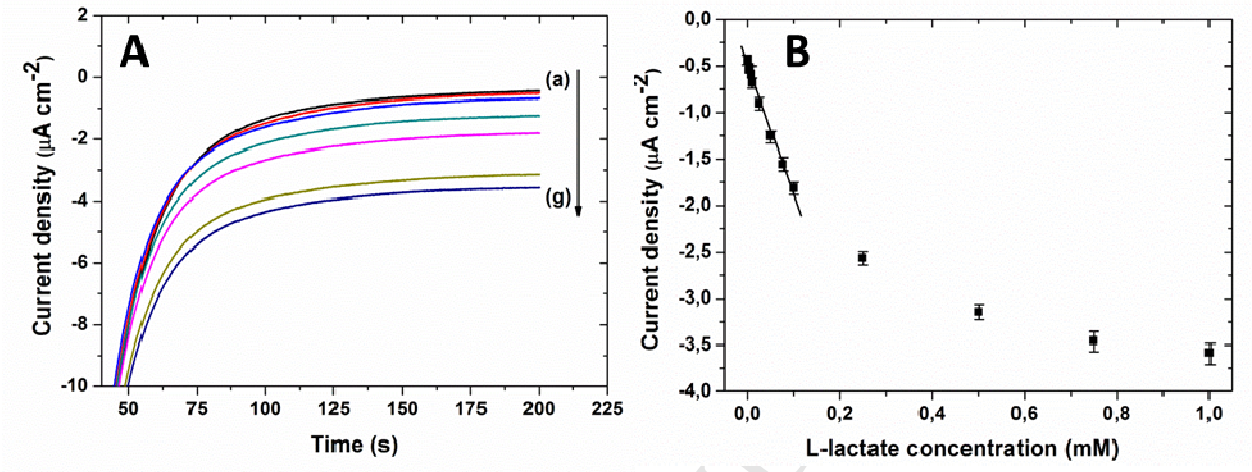


Fig. 3

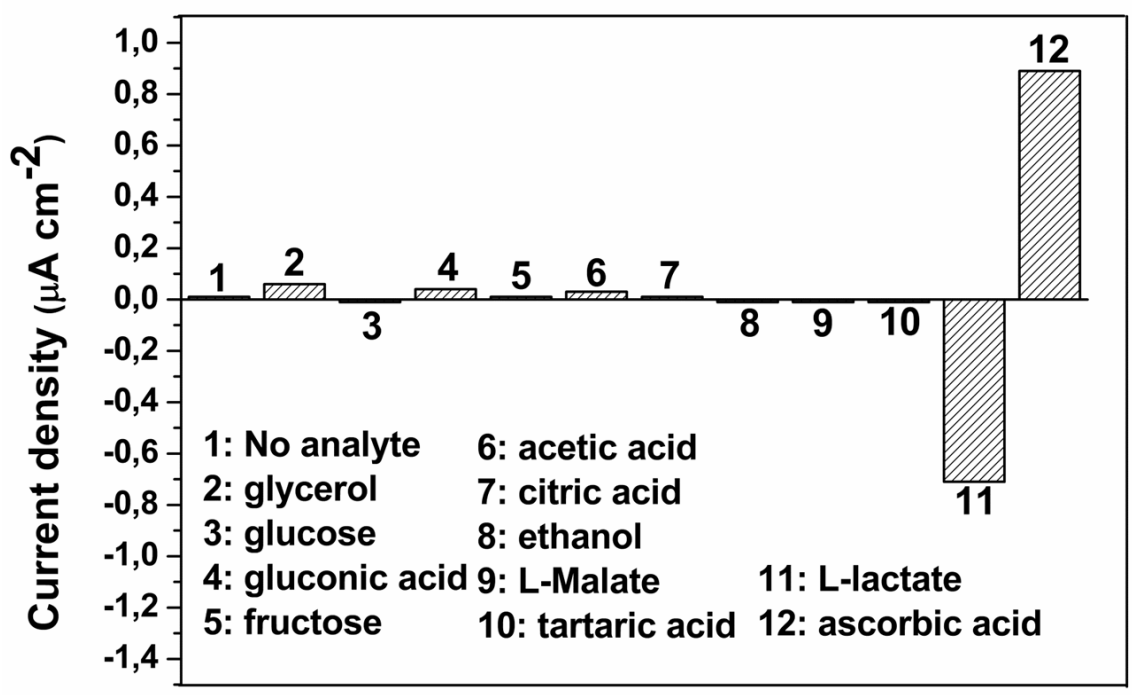


Fig. 4

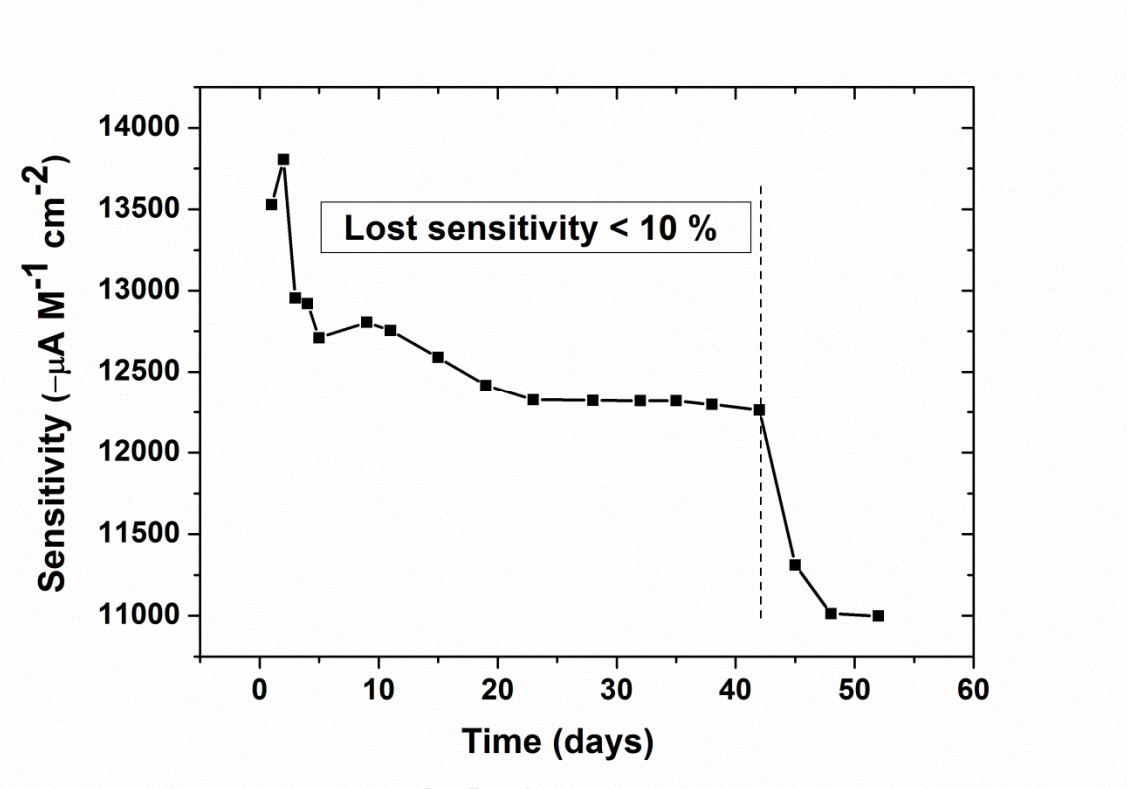
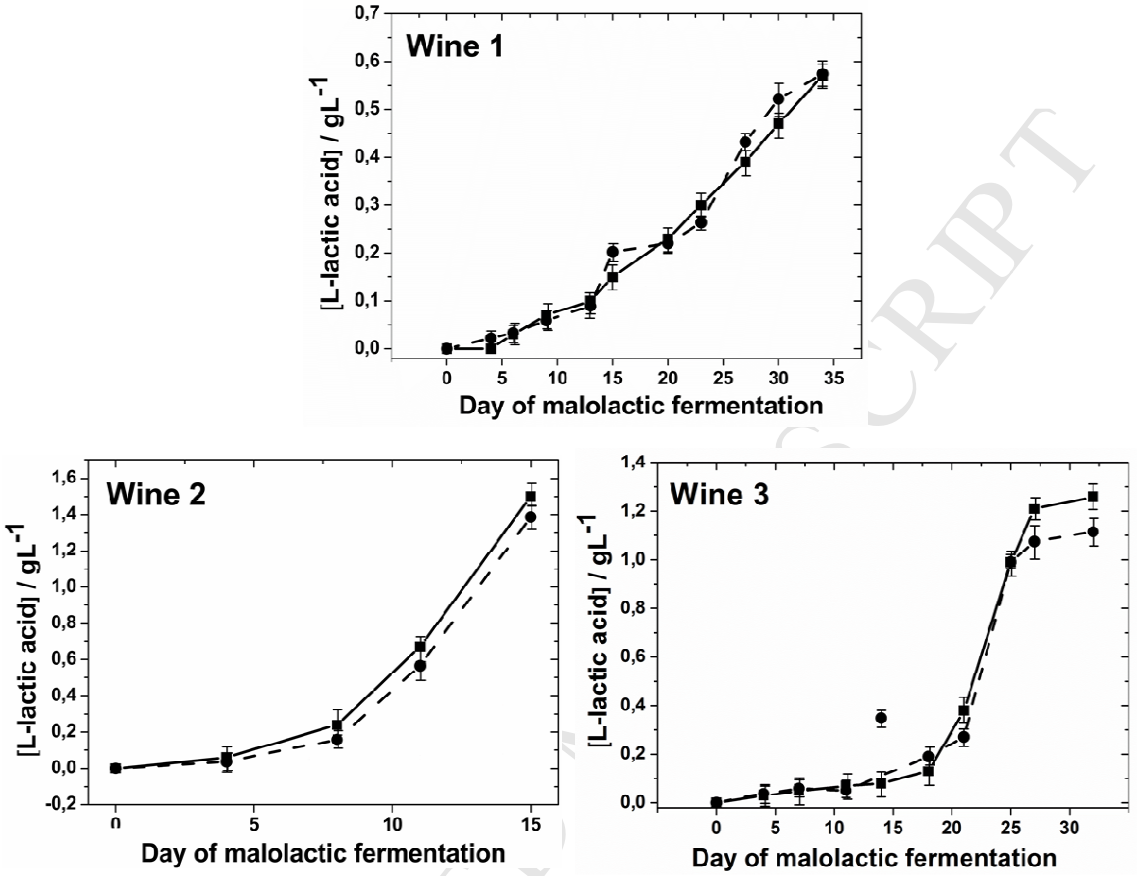


Fig. 5



Highlights

- Biosensor for L-lactate based on the co-immobilization of LOX and HRP in an electrosynthesized PPy film
- Extended lifetime of over 40 days
- Successful application to the L-lactic acid monitoring during the malolactic fermentation process of three different red wines