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Monitoring of malolactic fermentation in wine using an electrochemical bienzymatic biosensor for L-lactate with long term stability

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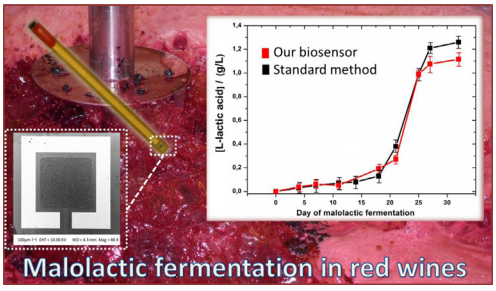
HIGHLIGHTS

- Biosensor for L-lactate based on an electrosynthesized PPy film.
- Extended lifetime of over 40 days.
- Application to L-lactic acid monitoring during the malolactic fermentation process.

GRAPHICAL ABSTRACT



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ABSTRACT

L-lactic acid is monitored during malolactic fermentation process of wine and its evolution is strongly related with the quality of the final product. The analysis of L-lactic acid is carried out off-line in a laboratory. Therefore, 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, 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 $-(13500 \pm 600)$ 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.

1. Introduction

Measuring and controlling L-lactic acid concentrations is

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

One of the main drawbacks of biosensor devices is the limited lifetime mainly related to the biomolecules stability and the procedure applied for their incorporation onto the transducer surface. Therefore, the chosen immobilization procedure has to be studied in detail and optimized in order to maximize the working stability over time. The enzyme entrapment in three-dimensional matrices [19,20] proved to be a good alternative due to the simple fabrication and the not required modification of the enzyme structure, which improves the lifetime of the biosensor. Besides, the enzyme entrapment in a membrane of a conducting polymer by electropolymerization is a common strategy in amperometric biosensors [21–23]. A one-step controlled process is carried out under potentiostatic (set potential) or galvanostatic (set current) conditions in a solution containing the monomer and the enzymes. Both approaches induce the oxidation of the monomer and the formation of an electrogenerated polymer layer, 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 °C. Horseradish peroxidase (HRP, type VI-A, essentially salt-free, lyophilized powder, 250–330 U mg^{-1} solid) was stored in a refrigerator at 4 °C and used as received. Pyrrole (reagent grade, 98%) was distilled every week and stored in a freezer at −20 °C. 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.