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PAPER

Amperometric bienzymatic biosensor for L-lactate analysis in wine and beer samples†

Sandra Pérez* and Esteve Fàbregas

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A novel amperometric bienzymatic biosensor has been developed based on the incorporation of Lactate Oxidase (LOx) and Horseradish Peroxidase (HRP) into a carbon nanotube/polysulfone membrane by the phase inversion technique onto screen-printed electrodes (SPEs). In order to improve the sensitivity and reduce the working potential, experimental conditions have been optimized and ferrocene has also been incorporated into the membrane as a redox mediator of the enzymatic reactions, which allows the reduction of H_2O_2 at −100 mV. Measurements were carried out in phosphate buffer solution at pH 7.5 and under batch conditions. The biosensor response time to L-lactate was only 20 s and showed a good reproducibility (RSD 2.7%). Moreover, the detection limit was 0.05 mg L^{-1} of L-lactate with a linear interval range from 0.1 mg L^{-1} to 5 mg L^{-1}. Finally, the biosensor has been applied to the determination of L-lactic acid in different wine and beer samples. Then, the results obtained with the biosensor were compared with the ones obtained using, as a reference method, a commercial kit based on spectrophotometric measurements, obtaining an excellent agreement between the results, validating our approach.

Introduction

Determination of L-lactic acid is important in several areas such as clinical diagnostics,[1] sports medicine[2] and food analysis.[3] Particularly in wine, L-lactic acid is mainly produced from the decomposition of L-malic acid during malolactic fermentation.[4] In the winemaking industry, the course of malolactic fermentation is monitored by the decrease of L-malic acid concentration and the increase of L-lactic acid level. The control of this process is important given that it contributes to the mouthfeel of wine, enhancing the body and flavour persistence of wine, and producing wines of greater palate softness and roundness. If this process occurs in the bottle and is uncontrolled the wine will appear to the consumer to still be fermenting; thus being not suitable for commercialization, and preventing significant economic losses with its control. The primary objective of this transformation is to deacidify the wine, at the same time it also increases the biological stability of the wine and improves its organoleptic properties. For these reasons, following the levels of malate and lactate in wines could be considered as a quality test.

Several analytical procedures for the determination of L-lactic acid, such as liquid,[5] ion-exchange[6,7] and gas chromatography[8,9] or colorimetric methods[10] based on enzymatic reactions, have been reported. Generally, the problem of these methods is that they are arduous, time-consuming and laborious, and some of them also need some sample pre-treatment and reagent preparation.[11] Therefore, biosensors represent an alternative to classical methods of analysis with huge advantages; *i.e.* a rapid, simple and direct measurement besides their low cost production. Usually, developed lactate biosensors are based on lactate dehydrogenase (LDH) or oxidase (LOx) enzymes.[11–17] On the one hand, biosensors based on dehydrogenases require the presence of NAD^+, a cofactor which is reduced to NADH and makes the enzymatic system more sophisticated and expensive.[16] Besides this, the incorporation of this cofactor into the biosensor is not easy and could cause a limitation in the lifetime if not enough NAD^+ was immobilized. Otherwise, in the case where it is not possible its incorporation results in the usage of large amounts of this cofactor to be added to the measuring cell, increasing in this way the cost. On the other hand, lactate oxidases contain the cofactor FAD in its structure, which catalyze the conversion of lactate and O_2 to pyruvate and H_2O_2, respectively. Given that the oxidation of H_2O_2 occurs at high potentials, other species can be oxidised and act as interferences, ascorbate being the most typical one.[18–20] In order to avoid these problems, there are some strategies to reduce the working potential. Usually this could be achieved with the development of bienzymatic biosensors using Horseradish Peroxidase (HRP),[21–23] with the use of redox mediators[16,23–25] or even combining both approaches.[23] In this manner, bienzymatic biosensors combine the enzymes lactate oxidase and horseradish peroxidase, using Ferrocene (Fc) as a mediator for the amperometric detection of H_2O_2. LOx catalyzes the oxidation of lactate to pyruvate through the reduction of O_2 to hydrogen peroxide, which is then reduced to H_2O by HRP and finally, detected at the

Sensors and Biosensors Group, Department of Chemistry, Universitat Autònoma de Barcelona, Edifici Cn, 08193 Bellaterra, Barcelona, Spain
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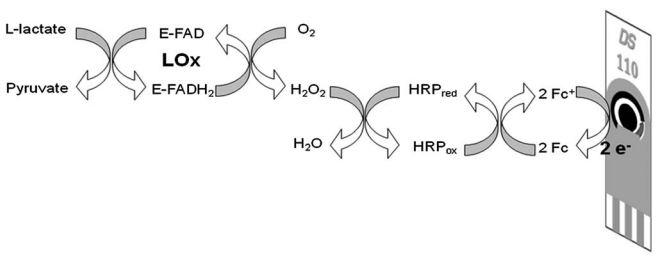
electrode surface through the reduction of ferrocenium generated (Scheme 1). In that case, a reduction potential might be applied to detect the H_2O_2 produced. Some of the analytical characteristics of these biosensors are summarized in Table 1.

Moreover, in the last few years, new strategies for the determination of L-lactate based on biochemical logic gates have appeared in the literature. These strategies combine the specificity of enzymes with digital information processing, obtaining a binary response (1/0) instead of a quantitative value. For instance, an AND/IDENTITY logic gate based on lactate oxidase, horseradish peroxidase and glucose oxidase was designed with the aim of processing biochemical information related to the normal and pathophysiological conditions.[29]

Enzymes have been incorporated into polysulfone (PS) membranes by phase inversion (PI). This technique has been used in recent years for the immobilization of different biomolecules.[16,24,30–35] A thin film of polymer solution (in DMF) is deposited onto the surface of the electrode and then immersed into an aqueous solution (non-solvent) that contains the biomaterial. Thus, causing an exchange of the solvent by the non-solvent and the precipitation of the membrane, biomolecules present in aqueous solution are trapped and incorporated into the polysulfone membrane. Polysulfone[36–39] is an attractive material because of its chemical and biological stability,[40] high resistance in extreme pH, thermal stability and porosity. Also its porous size is controllable depending on the polymer concentration, batch conditions in the phase inversion process, *etc.* Due to all these properties, it has been used for many applications as a polymer for commercial microfiltration and ultrafiltration membranes.

The combination of a PS polymer membrane with Multi-Walled Carbon Nanotubes (MWCNTs) as a transducer[30–32,36] offers unique properties for the easy incorporation of biological moieties, providing a composite material with a high electrochemical response to the corresponding analyte. Since carbon nanotubes were discovered by Iijima,[41] they have received a great deal of attention as an electrode material. CNTs have been used in sensing applications because of their excellent electrical properties, high surface-to-volume ratio, high chemical stability and minimization of surface fouling onto electrochemical devices. However, CNTs also display remarkable catalytic properties towards the oxidation of numerous biological substances such as acetaminophen, dopamine, urate, ascorbate, *etc.* and as a consequence of this, their selectivity decreases.[42]

Herein, we report the development of an amperometric bienzymatic biosensor for the determination of L-lactate based on a MWCNT/PS membrane deposited onto screen-printed electrodes, where LOx and HRP were incorporated by the phase



Scheme 1 Schematic diagram of the reactions involved for the lactate determination.

Table 1 Some analytical characteristics of L-lactate biosensors

Enzymes	Material working electrode	Mediator, cofactor	Potential	pH	Linear interval range (M)	LOD (M)	Sensitivity ($\mu A\ M^{-1}\ mm^{-2}$)	Stability	RSD (%)	Samples	Reference
LOx (EC. 1.13.12.4); *Peddiococcus* sp./HRP type II from *horseradish*	MWCNT/PS onto carbon screen printed electrodes	Ferrocene	−100 mV	7.5	1.1×10^{-6} to 5.6×10^{-5}	5.6×10^{-7}	1168.8	40% of its original response after 2 weeks	2.7	Wine and beer	This work
LOx (EC. 1.13.12.4); *Peddiococcus* sp.	MWCNT/PS onto carbon screen printed electrodes	Cobalt(II) phthalocyanine	350 mV *vs.* Ag/AgCl	7.5	5.0×10^{-6} to 5.0×10^{-4}	3.4×10^{-6}	104.7	30% of initial signal after 20 days	5.3	—	16
LOx (EC 1.1.3.2); *Peddiococcus* sp./HRP type II from *horseradish*	3-mercaptopropionic acid self-assembled monolayer SAM-gold electrode	Tetrathiafulvalene	−50 mV *vs.* Ag/AgCl	6.5	4.2×10^{-7} to 2.0×10^{-5}	4.2×10^{-7}	383.7	91% of initial signal after 5 days	8.3	Synthetic wine	26
LOx (EC. 1.1.3.2); *Peddiococcus* sp.	Glutaraldehyde/ polypyrrole onto Pt	—	—	7	0–2 × 10^{-2}	—	1.0	86% of initial signal after 2 days	1.6	Tomatoes juice	27
LOx (EC. 1.1.3.2); *Peddiococcus* sp./HRP type II from *horseradish* LOx/HRP	Chitosan/MWCNT onto gold electrodes	Ferrocyanide	−50 mV *vs.* Ag/AgCl	7	5.0×10^{-6} to 3.4×10^{-4}	1.66×10^{-6}	925.5	90% of initial sensitivity -after 15 months	3.7	Wine and food samples	28
LOx *Peddiococcus* sp./ HRP from *horseradish*	Nylon/polyazetidine onto an oxygen Clark electrode	—	−650 mV *vs.* Ag/AgCl	7	5.6×10^{-5} to 3.4×10^{-3}	2.8×10^{-5}	231.6 $\mu A\ M^{-1}$ (area not declared)	180–200 assays	2.8	Wine	22
LOx (EC. 1.1.3.2); *Peddiococcus* sp./HRP (EC. 1.11.1.7); type II	Graphite/Teflon	Ferrocene	0.0 mV *vs.* Ag/AgCl	7.4	5.0×10^{-6} to 1.0×10^{-4}	1.4×10^{-6}	60	60 days	6.6	Yogurt, red wine	23

inversion technique. In this fashion, several works have demonstrated the advantages of this PS/CNTs composite matrix and its ability to easily immobilize biomolecules in a few minutes.[30] Hence, the benefits of these membranes combined with the use of commercial SPEs offer the possibility of developing a more portable system which in addition could allow carrying out on-field analyses. Now is the first time that a MWCNT/PS based biosensor has demonstrated its applicability towards the determination of compounds in real samples, in this case, the quantification of L-lactate in wine and beer samples, comparing its results with those obtained by a reference method.

Experimental

Reagents and solutions

Lactate oxidase from *Pediococcus* species (20 units mg^{-1}), peroxidase, type II from horseradish (188 units mg^{-1}), sodium L-lactate, sodium phosphate dibasic anhydrous, potassium chloride, Bovine Serum Albumin (BSA) and Multi-Walled Carbon Nanotubes (MWCNTs; length 0.5–200 μm and outer diameter 30–50 nm), sodium citrate tribasic dehydrate, L(+)-ascorbic acid, sodium succinate dibasic hexahydrate, gallic acid, L-malic acid, D(+)-glucose monohydrate, ethanol, and acetic acid were purchased from Sigma-Aldrich© Chemie (Steinheim, Germany) and sodium tartrate from Alco (Terrassa, Spain). Polysulfone was obtained from BASF (BASF Ultrasons S 3010 natur, Frankfurt, Germany). *N*,*N*-Dimethylformamide (DMF) and nitric acid were purchased from Panreac (Barcelona, Spain). Ferrocene (Fc) was purchased from Alfa Aesar (Germany). Kit acid L-lactic liquid (Steroglass SQPE052029) was purchased from RAL, *técnica para el laboratorio*, S.A. (Barcelona, Spain).

All solutions were prepared with deionised water obtained from PURELAB® Ultra Laboratory Water Purification Systems, and measurements were taken in a phosphate buffer (0.1 M $HPO_4^{-2}/H_2PO_4^-$, 0.1 M KCl, pH 7.5). MWCNTs were previously purified by stirring them in 6 M nitric acid for 2 hours and dried at 80 °C.

Wine and beer samples

In order to test the applicability of the developed biosensor to food industry analysis, a total of 15 samples were acquired at the local supermarket and analyzed. The formed set includes 8 wines (6 red, 1 rosé and 1 white) and 7 beer samples. Only one sample of white and rosé wine was considered given this type of wine is not usually subjected to malolactic fermentation, therefore it was very difficult to find some samples with a significant amount of lactic acid. Moreover, this parameter is not of interest in these cases given it does not provide relevant information to the wine. Detailed information of the wines and beers used as well as the results obtained are summarized in Table 2.

Apparatus

Amperometric and voltammetric measurements were taken using a μStat 200 Bipotentiostat from Dropsens (Oviedo, Spain). DropView software package was used to control the instrument, register and perform the analysis of the results. Carbon screen-printed electrodes (SPEs) were supplied by DropSens (Ref. 110) (Oviedo, Spain), which consist of a carbon working electrode (4 mm diameter), a carbon counter electrode and a silver reference electrode in the same support. Spectrophotometric measurements were carried out using a Spectronic Helios Epsilon spectrophotometer from Thermo Electron Scientific Instruments LLC (Madison, WI, USA). "Ultrasons 3000683" from J.P. Selecta (Barcelona, Spain) has been used for sonication.

Biosensor preparation

In a previous step prior to biosensor preparation, SPEs were electrochemically activated using cyclic voltammetry in 0.05 M potassium ferricyanide ($K_3[Fe(CN)_6]$) in order to improve their signal-to-noise ratio (*S*/*N*).[5,43] For this, the potential sweeps between −0.6 V and +0.6 V *vs.* Ag/AgCl pseudoreference electrode, with a scan rate of 100 mV s^{-1} and a step potential of 9 mV (Fig. S1A, see ESI†). In this manner, only five cycles were necessary to activate the electrodes and get stable responses. In this way, the noise in current signals was drastically reduced as can be seen in Fig. S1B†, where chronoamperometric measurements corresponding to successive additions of $K_3[Fe(CN)_6]$ were done before and after SPE activation. These activation processes are commonly used and achieved with pre-anodization treatments, where the improvement in *S*/*N* is usually attributed to the role of increased surface functionalities and roughness or removal of the surface contaminants. These activations have a stripping or renewing effect on the surface of the carbon electrodes, in a similar manner as the polishing step carried out with conventional carbon electrodes in order to renew its surface, not applicable in SPEs due to its nature.

The polysulfone composite membranes were prepared by Phase Inversion (PI) following the methodology previously established.[33] To assemble the PS/MWCNT/Fc/LOx/HRP membrane, the first step was to prepare the PS solution. For this, 84 mg of PS were dissolved in 1 mL of DMF. Then, 50 μL of this solution were mixed with 5 mg of ferrocene and 1 mg of MWCNT. The as-prepared CNTs usually aggregate into bundles because of the van der Waals forces, therefore it was necessary to sonicate for 1 hour to obtain a homogeneous dispersion. Next, 0.6 μL of PS/MWCNT/Fc/DMF paste was deposited onto the Dropsens SPE working electrode. Finally, to incorporate LOx and HRP, 5 μL of an aqueous solution of these enzymes (phase inversion solution) were added over the previous paste, causing the coagulation of the membrane through the phase inversion process. The phase inversion solution contains 10 mg mL^{-1} of LOx, 38 mg mL^{-1} of HRP and 38 mg mL^{-1} of BSA in phosphate buffer solution at pH 7.5. The phase inversion process only took 5 minutes, and afterwards the biosensor was dipped for 5 minutes in a buffer phosphate solution under continuous stirring to clean the sensor. The modified electrodes with films incorporating the enzyme were stored in phosphate buffer solution pH 7.5 at 4 °C.

Amperometric measurements

Amperometric measurements were carried out in batch mode in an open vessel at room temperature (25 °C). First, the bienzymatic biosensor was immersed in the electrochemical cell containing 10 mL of PBS buffer (0.1 M $H_2PO_4^-/HPO_4^{-2}$ and 0.1 M KCl at pH 7.5) under forced convection by stirring the solution

Table 2 Concentrations of L-lactate in various beverage samples determined using amperometric and spectrophotometric methods

	Samples	Sensor concentration (g L^{-1})	Confidence interval[a] (g L^{-1})	RSD (%)	Kit concentration (g L^{-1})	Difference (%)
Red wines	*Serrasegué*	1.06	0.08	2.8	1.03	−2.9
	Valls	0.53	0.03	1.9	0.55	3.6
	Terres negres 1	0.76	0.03	1.3	0.79	3.8
	Terres negres 2	0.79	0.03	1.3	0.81	2.5
	Terres negres 3	0.76	0.07	1.3	0.77	1.3
	Campo viejo	1.11	0.03	2.7	1.14	2.6
White wines	*Gandesa*	0.93	0.02	0.9	0.94	1.1
Rose wines	*Gourmet*	0.88	0.04	2.3	0.88	0.0
Beers	*Voll Damm*	0.067	0.004	3	0.071	5.6
	Carrefour	0.16	0.01	2.5	0.17	5.9
	Moritz	0.277	0.004	0.6	0.28	1.1
	San Miguel	0.030	0.001	1.7	0.029	−3.4
	Adlerbrau	0.047	0.002	2.1	0.048	2.1
	San Miguel (*alcohol-free beer*)	0.043	0.003	3	0.044	2.3
	Xibeca (*Damm*)	0.053	0.003	2.4	0.055	3.6

[a] Confidence intervals calculated with a confidence level of 95% $n = 3$ and $t_{tab} = 4.303$.

for 3 minutes until the current intensity became stable. Then, additions of L-lactate of known concentration were done, with a biosensor response time of only 20 seconds, and readings were taken under steady-state conditions as the average of the last 5 seconds (10 measurements with an interval of 0.5 s). Measurements were done in replicate, and the results given are the averages of the repeated measurements with their corresponding Relative Standard Deviations (RSD).

As shown in Scheme 1, lactate is oxidised to pyruvate by LOx generating H_2O_2, which is then reduced to H_2O by HRP and finally detected at the electrode surface through the reduction of ferrocenium generated. After the study of the optimal working potential, measurements were carried out at −100 mV *vs.* reference electrode.

Spectrophotometric measurements

For comparison purposes, wine and beer samples were also analyzed using a spectrophotometric commercial kit for the detection of L-lactate as the reference method. The kit was also based on enzymatic reactions, where L-lactic acid is oxidized to pyruvate by means of the enzyme L-Lactate Dehydrogenase (LDH) and the cofactor Nicotinamide Adenine Dinucleotide (NAD^+). As this reaction is in equilibrium, and in the presence of pyruvate and NADH it is far in favour in the formation of lactate, it is needed to remove the pyruvate from the system in order to dehydrogenate lactate completely. For this, pyruvate is withdrawn from the media by its conversion to alanine in the presence of the enzyme D-Glutamate Pyruvate Transaminase (D-GPT). Finally, the amount of NADH formed in the above coupled reactions is measured by the increase in absorbance at 340 nm, and stoichiometrically related to the amount of L-lactic acid present in the sample.

Results and discussion

Amount of enzyme in membrane

First of all, the amount of LOx enzyme used for the PI process in the biosensor preparation was evaluated.[16] For this, three different PI solutions containing different amounts of enzyme were prepared: 5, 10 and 20 mg mL^{-1} LOx in PBS. Then, as described in the biosensors preparation section, but without the incorporation of HRP and Fc, three different PS/MWCNT/LOx biosensors were constructed and its response was evaluated by means of their amperometric calibration curves towards L-lactate at 600 mV *vs.* reference electrode to detect the H_2O_2 produced. The linear interval and sensitivity of calibrations increase with the amount of enzyme incorporated into the membrane,[16,44] with a high improvement from 5 to 10 mg mL^{-1} of LOx, but a less significant effect from 10 to 20 mg mL^{-1}. Thus, 10 mg mL^{-1} of LOx was chosen as the optimal enzyme concentration due to the gain obtained in its response when employing more enzyme does not justify its increase.

Once optimized the amount of LOx used for the biosensor construction, an excess of HRP was also incorporated[31] into the PI solution. As said, this enzyme catalyzes the conversion of hydrogen peroxide to water, allowing the reduction of the working potential from 600 mV to −100 mV *vs.* reference electrode. Given that in the bienzymatic system the response of the biosensor will depend on HRP_{ox} generated in the second enzymatic reaction, and to guarantee that our signal is kinetically related to the substrate we want to determine (L-lactate acid), we must have the system controlled by the first reaction. Therefore, a higher proportion of HRP (*ca.* 500 units, 2.5 times more than LOx[31]) was added to the phase inversion solution. This was to make sure that in the case of enzyme saturation, all the lactate that reacts will be detected. In addition, the PI solution also contains 10 mg mL^{-1} of BSA[45] to improve the retention of enzymes into the PS membrane and as a consequence, the biosensor reproducibility. This is because the protein provides a more "comfortable" environment for the enzyme; since it could be expected that the immobilization of enzymes on a membrane will at least change its hydrophobicity, its surface charge and morphology; then, the use of BSA would be beneficial to improve these conditions, hence enhancing its immobilization and increasing at the same time its stability.

Electrochemical behaviour

In order to study the optimal working potential to carry out the L-lactate determination, cyclic voltammetry and amperometry measurements were performed. First, the enhancement of the signal when incorporating ferrocene as a mediator into the membrane was evaluated. For this, two different biosensors were prepared, one without the mediator in its membrane (PS/MWCNT/LOx/HRP) and the other with the mediator incorporated into it (PS/MWCNT/Fc/LOx/HRP). Then, the two biosensor responses were evaluated by cyclic voltammetry in a 0.1 g L^{-1} solution of L-lactate in PBS buffer pH 7.5. As can be seen in Fig. 1A, the reduction potential when ferrocene is absent is around −0.1 V, whereas in the case that the sensor contains ferrocene, this is around 0.1 V. Despite working without ferrocene is possible, the current intensity obtained in L-lactate calibrations is much higher in the case of PS/MWCNT/Fc/LOx/HRP. Therefore, this configuration was the one chosen for further experiments with lactate.

Once the benefits of the incorporation of ferrocene into the membrane were confirmed, the next step was to carry out an amperometric study in order to find the optimal working potential. Fig. 1B shows the maximum reduction signal at 0 V. Although even at this potential it was possible to obtain a good L-lactate response, owing to the fact that in real samples other species can be oxidized and act as interferences at this potential, −100 mV was selected as the working potential. At this potential, the reactions showed in Scheme 1 take place and L-lactate is detected.

Effect of pH buffer solution

Lastly in order to find the optimal measurement conditions, the influence of the pH in the biosensor response was also studied. In this case, pH has two different effects that must be considered: the enzymes activity (both for LOx and HRP) and the mediator (Fc). In order to study the biosensor response at different pHs, amperometric measurements of a 1 mg L^{-1} L-lactate solution were taken in PBS buffer from pH 6.5 to 8.5. For each pH, two replicas of the measurements were done. As shown in Fig. 2, for pH values between 6.5 and 7.5 the reduction current increases steeply as the pH increases, until pH 7.5 when it reached the maximum value; then for pH values higher than 7.5, the response decreased again. Therefore, this pH was selected as the optimum

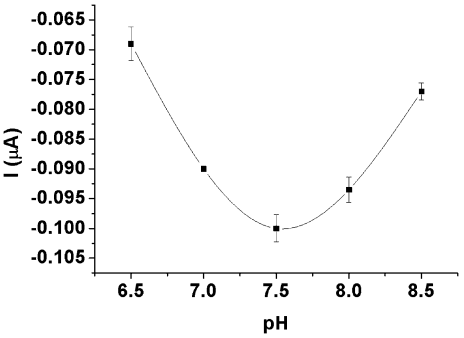


Fig. 2 Study of the pH of the phosphate buffer solution. Batch amperometric measurements were carried out in duplicate at a constant concentration of 1 mg L^{-1} of L-lactate, 0.1 M $H_2PO_4^-/HPO_4^{-2}$ and 0.1 M KCl, at −0.1 V.

and further experiments with the biosensor were carried out at pH 7.5.

Storage stability and linear interval range

Two different strategies to store the prepared biosensors were tested in order to attain longer lifetime and enzyme activity for the biosensor. The first strategy was to keep the biosensor dried at 4 °C. However, under these conditions biosensors lost almost all of its activity in one day, *i.e.* the sensitivity of the current response decreased *ca.* 90%. Hence, another strategy was attempted to improve its storage stability. For this, biosensor was kept at 4 °C as before, but immersed in 0.1 M PBS (pH 7.5) solution. With this, results improved significantly, although the response still decays slightly compared with the first day. Then it decreased slowly every day as shown in Fig. 3, keeping up to *ca.* 40% of its original response after 2 weeks of storage. Therefore, all electrodes were stored in buffer solution at 4 °C between measurements.

Once all the experimental conditions and the membrane composition were optimized, the next step was to evaluate the linear behaviour of the biosensor towards L-lactate. Thus, the calibration plot between 0.1 and 25 mg L^{-1} was built from the batch measurements based on addition of different microvolumes of a L-lactate 0.1 g L^{-1} solution to PBS buffer. Then, linear least-squares regression was fitted and it was observed that

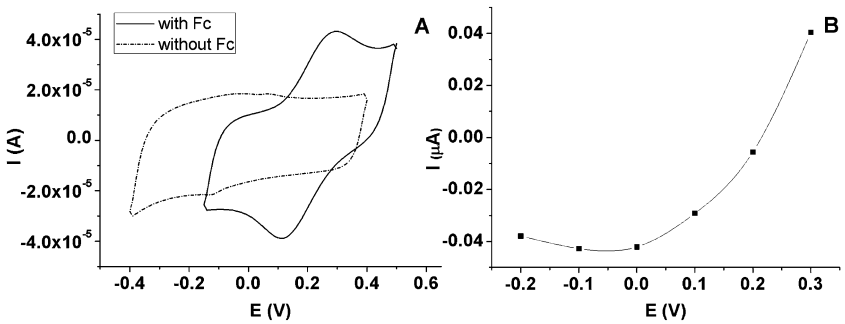


Fig. 1 Study of the working potential. (A) Cyclic voltammetric measurements were performed with a biosensor in the presence or absence of ferrocene as a mediator inside the membrane (scan rate = 100 mV s^{-1}). (B) Batch measurements were carried out by varying the working potential. All measurements were carried out at a 0.5 mg L^{-1} L-lactate solution in PBS (pH 7.5).

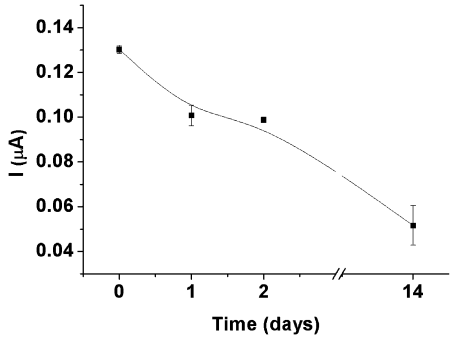


Fig. 3 Lifetime stability. Amperometric measurements were performed at 0.1 M phosphate buffer solution at pH 7.5 and 1 mg L^{-1} of L-lactate solution.

for values higher than 5 mg L^{-1} there was a loss of linearity as shown in Fig. 4. Therefore, it was chosen the interval between 0.1 and 3.5 mg L^{-1} as the linear interval range for the PS/MWCNT/Fc/LOx/HRP biosensor towards L-lactate and the one used to carry out further measurements.

Reproducibility

The reproducibility of the developed biosensor was evaluated in two different manners. First, the reproducibility of its response towards a standard solution of L-lactate was compared. In this manner, five replicates of the biosensor response towards a 1 mg L^{-1} L-lactate solution were taken. Results showed a good reproducibility between measurements, with an RSD of 2.7%. In addition, the reproducibility between calibrations was also tested performing three successive replicates of L-lactate calibration between 0.5 and 3.5 mg L^{-1} (Fig. 5). Results were also highly reproducible in the chosen working range with a RSD of 1.4% for the slopes of the different calibrations, and a Limit of Detection (LOD) of 0.053 mg L^{-1} of L-lactate. LOD is defined as the concentration corresponding to three times the blank standard deviation. The Standard Error of Estimate (SEE) could be taken as an estimation of the blank standard deviation, that it is the standard deviation of the residuals from the regression calculation. Therefore, LOD was calculated from the regression of the five calibration curves. In Fig. 5 a calibration curve for L-lactate has been represented, where values of each concentration were taken as the average of 5 replicates.

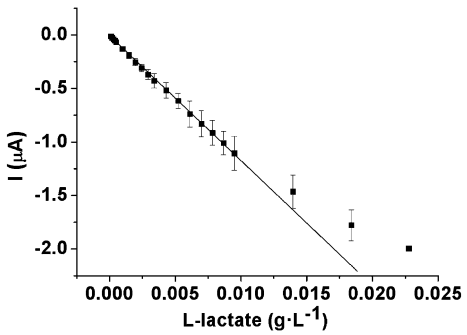


Fig. 4 Evaluation of the biosensor response and linear range towards L-lactate. Measurements were carried out at a 0.1 M phosphate buffer solution at pH 7.5.

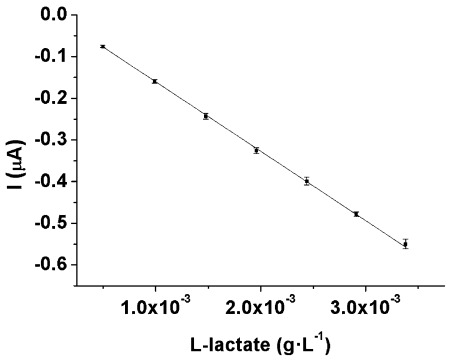


Fig. 5 L-Lactate calibration curves as average of five replicates for successive calibrations with the same biosensor. Measurements were performed at phosphate buffer solution (pH 7.5).

Biosensor's response

In order to evaluate our biosensor behaviour, a comparison of different performance parameters with other reported amperometric L-lactate biosensors has been carried out (Table 1). These biosensors have been developed using different types of supports, such as gold, platinum or carbon electrodes. The vast majority also used mediators such as ferrocene, ferrocyanide, tetrathiafulvalene or cobalt(II) phthalocyanine in order to decrease the working potential, otherwise the potential to carry out the measurements would be much higher, around +650 mV *vs.* Ag/AgCl. Moreover, as can be seen, HRP is frequently used as a second enzyme to catalyze the reduction of H_2O_2 produced in the first reaction. Regarding the linear interval range for L-lactate, biosensors reported present similar values, between 1.5 and 2 orders of magnitude, and normally starting nearly 10^{-6} M. In this sense, developed biosensor displayed an excellent sensitivity of 1168.8 mA M^{-1} mm^{-2}, combined with one of the lowest LOD and RSD values in comparison with the other reported ones. However, its main drawback is its stability, keeping only 40% of its initial signal after 2 weeks, whereas in the case of other published L-lactate biosensors this value is higher. Despite this disadvantage, this does not represent a significant problem since SPEs are meant to be used in a disposable manner.

Interferences study

In order to ensure its applicability to real samples, potentially interfering substances contained in wine and beer samples were evaluated, namely mallic acid (which is converted to lactate during malolactic fermentation); gallic acid (as one of the major phenolic compounds found in wine and beer); glucose (along with fructose, which is one of the main fermentable sugars in wine grapes); ethanol (since wine and beer have a 12–16% v/v and 3–9%, respectively); ascorbic acid (naturally present and often added as anti-oxidant); and also tartaric acid, succinic acid, citric acid and acetic acid (as part of the main compounds found in wines and usually considered for the preparation of synthetic wine).[46,47] The effect of all these compounds was checked carrying out amperometric measurements in PBS buffer with a concentration of 2 mg L^{-1} of each one and comparing the results with those obtained for the same L-lactate concentration. As can be seen in Fig. 6, under the optimized experimental conditions

selected for the determination of L-lactate, only gallic and ascorbic acids showed some response, representing 1.7 and 10.2% of L-lactate current signal, respectively. In the case of gallic acid, the studied concentration was close to the total phenolic content in red wine (2.16 g L^{-1}) and in beer samples (0.52 g L^{-1}) once the dilution factor applied to those samples for the determination of L-lactate has been taken into account, and in the case of ascorbic acid, its usual concentration in wine varied between 5 and 12 mg L^{-1}, lower than the content of L-lactate. For both interferences, although some response was obtained, taking into account the dilution step prior to L-lactate determination in wine (1 : 1000–2000) and in beer samples (1 : 200), they could not be considered as significant interferences in the L-lactate determination. Nevertheless, it must be reckoned that some matrix effect was observed when the biosensor was applied to real sample measurements. In this way, it was studied that the presence of ethanol, normally 12% v/v in wine, reduced *ca.* 20% the sensitivity of the biosensor (Fig. 7), also considering the dilution step.

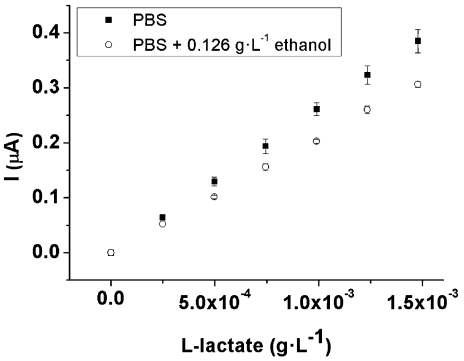


Fig. 7 Study of the influence of ethanol in the sensitivity of calibration curve to L-lactate. Measurements were carried out with the same biosensor in phosphate buffer solution at pH 7.5 in the presence or absence of ethanol. 0.126 g L^{-1} was the concentration evaluated, which corresponds to a 1 : 1000 dilution of the normally content in wine (12% v/v).

Assay in real samples

Finally, to evaluate the biosensor applicability in food analysis, concretely in the analysis of lactic acid in alcoholic beverages, it was tested with real wine and beer samples. As the subject of investigation, 8 wines and 7 beer samples were analyzed separately both with the biosensor and a spectrophotometric commercial kit for the detection of L-lactate as a reference method, and the results obtained were compared in order to validate its performance.

As previously said, the optimal working potential for amperometric determinations with the developed biosensor was between −100 and 0 mV *vs.* reference electrode, since the electrochemical interferences are minimum in this range. Given that, applying lower potentials (more negatives ones) contributes to the diminution of the electrochemical interferences, −100 mV was the one chosen to carry out L-lactate measurements. This is mostly important in wine samples, where phenolic compounds are easily oxidable.

The electrochemical assay of real samples was performed by means of the standard addition method to avoid any matrix effect, given a slight decrease was observed in the sensitivity when calibrations were carried out in wine or beer. Moreover, sample dilution was required in order to fit the expected concentration value with the biosensor linear range. In this way, the dilution factor was from 1 : 1000 to 1 : 2000 for wine samples and 1 : 200 for beers. All amperometric determinations were done in triplicate analysis for each sample, calculating L-lactate concentration from the calibration plot built after the addition of 6 stocks. Low L-lactate concentration values were able to be determined with our biosensor, as we can see for example in beer samples. However, the results obtained for other white wines assayed are not reported here since, in general, they do not present significant L-lactate levels. This is due to this type of wines are not usually subjected to malolactic fermentation[4] and therefore, L-lactic acid concentration in white wines is not significant. Results obtained with the biosensor are summarized in Table 2 and expressed as g L^{-1} of L-lactate.

Then, these results were plotted *vs.* those obtained with the commercial kit in order to compare both methodologies and to validate biosensor results; in addition, this comparison would be also useful to detect any interference effect if there was observed some lack of correlation between the two methodologies. From Fig. 8, it could be seen that the results obtained by our biosensor showed a good agreement with those obtained with the spectrophotometric method based on the determination of NADH formed. Linear least-squares regression was fitted and the characteristic parameters were calculated, obtaining a satisfactory

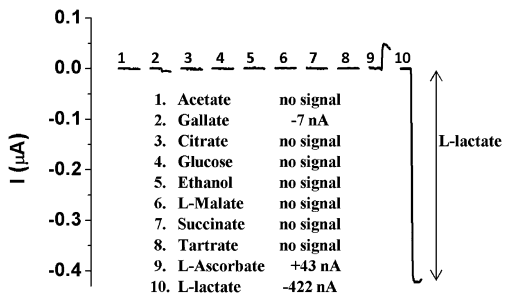


Fig. 6 Study of interferences. All the compounds evaluated have been studied at a concentration of 2 mg L^{-1} in a phosphate buffer solution (pH 7.5), also a comparison with the current response obtained with the same concentration of L-lactate is shown.

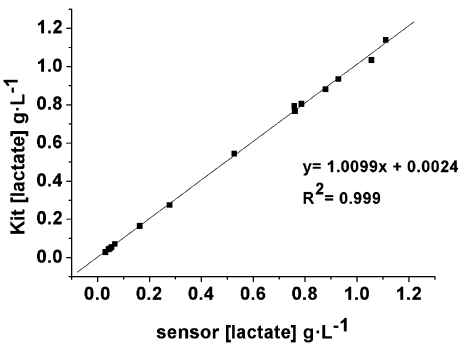


Fig. 8 Comparison of the results obtained with the biosensor (*y* axis) and those obtained with the reference spectrophotometric method (*x* axis) for L-lactate determination. Error bars correspond to biosensor replicate analysis ($n = 3$).

correlation ($r^2 = 0.999$) with slope value near one (1.01 ± 0.02) and intercept near zero (0.002 ± 0.012), containing both theoretical 1.0 and 0.0 values in the confidence interval. These parameters show that there are no significant differences between the results obtained with both methodologies and validate the applicability of the developed biosensor for L-lactic determination in wine and beer samples.

Conclusions

A bienzymatic biosensor for L-lactate determination based on the coimmobilization of LOx and HRP, and the usage of ferrocene as a mediator has been developed. The immobilization onto the SPE was achieved by a polysulfone/carbon nanotubes membrane. Besides its easy and fast preparation in only one step and considering the excellent property of polysulfone to incorporate all of the components involved in the amperometric measurement into a single membrane, this device has proven to have high accuracy, high sensitivity, low limit of detection and a fast throughput. Nevertheless, the shelf-life stability is not very high and might improve in future experiments.

Finally, to assess biosensor applicability to the food analysis field, this latter was applied to the determination of L-lactate in wine and beer samples. Since wine samples contain different substances that may be able to act as interferences, a low negative potential was applied thanks to the mediator effect. Measurements were carried out employing the standard addition method, and compared with the ones obtained using a spectrophotometric commercial kit for the determination of L-lactate as a reference method. This comparison demonstrated the applicability of the biosensor through the validation of the results obtained, demonstrating the reliability of our biosensor that besides reduces the analysis time to 18 min (5 min for the biosensor *vs.* 23 min for the spectrophotometric kit). Although in this case we have only focused on beverage field analysis, additional applications for this biosensor would be also possible employing the same strategy with different types of samples.

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