An L-Lactate Amperometric Enzyme Electrode Based on L-Lactate Oxidase Immobilized in a Laponite Gel on a Glassy Carbon Electrode. Application to Dairy Products and Red Wine

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Abstract
A biosensor based on the immobilization of Lactate oxidase in laponite – organosilasesquioxane films on glassy carbon electrode for the quantification of L-lactate in wine and dairy products is presented. The bioelectrode showed a very high sensitivity (0.33 ± 0.01) A cm^{-2} M^{-1} and a short time response (10 s) for less than 1 U of enzyme. No significant interferences, including ascorbic acid, were detected. For red wine, matrix effects assigned to polyphenols and anthocyanins were observed, which ware easily overcome by sample dilution. Our L-lactate determinations were in good agreement with those of two standard methods.

Keywords: Lactate oxidase biosensor, Laponite gel, L-lactate, Ferrocene-methanol, Wine, Dairy products, Biosensors, Enzymes

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1. Introduction

Since lactic acid has an influence on flavor, freshness, and storage quality of milk, dairy products, sausages and wines, it is an important analyte in food industry. It originates from lactose fermentation and is responsible for the characteristic sour taste of these foods. Lactic acid in wine develops from malo-lactic fermentation [1, 2], and its concentration in most cases varies from 1.0×10^{-3} to 6.7×10^{-2} mol L^{-1} [3]. For higher concentrations, wine acquires a disagreeable bitter flavor.

Lactic acid analysis in wine is performed mainly by liquid chromatography [4, 5] or capillary electrophoresis [6]. These techniques, although highly sensitive and accurate, may require laborious sample treatments and expensive instrumentation. For the detection of L-lactate, four enzymes are commonly used: Lactate dehydrogenase [7 – 9], Lactate oxidase (LOx) [10], Cytochrome b2 [11, 12] and Lactate monooxidase [13]. For the first three biocatalysts, pyruvate is the enzymatic product, whereas for the last enzyme, acetate is obtained.

In general, enzymatic biosensors based on the catalytic oxidation of L-lactate by LOx use either the natural cofactor oxygen or artificial mediators. In the former,

$$\text{L-lactate} + (\text{LOx})_{ox} \rightarrow \text{pyruvate} + (\text{LOx})_{red} \quad (1)$$

$$(\text{LOx})_{red} + O_2 \rightarrow (\text{LOx})_{ox} + H_2O_2 \quad (2)$$

Hydrogen peroxide can be amperometrically quantified by oxidation at potentials dependent on the electrode material. As an example, 0.5 V (vs. saturated calomel electrode) is applied in [14], and 0.65 V (vs. Ag | AgCl) in [15]. Due to the high potential usually necessary for hydrogen peroxide electrooxidation, other oxidizable compounds usually present in real samples may affect the amperometric quantification of L-lactate. Thus, it is a common practice for overcoming this difficulty to replace the natural electron acceptor by artificial mediators such as ferrocene derivatives [16 – 18], osmium redox polymers [19] or indoaniline derivatives [17].

In addition, mediators offer other advantages such as an increased linear response and perhaps an extended biosensor lifetime, because hydrogen peroxide, which can contribute to the deactivation of the enzyme, is not being generated [20].

Although FcMe has not been reported as mediator for LOx, it has been widely used for Glucose Oxidase, which has the same redox center (FAD). In this work we have employed the artificial mediator ferrocene-methanol (FcMe), since we found that it has a very good performance from the enzymatic and from the electrochemical points of view. Under our experimental conditions, Reaction 1 is followed by:

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$$(LOx)_{red} + (FcMe)_{ox} \rightarrow (LOx)_{ox} + (FcMe)_{red} \quad (3)$$

The electroanalytical signal is given by:

$$(FcMe)_{red} \rightarrow (FcMe)_{ox} + e \quad \text{formal potential 0.19 V vs. Ag} \mid \text{AgCl} \quad (4)$$

A lot of efforts have been devoted to find new and effective strategies for enzyme immobilization. Thus, it has been reported the direct incorporation of LOx on gold surfaces by either direct adsorption or covalent binding [18]; LOx covalently attached to an electropolymerized copolymer film [21]; inclusion of the biomolecule in a graphite Teflon composite together with Peroxidase and ferrocene in batch- and flow-injection modes [3]. LOx was incorporated in screen-printed graphite coated in Nafion [22], and this anionic polymer was also used for the incorporation of the enzyme together with Prussian Blue [23]. Other approaches involved entrapment of the enzyme in polymeric films[15, 24] and glutaraldehyde and polyaniline-*co*-fluoroaniline film deposited on an Indium tin oxide [25]. In other work, LOx, together with Glucose oxidase and Galactose oxidase was immobilized in polypyrrole and poly(ethylene glycol) [26]. It has also been described in literature the use of semipermeable membranes to keep the enzyme close to the electrode surface [27]. The immobilization of LOx together with Lactate dehydrogenase and Horseradish peroxidase on an oxygen selective Clark electrode with a Teflon membrane, working either in batch or in a flow injection mode was also reported [28]. Other work involved the inclusion of LOx in a composite film of Platinum nanoparticles and multi-walled carbon nanotubes [14]. Biosensors reported in [10, 14, 15, 24–27] were based on hydrogen peroxide.

Among the different matrices reported in literature, clays are a convenient choice because of their ion-exchange capacity, a well-known layered structure, and mechanical and thermal stability [29]. Laponite is a synthetic layered silicate, with a structure and a composition close to that of the natural hectorite mineral. Due to a swelling phenomenon in water, laponite provides host matrices with high hydrophilic character very suitable for enzyme immobilization [30]. Labbé and col. have described the physical confinement of Glucose oxidase and Polyphenol oxidase in a laponite gel matrix in an attempt to develop amperometric enzyme electrodes [30, 31]. The strategy involves the deposition of an aqueous mixture of laponite, enzyme, and an oligosilasesquioxane octamer on an electrode surface. The drying of this mixture and its successively swelling step in an aqueous electrolytic medium leads to the formation of an adherent octamer-laponite film in which the enzyme is entrapped. This constitutes a simple and reproducible one-step procedure, which does not denature the enzyme.

Despite the important number of publications related to L-lactate amperometric biosensors, there is still a great necessity to improve the detection limit and the sensitivity of such devices. Considering some representative works published in the last decade, most detection limit values were around 1×10^{-5} M [14, 18, 22, 25]; lower values were reported in few cases [3, 10, 23], from 5×10^{-7} M [10] to 1.4×10^{-6} M [3]. In relation to sensitivities, a wide range of values were informed. For comparison purposes, we put them in the same units, μA mM^{-1}. Some of them were lower than 2×10^{-2} [15, 22, 28]; most of the reported sensitivities ranged from 2×10^{-2} to 2 [10, 18, 23, 25, 28]. In [21, 26], 0.110 ± 0.040 μA mM^{-1} cm^{-2} and 0.151 μA mM^{-1} cm^{-2} were informed. The highest sensitivities we found were (2.98 ± 0.06) [3]; 6.36 [14] and approximately 12 μA mM^{-1} [24] (we estimated the last value from the calibration plot).

In this work, we report, for the first time, the immobilization of lactate oxidase in a mixed membrane material formed from laponite and oligosilasesquioxane octamer. We study the electrochemical behavior of the mediator FcMe on a glassy carbon electrode modified with the enzyme-octamer-laponite film. We investigate the analytical performance of the laponite modified LOx electrode, as well as the influence of potential interferents. Special attention is paid to improve both, the detection limit and the sensitivity. First the analytical performance of the enzymatic biosensor is analysed, and afterwards we use it for the quantification of L-lactate in red wine, fermented milk and yogurt.

2. Experimental

2.1. Solutions and Reagents

Laponite R. D., a synthetic hectorite (monovalent cation exchange capacity: $cec = 0.74$ mequiv g^{-1}) was obtained from Laportes Industries. The colloidal suspensions were prepared by dispersing 2 g L^{-1} laponite in water overnight.

The oligosilasesquioxane pillar precursor, PS^+, was synthesized from (trimethoxysilylpropyl) trimethyl ammonium (monomer S^+), according to [30]. In brief, a diluted solution of the monomer was stirred for 24 h to initiate a hydrolytic polycondensation in alkaline medium. Then, NaOH was added up to pH 12.5 in order to induce the condensation process. The PS^+ oligomer was obtained after another 24 h of stirring.

Lactate oxidase, LOx, (EC 232-841-6 from Pediococcus species) lyophilized powder containing 20 units mg^{-1} solid and L-(+)-lactic acid lithium salt 97% was purchased from Sigma Chemical Co. Enzyme stock solutions were prepared dissolving 2 mg of LOx lyophilized powder in 500 μL of 0.1 M phosphate buffer, pH 7.0.This enzyme solution was separated in five aliquots of 100 μL each one and stored at $-20\,^{\circ}C$. Under these conditions, the enzymatic activities remained stable for several weeks [18].

The background electrolyte solution was 0.1 M phosphate buffer prepared with NaH_2PO_4 and Na_2HPO_4 (J. T. Baker), pH 7.0, measured with a potentiometric glass electrode. Solutions of FcMe, (Aldrich) were prepared at concentrations indicated in each case. The enzyme substrate, L-lactate, was added to the background electrolyte in various concentrations. Gelatin gels were prepared dissolving 0.1 mg mL^{-1}

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of gelatin from bovine skin (Sigma-Aldrich) in buffer pH 7.0.

Other reagents were D(+)-glucose, L(+) tartaric acid dipotassium, succinic acid 99%, citric acid, DL-malic acid (all from Sigma), D-fructose (Fluka), acetic acid, formic acid, methanol and ethanol (Cicarelli). All chemicals were of reagent grade and used as received. Anthocyanins, which have very well established antioxidant properties, were used for the analysis of matrix effects in red wine samples. For this purpose, we used a mixture of anthocyanins from grape skins, purified according to [32]. A brief description of the purification procedure is given as supplementary material.

Wines, yogurt and fermented milk (containing *Lactobacillus casei spp.*) were purchased in a supermarket.

All solutions were prepared with ultra-pure water (18 MΩ cm^{-1}) from a Millipore Milli-Q System. All experiments were performed at room temperature.

2.2. Electrochemical Measurements

Cyclic voltammetry (CV) and amperometry studies were carried out with an Autolab (Eco-Chemie, Utrecht, Netherlands) equipped with a PGSTAT 30 potentiostat and GPES 4.9 software package. Convective conditions in amperometric measurements were maintained with a magnetic stirrer. The working potential value chosen for current–time curves corresponded to stationary state conditions.

Experiments were carried out in a three-compartment electrochemical cell with standard taper joints. Working electrodes of different characteristics were prepared on commercial glassy carbon-disc electrodes from CH Instrument (CHI104). The carbon disk geometric area of 0.079 cm^2 was determined by chronoamperometry. They are referred as glassy carbon electrodes (GCE). A large area platinum wire was employed as a counter electrode. All potentials are reported against the reference electrode Ag | AgCl | Cl^- (3 M). Solutions were deoxygenated with high-purity nitrogen for at least fifteen minutes prior to electrochemical measurements, and the gas flow was kept over the solution during the experiments. Analyte concentrations were determined by the standard addition method.

2.3. Validation Process

So as to validate the proposed sensor, two independent reliable analytical techniques based in the generation of hydrogen peroxide from L-lactate in the presence of lactate oxidase were used. The first, electrochemical in nature, was performed with a Cobas b 221 Blood Gas System, from Roche, USA. It is equipped with a measuring L-lactate chamber in which hydrogen peroxide was measured amperometrically on a carbon/platinum electrode. The second method was colorimetric, and the equipment used was a Hitachi 917 Modular P CAN 040 analyzer. In this case, hydrogen peroxide, in presence of 4-aminoantipyrine, peroxidase and a proton donor, developed a chromophore whose absorbance was measured at 540 nm [33].

2.4. Electrode Conditioning and Biosensor Preparation

Prior to each experiment, GCE were polished successively with alumina powder (Buehler) of particle size 1.0, 0.3, and 0.05 μm, copiously rinsed with ultrapure water and sonicated for 1 min between each polishing step.

The GCE surface was modified depositing gel layers of different composition. For gelatin modified electrodes (GeME), 30 μL of gel solution containing 0.1 mg mL^{-1} of gelatin in buffer solution were deposited on the GCE and dried at room temperature. Laponite modified electrodes without enzyme (LME) were used for blank experiments. They were prepared as follows: a mixture (30 μL) containing 30 μg of laponite and different amounts of PS^+ (0.5; 1; 2 and 3 *cec*) was deposited on the electrode surface.

For laponite modified enzymatic electrodes (LMEE), 20; 40 or 60 μg of LOx were incorporated into the mixture of laponite and PS^+. The initial pH of mixture laponite/PS^+/LOx was nearly 11 so it was adjusted by addition of concentrated HCl solution. Water from the laponite suspension was removed under reduced pressure, leading to the formation of the enzymatic oligomer-laponite clay film. After drying, the film was immersed for 45 min in buffer pH 7.0 for the swelling step. The loading of PS^+ in the suspension was quantified in molar number of amino groups and expressed as a multiple of the cation exchange capacity of laponite clay.

The mechanical properties and the adherence of the film were dependent on the rate of the dehydration process. Best results were obtained under mild dehydration under the low pressure produced by a water pump for 45 minutes. Films prepared under high vacuum pressure (mechanical pump) showed poor adherence and they peeled off. On the other hand, when the electrodes were dried at room pressure, there was a significant loss of enzyme activity. When not in use, the electrodes were stored at 4 °C.

3. Results and Discussion

3.1. Electrochemical Behavior of the Artificial Mediator

The electrochemical response of FcMe under our experimental conditions was checked by CV. Freshly polished GCE, GeME and LME were used, and possible film effects on the reversibility of the FcMe/$FcMe^+$ redox couple were analyzed.

The corresponding $i-E$ profiles for each electrode at different potential sweep rates v in buffer solution pH 7.0 + 0.2 mM FcMe were recorded (not shown). In the case of bare GCE, maximum currents were proportional to the scan rates v, for values below 0.050 V s^{-1}. For higher v, currents were independent of v. These results are in agreement with those reported in the literature [34], where it has been

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demonstrated that FcMe on polished GCE exhibits a self-inhibiting adsorption process. The authors resolved this inhibition with a gelatin film deposited on the GCE surface. We observed the same behavior under our experimental conditions. Voltammetric waves for GeME showed a reversible behavior, with a linear response of peak current I_p versus $v^{1/2}$ in the range $(0.001 \le v \le 0.250)$ V s^{-1}. From the slope of this plot, a diffusion coefficient $D = 5.9 \times 10^{-6}$ cm^2 s^{-1} was estimated. This linear response was characterized by the following regression data: slope $= (1.362 \pm 0.006) \times 10^{-4}$ A cm^{-2} $V^{-1/2}$ $s^{1/2}$; intercept $= (7 \pm 1) \times 10^{-7}$ A cm^{-2}; standard deviation $SD = 1.4 \times 10^{-7}$ A cm^{-2} and a regression coefficient of 0.99996. When the same type of experiments was performed on a LME, a similar behavior was observed: peak potentials $E_{pa} = 0.213$ V and $E_{pc} = 0.148$ V remained constant in the v interval $(0.001 \le v \le 0.250)$ V s^{-1} and a linear relationship between I_{pa} and $v^{1/2}$ was obtained. The slope was $(1.29 \pm 0.02) \times 10^{-4}$ A cm^{-2} $V^{-1/2}$ $s^{1/2}$, the intercept, $(2.1 \pm 0.6) \times 10^{-7}$ A cm^{-2}, with a SD of 5.4×10^{-7} A cm^{-2} and a regression coefficient of 0.99432. An apparent diffusion coefficient $D = 5.7 \times 10^{-6}$ cm^2 s^{-1} was estimated from the slope. This D value is not far from that obtained for FcMe on GeME. Furthermore, it is close to the D value in solution reported 6.7×10^{-6} cm^2 s^{-1} [35]. It can be concluded that the laponite film affects the FcMe redox couple in a similar way to gelatin, hindering FcMe adsorption and favoring the reversibility of the mediator electrochemical response.

Precipitation of the mediator into the gel matrix at concentrations higher than 0.3 mM was confirmed by CV (see supplementary material.). I_{pa} values, recorded in the absence of mediator in solution, indicated that its concentration in the film would be estimated as equivalent to 6×10^{-3} mM in solution. It should be noted that this concentration was very low in order to regenerate the enzyme, and accordingly, no catalytic response was observed in the presence of L-lactate. Furthermore, the amperometric signal became noisier, probably due to a decrease in film permeability. Because of this, FcMe concentrations higher than 0.3 mM were avoided.

3.2. Effect of the Laponite Gel Composition on the Enzymatic Response

It is expected that the gel suspension pH and the laponite/octamer ratio would have a strong influence on the ion-exchange properties, the permeability and the catalytic response of the LMEE. Figure 1 shows CV results at 0.001 V s^{-1} in 0.2 mM FcMe $+ 7.5 \times 10^{-4}$ M L-lactate in buffer solution for LMEE prepared with gel suspensions of different pH, from 5.5 to 8.5 with constant amounts of laponite, enzyme and octamer loading. In all cases, well-defined stationary catalytic currents were observed. Although the electrodes were stabilized in buffer pH 7.0 before each electrochemical experiment, pH in the gel during preparation (around 45 min) had a strong influence on the enzymatic response. The highest catalytic currents were observed at gel pH 6.5, voltammogram III, with some diffusion contribution to the maximum current. Significant activity losses were observed when gel pH was changed from 6.5 to 8.5 (compare voltammograms III, IV and Vin Fig. 1).

We measured the variation of the catalytic currents as a function of gel pH for LMEE prepared with varying PS^+ loading from 0.5 *cec* to 3 *cec*. The highest stationary currents were observed for gels containing PS^+ equivalent to 2 *cec*.

The relative amount of PS^+ incorporated in the film influenced both the structure and the net charge density of the enzyme matrix. In fact, it has been demonstrated that the increasing amounts of octamer is accompanied by the increase in the basal spacing between laponite platelets [30]. On the other hand, for PS^+ loading exceeding 1 *cec*, the gel becomes positively charged and in good conditions for stabilizing anionic enzymes by electrostatic interactions. For Glucose Oxidase, p$I = 4.3$ [26], the enzyme could not only induce ionic or physical interactions with the clay platelet surface, but it could also be entrapped sterically within the laponite hydrogel mesopores [30]. These effects have to be operative in the case of LOx, with a pI value of 4.6 [26]. A compromise between electrostatic and steric effects would explain the good responses observed for PS^+ loading equivalent to 2 *cec*.

According to the results described above, laponite gels for LOx entrapment were prepared at pH 6.5 and 2 *cec* octamer.

3.3. Analytical Performance of LMEE

3.3.1. Amperometric Quantification of L-Lactate

The catalytic response of the LMEE was studied by amperometry. Increasing amounts of L-lactate were added to 0.3 mM FcMe in phosphate buffer pH 7.0, and the

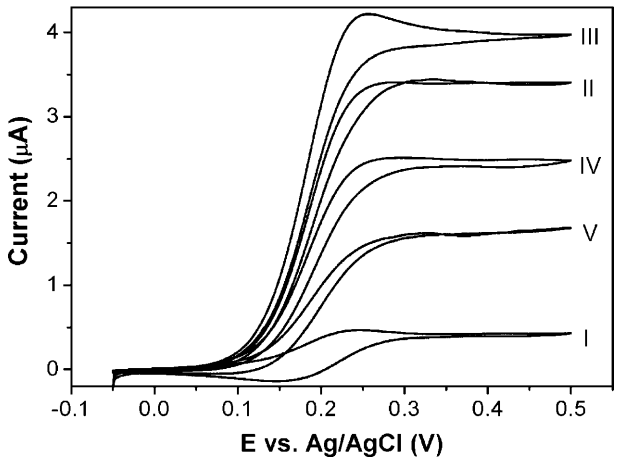

Fig. 1. Effect of gel composition on the electrocatalytic response of LMEE. $i-E$ profiles performed at 0.001 V s^{-1} on LMEE in 0.1 M phosphate buffer pH 7.0 + 0.2 mM FcMe, without (curve I) and with (curves II – V) 7.5×10^{-4} M L-lactate, at various gel pH: (II) 5.5; (III) 6.5; (IV) 7.5; (V) 8.5. Gel composition: 30 μg laponite + 20 μg LOx + 2 *cec* PS^+.

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current – time variations at 0.400 V were recorded. Figure 2 depicts typical results.

Figure 2a illustrates reproducible stationary currents reached after successive additions of 0.025 M L-lactate. Well-defined stationary currents were obtained, with response time around 10 s, very convenient for analytical purposes. In the same figure, the blank experiment (on LME without enzyme) clearly indicates that no detectable electroactive species were present in the solution or in the gel. The corresponding calibration and Eadie – Hofstee plots are shown in Figure 2b. For a given enzyme concentration, the latter representation is useful for estimating the substrate concentration range for which catalytic or diffusion control is operative [36]. For the same experimental conditions of Figure 2a, the stationary currents were diffusion controlled when substrate concentrations were below 2×10^{-4} M. As no current increase was observed for higher solution stirring speeds, the main contribution to L-lactate diffusion should take place within the film. On the other hand, enzyme kinetics determined the rate process for substrate concentration over 3.7×10^{-4} M. The regression analysis of the linear interval in the Eadie – Hofstee plot showed: slope $(2.3 \pm 0.1) \times 10^{-4}$ M; intercept $(2.10 \pm 0.03) \times 10^{-4}$ A cm^{-2}, *SD* 1.7×10^{-6} A cm^{-2} and regression coefficient of 0.99813. From the linear intervals of a set of plots similar to that of Fig. 2b but obtained for different enzyme/mediator ratios, the apparent Michaelis – Menten constant $K_M' = (2.2 \pm 0.2) \times 10^{-4}$ M was estimated. This value is almost equal to the one for the free enzyme (2.3×10^{-4} M) [25], indicating that no significant conformational change takes place as a result of the enzyme immobilization.

The analytical response of the biosensors was determined in electrolytic solutions of L-lactate prior to the analysis of real samples of wine and dairy products.

Ten consecutive potential steps at 0.400 V for 5.0×10^{-5} M L-lactate on a LMEE yielded reproducible stationary currents with a *RSD* value of 4.3%. From calibration plots similar to those shown in Figure 2b, a linear range from 3×10^{-6} M to 3.0×10^{-4} M was observed. Parameters of the linear range were as follows: slope and intercept were (0.33 ± 0.01) A cm^{-2} M^{-1} and $(6 \pm 2) \times 10^{-6}$ A cm^{-2} respectively, with $SD = 1.8 \times 10^{-6}$ A cm^{-2} and a regression coefficient of 0.99281.

The detection limit, 1.0×10^{-6} M, was considered as three times the signal-to noise ratio and was calculated from the standard deviation ($n = 10$) of the stationary currents for 1.25×10^{-5} M L-lactate, and the slope of the linear calibration plot [37]. It should be noticed that our biosensor yielded a sensitivity of (0.33 ± 0.01) A cm^{-2} M^{-1}, equivalent to (30.3 ± 0.9) μA mM^{-1}, which is higher than other L-lactate biosensors with the enzyme immobilized in sol-gel matrixes, i.e. 6.36 μA mM^{-1} [14], and approximately 12 μA mM^{-1} [24], the highest values mentioned in the bibliographic revision commented above. In both reports, no mediator was used and the analytical signal was based on the electrochemical oxidation of hydrogen peroxide.

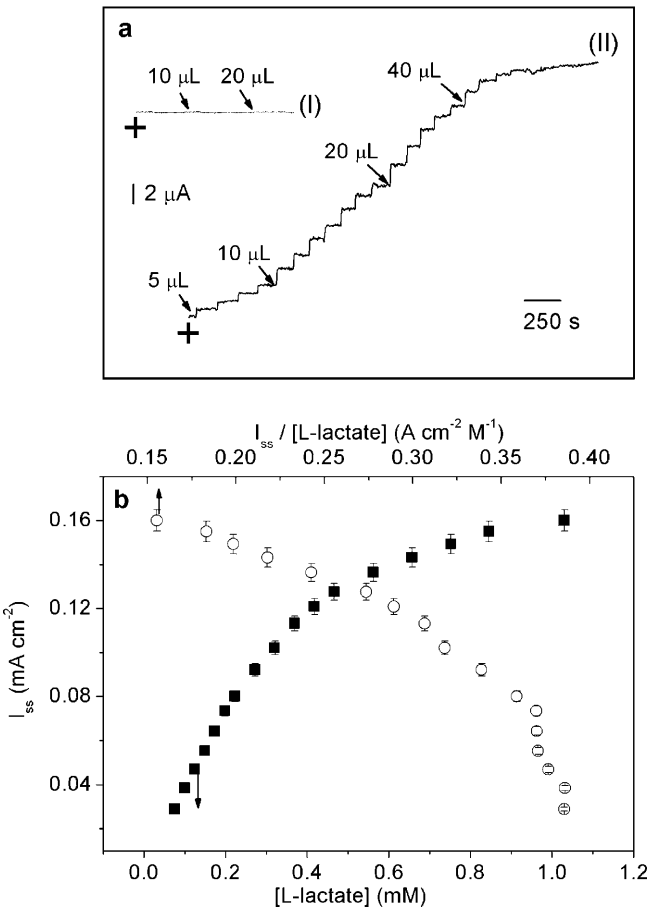

Fig. 2. a) Chroamperometric response at 0.400 V on a LME without (I) and with (II) 40 μg of LOx for successive additions of 0.025 M L-lactate to 10 mL 0.1 Mphosphate buffer pH 7.0 + 0.3 mM FcMe. b) Calibration and Eadie – Hofstee plots for data of Fig. 2a.

3.3.2. Stability and Variability of the LMEE

We have analyzed the stability of the LMEE with the storage time and with the number of analytical cycles. The relative activity loss was followed by the registration of one calibration plot every three days. Aliquots of 12.5 μM L-lactate were added to 0.3 mM FcMe pH 7.0 solution until no variation in the stationary current was observed. After the first week, an activity of 90% was preserved (sensitivity 0.30 ± 0.01 mA cm^{-2} mM^{-1}), without significant changes in K_M'. Ten days afterwards, a sensitivity drop of 35% was observed (0.22 ± 0.01 mA cm^{-2} mM^{-1}). Nevertheless, the sensitivity and the linear range were compatible for average L-lactate content in real samples. The electrodes could still be used after three weeks, provided a calibration plot was recorded each working day. It could be probed that the storage time, more than the number of cycles, was responsible for the decrease in sensitivity.

Electrodes prepared in a controlled way were very reproducible and the variability was low. Sensitivities obtained from three calibration plots corresponding to three electrodes prepared in the same way were 0.332, 0.320,

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and 0.338 A cm^{-2} M^{-1} respectively, with a standard deviation of 2.8%.

3.3.3. *Interference Analysis for the Quantification of L-Lactate*

In the quantification of L-lactate in food samples, it is desirable to avoid sample pretreatments. For this purpose, possible interferents commonly present in wine and dairy products were investigated. In principle, electroactive substances in the working potential range as well as those which interact with either the enzyme or the mediator have to be considered as potential interferents.

Fructose and glucose, alcohols and organic acids such as citric, tartaric acetic, malic, formic and succinic are present in wine in amounts ranging from 0.5 to 1 g L^{-1} and they have been reported as potential interferents. Ethanol is the second wine component, derived from sugar. Also methanol, an undesired component, is a matter of concern.

It is well known that red and white wines contain variable amounts of polyphenols and related substances with already established antioxidant properties. Red wines, in addition, are rich in anthocyanins not present in white wines. Nevertheless, to our knowledge, these substances have not been considered in the literature as potential interferents in electroanalytical techniques.

In the case of milky products, glucose as well as citric, acetic, ascorbic and uric acids would interfere [3]. In order to check the possible electrochemical activity of those substances, CV profiles were recorded for a LME in 0.3 mM FcMe in the absence (blank experiments) and in the presence of 8×10^{-4} M of each possible interferent in our working potential range. No differences in relation to blanks were detected in the case of acetic, citric, formic, tartaric, malic and succinic acids. Similarly, no electrochemical activity was observed for methanol, ethanol, fructose and glucose (voltammograms not shown).This was not the case for ascorbic acid, which is electroactive in the same potential range as FcMe. Its contribution to the total analytical current was evaluated by amperometry. Experiments at 0.400 V for increasing amounts of interferent were performed on GCE, LME and LMEE.

Sensitivities were evaluated from the linear range in the calibration plots. The corresponding values in A M^{-1} cm^{-2} were as follows: 0.264 ± 0.006 for clean GCE; 0.045 ± 0.002 for LME and 0.065 ± 0.003 for LMEE. The regression analysis of the GCE linear interval showed: slope = (0.264 ± 0.006) A cm^{-2} M^{-1}; intercept = $(2.8 \pm 0.9) \times 10^{-5}$ A cm^{-2}, $SD = 1.4 \times 10^{-5}$ A cm^{-2} and regression coefficient of 0.99653. For LME the regression parameters were as follows: slope = (0.045 ± 0.002) A cm^{-2} M^{-1}; intercept = $(7 \pm 2) \times 10^{-7}$ A cm^{-2}, $SD = 2.6 \times 10^{-7}$ A cm^{-2} and a regression coefficient of 0.99709. In the case of LMEE, slope = (0.065 ± 0.003) A cm^{-2} M^{-1}; intercept = $(7 \pm 1) \times 10^{-6}$ A cm^{-2}, $SD = 1.4 \times 10^{-5}$ A cm^{-2} and a regression coefficient of 0.98749. The detection limit for ascorbic acid on this electrode was 1.3×10^{-6} M. According to these values, the laponite film causes a noticeable decrease in sensitivity and contributes positively to reduce ascorbic acid interference. In addition, the LMEE sensitivity for lactic acid, 0.33 ± 0.01 A $M^{-1}cm^{-2}$, was over five times higher than that of ascorbic acid (0.065 ± 0.009) A M^{-1} cm^{-2}. This result can be compared with the one obtained on an electrode with the enzyme entrapped in an electropolymerized layer of overoxidized polypyrrole. In this case, 1×10^{-4} M ascorbic acid gave a bias around 3×10^{-5} M L-lactate [15].

Once the lack of electrochemical reactivity of each potential interferent was verified by CV, other possible interactions were investigated under amperometric conditions on a LMEE. Therefore, each substance was added to electrolytic solutions containing 0.3 mM FcMe + 1×10^{-4} M L-lactate, in the L-lactate/interferent molar ratios 1:1; 1:10 and 1:20. Glucose, fructose, methanol, ethanol and citric and formic acids did not modify the stationary current for L-lactate at 0.400 V. It is worthwhile noticing that tartaric, malic and succinic acids were reported as interferents for a graphite-teflon composite bienzyme electrode at 0.100 V [3]. On the contrary, steady state currents at 0.400 V on LMEE for ascorbic acid showed a significant oxidation current. For L-lactate/ascorbic acid molar concentration ratios of 1:1, 1:10 and 1:20, the observed steady state current were 0.8, 7.5 and 12 times higher than in the absence of ascorbic acid. Nevertheless, the L-lactate/ascorbic acid concentration ratio in real samples is usually above 10^3. This high value, together with sample dilutions usually needed in L-lactate analysis, determines that the maximum ascorbic acid concentrations in the working cell would be in the order of 3×10^{-8} M. This concentration lies well below the ascorbic acid detection limit under these analytical conditions. Accordingly, ascorbic acid interference could be considered not of concern.

Current – time curves indicated a very weak interference of acetic acid, but only when the analyte/interferent molar ratio was at least 1:20 (data not shown). Even so, the oxidation currents represented only 2% of the analytic signal. Hence, usual sample dilution would make this interference undetectable, except for very sour samples. In all cases, a blank experiment recorded on a LME without LOx would be necessary in order to verify the absence of acetic acid interference.

3.3.4. *Lactic Acid Determination in Real Samples*

Figure 3 shows current-time profiles obtained at 0.400 V in electrolytic solution containing 0.3 mM FcMe after the addition of increasing volumes of the following real samples: red wine (curves I), yogurt (curves II) and fermented milk (curves III), registered on a LMEE. Samples had no pretreatment, except milk products that were diluted 1:4 with buffer solution prior to its addition to the electrolytic cell, where all samples were diluted in 10 mL electrolytic solution.

Blank experiments (not shown here), were recorded on a LME, in the absence of the enzyme. No changes in the stationary currents for FcMe oxidation were observed after the addition of successive aliquots of real samples to the

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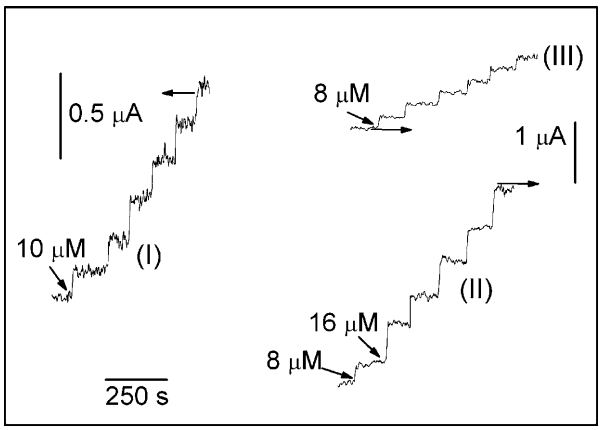

Fig. 3. Chronoamperometric response at 0.400 V on a LME with 40 μg of LOx, for successive additions of (I) red wine, (II) yogurt and (III) fermented milk, to 10 mL of 0.1 M phosphate buffer pH 7.0 + 0.3 mM FcMe. Volume aliquots were 5 μL for curves I and III, and 10 μL for curve II, except the first addition of 5 μL. The corresponding concentrations of L-lactate in each sample are indicated on the figure.

electrolytic cell. This is a clear indication that these samples did not contain detectable amounts of acetic or ascorbic acids. Any other detectable electroactive species could also be discarded.

Current – time profiles obtained in the presence of LOx (LMEE), in response to sample additions, showed well defined stationary currents, which were assigned to the enzymatic oxidation of lactic acid present in wine and dairy products.

At first sight, it can be claimed that the LMEE would be a very good alternative for L-lactate analysis in real samples without pretreatment procedures except dilution. However, undesired effects associated with chemical reactions involving matrix components, especially antioxidant species, would be of particular concern, and a careful analysis would be necessary. New experiments related to this point are described in the following section.

3.3.5. *Matrix Effects in the Quantification of* L-*Lactate in Red Wine*

In order to analyze possible matrix effects on L-lactic acid determination in red wine, amperometric profiles on LMEE were recorded. Three different sample volumes were added to 10 mL of buffer solution + 0.3 mM FcMe. Afterwards, successive aliquots of 0.025 M L-lactate solution were added to the cell, until saturation of the enzyme was reached. For evaluating the matrix incidence of each sample volume, the slope of each calibration plot (sensitivity) obtained in presence (B) and absence (B_0) of a volume of each food sample, recorded on the same electrode, were compared as $(B/B_0) \times 100 = SC$, sensitivity comparison. Data of all B and B_0 values are provided as supplementary material. SC values of 100% would indicate that no influence of matrix components was detected.

For red wine, SC values were 91.6%, 77.1% and 50.1% for sample volumes of 25.0 μL, 50.0 μL and 75.0 μL, respectively. According to these results, the existence of some type of interference, not electrochemical in nature, could be inferred. Nevertheless, the SC value of 91.6%, for the lowest sample volume, 25.0 μL, can be considered adequate for analytical purposes. From calibration plots a detection limit of $(2.6 \pm 0.2) \times 10^{-6}$ M was evaluated, and the corresponding K_M' value was $(2.02 \pm 0.05) \times 10^{-4}$ M. The L-lactate content in the real sample was $(2.0 \pm 0.1) \times 10^{-2}$ M.

The anti-oxidant properties of wine have been clearly demonstrated and attributed to its high content of natural polyphenolic compounds. Main representatives of such components are stilbenes, flavonoids, procyanidins and phenolic acid derivatives, cynamic acid and tyrosine [38].

The total concentration of polyphenolic compounds ranges from 1.80 to 4.06 g L^{-1} in galic acid equivalent, with an average of 2.57 g L^{-1}. Anthocyanidins, cyanidin and malvidin amount to 90 mg L^{-1} in red wines. Probably, this family of compounds would be responsible for the matrix effects observed in red wine.

In order to investigate this possibility, calibration plots for L-lactate were performed, as shown in Figure 4, (curve a) without and (curve b) with 5 mg L^{-1} of a mixture of anthocyanins. These antioxidants were a purified extract from red wine grape skins. A decrease of 7.8% in sensitivity and a 24.9% in the maximum current were evident. This clearly indicates that anthocyanins influence the determination of L-lactate.

3.3.6. *Matrix Effects in the Quantification of* L-*Lactate in Dairy Products*

The matrix effect for the L-lactate quantification in dairy products was determined by amperometry on LMEE in the same way as that described for wine. Volumes of previously

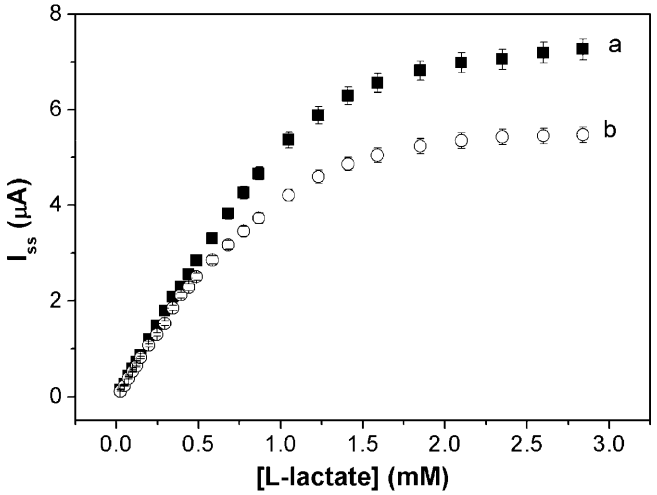

Fig. 4. Calibrations plots obtained from chronoamperometric measurements ($E = 0.400$ V) for successive additions of 0.025 M L-lactate to 10 mL 0.1 M phosphate buffer pH 7.0 + 0.3 mM FcMe (a) without and (b) with 5 mg L^{-1} of a mixture of anthocyanins.

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diluted samples were 10.0, 25.0 and 50.0 μL for yogurt and 25.0, 50.0 and 75.0 μL for fermented milk. Each sample volume was added to 10 mL of buffer solution + 0.3 mM FcMe. Then, successive aliquots of 0.025 M L-lactate solution were added to the cell up to enzyme saturation. Comparisons between sensitivities with and without sample addition were performed as described above for red wine. *SC* values obtained for each sample volume are summarized in Table 1.Values of 91.5% for yogurt and 95.3% for fermented milk can be considered as very satisfactory, and the corresponding sample volumes (10.0 and 25.0 μL respectively) were used for determining L-lactate levels in each sample. They were $(6.8 \pm 0.2) \times 10^{-2}$ M and $(1.2 \pm 0.1) \times 10^{-1}$ M for yogurt and fermented milk, respectively. Based on calibration curves, we determined detection limits of $(1.5 \pm 0.1) \times 10^{-5}$ M and $(1.06 \pm 0.07) \times 10^{-5}$ M for yogurt and fermented milk respectively.

In order to validate our method, the results obtained with the LMEE were compared with those of two automatic chemical analyzers, one electrochemical and the other colorimetric, both containing Lactate oxidase. Some results obtained on different food samples are summarized in Table 2. According to a *t*-test at the 95% confidence level, they were found not to be significantly different, except enzymatic colorimetric results for red wine. The observed differences would be associated to matrix absorbent components. According to these results, we can conclude that our LMEE can be used to monitor L-lactate in food samples such as red wine and dairy products.

4. Conclusions

All the above results demonstrate fairly well that the laponite modified LOx electrode (LMEE) is a very useful amperometric biosensor for the selective determination of L-lactate in food samples such as red wine and dairy products without sample pretreatment except dilution in some cases. The biosensor is easily and reproducibly prepared, with a good catalytic activity for at least twenty days after preparation. Furthermore, the laponite modified glassy carbon electrode showed a reliable electrochemical response for the FcMe mediator couple. This mediator proved to be a very convenient choice for LOx.

Comparison of calibration plots in the presence and absence of possible interferents usually found in food samples clearly indicate that none of them poses a real problem in the analyte quantification. On the one hand, most of these compounds are not electroactive on the laponite modified electrode; on the other hand, the rather high sample dilutions, compatible with the high sensitivity of the method, minimize the matrix effects. Even ascorbic acid, a well known electroactive species in the potential window used here, is not of concern due to its final concentration in the electrolytic cell, well below its electrochemical detection limit. In the particular case of red wine, analyses revealed matrix effects, which would be assigned to polyphenols in general and to anthocyanins in particular, as observed after the addition of a mixture of anthocyanins to the working cell. Nevertheless, the high sensitivity of the enzymatic electrode enables us to work with properly diluted samples and to achieve very satisfactory percentages of recovery. The same applies to the case for yoghurt and fermented milk. The analytical properties of this biosensor would enable its application to the analysis of other beverages such as beer.

It would also be useful for milk serum analysis, usually required in cheese manufacturing.

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Table 1. Matrix Effect evaluated as comparison of sensitivities for L-lactic acid quantification in presence and absence of a given volume of sample of dairy products on LMEE.

	Sample volume (μL)	Sensitivity comparison [a] (%)
Yogurt	10.0	91.5
	25.0	86.6
	50.0	71.7
Fermented milk	25.0	95.3
	50.0	84.1
	75.0	74.8

[a] slope of each calibration plot (sensitivity) obtained in presence (B) and absence (B_0) of a volume of each food sample. $SC = (B/B_0) \times 100$. See text for details.

Table 2. L-Lactate contents in different food samples; average values and standard deviations are calculated on three replicates.

Sample	L-Lactate content/10^2 mol L^{-1} LMEE biosensor	L-Lactate content/10^2 mol L^{-1} Cobas electrochemical System	L-Lactate content/10^2 mol L^{-1} Enzymatic colorimetric method
Red wine	1.88 ± 0.08	1.83 ± 0.09	2.55 ± 0.04
Fermented milk	16.2 ± 1.7	17.4 ± 0.6	17.8 ± 0.3
Yogurt	8.1 ± 0.9	9.2 ± 0.3	8.7 ± 0.6

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