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An amperometric biosensor based on lactate oxidase immobilized in laponite–chitosan hydrogel on a glassy carbon electrode. Application to the analysis of L-lactate in food samples

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ABSTRACT

A biosensor based on the immobilization of lactate oxidase (LOx) on a glassy carbon electrode modified with laponite/chitosan hydrogels for the quantification of L-lactate in alcoholic beverages and dairy products is presented. Ferrocene–methanol (FcMe) is used as artificial mediator. The purpose of this work is to determine the best hydrogel composition from the analytical point of view. The characterization of the hydrogels was carried out by CV, amperometry and EIS. According to permeabilities and charge transfer resistances for ferrocyanide (used as molecular probe) as well as the enzymatic behavior of the enzyme for L-lactate, the best laponite/chitosan mass ratio found was 25/50. The distinct features of the bioelectrode are its long stability, its ability to reject or minimize most interferents including ascorbic acid, and its excellent analytical response, which allowed the reduction of the enzyme content below 0.5 U, for a sensitivity of $(0.326 \pm 0.003)\,A\,cm^{-2}\,M^{-1}$, with a time response lower than 5 s and a detection limit of $(3.8 \pm 0.2) \times 10^{-6}$ M. Our L-lactate biosensor was validated by comparison with a standard spectroscopic method.

1. Introduction

L-lactate is an important analyte in foods due to its incidence on flavor, freshness and other properties. It comes from lactose (dairy products) or malo-lactic (alcoholic beverages) fermentation [1,2]. Lactic acid analysis in alcoholic beverages was performed mainly by liquid chromatography [3,4], or capillary electrophoresis [5]. These techniques, although highly accurate, require laborious sample treatment and expensive instrumentation.

Biosensors based on the catalytic oxidation of L-lactate by LOx use either the natural cofactor oxygen or artificial mediators. In the former case, hydrogen peroxide is produced, which is quantified by oxidation at potentials usually above 0.5 V. Artificial mediators usually require lower oxidation potentials which are desirable for avoiding possible interferences from other oxidizable compounds [6]. Artificial mediators such as derivatives of ferrocene [7–9], p-phenylenediamine [10], osmium redox polymers [11,12], indophenol [13] or indoaniline [8], have been widely used.

In a previous publication we reported for the first time the use of FcMe in LOx regeneration in an amperometric biosensor, showing evidence for its very good performance [6].

The electrode reaction is as follows:

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

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

The electroanalytical signal is given by:

$$(\text{FcMe})_{red} \rightarrow (\text{FcMe})_{ox} + e, \quad \text{formalpotential} 0.19\,\text{V vs.Ag|AgCl} \quad (3)$$

Among the different enzyme immobilization matrices reported, clays are a convenient choice because of their ion-exchange capacity, layered structure, and mechanical and thermal stability [14]. Laponite is a synthetic layered silicate similar to the natural hectorite mineral, which provides highly hydrophilic host matrices suitable for enzyme immobilization [15]. The confinement of glucose oxidase and polyphenol oxidase (PPO) in a laponite matrix on amperometric electrodes has been described [15–19]. Also, we have used the same mineral clay to immobilize LOx. The methodology involved the deposition of an aqueous mixture of laponite, enzyme, and an oligosilasesquioxane octamer on an electrode surface. It was proved that the laponite-octamer modified LOx

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biosensor was very convenient for the determination of L-lactate in foods without analytical problems associated with common interferents, including ascorbic acid, AA [6]. However, sensibility and stability improvement, as well as biopolymers more enzyme-compatible and easier to prepare than oligosilasesquioxane are desirable.

In this sense, it has been proven that chitosan (CHT) is very suitable for immobilization matrices, due to its biocompatibility with proteins in general and enzymes in particular. Its structure is based on linear copolymers of (1,4)-2-amino-2-deoxy-β-D-glucan and (1,4)-2-acetamido-2-deoxy-β-D-glucan. It has been also demonstrated that the CHT matrix supports fast ion transport, since the apparent diffusion coefficients of $Ru(NH_3)_6^{3+}$ and dopamine in the films are close to the aqueous values [20]. This fact, together with its high film-forming ability, water permeability, mechanical strength and good adhesion, makes this biopolymer an excellent choice for biosensors [20–25]. The use of CHT integrated to gold nanoparticles has also been reported [26,27].

Laponite/CHT hydrogel has been proposed as immobilization matrix for PPO. FT-IR spectra indicating molecular interactions between PPO and CHT in the laponite gel, preventing the release of PPO. The biocomposite exhibited enhanced biocompatibility, mechanical strength and adhesion to glassy carbon electrodes [28].

CHT/LOx biocomposites with or without other additives have been reported. In the first case, the formation of polyelectrolyte complexes between LOx and polysaccharide chains of CHT was proposed, with a stoichiometry ratio of 1 LOx molecule per 150 monomeric units of CHT [29]. Wang et al. [30] described a bioelectrode resulting from the self-assembly of negatively charged LOx and oppositely charged cobalt phthalocyanine colloid, covered by a CHT film containing MnO_2 nanoparticles. Cui et al. [31] reported a network nanocomposite containing CHT, an osmium redox polymer, carbon nanotubes and LOx deposited on a gold electrode. In all cases, the high biocompatibility of LOx and CHT was demonstrated.

In this work we report for the first time the immobilization of LOx in laponite/CHT films. We characterized the conductivities of hydrogels prepared with varying proportions of CHT by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). We investigated the analytical performance of the laponite/CHT modified LOx electrode, the influence of interferents and afterwards we used it for the quantification of L-lactate in wine, beer and fermented milk.

2. Experimental

2.1. Solutions and reagents

Laponite R. D. (monovalent cation exchange capacity: cec = 0.74 mequiv g^{-1}) was obtained from Laportes Industries. Its suspensions were prepared by dispersing 1.5 g L^{-1} in water overnight.

LOx (EC 232-841-6 from *Pediococcus* species) lyophilized powder containing 20 units mg^{-1} solid and L-(+)-lactic acid lithium salt 97% were from Sigma Chemical Co. Stock solutions containing 1 mg mL^{-1} were prepared dissolving 2 mg of the enzyme in 500 μL of 0.1 M phosphate buffer, pH 7.0.This solution was separated in aliquots of 100 μL and stored at −20 °C. Under these conditions, the enzymatic activities remained stable for several weeks [9].

The electrolyte solution was 0.1 M phosphate buffer prepared with NaH_2PO_4 and Na_2HPO_4 (J. T. Baker), pH 7.0. Solutions of 0.2 mM FcMe (Aldrich) were used as artificial mediator, and L-lactate was added to the background electrolyte in various concentrations.

Other reagents were CHT, D(+)-glucose, L(+) tartaric, succinic, citric and DL-malic acid (Sigma), D-fructose (Fluka), acetic and formic acid, methanol and ethanol (Cicarelli). All chemicals were of reagent grade and used as received. CHT flakes were dissolved in 1.0 wt.% acetic acid and then diluted with water to a final concentration of 1.0 wt.%. White wine, beer 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. Experiments were carried out at room temperature.

The proposed sensor was validated by comparison with a reliable standard spectrophotometric method (Boehringer Mannheim/R-Biopharm, Enzymatic Bio Analysis/Food Analysis, Cat. No. 10 139 084 035) based on the absorbance at 340 nm of NADH formed in the presence of L-lactate by L-lactate dehydrogenase.

2.2. Electrode conditioning and biosensor preparation

Prior to each experiment, glassy carbon disk electrodes, GCE, were polished with 1.0, 0.3, and 0.05 μm alumina particles (Buehler), copiously rinsed with ultra-pure water, sonicated for 1 min between each polishing step and air-dried. Hydrogel-modified enzymatic electrodes (HMEE) were prepared depositing aliquots of a mixture containing 25 μg of laponite, 20 μg of LOx and different amounts of CHT (25; 50; 75 and 100 μg) on the GCE surface and air-dried at 4 °C. Hydrogel-modified electrodes without enzyme (HME) were use for blank experiments. When not in used, electrodes were stored at 4 °C.

2.3. Electrochemical measurements

CV, EIS and amperometry were performed with an Autolab (Eco-Chemie, Utrecht, Netherlands) with a PGSTAT 30 potentiostat and GPES 4.9 software. EIS was carried out in 5 mM $K_3[Fe(CN)_6]$ + 0.1 M KCl, with the following conditions: sine wave potential amplitude 5 mV, bias potential 200 mV and frequency range 0.05 Hz–10 KHz. Convective conditions in amperometric measurements were maintained with a magnetic stirrer.

Working electrodes were prepared on GCE from CH Instruments (CHI104). The geometric area (0.035 cm^2) was determined by chronoamperometry. A platinum wire was the counter electrode. All potentials are reported against Ag|AgCl|Cl^- (3 M). Solutions were deoxygenated with high-purity nitrogen for at least 15 min prior to measurements, and the gas flow was kept over the solution. Analyte concentrations were determined by the standard addition method.

3. Results and discussion

3.1. Effects of composition of laponite/chitosan hydrogels on the film properties

We recorded CV experiments on electrodes prepared with 25 μg of laponite, 20 μg of LOx and 25, 50, 75 or 100 μg of CHT in 0.1 M KCl + 5 mM $K_3[Fe(CN)_6]$ at potential sweep rates ν in the range ($0.010 \leq \nu \leq 0.300$ V s^{-1}) (not shown). In all cases, a linear response of peak current I_p vs. $\nu^{1/2}$ was observed. The slope of these plots, dependent on hydrogel composition, allowed us to estimate the permeability of each film according to $P = (I_{film}/I_{bare}) \times 100\%$ [20]. In our case, I stands for the normalized maximum currents $I_p/\nu^{1/2}$ for voltammograms corresponding to $Fe(CN)_6^{3-}$ electro-oxidation (Table 1). Slopes, intercepts, standard deviations and regression coefficients are provided as Supplementary Material.

Laponite posed a barrier for ferrocyanide permeation, which was partially overcome by the further addition of CHT. The relative increase in permeability pointed to a preferential partitioning of ferrocyanide into the CHT film. This is in qualitative agreement

Table 1
Charge transfer resistances R_{TC} and permeabilities P for electrodes modified with hydrogels containing 25 μg laponite (HME) and 25 μg laponite + 20 μg LOx (HMEE) with different CHT contents. The R_{TC} value for bare glassy carbon electrode was 0.092 ± 0.002 kΩ.

CHT (μg)	HME R_{TC} (kΩ)	HMEE R_{TC} (kΩ)	HMEE P
0	3.14 ± 0.03		66.0 ± 0.8
25	0.1664 ± 0.0008	5.6 ± 0.5	151.6 ± 0.9
50	0.069 ± 0.007	1.0 ± 0.4	152.0 ± 0.7
75	0.116 ± 0.002	1.6 ± 0.2	135.2 ± 1.1
100	0.229 ± 0.001	2.0 ± 0.3	117.5 ± 0.8

with Cruz et al. [20], although in our case, P values varied due to the incorporation of laponite and LOx in the hydrogel. Maximum values in P were evident for CHT content in the range 25–50 μg.

We used EIS for following the incidence of hydrogel composition on the electron transfer resistance (R_{CT}). R_{CT} gives direct information of the electron transfer reaction [32].

Except in the case of films containing 25 μg laponite + 25 μg CHT, experimental Nyquist and Bode diagrams were satisfactorily modeled with the Randles equivalent circuit, Boukamp [33]. Theoretical and experimental impedance spectra and the corresponding circuit parameters are provided as Supplementary Material.

Fig. 1 displays the Nyquist plots and the corresponding voltammograms for GCE; GCE + 25 μg laponite; GCE + 25 μg laponite + 50 μg CHT; and GCE + 25 μg laponite + 50 μg CHT + 20 μg LOx.

For bare GCE (curve a) as well as for GCE + 25 μg laponite + 50 μg CHT (curve c) the semicircle in the Nyquist plot is not defined. Only the linear response at 45° (Warburg) was observed, corresponding to a reversible charge transfer reaction. In our case, both electrodes gave reversible voltammograms, as shown in Fig. 1B curve a, and Fig. 1B curve c, with peak currents $I(c) > I(a)$. This has to be expected considering that, in the presence of cationic CHT, the anionic probe is partitioned in the gel, increasing the local concentration and the corresponding oxidation current.

For the other film-modified electrodes, one semicircle followed by a straight line was defined, indicating the predominance of either charge transfer or diffusion control, depending on frequency. The incorporation of laponite (Fig. 1A, curve b) was responsible for a notable increase in R_{CT}. Further inclusion of CHT in the gel contributed to the formation of a network like structure with higher porosity than pure laponite film [28], and accordingly, an important decrease in film resistivity was observed.

Higher redox currents have to be expected for lower transfer resistances, if no other factors are involved. As the consequence of the deposition of laponite on the electrode surface, $R(b) > R(a)$ and accordingly $I(b) < I(a)$. The incorporation of CHT into the compact laponite layer decreased the resistance and increased the corresponding oxidation current: $R(c) < R(b)$ and $I(c) > I(b)$.

Another increase in resistance was observed after LOx incorporation, $R(d) > R(c)$ and $I(d) < I(c)$. This effect has been explained in the case of laponite/CHT/PPO films in terms of enzyme hindrance to the electron-transfer [28]. A similar explanation could be given in our case for LOx. Also here, the increase in R_{CT} values confirmed the successful immobilization of LOx.

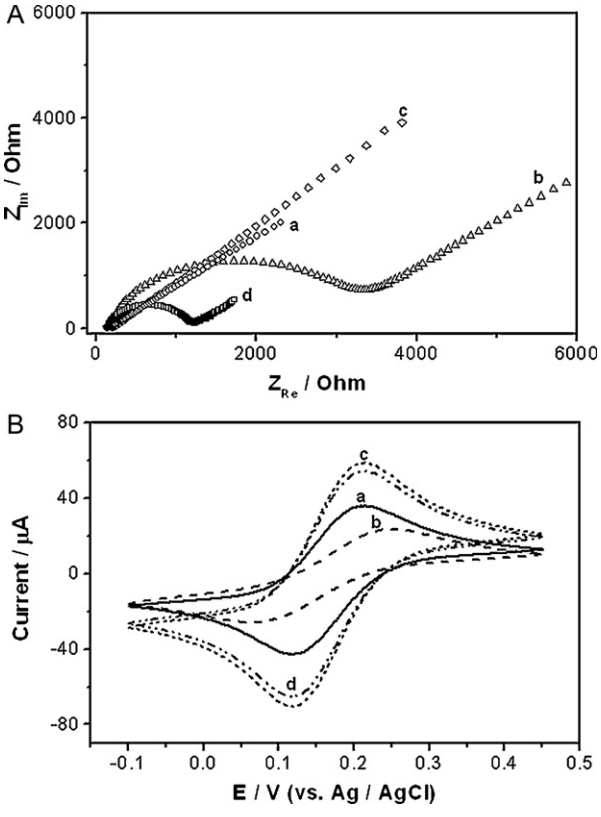

Fig. 1. (A) Nyquist plot of (a) bare GCE; (b) GCE + 25 μg laponite; (c) GCE + 25 μg laponite + 50 μg CHT, (d) GCE + 25 μg laponite + 50 μg CHT + 20 μg LOx in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl. The biased potential was 0.200 V (vs. Ag/AgCl), sine wave potential amplitude 5 mV and frequency range 0.05 Hz–10 kHz. (B) Cyclic voltammograms of 5 mM $K_3Fe(CN)_6$ in 0.1 M KCl solution at scan rate of 0.050 V s^{-1}. Voltammetric waves recorded on the same electrodes indicated in A(a–d).

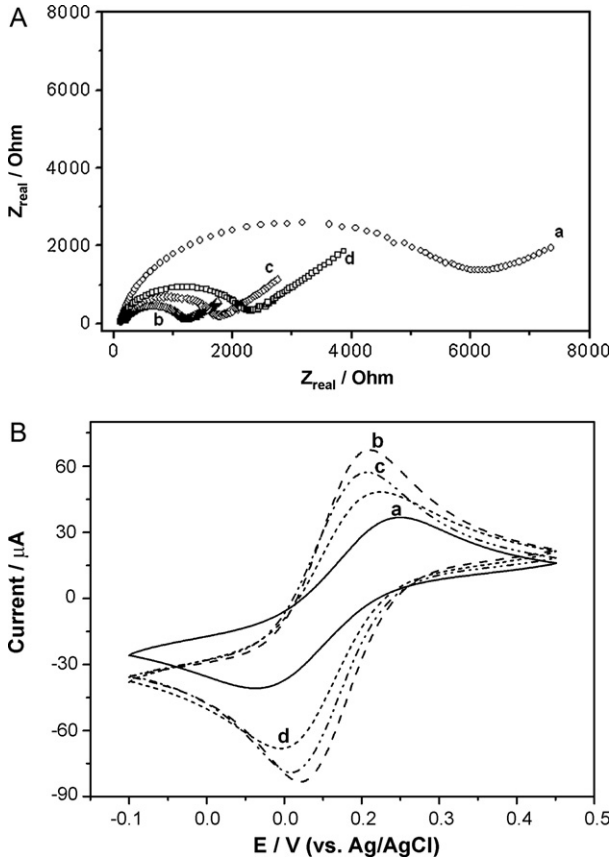

Fig. 2. (A) Nyquist plot of GCE + 25 μg laponite + 20 μg LOx and different amounts of CHT/μg, (a) 25, (b) 50, (c) 75 and (d) 100 in the presence of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl. The biased potential was 0.200 V (vs. Ag/AgCl), sine wave potential amplitude 5 mV and frequency range 0.05 Hz–10 kHz. (B) Cyclic voltammograms of 5 mM $K_3Fe(CN)_6$ in 0.1 M KCl solution at scan rate of 0.050 V s^{-1}. Voltammetric waves recorded on the same electrodes indicated in A(a–d).

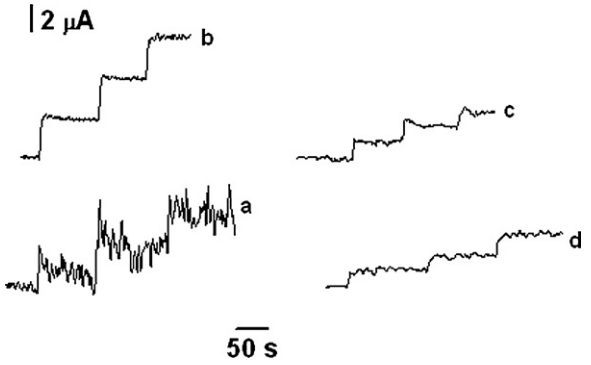

Fig. 3. Amperometric response at 0.400 V on a HMEE prepared with GCE + 25 μg laponite + 20 μg LOx and different amounts of CHT/μg (a) 25, (b) 50, (c) 75 and (d) 100 for successive additions (20 μL) of 0.011 M L-lactate to 10 mL 0.1 M phosphate buffer pH 7.0 + 0.2 mM FcMe.

Fig. 2A shows the Nyquist diagrams for films containing 25 μg laponite and 20 μg LOx with varying amounts of CHT. The lowest value in R_{CT} for HMEE corresponded to films containing 50 μg of CHT. These results were in good agreement with CV (Fig. 2B).

EIS and CV results were consistent with the corresponding *P* values reported above. Table 1 summarizes R_{CT} for laponite/CHT films with different amounts of CHT, with and without LOx. EIS for HMEE clearly indicated that laponite/CHT/LOx hydrogels behaved like a homogeneous medium in which only one charge transfer reaction took place. The R_{CT} value for laponite/CHT ratio = 25/50 was the lowest with or without enzyme, indicating that the relative amount of laponite and CHT, was responsible for the observed behavior.

3.2. Analytical performance of HMEE as a function of film composition

In order to analyze the influence of hydrogel composition on the catalytic response of the HMEE, amperometric studies were performed with bioelectrodes containing 25 μg of laponite, 20 μg of LOx and varying amounts of CHT (25; 50; 75 or 100 μg). Fig. 3 depicts reproducible stationary currents at 0.400 V obtained after successive additions (20.0 μL) of 0.011 M L-lactate to 0.2 mM FcMe in phosphate buffer pH 7.0, for each hydrogel composition. Time responses were shorter than 5 s. Stationary currents and signal to noise ratios were dependent on film composition. According to blank experiments (HME without enzyme) for each film composition, electroactive species were not detected (not shown).

From the calibration plots (see Supplementary Material) the analytical parameters were estimated for each CHT content (Table 2). The hydrogel containing 50 μg of CHT presented the most favorable linear range $(1.0–7.0) \times 10^{-5}$ M, detection limit $(3.8 \pm 0.2) \times 10^{-6}$ M, the shortest response time (4 s) and a very satisfactory sensitivity. This parameter was greatly improved in relation to our previous work [6], although it is not evident for the first sight. The reported sensitivities for both types of electrodes, laponite/sesquioxane [(0.33 ± 0.01) A cm^{-2} M^{-1}] and laponite/CHT [(0.326 ± 0.003) A cm^{-2} M^{-1}], were almost the same, but enzyme content for the latter was markedly lower. If enzyme content is taken into account, CHT electrode has a sensitivity of 0.815 A cm^{-2} M^{-1} unit^{-1}, which is almost 100% higher than the value obtained in the cases of sesquioxane (0.421 A cm^{-2} M^{-1} unit^{-1}).

Apparent Michaelis–Menten constants K'_M were estimated from Lineweaver–Burk plots from L-lactate calibration curves obtained for bioelectrodes with different CHT contents (Table 2). K'_M for the film with the lowest CHT concentration was almost equal to K_M in solution (2.3×10^{-4} M) [34], indicating that no significant conformational change took place after enzyme immobilization [6]. Electrodes with CHT content >25 μg showed increased K'_M values. This would be explained if the enzyme immobilization in the laponite/CHT hydrogel was accompanied by molecular interactions with some specific site of CHT, as was demonstrated by FT-IR spectra for PPO [28].

We have analyzed the LMEE stability after 30 days of storage in relation to CHT content. For this purpose, the amperometric currents we recorded every 4 days. Values in Table 2 clearly indicate that sensitivities were proportional to the CHT content. Films containing 100 μg of the biopolymer retained 100% of its original sensitivity. The relative low variation of sensitivity over time was considered as an indication of the film's ability to prevent the leaching of the immobilized enzyme. From data in Table 2, we concluded that the best choice from the analytical point of view was the hydrogel containing 50 μg of CHT.

The reproducibility of the electrode preparation was excellent, as can be deduced from currents obtained after L-lactate addition in three separated cells with HMEE containing 50 μg of CHT and prepared in a controlled way. Sensitivities obtained from the corresponding calibration plots were 0.327 ± 0.005; 0.326 ± 0.004 and 0.323 ± 0.004 A cm^{-2} M^{-1} respectively. Slopes, intercepts, correlation coefficients and RSD of slope and intercept of each calibration curve are provided as Supplementary Material.

3.3. Interference analysis for the quantification of L-lactate

Possible interferents commonly present in some alcoholic beverages and dairy products were investigated. Fructose, glucose, methanol, ethanol and citric, tartaric, acetic, malic, formic and succinic acids have been reported as interferents in wine and beer analysis. Ethanol is the second wine component. Also methanol, an undesired component, is a matter of concern. In the case of milk products, besides glucose and citric, acetic, and uric acids, AA is a ubiquitous interferent [35]. None of the analyzed substances, except AA, presented a significant electrochemical response on HME (not shown).

AA is electro-oxidized at 0.050 V. Its contribution to the analytical current was evaluated by amperometrically at 0.400 V for increasing amounts of interferent on GCE, HME and HMEE. Sensitivities, in A M^{-1} cm^{-2}, evaluated from the calibration plots, were $5.1 \pm 0.1 \times 10^{-3}$, $3.3 \pm 0.1 \times 10^{-3}$ and $2.5 \pm 0.1 \times 10^{-3}$ respectively. The detection limit for AA on HME was 7.4×10^{-5} M and on HMEE it was 4.0×10^{-6} M. According to these values, the laponite/CHT and the laponite/CHT/enzyme hydrogels were responsible for a 30% and 50% decrease in sensitivity respectively, and accordingly, for

Table 2
Analytical parameters for HMEE containing 25 μg laponite + 20 μg LOx and variable amounts of CHT.

CHT (μg)	Sensitivity (A M^{-1} cm^{-2})	10^6 Detection limit (M)	10^5 Quantification limit (M)	10^5 Linear range (M)	Response (time/s)	10^4 ($K\tilde{i}_M$/M^{-1})	% S[a]
25	0.43 ± 0.01	22 ± 3	7.33 ± 0.09	6–70	7	2.8 ± 0.5	80
50	0.326 ± 0.003	3.8 ± 0.2	1.26 ± 0.07	1–70	4	4.2 ± 0.4	85
75	0.317 ± 0.006	6.1 ± 0.4	2.03 ± 0.13	2–60	7	11 ± 0.3	94
100	0.281 ± 0.008	8.4 ± 0.5	2.80 ± 0.16	2–60	11	8.0 ± 0.4	100

[a] % of initial sensitivity after 30 days.

a positive reduce in AA interference. Together with this advantage, the sensitivity ratio for lactic/ascorbic acid for HMEE was 0.326/0.0025 = 130.4. This result indicates that electrode sensitivity to AA was low in comparison to that of L-lactate.

Interferents were investigated by amperometry on a HMEE. Therefore, each substance was added to electrolytic solutions containing 0.2 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 tartaric acids did not modify the stationary current for L-lactate at 0.400 V. Only small variations were observed in the case of the following acids: acetic (−2.3%), citric (3.0%), malic (2.9%) and succinic (2.1%). Nevertheless, these were observed only for 1:20 molar ratios, not a realistic situation.

CV experiments related to the oxidation of ascorbic acid (AA) on hydrogel modified electrode without enzyme (HME) and on hydrogel-modified enzymatic electrode (HMEE) showed a decrease in sensitivity of 30% and 50% respectively with respect to on bare GCE.

For L-lactate/AA molar concentration ratios of 1:1, 1:10 and 1:20, the observed steady state currents on HMEE were 0.6, 2.4 and 4.3 times higher than in the absence of this interferent. These results were better than those previously published [6]. L-Lactate/AA concentration ratio in real samples is usually above 10^3. Besides, sample dilutions in the electrolytic cell caused the effective AA concentrations to fall below 3×10^{-8} M, which lies well below our AA detection limit. Because of this, AA interference could be considered not of concern.

3.4. Quantification of L-lactate in alcoholic beverages and fermented milk: possible matrix effects

L-lactate content in white wine, beer and fermented milk was determined without pretreatment, except fermented milk that was diluted 1:4 with buffer prior to its addition to the cell. Current–time profiles were recorded on HMEE and HME. In the latter case, the lack of measurable oxidation currents was a clear indication that electroactive interferents, if present, were undetectable. Steady state currents on HMEE showed well-defined stationary currents, due to the enzymatic oxidation of L-lactate present in each sample.

Matrix effects on L-lactate determination were analyzed. In separate experiments, three different sample volumes were added to 10 mL of buffer solution + 0.2 mM FcMe, and for each one, successive aliquots (20.0 μL) of 0.011 M L-lactate solution were added. The matrix incidence was estimated from the slope of the corresponding sensitivities obtained in the presence (B) and absence (B_0) of a volume of each food sample, recorded on the same electrode, and 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 no matrix influence. Calibration plots obtained for the smallest sample volume were used to evaluate detection limits and L-lactate concentration in each beverage.

For white wine, SC values were 95.0%, 96.9% and 93.3% for 15.0 μL, 30.0 μL and 60.0 μL sample volumes, respectively, with a detection limit of $(4.1 \pm 0.3) \times 10^{-6}$ M and a L-lactate concentration of $(1.8 \pm 0.1) \times 10^{-2}$ M. For beer, SC values were 93.2%, 94.1% and 91.8% for 500.0 μL, 700.0 μL and 1000.0 μL of sample respectively. The detection limit was $(3.2 \pm 0.4) \times 10^{-6}$ M and the L-lactate content was $(2.5 \pm 0.2) \times 10^{-4}$ M. Fermented milk, for 1:4 diluted samples, presented SC values were 96.1%, 91.9% and 92.7% for 25.0 μL, 50.0 μL and 75.0 μL respectively. L-Lactate level was $(5.2 \pm 0.5) \times 10^{-2}$ M, with a detection limit of $(9.0 \pm 0.5) \times 10^{-6}$ M (figures showing the detection of L-lactate are provided as supplementary material). From the SC values reported above, we can conclude that matrix effects would be irrelevant for all samples, and accordingly, the smallest volumes employed in each case can be considered as very satisfactory.

Table 3
Validation of the amperometric method with a commercial spectrophotometric kit.

Sample	Volume (μL)	10^2 [L-Lactate] (M) Amperometry[a]	10^2 [L-Lactate] (M) Commercial kit[b]
White wine	15.0	1.8 ± 0.1	1.7 ± 0.2
Beer	500.0	0.025 ± 0.002	0.029 ± 0.004
Fermented milk	25.0[c]	5.2 ± 0.5	5.5 ± 0.4

[a] Reported values are the average of three replicates.
[b] Reported values are the average of five replicates.
[c] After 1:4 sample:buffer dilution.

In order to validate the proposed methodology, L-lactate content in wine, beer and fermented milk was also determined with a standard spectrophotometric method (average of five replicates), and the corresponding results were compared with those obtained amperometrically with the HMEE (three replicates), Table 3. According to a t-test and a F-test at the 95% confidence level, they were found not to be significantly different, and accordingly, the amperometric HMEE can be used to monitor L-lactate in food samples such as alcoholic beverages and dairy products.

4. Conclusions

CHT has clear advantages compared to oligosilasesquioxane. EIS, CV and amperometric results proved that Laponite/CHT hydrogels are a very suitable immobilization matrix for LOx in amperometric biosensors. Multipoint attachment of the protein, based on possible molecular interactions with CHT, film porosity enhancement, positive partition effects or film diffusion barriers have to determine the optimum ratio of laponite/CHT/LOx. Laponite/CHT = 25/50 gave the best values of P and R_{CT} for films with and without enzyme, stressing the importance of the interactions between both polyions in the development of a network-like structure. This, in turn, would explain the good electrochemical and enzymatic behavior of the biosensor, its high sensitivity and its operational stability.

Our results demonstrate that the HMEE is a very useful amperometric biosensor for the selective determination of L-lactate in food samples such as white wine and fermented milk without sample pretreatment. The biosensor is easily prepared and reproduced, with a high catalytic activity maintained for more than 30 days.

The low applied potential and permeability properties of the film allowed for the interference-free determination of L-lactate, as was demonstrated by comparison of calibration plots registered with and without interferents usually found in food samples. Matrix effects were minimized by sample dilutions compatible with the high sensitivity of the method and by the lack of electrochemical activity of most sample components at the sensing potential. Even AA is not of concern due to its final concentration in the electrolytic cell, well below its electrochemical detection limit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2010.11.026.

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