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Dual range lactate oxidase-based screen printed amperometric biosensor for analysis of lactate in diversified samples

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Abstract

Lactate concentration is studied as an indicator of physical performance in sports activities, and is also analyzed in health care applications, as well as in the food and cosmetic industries. This organic acid is routinely determined in different concentration ranges, depending on the type of samples for analysis. This paper describes the development of a screen-printed lactate oxidase (LO_x) based biosensor to determine lactate in broad concentration range. The Cu-MOF (copper metallic framework) crosslinking of 0.25U LO_x in a chitosan layer, allows to determine the enzymatic product generated on a platinum modified working electrode, at 0.15 V (vs SPE Ag/AgCl). The biosensor responds linearly in two different concentration ranges: a first catalysis range of 14.65 µA mM^{-1}, from 0.75 µM to 1 mM, followed by a saturation zone from 1 to 4 mM, after which a substrate enzymatic inhibition of 0.207 µA mM^{-1}, is observed up to 50 mM. These two ranges of analysis would allow the biosensor to be used for the determination of lactate in different types of samples, with low and high content of lactate. The method reproducibility was kept below 7% and a limit of detection of 0.75 µM was obtained. The device was successfully used in the determination of lactate in sweat and saliva, as a low cost noninvasive analysis, and also in wine samples.

Keywords: Lactate oxidase; Dual range biosensor; Noninvasive analysis; Sweat; Saliva; Wine.

1. Introduction

Lactate is known as a critical metabolite biomarker of tissue oxygenation, and is studied when assessing physical performance in sports activities, to prescribe appropriate exercise intensities [1,2]. It is also analyzed in health care applications to evaluate an underlying pathology during a routine stress test [3,4].

Due to a physiological accumulation, lactate relies in a process known as lactate acidosis, which takes place when the organism requires energy. This process can outcome in muscle fatigue, especially during endurance-based activities [5,6], result of pH decrease due to the levels of lactate in the blood and muscles [7]. Therefore, in clinical exercise and performance testing, blood lactate is the most frequently measured parameter, once it is considered as the key metabolite to finding out the degree of exhaustion of the athletes [2]. In view of the wide demand for the development of noninvasive, fast and reliable methods for monitoring lactate [8], saliva and sweat are suitable matrices for its determination, especially in sports medicine, since blood sampling can be avoided.

During exercise, lactate assessment through saliva samples has proven to be very attractive due to the high correlation with the blood levels [9,10], and also given the continuous and convenient availability of this metabolite, that can vary from 0.1 to 2.5 mM [11]. In turn, blood and sweat lactate levels have an unclear relation, although the sweat lactate is increased with exercise intensity [12–15]. Lactate constitutes about 0.28 % of the organic acids content in sweat, and is described to be present in concentration range from 3.7 mM up to 50 mM [16,17]. However, this dynamic range usually reaches a maximum of 25 mM [18].

The importance of lactate is not limited to the medical sector. This acid is found in many foods and beverages, in which concentration level indicates the freshness, stability, and quality of several products, being also added as acidulant, and flavoring agent (E270).[19]. In the winemaking industry, the course of malolactic fermentation is monitored by following the falling of L-malic acid and the rising of L-lactic acid. Therefore, this continuous monitoring is an important control for wine commercialization, preventing significant economic losses [20,21].

Among the various conventional analytical tools, colorimetric and chromatographic analysis of lactate are the standard methods. However, given the high complexity of these, simplest alternative such as, the use of screen-printed amperometric biosensors (SPE), have been developed for monitoring lactate [19,22–25].

The screen-printing technology offers high sensitivity and allows the easy electrode modification with nanomaterials and biocomponents, in order to attain reliable and selective

devices of analysis [26]. Platinum has an important role in electrode modification, especially for oxidase enzyme based-biosensors due to their catalytic effect on their product, allowing higher sensitivities for the electrochemical measurement [27–34].

Chitosan (CS) is a biocompatible cationic polymer, successfully applied in the biomedical domain [35–37]. Heightened interest in biosensing has prompted the extensive application of CS as a matrix for enzyme immobilization in SPE [38–43], providing an excellent microenvironment for biomolecules, emerging in highly sensitive and stable devices. Moreover, it possesses an excellent membrane-forming ability, high permeability to water, good adhesion, high mechanical strength and susceptibility to chemical modifications. Cruz et al., use the permselectivity properties of this polymer to develop a fast, selective, and reversible electrochemical sensor [44].

Furthermore, a new class of porous materials called Metal-Organic Frameworks (MOF) possess several advantageous properties for sensing applications. These materials, are recognized as a low-density network formed by a metallic center and an organic ligand. MOFs hold interesting properties such as ultrahigh and permanent porosity, high thermal and chemical stability, tunable pore structure and dimension, and adjustable internal surface characteristics. They have been applied to numerous areas of study, like catalysis, separation, biomedical imaging, clean energy, drug delivery and gas storage and separation [45,46]. In biosensing applications, these materials play a the role as biomimetic components [47], offering improved sensitivities for detection of certain analytes [48]. Gamero *et al*. reported an electrochemical biosensor based on three-dimensional ordered macroporous gold film electrodes, using covalently immobilized lactate oxidase, to obtain an increased conductivity and stability for lactate sensing [49].

In the last decade, numerous reports for lactate biosensing in several matrices have been published. As an alternative for a noninvasive analysis, sweat [33,34,50–53] and saliva [54–57], are target matrices for several biosensors. Also, numerous biosensors were also released for wine and other fermented beverages [20,58,59]. However, the applicability of these devices seems to be limited to one type of matrix sample, requiring a considerable number of enzymatic units, and/or a high potential for amperometric measurement to follow the enzymatic response. Also, the range of analysis seems to be limited to values under 28 - 30 mM of lactate [50,60].

In the present work, a novel approach was exploited for lactate analysis. Based on the enzymatic kinetic of immobilized lactate oxidase (LO_x), the biosensor responds linearly to lactate concentrations in two different ranges. Additional to the normal catalytic process, an

inhibition process at high substrate concentrations was observed, drawing a second linear zone, able to be used directly in the analysis of samples with high lactate content. As far as we know, it is the first time that a single LO_x based biosensor has demonstrated its applicability in the determination of lactate in diversified samples, using a low number of enzymatic units, with two distinct analysis ranges, allowing in this case, the quantification of lactate in wines, saliva and sweat samples. The design, characterization and evaluation of the dual range LO_x biosensor are described in the following sections.

2. Materials and methods

2.1.Materials

Lactate oxidase (LO_x) from *pediococcus sp.* (E.C. 1.1.3.2.) lyophilized powder, 28 units/mg solid, chitosan, glutaraldehyde (GA), bovine serum albumin (BSA), terephthalic acid (1,4 – H_2BDC), triethylenediamine (TED), N,N-Dimethylformamide (DMF), L-(-)-malic acid, maleic acid, gallic acid, sodium pyruvate, resveratrol and D(+)-Glucose monohydrate, were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium hexachloroplatinate (IV), L-histidine monohydrochloride monohydrate, sulfuric acid, urea, ascorbic acid, sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, copper nitrate trihydrate, ethanol and potassium chloride were obtained from Merck (Darmstadt, Germany). From Panreac-Applichem (Darmstadt, Germany) were purchased L-lactic acid (85%), succinic acid, citric acid anhydrous, ortho-phosphoric acid (85%), sodium chloride and sodium hydroxide. Creatinine was obtained from (Barcelona, Cromatest), and Uric Acid from Alfa Aesar. Quercetin dihydrate from Extrasynthese (Genay, France). Acetic acid from VWR (France).

In the fabrication of screen-printed electrodes inks used, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink), Electrodag 418 (silver ink) and Electrodag 452 SS (dielectric ink), were supplied by Achenson Colloiden (Scheemda, Netherlands).

The cooper metal framework (Cu-MOF) was synthesized according to the previously reported method by [61] and [48]. Briefly, copper nitrate trihydrate (0.493 g), 1.4-H_2BDC (0.453g) and TED (0.32 g) were dissolved in 100 mL of DMF, and the mixture was sonicated to obtain a homogenous solution. Then the solution was heated at 120ºC for 36 h. After cooling at room temperature, the obtained blue crystalline power was collected, washed with DMF and water several times and dried under vacuum overnight.

All the reagents used were of analytical grade and Milli-Q water (Millipore, Bedford, USA) was employed for preparing all solutions.

Human saliva and sweat samples were collected from healthy volunteers and were stored at -20ºC. The sweat samples were collected in vials, from volunteers front head, immediately after running. Also, saliva samples were collected directly in vials and stored until used. The wine samples were obtained from the local market and stored at 4ºC.

2.2.Apparatus

Voltammetric measurements were performed using an Autolab PGSTAT128N electrochemical system with the General Purpose Electrochemical System (GPES) software version 4.9 (Echo Chemie, Utrecht, The Netherlands). A PalmSens® portable electrochemical potentiostat with the PS Trace program (PalmSens® Instruments BV, Houten, The Netherlands) was used for chronoamperometric measurements. Reversed phase HPLC analysis was performed with a liquid chromatograph Flexar PerkinElmer® (Connecticut, U.S.A), consisting of a binary pump, manual sampler, and UV detector. Chromatographic separation was carried out at a ACE 5 C18 Column (ACE® HPLC Columns, Aberdeen, Scotland), 250 mm×4.6 mm and with particle size of 5 µm.

Microscopy imaging and elemental composition analysis were performed using a scanning electron microscope (SEM) JEOL JSM-6460LV with an INCA elementary X-ray analysis system Oxford Instruments (Abingdon-on-Thames, UK). The X-ray analysis was performed at three different zones of each working electrode with a zoom of x500.

2.3.SPE manufacturing

Screen printed electrodic systems, SPE, were produced following a previously described procedure [62]. Sequential deposition of the inks, which defines conductive silver tracks, Ag/AgCl reference electrode, carbon counter and working electrodes and dielectric layer, was performed on polyester films (HIFI Industrial Film, Dardily France).

2.4.Electrode Modification

2.4.1. Platinum coating (Pt/SPCE)

To achieve a homogenous Pt coating onto the carbon surface of the working electrode, a volume of 200 µL of a 1 mM platinum solution in 0.1 M KCl was deposited onto the electrodic system and cyclic voltammetric measurements were performed from 0.5 to -0.7 V (vs SPE Ag/AgCl), with a scan rate of 0.002 V s^{-1}. The experiments were conducted for 5, 10,

15, 20, 25, 30, 45, 55, 80 and 100 scans. The procedure, a modification of the one described for Compton [63], proves to be more efficient for the developed system (section 3.1).

2.4.2. *Preparation of enzymatic electrodes (LO_x–Cu-MOF/CS/Pt/SPCE)*

After the platinum modification, the Pt/SPCE electrode was rinsed with Milli-Q water. Then the electrode was dried and 2 µL of chitosan (0.6 % (w/v) in 0.5 % acetic acid) were cast onto the electrode surface, and dried at room temperature. Following, the enzyme was crosslinked on the CS/Pt/SPCE surface, using an immobilization solution, containing 3 µL of LO_x (1.5 U μL^{-1}, in 0.01 M phosphate buffer, pH 8.0), 3 µL of Cu-MOF (2 mg mL^{-1}), 0.56 µL of BSA (3 % (w/v)) and 0.56 µL of GA (2.5 % (v/v)). 1.20 µL of this solution, was cast onto the electrode surface, and the modified electrode, LO_x–Cu-MOF/CS/Pt/SPCE, was dried and stored at 4 ºC in a refrigerator until use.

2.5. Electrochemical measurements

Amperometric measurements were carried out in a bath system. All measurements were made at room temperature in a cell containing 5 mL of supporting electrolyte (phosphate buffer at 0.1 M, pH 7.0), with constant stirring. The working electrode operated at 0.60 and 0.15 V (vs SPE Ag/AgCl), in the case of LO_x/SPCE and LO_x/Pt/SPCE, respectively. The corresponding sample was added after reaching a stable baseline.

Wine and sweat samples were spiked and analyzed without any pretreatment. Only the saliva sample was spiked after centrifuging, to avoid high viscosity. Biosensing analysis of the real samples was carried out using the standard addition method.

Voltammetric measurements were recorded, between -0.2 V to 0.9 V (vs SPE Ag/AgCl) at different scan rates from 0.02 V s^{-1} to 0.15 V s^{-1} in a solution containing 2 mM of lactate in supporting electrolyte.

2.6. HPLC measurements

Before use, the column was cleaned with water and methanol/water (85/15), during 30 minutes for each step. Then, isocratic elution was performed using a phosphate buffer (0.2 % (v/v) of phosphoric acid and 0.16 % (w/v) of dihydrogen phosphate in water, pH 2.0), at 1.2 mL/min. Injection volume was 20 µL, and the quantification wavelength was set at 216 nm. The amount of lactate was quantified using Chromera chromatography software, determining the lactate/maleic acid peak area ratio, where maleic acid is the internal standard. The concentration of lactate in the samples was determined from the calibration curve of the peaks

at the retention time of 4.6 and 6.7 minutes, for lactate and maleic acid, respectively (see Fig. S1 in supplementary material) [64].

Prior to HPLC analysis, all samples were centrifuged at 13.2 rpm at 4 ^{0} C, during 30 min., and the supernatant filtered through sterile cellulose acetate 0.45 µm-membrane syringe filter. The samples were diluted; 7.5 times for red wine and sweat samples, and 2 times for white wine and saliva; and finally enriched with 10 ppm of maleic acid. All samples, standards and chromatographic phases were previously sonicated.

3. Results and discussion

Lactate Oxidase (LOx), is a flavoprotein recognized for its ability to catalyze the oxidation of lactate to pyruvate in the presence of oxygen, as shown in equation (1).

$$Lactate + O_2 \xrightarrow{LO_x} Pyruvate + H_2O_2 \quad (1)$$

In the proposed method, a biosensor based on the immobilization of LO_x on a platinum surface was used as a sensing interface to catalyze the electrochemical oxidation of hydrogen peroxide, equation (2). The biosensor allows the selective determination of lactate by monitoring the amperometric oxidation of the hydrogen peroxide, produced from the enzymatic reaction.

$$2\,H_2O_2 \xrightarrow{Pt\ electrode} O_2 + 2H^+ + 2e^-. \quad (2)$$

It was found that, depending on lactate concentration, 0.25 U of immobilized LO_x leads to different responses. In the first stage of enzymatic catalysis, a linear increment in the current signal was observed, for lactate concentrations from 0.75 µM to 1 mM. After a saturation zone, between 1 and 4 mM, a linear decrease in current intensity values takes place, for lactate ranging between 4 and 50 mM, as a consequence of a substrate inhibition process.

Copper metallic framework (Cu-MOF) and Chitosan (CS) were used to improve the biosensor, proving to considerably increase device performance. Therefore, a deep study of each component influence was carried out, as described in the next sections.

3.1.Electroactive surface area determination

Platinum surfaces are known to notably favor the oxidation of hydrogen peroxide since the potential required for oxidation corresponds to the platinum oxide (PtO) film formation [32]. The modification of the carbon working surface with a Pt coating improves the electrochemical signal for lactate determination. Therefore, in order to survey Pt influence in

the electrochemical response, the electroactive surface area (ESA) was determined for the different number of voltammetric cycles used in Pt coating of working electrodes [65].

Several theories have been proposed to determine the electroactive surface area (ESA) of electrodes. ESA measurements can be based on chemisorptions phenomena of a chemical bond between the catalyst surface and the adsorbate. As Pt provides a well-developed region for oxide monolayer reduction, the method applied evaluates the peak charge of PtO at the Pt/SPCE [65,66].

To determine the ESA of Pt/SPCE, cyclic voltammetric measurements were conducted in an acidic media (0.1 M H_2SO_4) from -0.3 to 1.3 V and the reduction peaks for platinum oxide (at approximately 0 V), were registered.

It was possible to calculate the ESA surface area by applying the equation (3) [66]:

$$ESA\ (cm^2) = \frac{Q_o(\mu C)}{420(\mu C\ cm^{-2})} \quad (3)$$

Where, Q_o is the charge associated with surface oxidation, and 420 μC cm^{-2} is the charge associated with the full monolayer of oxygen corresponding to PtO, which requires two electrons per site for its removal.

By increasing the number of voltammetric cycles the ESA value rises, given the amount of platinum deposited onto the carbon surface. The ESA value obtained by electrode modification with Pt is noticeably increased in comparison with the geometric area (0.126 cm^2) (see figure S2 in supplementary material). It has been previously found that the electroactive area can be increased about 1000 times comparatively to the geometric one [67]. Area evolution remained steady after 55 scans, and ESA value was estimated to increase hundredfold, compared to the geometric one. This plateau is defined by the electrode surface saturation for Pt deposition.

Besides the contribution in terms of electroactive area, which allows for a more effective determination of the enzymatic generated H_2O_2, this metallic coating also greatly contributes to an improvement of the optimal work potential since it decreases from 0.60 to 0.15 V. This new potential value introduces additional advantages in terms of electrochemical response specificity, also offering satisfactory current outputs.

Calibration curves were obtained to evaluate the effect of the number of scans in the Pt deposition process on the electrochemical behavior of the lactate biosensor. The higher the number of scans, the higher the current values that were found, increasing sensitivity for the first measurement range of biosensor (from 0.75 μM to 1 mM). However, for lactate concentrations from 4 to 50 mM, it was observed that the inhibitory response of the biosensor

was weakly altered by the amount of deposited Pt, indicating that the process is controlled by the decrease in enzymatic activity.

The Pt coating process is time consuming and does not greatly affect the inhibition process of LO_x. In light of this, 10 voltammetric cycles for SPCE modification with Pt were chosen, since it is less time consuming for electrode modification and the biosensor responds adequately to both low and high lactate concentrations, by increasing the ESA about 33 times relative to the geometric area. In agreement with electrochemical data, SEM and X-ray analysis shows an efficient nucleation of Pt onto working SPCE, exhibiting a satisfactory content of Pt nanostructures by using 10 voltammetric cycles of deposition, which favors the enzymatic product oxidation (see figures S3 and S4 in supplementary material).

3.2.Chitosan Influence in amperometric measurements

The presence of CS is proven to be essential for an adequate amperometric response, by affecting the enzymatic behavior in several aspects. It was observed that this membrane acts like a barrier, delaying the displacement rate of the enzymatic reaction product, H_2O_2, to the Pt surface and the amperogram resolution increased in the range of lactate concentrations corresponding to the enzyme inhibition (4 to 50 mM). When tested to evaluate enzymatic activity, the CS membrane marks a delay in electrochemical signal acquisition, proving that the polymeric film hinders the arrival of the analytes to the electrode surface. In fact, the linear relation between the cyclic voltammetric peak current and the square root of the scan rate according to Randles-Sevcik equation [68], demonstrates that a diffusion-controlled reaction is taking place on the Pt electrode surface.

The study of electroactive area was conducted in both the presence and absence of chitosan, since this membrane can lead to a lower Pt ESA available for the electrochemical oxidation of H_2O_2. In fact, the presence of a CS layer results in slightly lower ESA values, probably due to a partial surface covering with the polymeric molecules. Nevertheless, the same trend of the electroactive area evolution was attained for CS/Pt/SPCE (see figure S2 in supplementary material).

On the other hand, the enzymatic activity of LO_x is positively influenced by this compound, probably due to the biocompatibility of this natural polymer. The use of CS combined with a Pt coating on the electrode surface makes the developed biosensor more sensitive for both concentration ranges, 0.75 µM to 1 mM, and from 4 to 50 mM. Therefore, several CS concentrations of 0, 0.2, 0.6, 1 and 2 % (w/v) in 0.5 % acetic acid, were tested on the Pt surface, before the enzymatic immobilization (see Fig. S5, in supplementary material). A

considerable increase in generated current was observed in the presence of CS. Improved values for lactate biosensing in both analysis ranges were found for 0.6 and 1 % of CS. Nevertheless, a slow process for 1 % CS was observed, affecting the experimental time. Also, higher amount of CS seems to be connected to higher noise in the amperometric measurement. Bearing this in mind, 0.6 % CS was selected as the optimal membrane, given that it has a higher sensitivity for lactate with a good amperometric resolution (fig. 1 – A, orange line).

The role of this membrane was also studied when deposited in different steps of the immobilization procedure, and the effect in the amperometric signal registered. When deposited after enzyme immobilization, the registered current was lower than that observed when the biocomponent was first immobilized. This, probably reflects the LO_x activity loss as consequence of the pH of CS solution. The same behavior was found for the enzymatic sandwiching between two CS deposits resulting in a lag response for lactate sensing.

3.3.Optimization of immobilization procedure

3.3.1. Copper Metal-Organic framework influence in amperometric measurement

Additionally, in this work the use of Cu-MOF proves to be favorable in terms of the biosensor's inhibition signal sensitivity and stability. According with the literature, this MOF present a brick-like morphology, and is able to form a film layer in presence of chitosan, also this materials can influence the charge transfer process [48,49]. The study of the metallic framework influence was carried out using different amounts of Cu-MOF, ranging from 0, 1, 2, 3 and 4 mg mL^{-1}, in the immobilization solution (see Fig. S6, in supplementary material). Despite other Cu-MOF concentrations leading to higher slope values for lactate sensing through inhibition, they also show a higher variability of the electrochemical response. The optimal balance between stability and sensitivity for lactate biosensing was found to be 2 mg mL^{-1} of Cu-MOF. In fig. 1 – A (green line), we can observe an imperceptible influence in the catalytic zone barely affecting the sensitivity. This data contrast with a positive evolution of biosensor sensitivity for LO_x inhibition response. When added to the enzymatic immobilization step, Cu-MOF proves to result in higher sensitivity of immobilized enzyme to inhibitory concentrations of lactate, increasing the slope obtained in this second zone. Comparing with the normal glutaraldehyde crosslinking onto the working electrode, higher sensitivities and improved current decrease steps, were obtained.

Regarding the configuration of the sensing interface is noted that, by associating the individual contribution of each component with the all components used at time, the LO_x-

based biosensor sensitivity is increased in both ranges for analysis (fig.1 – A, blue line). It should be noted that the concentration ranges for analysis were maintained, indicating that the amount of enzyme is the responsible of this property. An enhanced signal is clearly observed when the Cu-MOF crosslinking is performed on a CS membrane, showing the metallic framework contribution in terms of sensitivity for the catalysis zone. This may be possibly attributed to a better orientation of the enzymatic structure in presence of the Cu-MOF by preventing the active center hinder by the CS membrane.

3.3.2. Enzymatic Crosslinking

The design and manufacture of enzymatic biosensors is deeply influenced by the immobilization of the enzyme on the electrochemical transducer, allowing an increase in stability and selectivity [69]. In this work, LO_x was immobilized onto the CS/Pt/SPCE by Cu-MOF crosslinking using GA as a bifunctional reagent mixed with a non-active protein, BSA. Despite the previously optimized amount of each crosslinking component (data not shown), elimination of GA fully from the immobilization procedure could result in higher biosensor performance since this bifunctional reagent is known for having denaturing properties. Furthermore, the recognized porosity of the MOF structures suggests that this type of components could also behave as an immobilization frame, avoiding the need of GA as bifunctional agent. Therefore, electrochemical measurements were carried out in the presence and absence of the crosslinking agents when Cu-MOF was present. However, the biosensor displays a non-favorable response when the crosslinking agents are absent. In fact, the biosensor's performance is altered, resulting in less sensitive in the inhibitory range. Also, an increase in measurement noise was observed, by signal to noise ratio decrease on those conditions, hindering an ideal visualization of amperometric steps.

3.4.Biosensor characterization

The accuracy of the developed method was calculated in terms of reproducibility and repeatability. Under optimum conditions, several calibration curves were obtained varying the concentration range from 0.75 µM up to 1 mM, and from 4 to 50 mM, to study reproducibility in both catalysis and inhibitory zones respectively (Fig. 1 – B and C). The reproducibility achieved in terms of RSD associated with the slopes of the calibration curves, recorded using different biosensors, was 5.03 % in the catalysis calibration range, while for the inhibitory lactate concentrations an RSD of 6.82 % was attained.

Despite the disposable nature, under optimum operating conditions, a single biosensor submitted to successive calibration measurements, in the first range, only lose more than 10 % of sensitivity after the tenth measurement (see Fig. S7, in supplementary material).

The linear regression parameters between lactate concentration and the analytical signal for catalysis range were used to determine the limit of detection (LOD). Using the equation LOD $=3S_{x/y}/b$, where $S_{x/y}$ is the estimated standard deviation of the minimum concentration detected and b the average slope of three catalysis calibration curves, the LOD obtained was of 0.75 µM [70,71].

In terms of lifetime, the biosensors prove to maintain approximately 100 % response sensitivity, reflected in terms of calibration curve slope, for both zones up to 50 days, when stored at 4ºC until use (see Fig. S8 in supplementary material). The long-term stability achieved with the LO_x–Cu-MOF/CS/Pt/SPCE, improve the commercial value of the sensor and the effectiveness in lactic acid monitoring during the malolactic fermentation, once this process usually takes place for up to 40 days in most wines [72]. The analytical parameters that characterize the sensor performance are shown in table 1.

In table 2, the main characteristics of the developed biosensor are compared with other LOx – based electrochemical biosensors reported in the literature. The approach presented in this work outperforms other devices in terms of operational stability and in terms of biosensor performance. The proposed biosensor is the only one that offers the possibility to determine lactate in different real samples, using two different concentration ranges of interest, a catalytic one from 0.75 µM to 1 mM, and an inhibitory range from 4 to 50 mM, both in the same device. Our device is the only able to determine high content of lactate, reaching an upper limit of analysis of 50 mM. In addition, the enzymatic units used of 0.25 U, are considerably lower than other approaches, that can reach similar ranges, resulting in an economic device. In this way, is possible to attend different end-users, once the biosensor can cover a broad range of analysis, being useful in both biological and food analysis, with possibility to perform continuous measurements.

3.4.1. Interference study

Since the biosensor is expected to be exposed to several types of electroactive compounds, its selectivity was examined to ensure applicability in the analysis of real life samples. Therefore, potential interferences were investigated under the same experimental conditions.

First, potentially interfering substances present in wine samples were evaluated; namely malic acid, glucose, ethanol, ascorbic acid, tartaric acid, succinic acid, citric acid and acetic acid.

Gallic acid, quercetin and resveratrol were also tested as possible phenolic interferents. For wine interference analysis, all evaluated compounds showed negligible electrochemical signals considering 100 % the lactate response (Fig. 2 – A). In fact, as shown in fig. 3, red wine gives an imperceptible signal when using a Cu–MOF/CS/Pt/SPCE based sensor (without the enzyme), compared to the amperometric signal obtained with a LO_x–Cu-MOF/CS/Pt/SPCE based sensor.

On the other hand, the plethora of metabolites and electrolytes present in perspiration and saliva potentially acting as biological interferents were considered (Fig. 2 – B). The amperometric responses of creatinine (84 µM), ascorbic acid (10 µM), glucose (0.17 mM), L-histidine (0.52 mM), pyruvic acid (0.18 mM), uric acid (24.5 µM), urea (10 mM) and sodium chloride (23 mM) were examined against the lactate signal in both catalysis and inhibitory concentration ranges. At physiological levels, negligible responses to creatinine, ascorbic acid, glucose and pyruvic acid, with respect to the lactate signal, were found.

Uric acid and urea were identified as interfering species, since they cause a small oxidation signal on platinum modified electrodes (fig. 2– B). Uric acid is present in saliva samples, however, as shown in table 3, high accuracy was achieved for lactate determination in this biological matrix analysis. Important interferences were found in the presence of L-histidine and sodium chloride (see Fig. S9 and Fig. S10 in supplementary material), given that these compounds inhibit the enzyme and their presence proves to significantly affect the performance of the developed biosensor, decreasing its sensitivity. The inhibitory effect of both species was found to be greater than that caused by the lactate. As a result, the slope values of the inhibitory calibration curves decreased by 49% and 65% in the presence of L-histidine and sodium chloride, respectively (see Fig. S10 in supplementary material).

More than an inhibitor of the LO_x, chloride can be interfering with the redox reaction, since this anion is described to inhibit the H_2O_2 oxidation onto the platinum surface, as described previously by Hall [73]. Regarding this, chloride influence in the catalysis zone was checked, resulting in a loss of sensitivity dependent of the anion concentration (see fig. S11 in supplementary material). Notwithstanding, the developed biosensor shows that can be used to determine variations in sweat lactate concentrations during physical exercise and to assess muscular fatigue by LO_x inhibition.

3.5. Application in biological and food samples

The lactate concentrations of four different samples were determined using the newly developed LO_x–Cu-MOF/CS/Pt/SPCE based biosensor and the results were compared with

those found using HPLC analysis as the reference method. The initial concentrations of lactic acid present in the samples were determined by applying the standard addition method. Table 3 shows the good correlation between chromatographic and electrochemical methods for lactate quantification in different samples, evidencing that the proposed lactate biosensor is suitable, accurate and reliable for analyzing this type of samples.

Conclusions

A new dual-range LO_x-based screen-printed biosensor was developed by a Cu-MOF crosslinking of only 0.25 U of LO_x on a platinum modified surface covered with chitosan. The device proved to be suitable for a broad range of lactate determination, offering two distinct ranges of analysis: an enzymatic catalysis range from 0.75 µM to 1 mM, and a substrate inhibitory concentrations range comprised between 4 and 50 mM. This inhibitory behavior is appropriate for determining sweat lactate as it encompasses all the possible ranges for analysis. Higher sensitivity in the modified platinum surface for lactate was found when working at 0.15V (vs SPE Ag/AgCl), avoiding the appearance of interferents. It has been proven that the Cu-MOF crosslinking used with CS membrane result in an improved performance of the final device for both zones. The biosensor presents good accuracy, since RSD value was kept below 7% for both, catalytic and inhibitory zones. A detection limit of 0.75 µM of lactate for the first zone was determined. The biosensor showed good repeatability for nine consecutive measurements. When stored at 4 ºC, biosensor long-term stability studies indicate that the sensitivity is maintained consistent over a period of 50 days.

The efficiency of the developed biosensor was tested in food samples such as white and red wines, as well as in biological samples, such as saliva and sweat. Regarding the inhibitory zone, after a convenient adaptation of the biosensor as a wearable device, could be useful in the direct measurement of high concentrations of sweat lactate. Overall, the biosensor can be potentially used to control malolactic fermentation in the winemaking process and would be highly useful for monitoring physical performance in sports medicine applications, as a noninvasive routine analysis, offering a low cost and easy to use analysis of lactate.

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Fig. 1. A: Amperograms obtained at different LO_x based biosensors in 5 mL of phosphate buffer pH 7.0, for successive additions of lactate ranging from 0.75 µM up to 50 mM. Eap: 0.15 V; (grey) LO_x/Pt/SPCE; (green) LO_x–Cu-MOF/Pt/SPCE; (orange) LO_x/CS/Pt/SPCE, and (blue) LO_x–Cu-MOF/CS/Pt/SPCE. Calibration curves in catalysis (B) and inhibition (C) zones obtained in optimal conditions, corresponding to amperogram (blue) LO_x–Cu-MOF/CS/Pt/SPCE.

Fig. 2. Percentages of amperometric responses of potential interferents against lactate response, at LO_x–Cu-MOF/CS/Pt/SPCE. Eap: 0.15 V (vs SPE Ag/AgCl). (A): Wine interferents. Spiked additions of 2 mg L^{-1} of each interferent compound against lactate: 0.5 mM, in phosphate buffer (0.1 M, pH 7.0); (B): Physiological interferents. Spiked additions of components at physiological levels, against 4 mM of lactate, in phosphate buffer saline (0.1 M, pH 7.0; with NaCl (23 mM)).

Fig. 3. Amperograms recorded at LOx–Cu-MOF/CS/Pt/SPCE (red curve) and at control Cu-MOF/CS/Pt/SPCE (blue curve), for the determination of lactate in wine samples. Successive additions of 10 µL of red wine in phosphate buffer (0.1 M, pH 7.0), at Eap: 0.15 V (vs SPE Ag/AgCl).

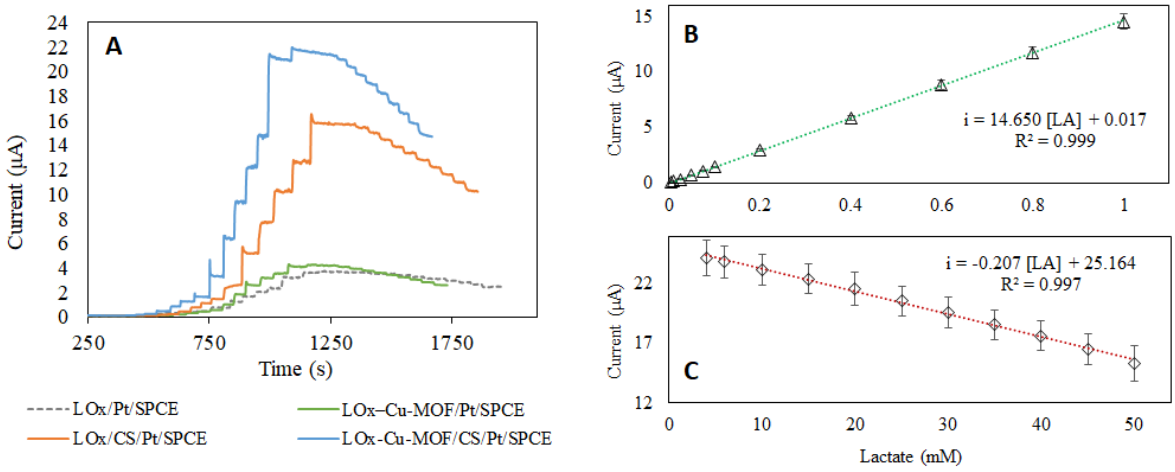


Fig. 1

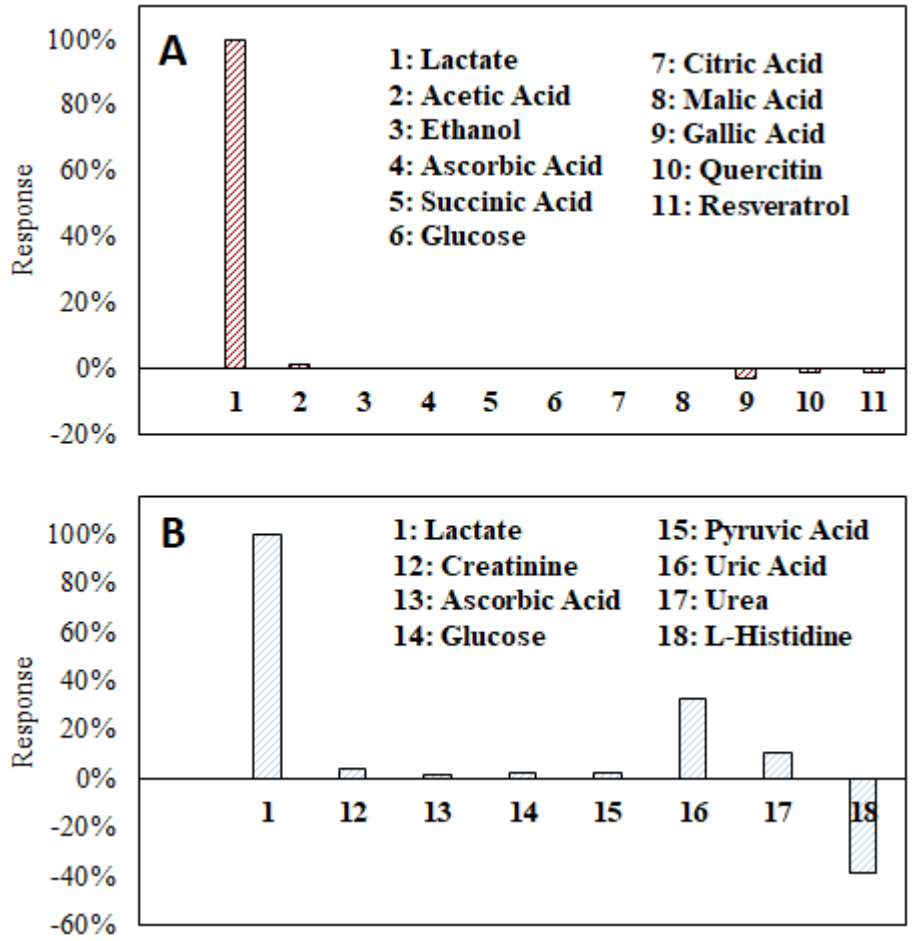


Fig. 2

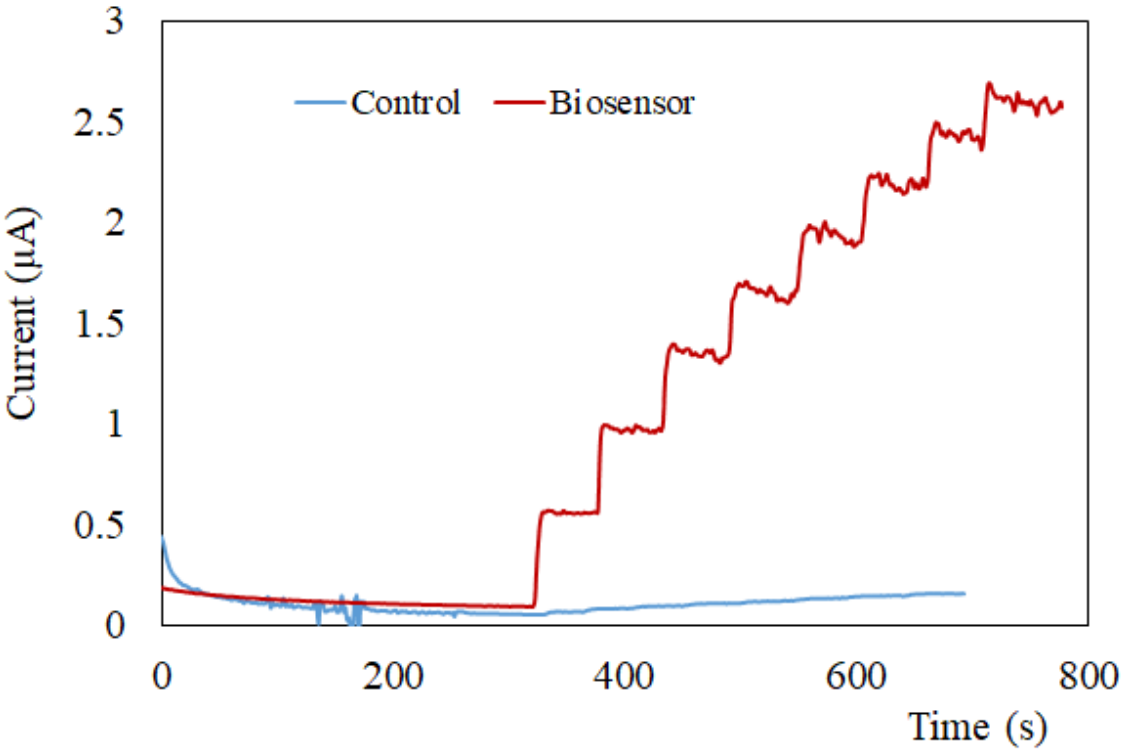
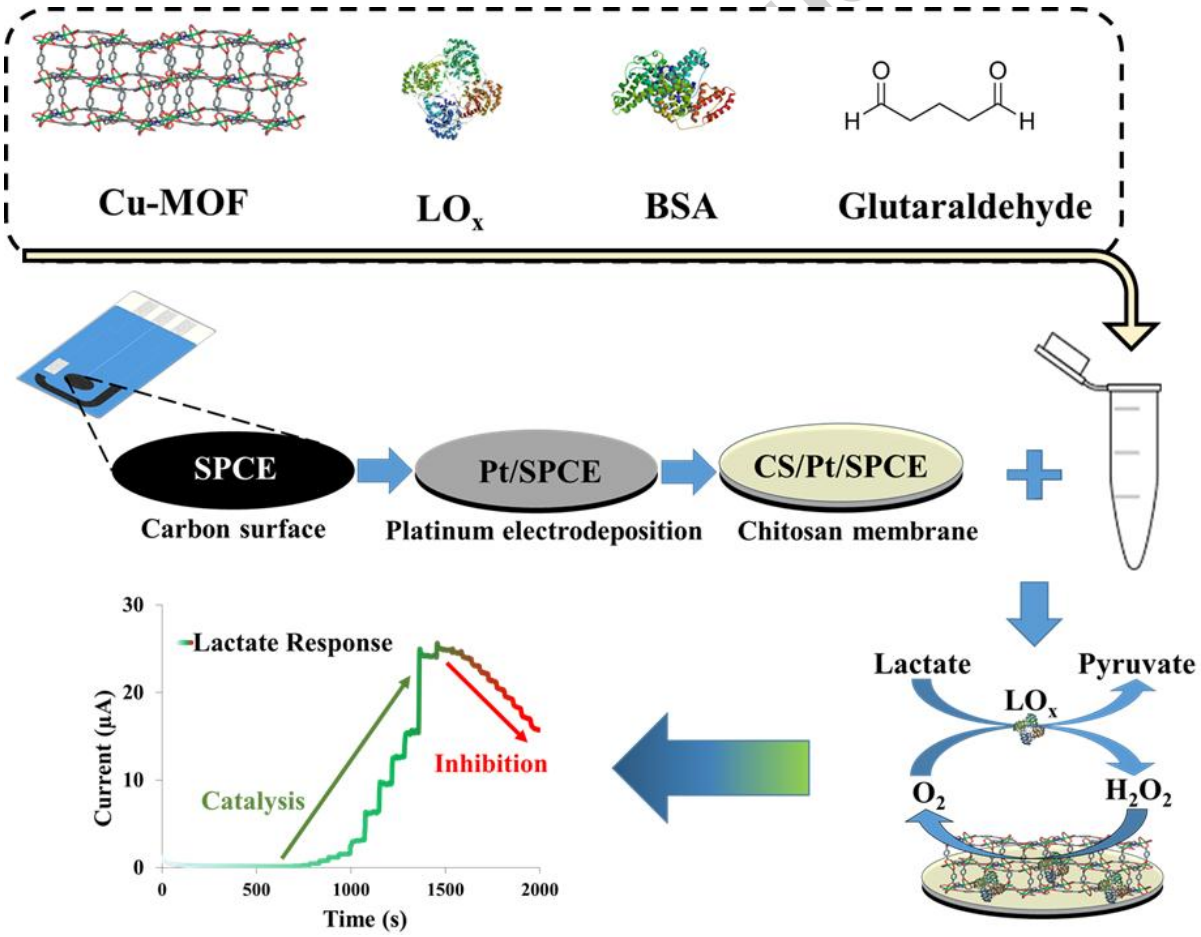


Fig. 3



Graphical Abstract

Table 1. Calibration parameters of LO_x–Cu-MOF/CS/Pt/SPCE biosensor for lactate determination in buffered media. (*) Inhibition response at physiological level of biological interferents.

Biosensor	**Working Potential (V)**	**Analysis range**	**Sensitivity Slope (μA mM^{-1}) n=3**	**R^2**	**Reproducibility (% RSD) n=3**	**LOD**
LO_x–Cu-MOF/CS/Pt/SPCE	+0.15 V	Catalysis	14.650 (±0.721)	0.999	5.03	0.75 μM
		Inhibition	0.207 (±0.016)	0.997	6.82	-
		Inhibition*	0.0123(±0.00051)	0.999	5.56	-

Table 2. Comparison of analytic characteristics of various lactate oxidase based – amperometric biosensors.

Enzymatic units used per Sensor	Working potential (V)	Electrode modification	Linearity (mM)	Detection limit (LOD) µM	Storage stability (Days)	Sensitivity (µA mM^{-1})	Reproducibility (RSD %)	Applications	References
0.25	0.150	LO_x – Cu-MOF/CS/Pt/SPCE	0.00075 – 1.0	0.75	50	14.650	5.03	Sweat, Saliva, Red and White Wines	This work
			4.0 – 50	-		0.207	6.82		
10.00	- 0.100	PS/MWCNT/Fc/LOx /HRP/SPCE	0.0011 – 0.056	0.56	15	1168.8 µA M^{-1} mm^{-2}	2.70	Wine and beer	[20]
0.20	0.075	PPY_{3DOM}/HRP/LO_x/ Au	0.01-0.1	0.52	40	13.500	4.00	Wines	[72]
2.13	0.730	LO_x/DTSP/Au_{3DOM}	0 – 1.3	3.93	30	1.630	3.50	Lack of real samples analysis	[49]
20.00	- 0.050	LO_x/PBnc/SPCE	0.01 – 0.5	10.00	21	6.380 mA M^{-1} cm^{-2}	NR	Blood	[74]
60.00[a]	0.650	LO_x/PPr-PPA/Ptw	0 – 1.0	NR	30	0.048	28.00	Saliva	[56]
800.00[a]	0.042	LO_x/PPD/PB/SPCE	0.1 – 1.0	50.00	NR	0.553	NR	Saliva	[57]
1.00	0.500	LO_x/PtNp-CNF-PDDA/SPCE	0.025 – 1.50	11.10	180	36.800 mA M^{-1} cm^{-2}	5.80	Blood and Sweat	[34]
6.40	0.00	CS – CNT/LO_x/ CS – CNT/PB/SPAuE	2.0 – 30.0	NR	32	0.22	5.00	Body sweat	[50]
12.12	0.050	CS/LO_x/CMCNT/TTF/SPCE	10.0 – 20.0	NR	150	0.644	3.60	Body sweat	[75]
16.16	- 0.100	PVC/LO_x-CS/SPPBE	0 – 28.0	NR	NR	0.096	NR	Body sweat	[53]

ABBREVIATIONS USED: 3DOM – Three-dimensional ordered microporous; [a] - Units used by mL of a polymerization solution; CMCNT – Carboxy-functionalized multiwalled carbon nanotubes; CNF – Carbon nanofibers; CNT – Carbon nanotubes; DTSP – Self-assembled monolayer of dithiobis-N-succinimidyl propionate; HRP – Horseradish Peroxidase; LO_x – Lactate oxidase; MWCNT – Multiwall carbon nanotubes; NR – not reported; PAP – poly(4-aminophenol); PB – Prussian blue; PBnc – Prussian blue nanocubes; PPD – poly(o-phenylenediamine); PDDA – poly(diallyldimethylammonium) chloride; PPr – poly-(phenol red); PPy – Polypyrrole; Pt – Platinum; PtNp – Platinum nanoparticles; Ptw – Platinum wire; PS – Polysufone; PVC – polyvinyl chloride; SPPBE – Screen-printed Prussian blue electrode; SPAuE – Screen-printed gold electrode; SPCE – Screen-printed Carbon electrode; TTF – tetrathiafulvalene.

Table 3. Comparison of results of lactate concentration obtained using amperometric and chromatographic methods in different samples. *N*=number of experiments.

Samples	N	LO_x – Cu-MOF/CS/Pt/SPCE	HPLC
		Lactate (mM)	
Red wine	5	38.82 ± 2.20	38.34 ± 2.93
White wine	5	7.90 ± 0.39	8.15 ± 1.00
Saliva	6	0.71 ± 0.08	0.73 ± 0.01
Sweat	6	34.56 ± 1.02	35.40 ± 0.60

Highlights

- New dual range amperometric LO_x biosensor to determine lactate in several matrices.
- Lactate determination through enzymatic catalysis and inhibition.
- Biosensor works with a very low number of enzymatic units.
- Pt, CS and Cu-MOF, reveal a synergetic effect in the biosensor sensitivity.