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Milk lactate determination with a rotating bioreactor based on an electron transfer mediated by osmium complexes incorporating a continuous-flow/stopped-flow system

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Abstract

The high sensitivity that can be attained using a bienzymatic system and mediated by the redox polymer [$Os(bpy)_2ClPyCH_2$NHpoly(allylamine)] (Os-PAA), has been verified by on-line interfacing of a rotating bioreactor and continuous-flow/stopped-flow/continuous-flow processing. When the hydrogen peroxide formed by LO_x layer reaches the inner layer, the electronic flow between the immobilized peroxidase and the electrode surface produces a current, proportional to lactate concentration. The determination of lactate was possible with a limit of detection of 5 nmol l^{-1} in the processing of as many as 30 samples per hour. This arrangement allows working in undiluted milk samples with a good stability and reproducibility. Horseradish peroxidase [EC 1.11.1.7] and Os-PAA were covalently immobilized on the glassy carbon electrode surface (upper cell body), lactate oxidase [EC 1.1.3.*x*] was immobilized on a disk that can be rotated.

Keywords: Milk; Lactate; Lactate oxidase; Horseradish peroxidase; Osmium; Bioreactor; Flow system; Biosensors

1. Introduction

The development of new approaches to determine low concentrations of analytes continues to receive great attention [1]. This is of importance in several areas such as the food industry, and clinical. Accurate and rapid determination of low levels of L-lactate has been determined using amperometric enzyme electrodes in several food samples [2–5]. In all the cases dilution of the sample is needed, also in well-established spectrophotometric method [6] and the fluorescent methods [7] need a pretreatment of the sample in the cases of milk and yogurt.

The use of multiple enzymes can enhance the sensor response characteristics and these enzyme arrangements have also constructed to amplify the signal by cycling the analyte [8]. Product accumulation, accomplished by a mediator, result in a very powerful tool to detect very low concentrations of a given substrate. Few approaches have been documented in the area of biosensing by exploiting bienzymatic amplification via substrate cycling and the use of a mediator [9].

In this paper, we show that the limit of detection for lactate can be lowered considerably if the double redox enzyme catalytic system and mediate by the redox polymer [$Os(bpy)_2ClPyCH_2$NHpoly(allylamine)]

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(Os-PAA) are utilized using a rather recently introduced strategy [10]. Our aim is develop a bioreactor able to analyze complex food, avoiding or minimizing the number of steps needed to assess the concentration of the analytes. We have chosen milk as the matrix example, and lactate as the possible analyte to quantify.

Such strategy allows an effective use of immobilized active centers and the effects of interferents can be also reduced by the low potential used [11]. The bioreactor presented here is formed by an inner layer of Os-PAA and horseradish peroxidase deposited on a glassy carbon electrode (top part) and lactate oxidase was immobilized in a rotating disk (bottom part), this part of the reactor works as the recognition element.

The approach described in this paper is important for the determination of lactate in a complex matrix like milk when the levels are very low and others methods are not suitable due to relatively-low sensitivity. It has to be pointed out that a large number of samples can be processed by means of the proposed method which shows adequate sensitivity, low-cost, versatility, simplicity and effectiveness.

The strategy proposed here, however, is capable of providing useful information where conventional monitoring procedures fail. Experimental data presented in this paper verifies this assertion.

2. Experimental

2.1. Reagents and solutions

All reagents used, were of analytical reagent grade. The enzyme lactate oxidase, LO_x, [EC 1.1.3.*x*], from *Pediococcus* sp, horseradish peroxidase, HRP [EC 1.11.1.7] Grade II, and the enzymatic method for the L-lactate determination kit (Catalog No. 735-10) were purchased from Sigma Chemical Co., St. Louis, Glutaraldehyde (25% aqueous solution) were purchased from Merk, Darmstadt. 3-Aminopropyl-modified controlled-pore glass (APCPG), 1400 Å mean pore diameter and $24\,m^2\,mg^{-1}$ surface area, was from Electro-Nucleonics (Fairfield, NJ) and contained $48.2\,\mu mol\,g^{-1}$ of amino groups. Synthesis of osmium redox polymer (Os-PAA) containing $[Os(bpy)_2Cl(pyCH_2–)]$ is described elsewhere [12]. Aqueous solutions were prepared using purified water from a Milli-Q-system and the samples were diluted to the desired concentrations using a 10 ml Metrohm E 485 burette.

2.2. Lactate oxidase immobilization

The rotating disk reactor (bottom part) was prepared by immobilizing lactate oxidase, LO_x, on 3-aminopropyl-modified controlled-pore glass. The APCPG, smoothly spread on one side of a double-coated tape affixed to the disk surface, and was allowed to react with an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M carbonate) for 2 h at room temperature. After washing with purified water and 0.10 M phosphate buffer of pH 7.00, the enzyme (10.0 mg of enzyme preparation in 0.50 ml of 0.10 M phosphate buffer, pH 7.00) was coupled to the residual aldehyde groups in phosphate buffer (0.10 M, pH 7.00) overnight at 5 °C. The immobilized enzyme preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C between uses. The immobilized lactate oxidase preparations were perfectly stable for at least 2 months of daily use.

2.3. Enzyme redox polymer reactor

The first layer was prepared by mixing a 20 μl aliquot of Os-PAA solution (0.3% (w/v)) onto a glassy carbon electrode of 8 mm diameter, then polymer was crosslinked with 15 μl of horseradish peroxidase solution (1% (w/v)) and 50 μl of poly(ethylene glycol) diglycidyl ether solution (1% (w/v), PEG 400, Polysciences, USA), the electrode was dried in a vacuum desiccators for at least 30 min. Then the second layer was formed 15 μl of a solution containing 18 μg of poly(allylamine) (PAA) and 60 μg of PEG. Then the electrode was dried. The stability of the preparation has been investigated. The biosensor was run continuously for 20 days. During that time the response declined by only 10%. The results show that the preparations were stable and robust also a high catalytic current was observed when this composition was used for the construction of the detector system.

2.4. Flow-through reactor/detector unit

The main body of the cell was made of Plexiglas. Fig. 1 illustrates the design of the flow-through

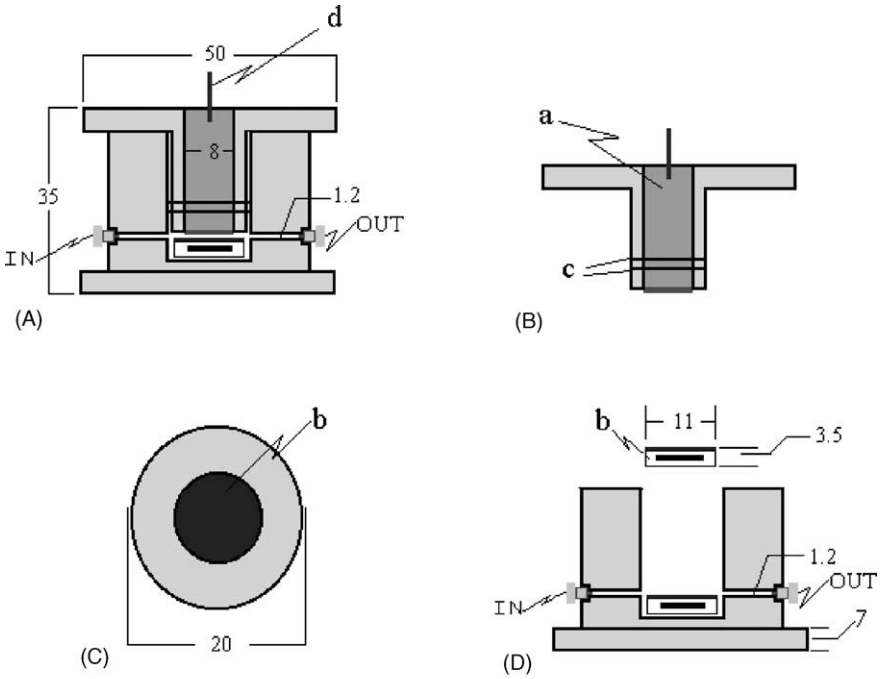


Fig. 1. Schematic representation of components in the bioreactor flow cell. (A) Assembled reactor. (B) Upper cell body. (C) Top view of lower cell body—b: rotating bioreactor (with immobilized LO_x). (D) Lower cell body. a: Glassy carbon electrode with enzyme/mediator immobilized; b: rotating bioreactor; c: O-ring; d: electrical connection. All measurements are given in millimeters.

chamber containing the rotating enzyme reactor and the detector system. Modified glassy carbon electrode is on the top of the rotating reactor. The rotating reactor is a disk of Teflon in which a miniature magnetic stirring bar (Teflon-coated Micro Stir bar from Markson Science, Inc. Phoenix, Az) has been embedded. Typically, a reactor disk carried 1.4 mg of controlled-pore glass on its surface. Rotation of the lower reactor was effected with a laboratory magnetic stirrer (Metrohm E649 from Metrohm AG Herisau, Switzerland) and controlled with a variable transformer with an output between 0 and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina).

The potential applied to the modified glassy carbon electrode for the functional group detection was +0.00 V versus Ag/AgCl, 3.0 M NaCl reference electrode. At this potential, a catalytic current was well-established, we expected less contribution of electroactive interferences that are present in milk.

2.5. Flow system

A pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics Inc., Middleton, WI) was used for pumping, sample introduction, and stopping of the flow. Fig. 2 illustrates schematically the components of the single-line continuous-flow setup. The pump tubing was Tygon (Fisher AccuRated, 1.0 mm i.d., Fisher Scientific Co., Pittsburgh, PA) and the remaining tubing used was Teflon, 1.00 mm i.d. from Cole-Parmer (Chicago, IL).

2.6. Spectrophotometric determination of L-lactate

The enzymatic Boehringer Mannheim UV method (D-lactic/L-lactic acid) was used, following the manufacturer's instructions [13]. A dairy sample clean-up was made by addition of EDTA to a final concentration of 2% (w/v). The concentration

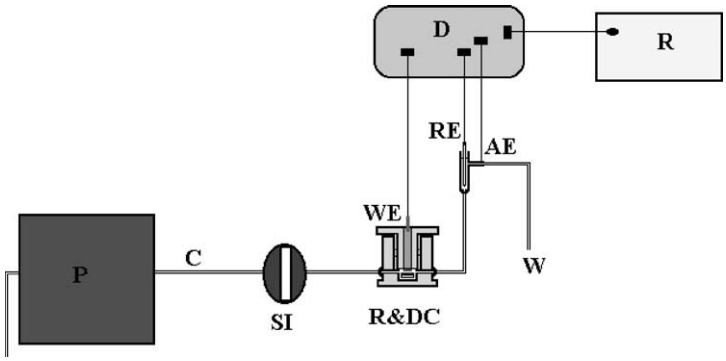


Fig. 2. Block diagram of the continuous-flow system and detection arrangement. P: Pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics Inc., Middleton, WI); C: carrier buffer line; SI: sample injection; W: waste line; R and DC: reactor and detector cell; WE: modified electrode; RE: reference electrode (Ag/AgCl, 3.0 M NaCl); AE: auxiliary electrode (stainless steel tubing); D: potentiostat/detection unit (LC-4C, Bioanalytical Systems, West Lafayette, IN); R: recorder (Varian, Model 9176, Varian Techtron, Springuale, Australia).

of L-lactic acid was calculated using the extinction coefficient of NADH [13].

Absorbance determinations were made at 340 nm using a single beam Beckman DU 350 UV-Vis spectrophotometer and glass cuvettes.

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc., Cambridge, MA).

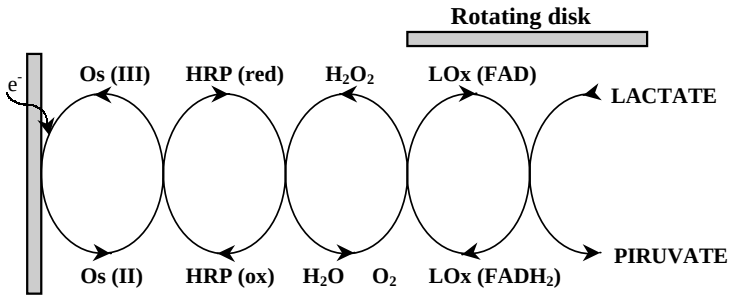
3. Results and discussion

The development of analytical system based on the use of immobilized multienzyme system and mediator represent one of the most rapid advancing areas of biosensors. Biosensors find a widespread application in scientific investigation as well as in medicine, biotechnological processes, food industries and environmental control.

The pioneering work of Kulys et al. [14] demonstrated that the combination of peroxidase and an oxidase makes possible to construct amperometric biosensors that work at more negative potentials avoiding electroactive interferences. This principle has also been used by other groups introducing an electroactive polymer to facilitate the HRP reduction [15,16]. Essentially, the electrodes are formed by an inner layer that contains peroxidase and the electroactive polymer and an outer one containing an oxidase. In our case, we have divided the enzymes in two compartments, peroxidase and the electroactive polymer onto the electrode, covered by a film of PEG and PAA that works as a filter to the diffusion of caseins toward the electroactive polymer. The second compartment in the rotating disk contains the lactate oxidase immobilized. The principle of operation is shown in Scheme 1. Advantages of this configuration are the stability that results from an excess of oxidase in the sensor and forced convection of the produced peroxide to the electrode.

Integrated rotating bioreactor/detector systems with hydrodynamic characteristics have received little attention in the development of biosensors with a mediator in a continuous-flow situation. This paper aims at establishing the characteristics of an assembly in a continuous-flow/stopped-flow programmed operation.

The implementation of continuous-flow/stopped-flow programming and the location of two facing independent reactors (Fig. 1), each containing one of the immobilized enzymes involved in the sequence illustrated earlier, permits: (a) utilization of relatively-low enzyme loading conditions (b) instantaneous operation under high initial rate conditions, (c) easy detection of accumulated products, and (d) reduction of apparent Michaelis–Menten constant, K'_M. A more complete reagent homogenization is achieved, because the cell works as a mixing chamber by facilitating the arrival of substrate at the active sites and the release of products from the same sites. The net result is high values of initial rates (see Table 2). The main advantages of this system are its simplicity, and



Scheme 1. Schematic representation of the biosensor.

the ease with which it can be applied to the determination of lactate at low levels. Moreover, this strategy is easily adapted to continuous-flow processing.

Consider that the following generalized chemical transformation occurs on the surface of the rotating disk:

$$A \xrightarrow{K} P$$

in which A is the analyte (lactate), and P the product H_2O_2. The H_2O_2 is carried from the bulk of the solution to the reactor under convective transport and a certain number of H_2O_2 reach the modified electrode, were the current developed at the detector should be directly proportional to the concentration of analyte in the bulk of solution and should increase with increasing rotation velocity. If the flow is stopped when the sample plug transported by continuous-flow reaches the center of the reactor, detection take place under conditions similar to those of batch detection [17,18].

3.1. Effect of reactor rotation and continuous-flow/stopped-flow operation

If the reactor in the cell is devoid of rotation, there is practically no response. If a rotation of 900 rpm is imposed on the reactor located at the bottom of the cell (with immobilized LO_x), the signal is dramatically amplified. As shown in Fig. 3, if the lower reactor is devoid of rotation, the response is lower because diffusional limitations control the enzyme-catalyzed reaction. The trend indicates that, up to velocities of about 900 rpm, a decrease in the thickness of the stagnant layer improves mass transfer to and from the immobilized enzyme active sites. Beyond 900 rpm, the initial rate is constant, and chemical kinetics controls the overall process. As observed earlier [19], although the mass transfer is being realized under conditions similar to a thin-layer bounded diffusion with imposed turbulence, the dependence seems to agree better with the response at a rotating disk electrode. Fig. 4 shows the effect of rotation under continuous and stopped-flow conditions. Response to 0.1 mM lactate under continuous-flow is relatively small but comparatively larger if the reactor is rotated (compare traces B and C in Fig. 4). A significant signal that increases almost linearly as time develops when the disk is rotated. Under stopped-flow conditions there is a response, but smaller than with rotation. These responses indicate that the utilization of the biocatalytic action of the immobilized enzyme preparations is better under rotation of the reactor at the bottom of the cell.

3.2. Effect of cell volume and sample size

Depending on the volume of the cell in contact with the reactors, the overall process becomes controlled by diffusion (large volumes) or by the chemical kinetics of the enzyme-catalyzed reactions (small volumes). The cell volume was changed from 300 μl to 1 ml by removing the O-rings between the upper and lower half of the cell. The rate of response, as expected, decreased linearly with an increase in cell volume, due to the dilution effect favored by rotation, and the fact that the measured current is directly proportional to bulk concentration. The smallest cell volume of 300 μl was adopted for further studies.

The rate of response increased linearly with sample size up to 200 μl in a cell with a volume of 300 μl. For convenience a sample size of 200 μl was used to

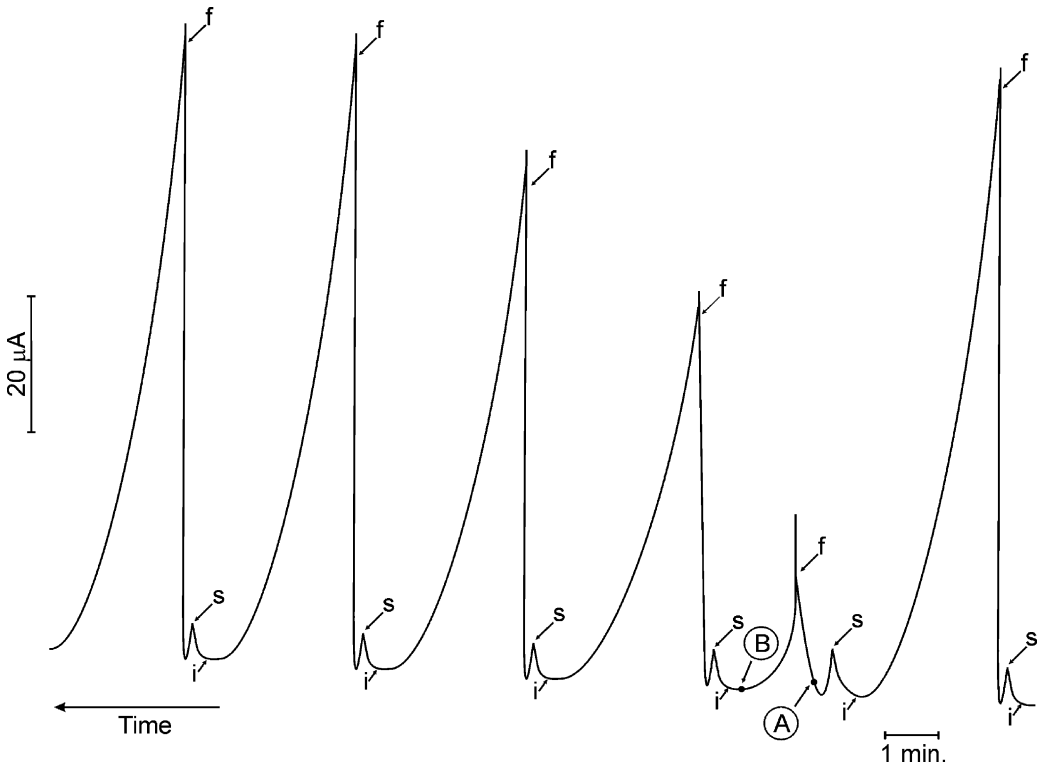


Fig. 3. Effect of reactor rotation under stopped-flow. At A the rotation of the lower reactor was interrupted and B the rotation was re-initiated (900 rpm). i: Injection; s: flow stopped; f: flow continued. Flow rate, 1.00 ml min^{-1}; cell volume, 300 ml; lactate concentration, 0.10 mM; 0.10 M phosphate buffer of pH 7.00.

evaluate other parameters. Sensitivity is almost tripled in the range between 50 and 200 μl (Table 1).

3.3. The apparent Michaelis–Menten constant

As noted at the beginning of this section, rotation is expected to decrease the values of the apparent Michaelis–Menten constant, K'_M, since the catalytic efficiency is increased. Michaelis constant which differ substantially from that measured in homogeneous solution and is not an intrinsic property of the enzyme, but of the system. This constant characterizes the reactor, not the enzyme itself. It is a measure of the substrate concentration range over which the reactor response is linear [20]. Table 2 summarizes the values of K'_M for the system obtained at five different rotation velocities and stopping the flow for 60 s during measurement. The calculation of K'_M was performed under conditions in which [substrate] $\gg K'_M$,

Table 1
Effect of sample size on initial rate measured under stopped-flow conditions

Sample size (μl)	Initial rate (μA s^{-1})	Linear regression standard deviation
50	7.03	±1.50
75	18.33	±1.23
100	39.00	±0.11
125	58.10	±1.30
150	72.20	±0.19
175	78.00	±0.02
200	82.05	±0.03
225	83.14	±0.10
250	82.22	±0.02
275	84.67	±0.12

Each value of initial rate based on triplicate of six determinations. In both cases: flow rate, 1.00 ml min^{-1}; cell volume, 300 μl; lactate concentration, 0.10 mM; 0.10 M phosphate buffer of pH 7.00. The flow was stopped for 60 s during measurement.

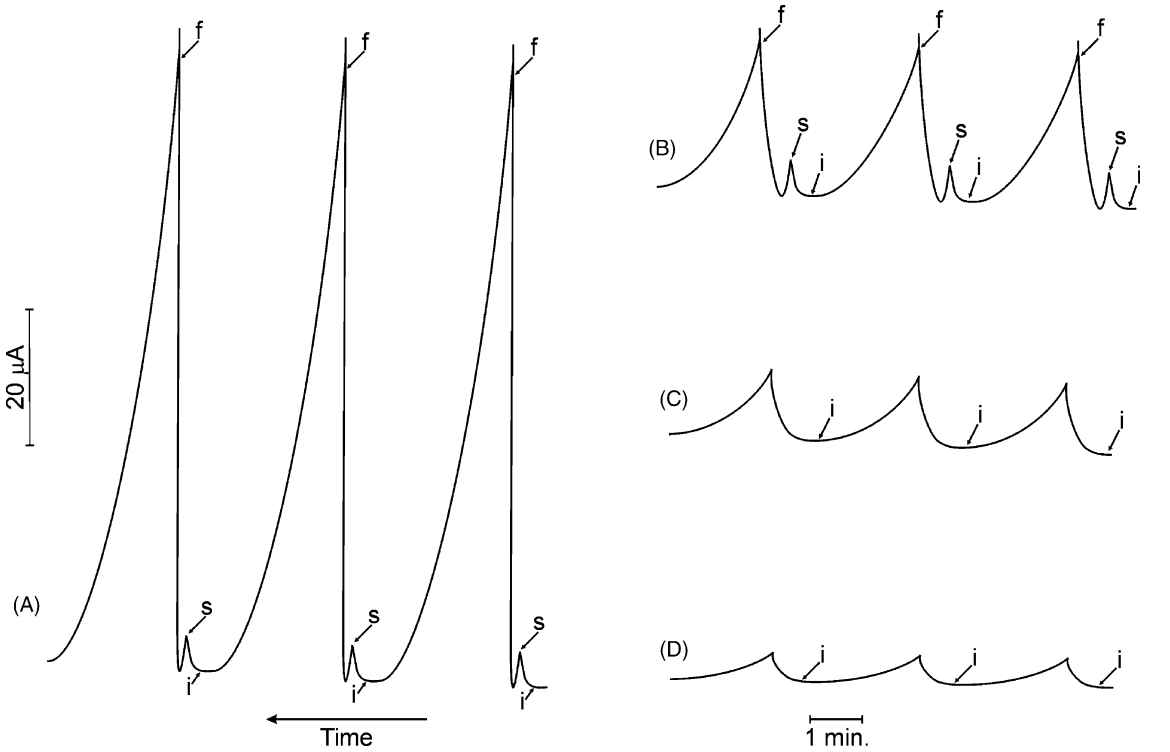


Fig. 4. Effect of reactor rotation under continuous- and stopped-flow conditions. (A) Stopped-flow with rotation. (B) Continuous-flow with rotation. (C) Stopped-flow without rotation. (D) Continuous-flow without rotation. Flow rate, 1.00 ml min^{-1}; cell volume, 300 ml; lactate concentration, 0.10 mM; 0.10 M phosphate buffer of pH 7.00.

as a consequence the following applies (Equation 1), assuming that the Briggs and Haldane scheme [21] is in operation:

$$\frac{1}{S} = \frac{m}{[\text{L-lactate}]} + n \quad (1)$$

where S = rate of response; $K'_M = m/n$. The apparent constant is thus obtained from the slope and intercept of the plot of $1/S$ versus 1/[L-lactate] plot. This is a graphical approach similar to the Lineweaver–Burk plot.

Table 2
Values of K'_M (apparent Michaelis–Menten constant)

Rotation velocity (rpm)	K'_M (mM)[a]	Linear regression standard deviation
170	22.74	±0.72
240	14.33	±2.35
420	10.37	±1.22
840	4.03	±1.30
900	2.80	±0.88

Determinated as discussed in the text (temperature 20 ± 1 °C).
[a] Each value of K'_M based on triplicate of six different substrate concentration.

3.4. Effect of pH, and lactate concentration

The rate of response, under stopped-flow conditions, dramatically increased (almost doubled) from pH 6.5 to 7.00 and continued a moderate increase up to pH 8.00, the highest value tested. The pH used to evaluate the variables was 7.00, provided by a phosphate buffer (0.10 M total phosphate).

A linear relation (Eq. (2)) was observed between the rate of response (μA min^{-1}) and the L-lactate concentration in the range of 0.010 and 2.50 mM (rotation 900 rpm).

$$\text{rate of response} = 0.553 + 440.0 C_{\text{L-lactate}} \quad (2)$$

The correlation coefficient for this type of plot was typically 0.993. Detection limit (DL) is the lowest

Table 3
Results obtained in the measurement of L-lactate in dairy products

Sample	Proposed method		Spectrophotometric method	
	Lactate ($mM\,l^{-1}$)	Linear regression standard deviation	Lactate ($mM\,l^{-1}$)	Linear regression standard deviation
Skim milk	0.75	±0.05	0.66	±0.21
Fresh milk (La Serenisima)	0.16	±0.08	N/D	±0.01
Skim yogurt (Ser)	102.35	±0.12	92.70	±0.03
Acidophilus milk (Sancor)	87.03	±0.01	N/D	–
Fruit yogurt (Ser)	33.72	±0.12	40.20	±0.11
Fresh milk (Milkaut)	0.22	±0.06	N/D	–
Fruit yogurt (Sancor)	35.33	±0.01	42.66	±0.01

concentration that can be distinguished from the noise level [22]. In this study, the concentration of L-lactate giving a signal-to-noise ratio of 3:1 was ca. 5 nM. Reproducibility assays were made using repetitive standards ($n = 6$) of 0.100 mM L-lactate; the percentage standard error was less than 5%.

The stability of the biosensor was tested for nearly 3 h of continuous use in the FIA system, after 30 min the system reached a stable response. The long-term stability of the bienzimatic system to undiluted milk samples was study. In this experiment, after every five milk samples, a standard of 0.5 mM L-lactate is injected to test the electrode response. In the FIA system using a bienzimatic reactor, there is practically no decay in the catalytic current after 20 milk samples.

3.5. Determination of lactate in milk

The matrix effect generated by substances which might interfere with the response of the biosensor was examinated. With this aim, an experiment using the standard addition method was carried out in the same flow system. Different know amounts of L-lactate were added to the milk sample prior to testing and injection; from this calibration curve the same milk sample gives a value of 0.23 mM. Therefore, even through the caseins do not foul the electrode, the matrix has an important effect on the response to lactate.

EDTA can dissolve micellar caseins; adding EDTA to milk to a final concentration of 2%, a new determination was carried out. Using the calibration curve in buffer, a value of 0.25 mM is obtained. The same value is obtained if the sample is centrifuged at 10,000 g for 15 min to eliminate micellar casein. This shows that caseins still produced an important effect in the signal that can be eliminated by using EDTA or making standard additions. When milk samples are used without dilution, even though there is an important effect in the signal that could be eliminated by using EDTA as part of the carrier.

We used this bienzimatic reactor to follow the first steps of a lactic fermentation. When we compared the change of pH during the lactic fermentation, there was a correlation between the two parameters, but the change in pH in the first stage of the fermentation was very low, as we expected considering the presence of buffer systems in the milk, mainly phosphate and carbonate.

The agreement with the standard spectrophotometric method was good (Table 3), mainly at L-lactate concentration higher than 1–2 mM. At low lactate concentration (milk samples), the reproducibility in the spectrophometric method was poor, and the values were not useful to test our method. The detection limit for the spectrophotometric method [6] is ca. 0.17 mM under the assay conditions used (100 μl sample in a final volume of 1 ml, and taking into account a 0.05 absorbance unit difference with respect to the blank).

4. Conclusions

The usefulness of multiple enzyme arrangements used for the determination of very low concentrations of lactate is demonstrated. In practice, the method is very simple and straightforward, it is the good applicability in food industry. The sensor developed in this work is able to operate as a fast, selective and sensitive

detection unit when is incorporated into a FIA system, and provides a fast and cost effective solution to the realization of quantitative information at extremely low levels of concentrations. The agreement with the standard spectrophotometric method was good, mainly at L-lactate concentrations higher than 1–2 mM. At the potential applied +0.00 V versus Ag/AgCl the electroactive species present in the milk did not cause any observable interference.

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