Talanta 152 (2016) 147–154

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Implementation of a new integrated D-lactic acid biosensor in a semiautomatic FIA system for the simultaneous determination of lactic acid enantiomers. Application to the analysis of beer samples

E. Vargas [a,b], M.A. Ruiz [b], S. Campuzano [a], G. González de Rivera [c], F. López-Colino [c], A.J. Reviejo [a], J.M. Pingarrón [a,*]

[a] Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040 Madrid, Spain
[b] InBea Biosensores S.L. Parque Científico de Madrid, C/ Santiago Grisolía 2, 28760 Tres Cantos, Madrid
[c] Escuela Politécnica Superior, Universidad Autónoma de Madrid, E-28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 18 December 2015
Received in revised form
22 January 2016
Accepted 30 January 2016
Available online 1 February 2016

Keywords:
Semiautomatic FIA system
Amperometric detection
Enzymatic biosensors
Simultaneous detection
Lactic acid enantiomers

ABSTRACT

An integrated amperometric D-lactic acid biosensor involving a gold film deposited by sputtering on a stainless steel disk electrode where the enzymes D-lactic acid dehydrogenase (DLDH) and diaphorase (DP) as well as the redox mediator tetrathiafulvalene (TTF) are coimmobilized by using a dialysis membrane, is reported in this work. Amperometry in stirred solutions at a detection potential of +0.15 V (vs Ag/AgCl reference electrode) provided a linear calibration plot for D-lactic acid over the 1.0×10^{-4} to 3.8×10^{-3} g L^{-1} concentration range, with a limit of detection of 3.1×10^{-5} g L^{-1}. The usefulness of the biosensor was demonstrated by determining D-lactic acid in beer samples with good results. Additionally, the biosensor was implemented together with a commercial L−lactic amperometric biosensor in a semiautomatic flow-injection analysis (FIA) system able to perform a rapid and simple stereo-specific determination of D- and D-lactic without a previous separation step. The operational characteristics of the biosensors under flow conditions were evaluated and its applicability was demonstrated through the simultaneous determination of both enantiomers in beer samples.

1. Introduction

Lactic acid is the end product of glycolysis in an anaerobic environment and exists as L-(+)- or D-(−)-isomers. The L-(+)-isomer is the normal intermediate in mammalian metabolism whereas the D-(−)-isomer is principally produced by micro-organisms and by some algae and plants. D-(−)- lactate consumption in humans must be controlled and the WHO recommends a daily intake of this isomer of ~100 mg kg^{-1} body weight [1]. Moreover, L-lactic acid is supplemented into foods and beverages (E270) where a tart flavor is desired, and is widely used as a non-volatile acidulant. The determination of content of the lactate isomers is of great interest in both food quality control and clinical diagnosis. In particular, it is important in fermentation processes, e.g. in beer technology, in yoghurt and cheese production and in milk, for the control of bacterial contamination, to ensure good storage of the beverage and to avoid an excessive acidification of the product which leads to unpleasant tastes with the consequent economic losses [1–3].

In view of this, and besides methodologies able to perform the determination of total lactic acid content, the development of novel analytical methods able to perform the stereo-specific quantification of the lactate isomers is of great importance to assure the quality of beverages like beer.

Apart from the commercially available spectrophotometric enzymatic assay kits, based on the specific reactions of enantiomers selective dehydrogenases and the co-factor NAD^+, a variety of analytical methods for the enantiomeric separation and determination of lactic acid isomers have been reported. These include gas chromatography (GC) [4–6], capillary electrophoresis (CE) [7–9], liquid chromatography with mass spectrometric detection (LC–MS) [10], high-performance liquid chromatography (HPLC) with chiral stationary phases [11–13] or chiral mobile phase [14] and HPLC with precolumn fluorescence derivatization and chiral column [15–17]. However, these methods often suffer from basic limitations in terms of performance, equipment cost, complexity, sample processing and run times, which create challenges or render them impractical for high-throughput routine

* Corresponding author.
E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).

http://dx.doi.org/10.1016/j.talanta.2016.01.063
0039-9140/

laboratory. Therefore, it is of interest the development of alternative, simple, and low-cost methods for the simultaneous determination of D- and L-lactic acids [18].

Regarding the analysis of beer samples, although several chromatographic and electrophoretic methods have been used to determine lactic acid [19–21], they need sample pretreatment and cannot distinguish between lactate isomer forms. To the best of our knowledge, there are only two reports to determine the D- and L-lactate content in beer samples. Girotti et al. [1] developed a bioluminescent flow sensor based on the monitoring of the reduced form of nicotinamide adenine dinucleotide, produced by bioluminescent bacterial D- and L-lactate dehydrogenases immobilized on separated nylon coil reactors. This approach allows the determination of both isomers in the 0.1–10 mM concentration range. On the other hand, a flow-injection dual biosensor involving microdialysis sampling was proposed by Nanjo et al. [22]. The dialysate is automatically injected into the flow-injection line and a dual enzyme amperometric electrode, consisting of two platinum disks modified with L–LDH/DP or DLDH/DP, was arranged perpendicularly to the flow direction. The proposed system allowed the simultaneous determination of D- and L-lactic acids in the 0.02–1.0 mM range.

In this work, we report for the first time an integrated amperometric biosensor for D-lactic acid determination based on the DLDH/DP/TTF system. The analytical performance of this biosensor was evaluated under batch conditions and its applicability for the analysis of real samples demonstrated by analyzing beer samples. Additionally, a simple, fast and sensitive electrochemical approach for the determination of the enantiomeric composition of lactic acid in beer samples, without a previous separation step, was successfully developed. This approach involved the appropriate integration of the above mentioned D-lactic acid biosensor with a commercial L-lactic acid biosensor in a semiautomatic FIA system. The proposed method showed satisfactory linearity and precision and successful applicability for the quantification of D- and L-lactic acid enantiomers in beer samples, thus circumventing the need for expensive and complex instrumentation to perform a previous separation step and therefore useful for routine analyses.

2. Materials and methods

2.1. Apparatus and electrodes

Amperometric measurements were carried with single and bichannel amperometric detectors purchased from InBea Biosensores S.L. (Madrid, Spain). A P-Selecta ultrasonic bath and a P-Selecta Agimatic magnetic stirrer were also used. Flow experiments were carried out using a Spetec Perimax-12 peristaltic pump.

D-lactic acid biosensor (fabricated as described in Section 2.3) and L-lactic acid commercial biosensors developed previously in our research group [23], involving the use of a lactate oxidase (LOD)/peroxidase (HRP)/ferrocene (Fc) system, and commercialized by InBea Biosensores S.L., were employed as working electrodes to carry out the amperometric detection. A BAS MF-2052 Ag/AgCl/KCl (3 M) reference electrode and a Pt wire counter electrode were also employed. A 20-mL electrochemical cell and a homemade methacrylate wall-jet cell (10 mL) were employed for amperometric measurements in stirred solutions and in the semiautomatic mode, respectively.

2.2. Reagents and solutions

Stock 3.0 g L^{-1} D- (Aldrich) and L- (Sigma) lactic acid solutions were prepared in 0.05 mol L^{-1} phosphate buffer of pH 7.4. More dilute standards were prepared by suitable dilution with the same phosphate buffer solution, which was also used as supporting electrolyte. A 0.5 U μL^{-1} DP solution (Sigma, EC 1.8.1.4 from *Clostridium kluyveri*, 8.0 U mg^{-1} solid) and a 5.0 U μL^{-1} DLDH solution (Sigma, EC 1.1.1.28 from *Lactobacillus leichmanii*, 148,92 U mg^{-1} solid) both prepared in 0.05 M phosphate buffer solution of pH 7.4, were used for the preparation of the D-lactic acid biosensor. Moreover, a 0.5 mol L^{-1} TTF (Aldrich) solution in acetone was prepared. Dialysis membranes (Snake Skin® Pleated Dialysis Tubing, 10K MWCO) were purchased from Thermo Scientific.

Other solutions employed were: 40 g L^{-1} stock solutions of D-glucose (Panreac), D-fructose (Sigma), L-arabinose (Sigma), D-galactose (Fluka) and D(+)maltose (Sigma–Aldrich); 3 g L^{-1} stock solutions of citric acid (Merck), acetic acid (Scharlab), L-malic acid (Merck), succinic acid (Sigma–Aldrich), ascorbic acid (Fluka) and glycerol (Panreac); 5 g L^{-1} stock solutions of glutamine (Sigma–Aldrich), 5% (v/v) stock solutions of ethanol (Sharlab), all prepared in 0.05 mol L^{-1} phosphate buffer of pH 7.4.

All chemicals used were of analytical-reagent grade and water was obtained from a Millipore Milli-Q purification system.

2.3. Procedures

2.3.1. D-lactic acid biosensor construction

Coimmobilization of the enzymes and the mediator onto gold sputtered-modified stainless steel disk electrodes (ϕ=6.0 mm) was carried out as follows: a 3-μL aliquot of the 0.5 U μL^{-1} DP solution and a 2-μL aliquot of the 5.0 U μL^{-1} DLDH solution were sequentially deposited onto the electrode surface and let dry at room temperature. Then, a 3-μL aliquot of the 0.5 mol L^{-1} TTF solution was dropped on the modified electrode waiting again for drying at room temperature. Finally, a 1.5 cm^2 piece of the dialysis membrane was fixed on top of the electrode surface and secured with an appropriate O-ring.

2.3.2. Amperometric detection

Amperometry in stirred solutions with the D-lactic acid biosensor was performed by applying a detection potential of +0.15 V (vs Ag/AgCl).

The semiautomatic FI system used for the simultaneous determination of lactic acid isomers is described in detail in Section 3.2. The carrier was a 0.05 mol L^{-1} phosphate buffer solution of pH 7.4 (containing NAD^+ 1.0 mmol L^{-1}). L-lactic and D-lactic acid biosensors were polarized at 0.00 and +0.15 V vs Ag/AgCl, respectively.

2.3.3. Analysis of beer samples

Commercial beers with, without and “0.0” alcohol content were de-gassed using an ultrasonic bath before sample uptake. The determinations in stirred solutions did not imply any other sample treatment and an aliquot of the sample was added to the electrochemical cell containing 20 mL of the supporting electrolyte required by each biosensor (0.05 mol L^{-1} phosphate buffer solution of pH 7.4 supplemented or not with NAD^+ 1.0 mmol L^{-1} for L- and D-lactic acid, respectively).

Regarding the determination of both isomers in beer samples, the developed D-lactic acid biosensor together with the commercial L-lactic biosensor purchased from InBea Biosensores S.L. were employed as amperometric detectors in the semiautomatic FIA system described in Section 3.2. The determination of each isomer was carried out by injecting into the semiautomatic FIA system the sample solution (prepared by diluting 10 times the degassed beer sample in the carrier solution), and interpolating the amperometric signal obtained into the calibrations plots constructed previously for each isomer under flow conditions.

3. Results and discussion

3.1. Bienzyme biosensor for D-lactic acid determination

The construction of a bienzyme biosensor for D-lactic acid determination based on the DLDH/DP system onto gold sputtered-modified stainless steel disk electrodes was addressed for the first time in this work. The involved enzyme reactions are depicted in Fig. 1b and imply oxidation of D-lactic acid to piruvate catalyzed by DLDH and simultaneous reduction of cofactor NAD^+ to NADH. The NADH is then re-oxidized by TTF^+, this reaction being catalyzed by the enzyme diaphorase [24]. The generated TTF is amperometrically monitored at the modified electrode with the resulting current being dependent on the D-lactic acid concentration. In this context, TTF is well known as an appropriate mediator for the NAD^+/NADH system [25,26] exhibiting suitable electrochemical properties.

3.1.1. Optimization of working variables

The optimization of all the experimental variables implied in the D-lactic acid biosensor performance was accomplished by comparison of the amperometric signals obtained at +0.10 V for 1.8×10^{-3} g L^{-1} D-lactic acid in stirred solutions. Regarding biosensor preparation, only the influence of the DLDH loading was checked (between 0.0 and 20.0 U), whereas both DP and TTF loadings were the same than those optimized previously for a glycerol biosensor [26]. The measured current increased with DLDH loading up to 10 U and showed a dramatic decrease for larger loadings, which is likely due to the blocking of the electrode surface and/or the active centers of the DP (previously immobilized) by the large amount of immobilized DLDH. Consequently, the selected composition of the bienzyme electrode for further work was: 10.0 U DLDH/ 1.5 U DP/1.5 μmol TTF.

Once the composition of the biosensor was established, the effect of pH and the applied potential on the D-lactic acid amperometric response was evaluated. The influence of pH was tested over the 5.5–8.5 pH range (data not shown). The integrated biosensor exhibited a maximal activity towards D-lactic acid at pH 7.4, in good agreement with optimal pH values of 8.0 and 7.0 reported for free DLDH [27] and DP [28]. Accordingly, a 0.05 mol L^{-1} phosphate buffer solution of pH 7.4 (containing 1.0 mmol L^{-1} NAD^+) was chosen as working medium.

The effect of the applied detection potential on the biosensor response was examined in the 0.05 to +0.25 V range (vs Ag/AgCl reference electrode). A catalytic effect was observed between 0.05 and +0.25 V, with the largest steady-state current measured at +0.15 V. The decrease of the amperometric response above this potential value was reported previously [26,29] and attributed to the non-reversible oxidation of TTF^+ to TTF^{2+} that decomposes and leaks from the electrode surface. Accordingly, a working potential of +0.15 V was chosen for further work to accomplish a sensitive detection, to ensure the stability of the integrated biosensor and also to minimize the effect of potential interfering substances able to be oxidized at the electrode surface.

Furthermore, the concentration of the cofactor was also optimized by performing experiments with various NAD^+ concentrations in the 0.05–5.0 mmol L^{-1} range. The measured current increased with the cofactor concentration up to 1.0 mM and decreased for concentrations higher than 2.5 mmol L^{-1}. In view of these results and in order to avoid a high consumption of the reagent a final concentration of 1.0 mmol L^{-1} was chosen for further experiments.

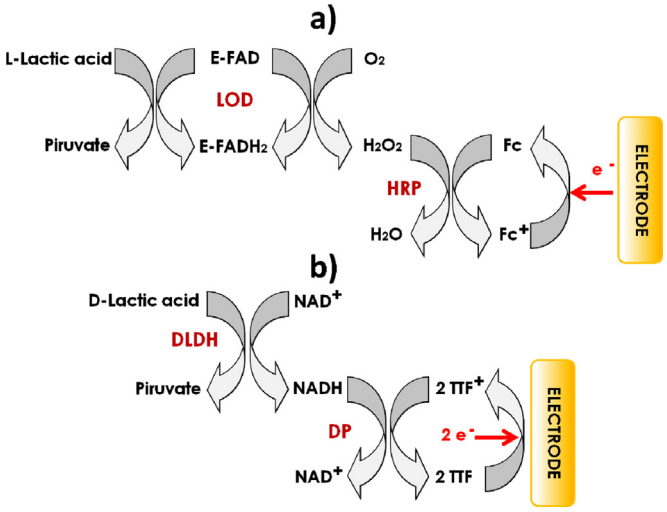

Fig. 1. Schematic diagram displaying the enzyme and electrode reactions involved in the L-lactic acid determination at the commercial LOD/HRP biosensor (a) and in the D-lactic acid determination at a DLDH/DP biosensor developed in this work (b).

3.1.2. Stability of the D-lactic acid biosensor

Taking into account that the stability of the biosensor response is one of the most critical factors for assessing the possibilities of a biosensor to be applied in control processes and routine monitoring, different aspects concerning this aspect were considered.

The repeatability of the measurements was evaluated from the steady-state current values corresponding to repetitive measurements ($n=10$) of 4.5×10^{-4} g L^{-1} D-lactic acid. A RSD value of 4.4% was obtained, indicating a good repeatability of the measurements with no need to apply a cleaning or pretreatment procedure to the biosensor.

Moreover, the reproducibility of the responses obtained with five different biosensors prepared in the same manner was also checked. A RSD value of 4.7% was calculated from the slope values corresponding to the different calibration plots constructed for D-lactic acid over the 4.5×10^{-4}–9.0×10^{-4} g L^{-1} concentration range. This RSD value suggested that the biosensor fabrication procedure was reliable and allowed reproducible amperometric responses to be obtained with different biosensors constructed following the described methodology.

In addition, the lifetime of one single biosensor was also evaluated by performing daily amperometric measurements for 4.5×10^{-4} g L^{-1} D-lactic acid standards solutions. After use, the biosensor was stored at 4 °C in phosphate buffer of pH 7.4. A control chart was constructed by setting the average current value calculated from 4 measurements made the first day of the study as the central value, while the upper and lower limits of control were set at $\pm 3 \times$ SD of this central value. The obtained results (Fig. S1 in the Supporting Information) demonstrated that the biosensor response remained within the control limits for 9 days indicating good storage stability. It is worth to mention also that, although with lower sensitivity (52% of the initial sensitivity), the biosensor was useful during about 22 days, providing adequate calibration plots (re-calibration is then needed) suitable to perform reliable determinations in commercial beers. It is interesting to note that other enzyme biosensors based on similar electrode configurations and immobilization strategies exhibited different storage stabilities (from 25 days for a malate dehydrogenase/diaphorase (MDH/DP) L-malic acid biosensor (unpublished results)) to 60 days for a fructose biosensor using fructose dehydrogenase (FDH) [30]). Therefore, it can be concluded that the stability of the enzymatic system seems to play a determinant role in the storage stability of the developed biosensor in comparison with the small TTF leakage (although the mediator was immobilized in excess and exhibits low solubility in water [31,32]) occurring from the electrode surface.

Table 1
Comparison of the analytical characteristics for different electrochemical enzymatic biosensors reported for D-lactic acid determination.

Electrode	Enzymes	Redox mediator	Immobilization	*E*, V	Sample	LR, g L^{-1}	LOD, g L^{-1}	Sensitivity, nA g^{-1} L	Reference
Gold microelectrode	DLDH/DP	$K_4Fe(CN)_6$	Entrapment by a dialysis membrane	+0.20 (vs Au)	–	$(4.504–1351)\times10^{-4}$	9.0×10^{-4}	–	[33]
SPCE modified with MB	DLDH	MB	Immobilization with NAD+ using polyethileneimine and nafion	−0.05 (vs Ag/AgCl)	Wine	$(9.0–90)\times10^{-3}$	4.5×10^{-3}	2.30×10^{3}	[34]
Gold microelectrode	DLDH/DP	$K_4Fe(CN)_6$	Entrapment by a semipermeable membrane	+0.20 (vs Au)	–	$(4.504–1351)\times10^{-4}$	4.5×10^{-4}	–	[35]
SPCE modified with MB–RS	DLDH	MB–RS	Entrapment in PVA-SbQ matrix, polyethileneimine and nafion	−0.15 (vs Ag/AgCl)	Wine	$(4.5–90)\times10^{-3}$	2.7×10^{-3}	2.30×10^{3}	[36]
SPCE modified with MB	DLDH	MB	Entrapment in PVA-SbQ matrix	0.00 (vs Ag/AgCl)	Wine	$(4.5–90)\times10^{-3}$	2.7×10^{-3}	–	[37]
Pt disk	DLDH/DP	$K_4Fe(CN)_6$	Polypyrrole and mediator film obtained by electropolymerization, immobilization of enzymes and NAD-dextrane by means of a semipermeable membrane	+0.10 (vs Pt)	–	$(9.0–45)\times10^{-3}$	2.7×10^{-3}	–	[38]
Clark electrode	LOx/HRP/DLDH	–	Nylon membrane functionalized with carboxylic groups	−0.65 (vs Ag/AgCl)	Wine	$(5.0–300)\times10^{-3}$	2.5×10^{-3}	1.20×10^{2}	[39]
–	DLDH	$K_4Fe(CN)_6$	–	–	Wine	$(1.80–495)\times10^{-3}$	9.0×10^{-5}	7.23×10^{3}	[40]
CPE	DLDR	PMS–RS	Entrapment with GA	0.00 (vs Ag/AgCl)	Beer	$(1.351–9008)\times10^{-3}$	–	2.12×10^{1}	[41]
SPE modified with SWCNTs and MB	DLDH	MB	Entrapment in sol–gel	−0.05 (vs Ag/AgCl)	–	$(9.008–2340)\times10^{-3}$	1.4×10^{-3}	2.04×10^{3}	[42]
Graphite electrode	DLDR (from yeast *Hansenula polymorpha*)	Cytochrome c	Entrapment of the DLDH enriched cells' debris within an Os-containing cathodic electrodeposition polymer	+0.25 (vs Ag/AgCl)	–	–	2.8×10^{-4}	–	[43]
Au-sputtered steel disk electrode	DLDH/DP	TTF	Entrapment in a dialysis membrane	+0.15 V (vs Ag/AgCl)	Beer	$(1.00–38.0)\times10^{-3}$	3.1×10^{-5}	4.55×10^{4}	This work

Abbreviations: CPE: carbon paste electrode; DLCR: D-lactate cytochrome C oxiderreductase; DLDH: D-lactate dehydrogenase; DP: diaphorase; GA: glutaraldehyde; HRP: horseradish peroxidase; LOD: limit of detection; LOx: lactate oxidase; LR: linear range; MB: Meldola Blue; NAD+/NADH: oxidized and reduced form of the dinucleotide of adenine and nicotinamide cofactor; PMS: phenazine metasulphate; PVA-SbQ: photocrosslinkable polyvinyl alcohol that contains stilbazolium groups; RS: Reinecke salt; SCE: saturated calomel electrode; SPCE: screen printed carbon electrode; SWNTs: single-wall carbon nanotubes; TTF: tetrathiafulvalene.

Table 2
Determination of the D-lactic acid content (in mg L^{-1}) in different beer samples using the developed amperometric biosensor and comparison with the results provided by a commercial enzymatic spectrophotometric kit.

Sample	Alcohol content	Biosensor	Enzymatic kit	t_{exp}
1	< 1%	24 ± 3 $RSD_{n=5}=9.7\%$	22 ± 2 $RSD_{n=3}=5.0\%$	2.462
2	~5%	71 ± 2 $RSD_{n=5}=2.3$	70 ± 6 $RSD_{n=3}=4.7\%$	1.668
3	< 1%	29.7 ± 0.8 $RSD_{n=5}=2.3$	29 ± 3 $RSD_{n=3}=6.0\%$	2.556

3.1.3. Analytical characteristics of the D-lactic acid biosensor

A linear calibration plot for D-lactic acid was constructed over the $(1.00–38.0) \times 10^{-4}$ g L^{-1} concentration range ($r=0.9994$) with a slope value of $(1.45 \pm 0.02) \times 10^{-2}$ A mol^{-1} L cm^{-2}, and an intercept of (2 ± 3) nA. The limits of detection (LOD) and quantification (LQ) were estimated according the $10 \times s_b/m$ and $3 \times s_b/m$ criteria, respectively, where m is the slope of the calibration graph and s_b is the standard deviation of the blank current, which was measured each second for a 100 s period of time before recording the analytical signal. The values obtained were 3.1×10^{-5} and 1.0×10^{-4} g L^{-1}, respectively. Table 1 summarizes a comparison of the analytical characteristics for different electrochemical enzyme biosensors reported in the literature for D-lactic acid. It is worth to mention that, to the best of our knowledge, the sensitivity and LOD achieved with the developed biosensor were the best reported to date using electrochemical enzyme biosensors.

3.1.4. Selectivity

The effect of potentially interfering compounds on the biosensor response was investigated under the experimental conditions specified above. The influence of substances normally present in beer samples was evaluated by adding to the electrochemical cell (containing 20 mL of the supporting electrolyte) 200 µL of standards solutions prepared at the maximum concentration level at which they are found in these samples: D-glucose, D-fructose, L-arabinose, D-galactose and maltose at 40 g L^{-1}; glycerol and citric, acetic, L-malic, L-lactic and succinic acids at 3.0 g L^{-1}; ethanol at 5% (v/v); glutamine at 5.0 g $L^{-1}$and ascorbic acid at 40 mg L^{-1}.

Among all of these substances, only ascorbic acid yielded an amperometric response under the working conditions which was due to the electrochemical oxidation of this compound at the applied potential and to the reported catalytic oxidation of ascorbic acid by TTF [44]. Additional experiments were performed to evaluate more exhaustively the potential effect of ascorbic acid. The comparison of the amperometric signals provided by the developed biosensor for a D-lactic acid concentration of 1.0×10^{-4} g L^{-1} and that measured for a mixture solution containing the same D-lactic acid concentration plus a 4-times higher concentration of ascorbic acid (1.0×10^{-4} g L^{-1} D-lactic acid + 4.0×10^{-4} g L^{-1} ascorbic acid) demonstrated that this latter was only 5% larger than the amperometric measurement obtained with no ascorbic acid. These results ensure the absence of any significant interference from the presence of this common naturally occurring acid and clearly demonstrate the appropriate selectivity of the developed biosensor for the determination of D-lactic acid in beer or in dairy products containing other saccharides or organic acids.

3.1.5. Determination of D-lactic acid in beer

D-lactic acid was determined in commercial beer samples with, without and "0.0" alcohol content. No matrix effect was found for any of these types of beer. Indeed, the slope values of the calibration graphs constructed for D-lactic acid by applying the standard additions method for the samples were not statistically different (by using the Student's test) to that of the calibration graph prepared with D-lactic acid standards (see Table S1 in the Supporting Information). Therefore, all samples could be analyzed simply by interpolation of the corresponding amperometric signals measured after adding the appropriate volume of the raw sample to the electrochemical cell (containing 20.0 mL of 0.05 M phosphate buffer pH 7.4, supplemented with 1.0 mM NAD^+) into the calibration plot constructed with standards solutions.

The results obtained from five replicates of three different beer samples were compared with those provided by a commercial enzymatic kit (according to the protocol described in the specifications) (Table 2). The confidence intervals were calculated for $\alpha=0.05$. A hypothesis test demonstrated the absence of statistically significant differences between the mean values found for all the analyzed samples at the significance level of 95% (t_{exp} values shown in Table 2 were in all cases lower than the t_{tab} value of 2.776). Taking into account the straightforwardness of the developed working protocol, this can be considered as a very good result, demonstrating the usefulness of the developed biosensor to perform the analysis just upon simple addition of the sample to the electrochemical cell.

3.2. Design and optimization of a semiautomatic FIA system for the simultaneous determination of lactic acid isomers

The simultaneous determination both lactic acid isomers was accomplished by implementing a semiautomatic flow system using the novel developed D-lactic acid biosensor and a commercially available L-lactic acid biosensor as amperometric transducers. The flow system used is schematically depicted in Fig. 2 and is composed of a two-channel peristaltic pump placed after a 3-way flow splitter, a manual injection valve and a double wall-jet cell with the electrodes connected to a bi-channel amperometric detector.

The double "wall-jet" large volume cell used was designed by the research group and keeps the two biosensors as well as the auxiliary and reference electrodes immersed in the same solution than the carrier (~10 mL). As it is shown in Fig. 2a, the entries of carrier or sample solutions incident perpendicularly to the surface of both bioelectrodes.

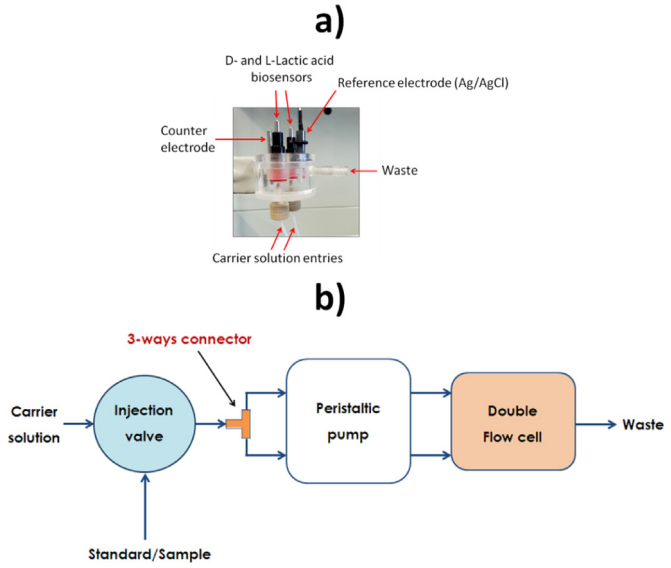

Fig. 2. Picture of the designed double "wall-jet" large volume cell (a) and scheme of the semiautomatic FIA system (b) for the simultaneous determination of lactic acid isomers.

The experimental variables affecting the responses of the biosensors under the flow conditions were optimized by measuring the amperometric responses for D- and L-lactic acids using 0.05 mol L^{-1} phosphate buffer of pH 7.4 supplemented with NAD^+ 1 mM as the carrier solution. Considering the results described in Section 3.1.1 as well as the recommendation of the L-lactic acid biosensor supplier, the detection potential values were +0.15 V and 0.00 V (vs. Ag/AgCl) for D- and L-lactic acids respectively.

The effect of the flow rate was checked by injecting 500 µL of standards solutions of both isomers with concentrations ranging from 3.0×10^{-3} to 9.0×10^{-3} g L^{-1} at different flow rates (0.5, 1.0, 2.0, 3.0 and 3.8 mL min^{-1}). The obtained results (see Fig. S2 in the Supporting Information) led us to select a flow rate of 3.0 mL min^{-1} as a compromise between sensitivity and sampling frequency. Considering this high flow rate, a sample volume of 500 µL was selected for further work in order to achieve sufficient sensitivity.

3.3. Stability studies in the semiautomatic FI system

The repeatability of the amperometric responses recorded with the semiautomatic FI system was evaluated from measurements of series of 20 repetitive injections for 8.0×10^{-3} g L^{-1} standard solutions of both isomers (Fig. 3). The RSD values from i_p measurements were 2.3 and 1.6% for D- and L-lactic acids, respectively, thus confirming an excellent repeatability of the flow amperometric responses in spite of the hydrodynamic conditions.

The stability of the biosensors upon storage and working under flow conditions was also evaluated. Storage conditions were 0.05 mol L^{-1} phosphate buffer solution, pH 7.4, at 4 °C for the D-lactic acid biosensor and −20 °C for the L-lactic acid biosensor. Each working day, the amperometric responses provided by the biosensors for 4.0×10^{-3} g L^{-1} D-lactic and 9.0×10^{-3} g L^{-1} L-lactic acid solutions were recorded. Although these responses showed a gradual decrease with time, both biosensors demonstrated to remain operational and suitable to perform the analytes quantification for about 12 days, period of time during which approximately 100 samples could be measured with each biosensor. In addition, a similar behavior was observed with 5 different biosensors for each target analyte.

3.4. Analytical characteristics for the simultaneous determination of lactic acid isomers with the semiautomatic FI system

Under the optimized flow conditions, linear calibration curves for D- and L-lactate acids could be obtained with the analytical characteristics summarized in Table 3. The limits of detection and quantification were calculated using the same criteria mentioned in Section 3.1.3. These characteristics demonstrated the usefulness of the biosensors for the simultaneous determination of the isomers with the flow system, exhibiting good analytical performance and sampling frequencies of around 40 samples per hour, which constitute relevant characteristics for applications in the food industry. It is worth to mention also that the developed methodology allows quantification of lower enantiomers concentrations than other approaches previously reported (0.009 [1] and 0.0018 g L^{-1} [22]). Moreover, in comparison with the approach developed by Nanjo et al. [22], it is worth to mention that the methodology developed in this work is much simpler and, although the sensitivity for D-lactic acid is similar (89.2 vs 91.5 nA mM^{-1}), it provides four-times higher sensitivity for L-lactic acid determination (454 vs 101 nA mM^{-1}).

3.5. Simultaneous determination of lactic acid isomers in beer samples

The usefulness of the semiautomatic FI system for the simultaneous determination of D- and L-lactic acids in beer samples was evaluated and the results compared with those provided using a commercial enzymatic spectrophotometric kit and also using the biosensors under batch conditions in stirred solutions. While the protocol described in Section 3.1.5 was used for D-acid lactic determination, L-lactic acid was quantified by applying the method "L-lactic acid bionalyzer in beer samples" validated by the Company InBea Biosensores S.L.

Similarly to that commented for the D-lactic acid biosensor, no matrix effects were observed in the analysis of these samples (as an example results obtained in the analysis of beer samples with ~5% alcohol are summarized in Table S2). Therefore, quantification of both enantiomers could be accomplished by simple interpolation of the measured current from the 10-times diluted samples into the calibration plot constructed over the 3.0×10^{-3}–1.2×10^{-2} g L^{-1} concentration range with D- and L-lactic standard solutions. The results achieved using the three different methodologies are summarized in Table 4. As an example, Fig. 4 shows the amperograms obtained in the simultaneous analysis of both isomers in a beer sample. As it is shown in Table 3, the developed approach provided levels of total lactate that varied from 92 to 190 mg L^{-1}, with D-lactate values between 58 and 98 mg L^{-1} and L-lactate values between 25 and 92 mg L^{-1}. These results are in agreement with the lactate contents found previously [45–48].

The results provided by the three methodologies were statistically compared by constructing the corresponding regression plot (Table 5).

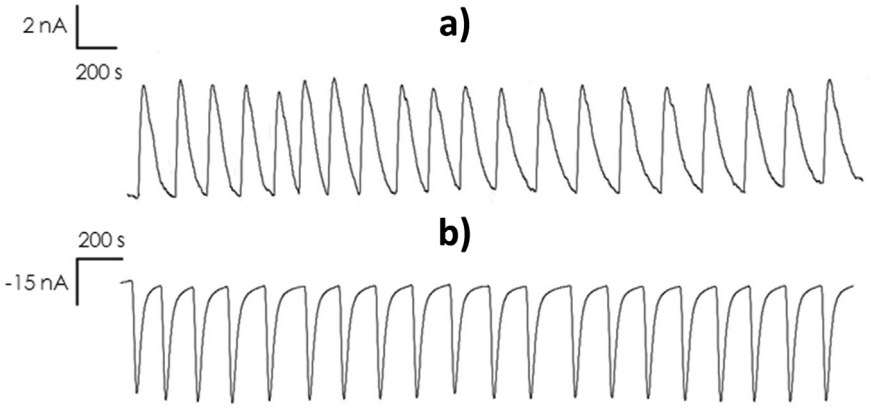

Fig. 3. Successive amperometric responses obtained for 8.0×10^{-3} g L^{-1} D- (up) and L- (down) lactic acid standard solutions with the semiautomatic FI system. Carrier solution: phosphate buffer 0.05 mol L^{-1} pH 7.4 containing 1 mmol L^{-1} NAD^+; flow rate: 3.0 mL min^{-1}; sample volume: 500 µL; E_{ap}=0.15 (D-lactic acid) and 0.00 (L-lactic acid) V vs. Ag/AgCl. The direction of recorded peaks reflects the oxidation or reduction currents measured.

Table 3
Analytical characteristic for the simultaneous determination of both acid lactic isomers with the semiautomatic FI system.

Isomer	L.R., g L^{-1}	Slope, A mol^{-1} L cm^{-2}	o.o, nA	r	LOD, g L^{-1}	LQ, g L^{-1}
D-	$(5.00–170)\times10^{-4}$	$(3.15\pm0.06)\times10^{-4}$	0.2 ± 0.2	0.9991	1.5×10^{-4}	5.0×10^{-4}
L-	$(3.900–1300)\times10^{-5}$	$(1.61\pm0.02)\times10^{-3}$	0.4 ± 0.3	0.9997	1.2×10^{-5}	3.9×10^{-5}

Table 4
D- and L-lactic concentration in beer samples with ~5% alcohol (with alcohol content) (in mg L^{-1}) obtained using the semiautomatic FI system, the batch methodologies under stirred conditions and the enzymatic kit.

	Batch		FIA system		Enzymatic kit	
Sample	D-	L-	D-	L-	D-	L-
1	65 ± 10 ($RSD_{n=3}$ =6.4%)	26 ± 4 ($RSD_{n=3}$ =6.7%)	67 ± 6 ($RSD_{n=5}$ =6.9%)	25 ± 2 ($RSD_{n=5}$ =7.0%)	68 ± 7 ($RSD_{n=3}$ =4.0%)	26 ± 5 ($RSD_{n=3}$ =8.0%)
2	96 ± 11 ($RSD_{n=3}$ =4.8%)	94 ± 6 ($RSD_{n=3}$ =2.8%)	98 ± 5 ($RSD_{n=10}$ =6.8%)	92 ± 3 ($RSD_{n=10}$ =4.2%)	99 ± 11 ($RSD_{n=3}$ =4.4%)	93 ± 12 ($RSD_{n=3}$ =5.3%)
3	84 ± 8 ($RSD_{n=3}$ =3.4%)	58 ± 9 ($RSD_{n=3}$ =6.1%)	84 ± 10 ($RSD_{n=5}$ =9.2%)	58 ± 5 ($RSD_{n=5}$ =6.9%)	86 ± 3 ($RSD_{n=5}$ =3.2%)	60 ± 4 ($RSD_{n=5}$ =5.9%)
4	78 ± 10 ($RSD_{n=5}$ =9.8%)	32 ± 4 ($RSD_{n=5}$ =9.9%)	78 ± 3 ($RSD_{n=10}$ =5.8%)	33 ± 2 ($RSD_{n=10}$ =9.6%)	77 ± 8 ($RSD_{n=3}$ =4.1%)	30 ± 4 ($RSD_{n=3}$ =6.0%)
5	59 ± 7 ($RSD_{n=5}$ =9.8%)	51 ± 5 ($RSD_{n=5}$ =8.4%)	58 ± 7 ($RSD_{n=5}$ =9.9%)	52 ± 6 ($RSD_{n=5}$ =8.6%)	59 ± 10 ($RSD_{n=3}$ =7.1%)	53 ± 14 ($RSD_{n=3}$ =8.6%)

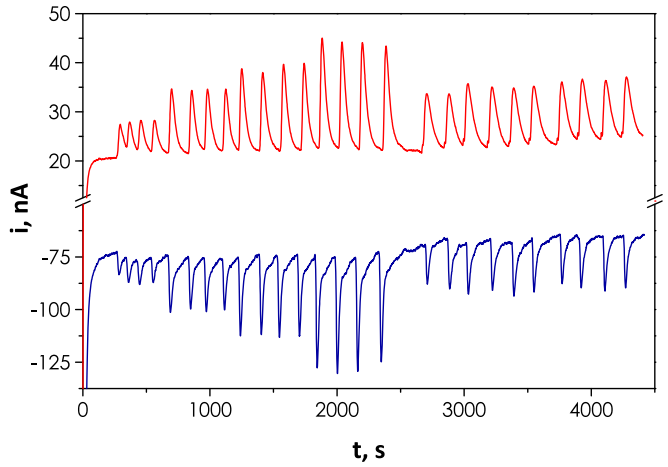

Fig. 4. Successive amperometric responses obtained for 4 successive injections of 3.0×10^{-3}, 6.0×10^{-3}, 9.0×10^{-3} and 1.2×10^{-2} g L^{-1} standards of D- (upper curve) and L-(lower curve) lactic acid and 10 successive injections of 10-times diluted beer sample (with alcohol) with the semiautomatic FI system. Carrier solution: 0.05 mol L^{-1} phosphate buffer, pH 7.4, containing 1 mmol L^{-1} NAD^+; flow rate: 3.0 mL min^{-1}; sample volume: 500 μL; E_{ap}=0.15 (D-lactic acid) and 0.00 (L-lactic acid) V vs. Ag/AgCl.

Table 5
Characteristic parameters of the corresponding linear least-squares regression plots.

Compared methodologies	Isomer	Slope	Intercept	r
Batch amperometry in stirred solutions vs. enzymatic kit (n=10)	D-	0.95 ± 0.06	3 ± 5	0.994
	L-	0.99 ± 0.04	0 ± 2	0.998
Semiautomatic FI system vs. enzymatic kit (n=10)	D-	0.99 ± 0.04	0 ± 3	0.998
	L-	0.97 ± 0.04	1 ± 2	0.998
Semiautomatic FI system vs. batch amperometry in stirred solutions (n=10)	D-	1.04 ± 0.05	-2 ± 4	0.997
	L-	0.97 ± 0.03	1 ± 1	0.999

As can be observed, the confidence intervals (for a 0.05 significance level) calculated for the slope and intercept values included the unit and the zero values in all cases, indicating that no systematic errors are apparent using the developed methodology when compared with individual methodologies based on amperometry under batch conditions in stirred solutions or using the enzymatic spectrophotometric kit. Therefore, the FI system using the biosensors as amperometric detectors can be successfully used for the quantification of lactic acid enantiomers in beer samples.

4. Conclusions

The novel integrated D-lactic amperometric biosensor prepared in this work shows great analytical performance and has been successful coupled with a commercially available L-lactic biosensor in a semiautomatic FI system able to perform the simultaneous determination of lactic acid isomers in a single experiment and without previous separation steps. The proposed methodology has demonstrated to be reliable and reproducible and has been successfully applied to lactic acid enantiomers quantification in commercial beer samples with inherent advantages of simplicity, sufficient sensitivity and assay time. These good analytical characteristics circumvent, for instance, the need for expensive columns with chiral stationary phases, and therefore show great promise as an affordable and useful analytical tool for routine food quality control. Moreover, the developed methodology can find application also in the clinical field to determine the enantiomeric composition of lactate in bodily fluids as a valuable diagnostic indicator of severe medical disorders such as infection, ischemia or traumatic sock.

Acknowledgments

The financial support of the Spanish Ministerio de Economía y Competitividad Research Project, CTQ2015-64402-C2-1-R, and the NANOAVANSENS Program from the Comunidad de Madrid (S2013/MT−3029) are gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.01.063.

References

[1] S. Girotti, M. Muratori, F. Fini, E.N. Ferri, G. Carrea, M. Koran, P. Rauch, Eur. Food Res. Technol. 210 (2000) 216–219.
[2] A. Querol, G.H. Fleet (Eds.), The Yeast Handbook. Yeast in Food and Beverages, Springer, Germany, 2006, ISBN-10 3-540-28388-9.
[3] M. Przybyt, J. Iciek, A. Papiewska, J. Biernasiak, J. Food Eng. 99 (2010) 485–490.
[4] B. Koppenhoefer, H. Allmendinger, Fresenius J. Anal. Chem. 326 (1987) 434.
[5] F. Betschinger, J. Libman, A. Shanzer, J. Chromatogr. A 746 (1996) 53–62.
[6] Y. Inoue, T. Shinka, M. Ohse, H. Ikawa, T. Kuhara, J. Chromatogr. B 838 (2006) 37–42.

[7] S. Kodama, A. Yamamoto, A. Matsunaga, T. Soga, K. Minoura, J. Chromatogr. A 875 (2000) 371–377.
[8] L. Saavedra, C. Barbas, J. Chromatogr. B 766 (2002) 235–242.
[9] L. Tan, Y. Wang, X. Liu, H. Ju, J. Li, J. Chromatogr. B 814 (2005) 393–398.
[10] E.J. Franco, H. Hofstetter, O. Hofstetter, J. Pharmaceut. Biomed. Anal. 49 (2009) 1088–1091.
[11] N. Ôi, H. Kitahara, F. Aoki, J. Chromatogr. 631 (1993) 177–182.
[12] N. Oi, H. Kitahara, F. Aoki, J. Chromatogr. A 666 (1994) 457–462.
[13] S. Okubo, F. Mashige, M. Omori, Y. Hashimoto, K. Nakahara, H. Kanazawa, Y. Matsushima, Biomed. Chromatogr. 14 (2000) 474–477.
[14] C. Olieman, E.S. De Vries, Neth. Milk Dairy J. 42 (1988) 111–120.
[15] T. Fukushima, S. Adachi, H. Ichihara, S. Al−Kindy, K. Imai, Biomed. Chromatogr. 13 (1999) 418–424.
[16] T. Fukushima, J.-A. Lee, T. Korenaga, H. Ichihara, M. Kato, K. Imai, Biomed. Chromatogr. 15 (2001) 189–195.
[17] H. Hasegawa, T. Fukushima, J.A. Lee, K. Tsukamoto, K. Moriya, Y. Ono, K. Imai, Anal. Bioanal. Chem. 377 (2003) 886–891.
[18] G. Cevasco, A.M. Piątek, C. Scapolla, S. Thea, J. Chromatogr. A 1218 (2011) 787–792.
[19] L. Bianco, M. Marucchi., Ind. Bevande 16 (1987) 1.
[20] S. Boyles, J. Am. Soc. Brew. Chem. 50 (1992) 61–63.
[21] K.J. De Vries, J. Am. Soc. Brew. Chem. 51 (1993) 155–157.
[22] Y. Nanjo, T. Yano, R. Hayashi, T. Yao, Anal. Sci. 22 (2006) 1135−−1138.
[23] B. Serra, A.J. Reviejo, C. Parrado, J.M. Pingarrón, Biosens. Bioelectron. 14 (1999) 505–513.
[24] J. Katrlík, V. Mastihuba, I. Vstiar, J. Šefčovičová, V. Štefuca, P. Gemeiner, Anal. Chim. Acta 566 (2006) 11–18.
[25] P.C. Pandey, S. Upadhyay, B.C. Upadhyay, H.C. Pathak, Anal. Biochem. 260 (1998) 195–203.
[26] M. Gamella, S. Campuzano, A.J. Reviejo, J.M. Pingarrón, Anal. Chim. Acta 609 (2008) 201–209.
[27] F. Gasser, M. Doudoroff, R. Contopoulos, J. Gen. Microbiol. 62 (1970) 241–250.
[28] S. Chakraborty, M. Sakka, T. Kimura, K. Sakka, Biosci. Biotechnol. Biochem. 72 (2008) 982–988.
[29] S. Campuzano, R. Galvez, M. Pedrero, F.J. Manuel de Villena, J.M. Pingarrón, J. Electroanal. Chem. 526 (2002) 92–100.
[30] A.J. Reviejo García, J.M. Pingarrón Carrazón, S. Campuzano Ruiz, M. Gamella Carballo, V.V. García-Echave, J. Manso Lorenzo, A. Guzmán Vázquez de Prada, F.J. Ferrero Martín, J. Campo Rodríguez, M. Valledor Llopis, Biosensor amperométrico desechable, método de fabricación del mismo y método de determinación de la presencia de analitos en alimentos, PCT/ES2009/000381, 2010.
[31] H. Gunasingham, C.-H. Tan, Electroanalysis 1 (1989) 423–429.
[32] F. Céspedes, E. Martínez-Fábregas, S. Alegret, Electroanalysis 6 (1994) 759–763.
[33] H. Tap, P. Gros, A.M. Gué, Sens. Actuators B 68 (2000) 123–127.
[34] A. Avramescu, T. Noguer, V. Magearu, J.L. Marty, Anal. Chim. Acta 433 (2001) 81–88.
[35] A.M. Gué, H. Tap, P. Gros, F. Maury, Sens. Actuators B 82 (2002) 227–232.
[36] A. Avramescu, T. Noguer, M. Avramescu, J.L. Marty, Anal. Chim. Acta 458 (2002) 203–213.
[37] A. Avramescu, S. Andreescu, T. Noguer, C. Bala, D. Andreescu, J.L. Marty, Anal. Bioanal. Chem. 374 (2002) 25–32.
[38] P. Gros, M. Comtat, Biosens. Bioelectron. 20 (2004) 204–210.
[39] F. Mazzei, F. Botrè, G. Favero, Microchem. J. 87 (2007) 81–86.
[40] N. Darie, C.F. Ognean, M. Ognean, A. Gafita, O.S. Petrenciu, J. Agroaliment, Proc. Technol. 13 (2007) 359–362.
[41] M. Pohanka, P. Zboril, Food Technol. Biotech. 46 (2008) 107–110.
[42] A. Arvinte, A.M. Sesay, V. Virtanen, C. Bala, Electroanalysis 20 (2008) 2355–2362.
[43] O.V. Smutok, K.V. Dmytruk, M.I. Karkovska, W. Schuhmann, M.V. Gonchar, A. A. Sibirny, Talanta 125 (2008) 227–232.
[44] A.S.N. Murthy, Anita, Biosens. Bioelectron. 11 (1996) 191–193.
[45] B. Maendl, A. Piendl, Proc. Eur. Brew. Conv. 13 (1971) 343−−354.
[46] N. Coote, B.H. Kirsop, J. Inst. Brew. 80 (1974) 474–483.
[47] S. Engan, Brygmesteren 36 (1979) 157–160.
[48] Heikki Suomalainen, Lalli Nikänen, Aroma of Beer, Wine and Distilled Alcoholic Beverages, Kluwer Academic Publishers, 1983, Springer, Netherlands, ISBN 90-77-553- (REIDEL).