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Automatic bionalyzer using an integrated amperometric biosensor for the determination of L-malic acid in wines

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ABSTRACT

A new automatic bioanalyzer for L-malic acid using an integrated amperometric biosensor as detector is reported for the first time in this work. The biosensor is constructed by gold film sputtering deposition on a stainless steel disk electrode and co-immobilization of the enzymes malate dehydrogenase (MDH) and diaphorase (DP) together with the redox mediator tetrathiafulvalene (TTF) by means of dialysis membrane. The analytical performance of the biosensor was evaluated when it was used as amperometric detector in three different analytical methodologies: stirred solutions, semiautomatic FIA system and automatic bioanalyzer. The bienzyme biosensor exhibited great analytical performance in terms of sensitivity, selectivity and reproducibility of the measurements and its usefulness was demonstrated by analyzing wine reference materials with certified content of L-malic acid. The attractive analytical and operational characteristics demonstrated by the automatic bioanalyzer make it a promising simple, rapid and field-based tool for routine wine and fruit control.

1. Introduction

Malic acid ($C_4H_6O_5$) is one of the major organic compounds contributing to the flavor and taste of fresh fruit, juices and other drinks. While L-malic acid occurs naturally in fruits and vegetables, D-malic acid can be found only when synthetic racemate is used as an additive in food industry [1]. Currently, excess of malic acid in grape musts is a concern to winemakers, since it significantly influences the sensory properties of wine. The understanding of the complex transformation of the grape must into wine allows the producers to monitor and control the different steps of this process in order to obtain more refined products. The winemaking process includes alcoholic fermentation conducted by yeast and a secondary fermentation performed by lactic acid bacteria, called malolactic fermentation (MLF) in which L-malic acid is converted into L-lactic acid and CO_2 by the lactic bacteria *Leuconostoc sp*. and *Lactobacillus sp*. MLF affects the quality and the future taste of wines by three major effects: acid reduction, flavor modification, and biological stability [2] and, depending of the wine type, the climatic zone of production or the requirements for commercialization, it should be avoided, controlled or even encouraged. For these reasons, the determination of L-malic and/or L-lactic acids in wines and during the MLF is of great interest and can be considered as a real "quality test", necessary to allow the winemaker to take the proper decisions [3,4]. Apart from its interest in wines samples, since significant increases in L-malic acid concentration have been shown to serve as a primary indicator of fruit maturity, the determination of this acid could provide a more objective means of determining the ripeness and hence 'shelf life' of horticultural produce than simple appearance and taste [5–7]. These findings make the quantitative analysis of L-malic acid highly desirable in the food industry.

Determination of L-malic acid is usually performed by chromatographic [8–15], electrophoretic [16,17] and enzymatic methods [18–20]. These methods are neither suitable for routine analysis nor adapted to the competences and financial constraints of small winemakers, for whom cheap and smart devices like biosensors represent an attractive alternative. Biosensors, and in particular electrochemical biosensors offer fast, cheap and smart easy-to-handle devices, amenable to miniatiaturization, able to detect selectively and quantify L-malic acid in real time and in situ. They can be envisaged as serious competitors for conventional techniques, representing an attractive alternative for small industries [4]. Numerous electrochemical biosensors involving L-malic acid-specific enzymes, such as malic enzyme (ME) [5,21–25], malate dehydrogenase (MDH) [2,4,6,7,26–28], and malate quinone

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oxidoreductase (MQO) [1,3] have been reported in the literature and used for the determination of malic acid in wines. However, to the best of our knowledge, no automatic analyzer for L-malic determination has been reported so far.

In this work, we report for the first time the development of an automatic bioanalyzer for L-malic acid using as a detector an amperometric enzymatic biosensor. The analytical performance of this biosensor, based on the MDH/DP/TTF system, was evaluated when used in three different analytical methodologies: stirred solutions, semiautomatic FIA system and automatic bioanalyzer. Moreover, its applicability for the analysis of real samples was successfully demonstrated by analyzing reference standards for wine samples.

2. Materials and methods

2.1. Apparatus and electrodes

Amperometric measurements in stirred solutions and in the semiautomatic FIA system were carried out with a single amperometric detector purchased from InBea Biosensores S. L. (Madrid, Spain) and with the developed automatic bioanalyzer. 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.

L-malic acid biosensor (fabricated as described in Section 2.3.1) was employed as working electrode to carry out amperometric detection. A BAS MF – 2052 Ag/AgCl/KCl (3 M) reference electrode and a Pt or a stainless steel wire as counter electrode were also employed. A 20-mL electrochemical cell was used for amperometric measurements in stirred solutions, while homemade methacrylate 10 mL wall – jet and 40 μL cylindrical cells were employed in the semiautomatic mode and with the automatic bioanalyzer, respectively.

2.2. Reagents and solutions

A stock 12.0 g L^{-1} L-(−)-malic acid disodium salt (Sigma) solution was prepared in 0.05 mol L^{-1} phosphate buffer of pH 8.0. 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 2.0 U μL^{-1} MDH solution (Sigma, EC 11.1.37 from *Thermus flavus*, 100 U mg^{-1} solid) both prepared in 0.05 M phosphate buffer solution of pH 7.4, were used for the preparation of the L–malic acid biosensor. Moreover, 0.5 mol L^{-1} TTF (Aldrich) and Fc (Fluka) solutions in acetone and ethyl acetate (Scharlau), respectively, were prepared. Dialysis membranes (Snake Skin® Pleated Dialysis Tubing, 10 K MWCO) were purchased from Thermo Scientific.

Other solutions employed were: stock solutions of 0.95 g L^{-1} D–glucose (Panreac), 0.84 g L^{-1} D – fructose (Sigma), 0.3 g L^{-1} L-arabinose (Sigma), 0.13 g L^{-1} D–galactose (Fluka), 15.0 g L^{-1} glycerol (Panreac), 1.0 g L^{-1} citric acid (Merck), 11.5 g L^{-1} tartaric acid (Merck), 3.0 g L^{-1} sodium gluconate (Sigma), 4.2 g L^{-1} D–lactic acid (Aldrich), 4.2 g L^{-1} L–lactic acid (Sigma), 0.9 g L^{-1} acetic acid (Scharlau), 15.0 mg L^{-1} ascorbic acid (Fluka), 14% (v/v) ethanol (Sharlab) and 2.0 g L^{-1} glutamine (Sigma – Aldrich), all prepared in 0.05 mol L^{-1} phosphate buffer of pH 7.8.

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. L-malic acid biosensor construction

Co-immobilization of the enzymes and the mediator onto gold sputtered – modified stainless steel disk electrodes was carried out as follows: a 3-μL aliquot of the 0.5 U μL^{-1} DP solution and a 3.0-μL aliquot of the 2.0 U μL^{-1} MDH 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.

Given the simple and rapid preparation protocol of this biosensor, when the corresponding quality control is not fine (see below), replacement for a new bioelectrode is recommended. Usually batches of 5 electrodes were prepared at the same time and stored at 4 °C in phosphate buffer pH 7.0. Therefore, replacement of the biosensor could be made in a rapid manner avoiding significant delay in the routine work.

2.3.2. Semiautomatic FIA system for L – malic acid determination

A semiautomatic flow system using the novel L-malic acid bienzyme biosensor as amperometric detector was implemented. The conventional semiautomatic FIA system was composed of a peristaltic pump, a manual injection valve and a “wall – jet” large volume cell (~10 mL), keeping the biosensor and the auxiliary and reference electrodes immersed in the same solution than the carrier.

2.3.3. Automatic bionalyzer for L-malic acid determination

Based on the semiautomatic FIA system, an automated bioanalyzer enabling the automatic "off-line" analysis of L-malic acid was developed. This development included both mechanical and electrical designs of the device as well as applications for different operating and control systems for monitoring malic acid determination. From a functional point of view, the automatic bioanalyzer can be considered to be composed of three systems: a flow system, an electronic system and application software controlling the instrument functionality. The most relevant features of each of these systems and a detailed description of the automatic bioanalyzer are given in the Supporting Information.

2.3.4. Amperometric detection

Amperometric detection with the L-malic acid biosensor was performed by applying a detection potential of +0.15 V (vs Ag/AgCl).

In stirred solutions, a 100–200 μL aliquot of the L-malic acid standard or diluted sample solution was added to the electrochemical cell containing 20 mL of the supporting electrolyte (0.05 mol L^{-1} phosphate buffer solution of pH 8.0 supplemented with NAD^+ 1.0 mmol L^{-1}).

When measurements were made in a conventional semiautomatic FIA system, a homemade methacrylate wall-jet cell (10 mL) connected to a peristaltic pump and an injection valve using a flow rate of 3.0 mL min^{-1} and a sample injection volume of 500 μL^{-1}. The carrier was a 0.05 mol L^{-1} phosphate buffer solution of pH 8.0 containing 1.0 mmol L^{-1} NAD^+.

The determination of L-malic acid with the automatic bioanalyzer was carried out as follows: first of all the reference electrode and the biosensor were introduced into the cylindrical flow cell. Next, the software application of the bioanalyzer is executed and the peristaltic pumps are activated to allow the carrier solution passing to the cell. Once verified the absence of air bubbles into the system, it is ready to determination of L-malic according to the configuration of the software application. The analysis method to

be executed by such "software" is the previously set from the configuration file and user interface thereof. The variation of the current intensity over time is recorded by the bioanalyzer under the established conditions, obtaining real-time analytical information on the L–malic acid content, depending on the programmed analysis method.

2.3.5. Analysis of wine reference materials

Lot 2009 of wine reference materials with certified contents of L-malic acid from Centre Œnologique de Bourgogne was analyzed. This lot contained four different wine reference materials: red wine with (1.5 ± 0.2) g L^{-1} L–malic acid (Reference 1); white wine with (1.02 ± 0.07) g L^{-1} L–malic acid (Reference 2); white wine with (0.96 ± 0.04) g L^{-1} L–malic acid (Reference 3) and rosé wine with (5.6 ± 0.6) g L^{-1} L–malic acid (Reference 4).

The determination of L-malic acid in these reference materials using amperometry in stirred solutions just implied a previous sample dilution with the supporting electrolyte (1/5 for References 1 and 2 and 1/10 for Reference 4) before adding the corresponding aliquot (200 µL for References 1 and 2 and 100 µL of Reference 4) to the electrochemical cell containing 20 mL of the supporting electrolyte (0.05 mol L^{-1} phosphate buffer solution of pH 8.0 supplemented with NAD^+ 1.0 mmol L^{-1}). The amperometric signals obtained were interpolated into a calibration plot constructed previously with L–malic acid standard solutions in the $2.0 \times 10^{-3} - 4.0 \times 10^{-3}$ g L^{-1} concentration range.

Regarding L-malic acid determination with the semiautomatic FIA system and the automatic bioanalyzer, samples were diluted with the carrier solution (25 times: References 1,2 and 3 and 100 times: Reference 4) and injected into the systems. Also, the amperometric signal obtained were interpolated into the calibrations plots constructed with standard L–malic acid solutions (0.03 – 0.12 g L^{-1}).

3. Results and discussion

3.1. Bienzyme biosensor for L–malic acid determination

A bienzyme biosensor for L – malic acid determination involving the MDH/DP enzymatic system co-immobilized onto sputtered gold film – modified stainless steel disk electrodes was addressed for the first time in this work. The choice of the electrode support was made with the aim of preparing low cost biosensors suitable to be routinely used in the food industry. The enzyme reactions are shown in Fig. 1 and imply oxidation of L-malate to oxaloacetate catalyzed by MDH and simultaneous reduction of cofactor NAD^+ to NADH. The NADH is then re – oxidized by TTF^+, this reaction being catalyzed by the enzyme diaphorase [29–31]. The electrochemical oxidation of the generated TTF is amperometrically monitored at the modified electrode with the resulting current being dependent on the L–malic acid concentration.

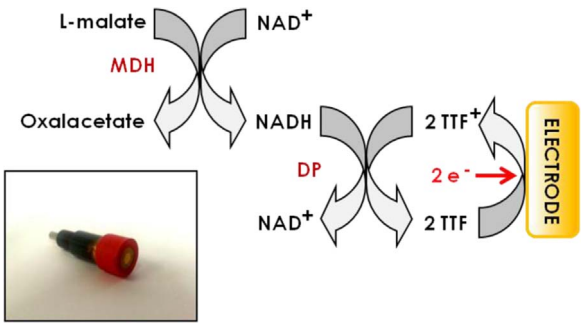

Fig. 1. Schematic diagram displaying the enzyme and electrode reactions involved in the developed bienzyme L-malic acid biosensor. Inset: picture of the biosensor.

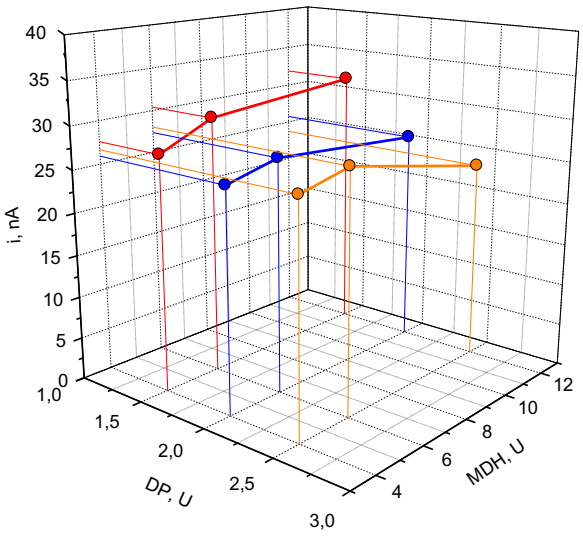

Fig. 2. Effect of the DP and MDH loadings on the amperometric responses measured with the bienzyme biosensor for 2.0×10^{-3} g L^{-1} L–malic acid; biosensors prepared with: 1.5 U DP and 4, 6 or 12 U MDH (●), 2.0 U DP and 4, 6 or 12 U MDH (●), 2.5 U DP and 4, 6 or 12 U MDH (●). Supporting electrolyte: 0.05 mol L^{-1} phosphate buffer solution of pH 8.0 supplemented with 1.0 mmol L^{-1} NAD^+. $E_{app} = +0.10$ V (vs Ag/AgCl).

3.1.1. Optimization of working variables

The optimization of all the experimental variables affecting the L-malic acid biosensor behavior was accomplished by measuring the amperometric signals obtained at +0.10 V (vs Ag/AgCl) for 2.0×10^{-3} g L^{-1} L–malic acid in stirred solutions. The composition of the enzymatic system, NAD^+ concentration, pH and applied potential were optimized.

The influence of the DP and MDH loadings (between 1.5 and 2.5 U for DP and between 4.0 and 12.0 U for MDH) on the measured current was checked (Fig. 2). As a compromise between cost and performance, loadings of 1.4 and 6.0 U were selected for DP and MDH, respectively. Moreover, the responses obtained with two different redox mediators, TTF and Fc, using MDH/DP biosensors prepared with 1.5 µmol of each redox mediator were also compared. Results obtained (not shown) indicated that biosensors constructed with TTF provided amperometric responses twice larger than those measured with the biosensor prepared with Fc. Consequently, the selected composition of the bienzyme electrode for further work was: 6.0U MDH/1.5 U DP/1.5 µmol TTF.

The influence of pH was tested over the 5.0 – 12.0 pH range (data not shown). The integrated biosensor exhibited a maximal activity towards L–malic acid at pH 8.0, in agreement with optimal pH value reported for free MDH [32] and DP [33]. Accordingly, a 0.05 mol L^{-1} phosphate buffer solution of pH 8.0 was chosen as working medium.

The concentration of the cofactor in solution was also optimized by performing amperometric measurements with various NAD^+ concentrations in the 0.05–5.0 mmol L^{-1} range. The current increased with the cofactor concentration up to 1.0 mM and leveled off for higher concentrations (results not shown). Therefore, in order to avoid a high consumption of this reagent a NAD^+ concentration of 1.0 mmol L^{-1} was chosen for further experiments.

The effect of the detection potential on the biosensor response was examined over the 0.00 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 (vs Ag/AgCl) and a decrease for more positive potentials due to the non-reversible oxidation of TTF^+ to TTF^{2+} that decomposes and leaks from the electrode surface [31,32].

Accordingly, a working potential of +0.15 V (vs Ag/AgCl) was chosen for further work to accomplish a sensitive detection, ensure the stability of the integrated biosensor and also minimize the effect of potential interfering substances able to be oxidized at the electrode surface.

3.1.2. Stability of the L-malic acid biosensor

Different aspects regarding the stability of the bienzyme biosensor were evaluated. Repetitive measurements (n=10) for 2.0×10^{-3} g L^{-1} malic acid carried out with the same biosensor yielded a RSD value of 5.2%, indicating a good repeatability of the measurements with no need to apply a cleaning or pretreatment procedure to the biosensor. The reproducibility of the responses obtained with different biosensors prepared in the same manner was also checked. Calibration plots constructed for malic acid in the 2.0×10^{-3}–4.0×10^{-3} g L^{-1} concentration range with five different biosensor yielded a RSD of 4.3% for the corresponding slope values, thus demonstrating that the biosensor fabrication procedure was reliable and allowed reproducible amperometric responses to be obtained with different biosensors constructed following the described methodology.

Finally, the lifetime of one single biosensor was tested by performing daily amperometric measurements for 2.0×10^{-3} g L^{-1} malic acid. After using, the biosensor was stored at 4 °C in phosphate buffer of pH 7.0. A control chart was constructed by setting the average current value calculated from 5 measurements made the first day of the study as the central value, while the upper and lower limits of control were set at $\pm3\times$SD of this central value (see Fig. S10 in the Supporting information). The test was performed in parallel with 5 different biosensors and the obtained results indicated that they provided responses inside the control limits for 25 days. It is worth to mention that during these 25 days, apart from the measurements for 2.0×10^{-3} g L^{-1} malic acid standard solutions, 45 samples (wine reference materials and commercial wines) could be also analyzed with each biosensor.

3.1.3. Analytical characteristics of the L-malic acid bienzyme biosensor

Under the optimized working conditions, a typical calibration curve for an enzyme system could be constructed. A linear calibration range for malic acid was obtained over the $(9.80–470)\times10^{-5}$ g L^{-1} concentration range (r=0.9991) (Fig. S11) with a slope value of $(1.78\pm0.02)\times10^{4}$ nA g^{-1} L, and an intercept of (1 ± 1) nA. The limits of detection (LOD) and quantification (LQ) were calculated 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 2.9×10^{-5} and 9.8×10^{-5} g L^{-1}, respectively. Table 1 compares these analytical characteristics with those reported for other electrochemical enzyme biosensors for malic acid. As it can be deduced, both sensitivity and LOD with the developed biosensor were the second best reported up to date, only slightly improved by the amperometric disposable biosensor reported by Molinero-Abad et al. [1], using screen-printed carbon electrodes (SPCEs) modified with AuNPs, TTF and MQO. It is important to remark also that operational lifetime of the developed biosensor is one of the best reported so far with the exception of that described by Mazzei et al. [26] which, however, exhibited 100-times poorer sensitivity than achieved with the biosensor prepared in this work.

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 currently present in wines was tested by adding to the electrochemical cell (containing 20 mL supporting electrolyte) 250 µL of interfering standards solutions prepared at the maximum concentration level at which they are found in wines: D−glucose (0.95 g L^{-1}), D−fructose (0.84 g L^{-1}), L−arabinose (0.3 g L^{-1}), D−galactose (0.13 g L^{-1}), glycerol (15.0 g L^{-1}) and citric acid (1.0 g L^{-1}), tartaric acid (11.5 g L^{-1}), gluconic acid (3.0 g L^{-1}), D−lactic acid (4.2 g L^{-1}), L−lactic acid (4.2 g L^{-1}), acetic acid (0.9 g L^{-1}), ascorbic acid (15.0 mg L^{-1}), ethanol (14% v/v) and glutamine (2.0 g L^{-1}).

None of these substances yielded a significant amperometric response under the employed working conditions (Fig. S12 in the Supporting information) thus clearly demonstrating the excellent selectivity of the bienzyme biosensor for the determination of malic acid in wines containing other saccharides or organic acids.

3.1.5. Determination of malic acid in wine reference materials with certified content of L-malic acid

L−malic acid was determined in three different wine reference materials with certified content of L-malic acid. As it is shown in Table S1 (in the Supporting information), the slope values calculated from the calibration graphs constructed for L−malic acid standards and by using the standard additions method for the three types of reference materials tested, showed no statistically significant differences. Therefore, no apparent matrix effect was found for any reference material and the samples could be analyzed simply by interpolation of the corresponding amperometric signals, measured after adding the appropriate volume of the previously diluted sample (as indicated in Section 2.3.4) to the electrochemical cell (containing 20.0 mL of 0.05 M phosphate buffer pH 8.0, supplemented with 1.0 mM NAD^+), into the calibration plot constructed with L-malic acid standard solutions.

The results obtained from five replicates of each reference material are summarized in Table 2, with the confidence intervals 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 and the RSD values obtained (in all cases $<5\%$), these results demonstrated the usefulness of the L-malic acid bienzyme biosensor for the reliable determination of the target acid in wine samples just upon a previous sample dilution.

3.2. L−malic acid determination at the semiautomatic FIA system

As it is usual in semiautomatic FIA systems, both the carrier solution and the injection volume were optimized. The effect of the flow rate was evaluated by comparing the amperometric signal obtained after injecting 500 µL of a 0.08 g L^{-1} L-malic acid standard solution. Results shown in Fig. 3a led us to select a flow rate of 3.0 mL min

The repeatability of the peak current measurements was evaluated from series of 10 repetitive injections of a 0.02 g L^{-1} L-malic acid standard solution as well as 30 repetitive injections of the wine reference material no. 4 (5.6 ± 0.6 g L^{-1} L-malic acid) which was previously diluted 200 times with 0.05 mol L^{-1} phosphate buffer, pH 8.0. The calculated RSD values were 2.5% and 5.2%, respectively, thus confirming an excellent repeatability of the flow injection amperometric responses despite the hydrodynamic conditions.

3.2.1. Analytical characteristics for L-malic acid determination with the semiautomatic FIA system

The calibration constructed for L-malic acid under the optimized conditions in the semiautomatic FIA system is shown in

Table 1.
Analytical characteristics reported for different electrochemical enzymatic biosensors for L-malic acid determination.

Electrode	Enzymes	Redox mediator	Immobilization	E, V (vs Ag/AgCl)	Sample	LR, g L^{-1}	LOD, g L^{-1}	Sensitivity, nA g^{-1} L	Operation lifetime	Storage stability	References
Rhodinised carbon SPE	ME	Rh	Adsorption	+0.30	Apple, potato and tomato	$(3.8-94)\times10^{-3}$	3.8×10^{-3}	6.89×10^{3}	–	–	[34]
Graphite SPE modified with MB	ME	MB	Entrapment in a polyethylenimine–glutaraldehyde cross-linking membrane	+0.20 V	Wine	$(1.34-134)\times10^{-3}$	1.34×10^{-3}	–	75% initial response after 15–20 measurements	–	[35]
Graphite SPE modified with MB	MDH	MB	Entrapment in PVA-AWP	−0.15	Wine	$(3.2-53)\times10^{-3}$	3.2×10^{-4}	5.26×10^{3}	20 successive determinations	3 months	[2]
Graphite SPE	MQO	PMS	Entrapment in PVA-SbQ	−0.01	Wine	$(6.70-201)\times10^{-4}$	6.7×10^{-4}	1.30×10^{4}	10 successive measurements	1 month	[3]
Clark electrode	MDH/HRP	–	Nylon membrane functionalized with carbonyl groups	−0.65	Wine	$(9.00-270)\times10^{-3}$	5.0×10^{-3}	1.00×10^{2}	70% initial activity after 200–300 determinations	–	[26]
GCE modified with CNTs	MDH	–	Adsorption, cross-linking, entrapment with nafion membrane	+0.30	–	$(1.3-8.0)\times10^{-2}$	6.7×10^{-3}	1.74×10^{2}	5 determinations	–	[27]
GCE modified with SWCNTs	MDH	MB	Adsorption, nafion entrapment, sol-gel entrapment	−0.05	–	$(4.56-181)\times10^{-2}$	4.0×10^{-4}	6.47×10^{3}	95% initial sensitivity after 10 repetitive measurements	–	[6]
Au-disk electrode	MDH/DP	TTF	Coimmobilization of enzymes and TTF on MPA-SAM modified AuE and entrapment with a dialysis membrane	+0.10	Wine	$(6.97-268)\times10^{-5}$	7.0×10^{-5}	1.18×10^{4}	30% original sensitivity after 7 days of continuous use	15 days	[4]
Au-SPE modified with MWCNTs	MDH/DP	Ferrocyanide	Entrapment between two layers of quitosan	+0.30	Wine	$(1.3-82)\times10^{-3}$	2.4×10^{-4}	9.10×10^{3}	No loss in sensitivity after 30 consecutive measurements	90% initial sensitivity after 1 year	[28]
SPCE modified with MWCNTs	MDH dependent of $NADP^{+}$	–	Cross-linking with EDC	−0.15	–	$(0.0-1.6)\times10^{-2}$	8.0×10^{-3}	–	–	–	[7]
SPCE modified with MWCNTs	MQO	TTF	Cross-linking with GA onto the electrode with deposited BSA	+0.10	Wine	$(1.5-15)\times10^{-5}$	1.5×10^{-5}	5.15×10^{4}	–	3 weeks (without use)	[1]
Au−sputtered steel disk electrode	MDH/DP	TTF	Entrapment in a dialysis membrane	+0.15 V	Reference standards for wine samples	$(9.80-470)\times10^{-5}$	2.9×10^{-5}	$(1.78\pm0.02)\times10^{4}$	45 samples/25 days	–	This work

Abbreviations: CNTs: carbon nanotubes; DP: diaphorase; EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GA: glutaraldehyde; GCE: glassy carbon electrode; HRP: horseradish peroxidase; LOD: limit of detection; LOx: lactate oxidase; LR: linear range; MB: methylene blue; ME: malic enzyme; MDH: malate dehydrogenase;MPA: mercaptopropionic acid; MQO: malate quinone oxidoreductase; PVA-AWP: photocross-linkable polyvinyl alcohol containing stilbazolium groups; PVA-SbQ: photo-cross-linkable poly(vinyl alcohol) polymer; PMS: phenazine methosulfate; SPE: screen-printed electrode; TTF: tetrathiafulvalene.

Table 2.
Determination of L-malic acid (in g L^{-1}) in three different wine reference materials using the L-malic acid bienzyme biosensor by amperometry in stirred solutions.

Reference standard	$[\text{L-malic acid}]_{certified}$	$[\text{L-malic acid}]_{found}$	t_{exp}
1	(1.5 ± 0.2)	(1.48 ± 0.08) $RSD_{n=5}=4.5\%$	0.667
2	(1.02 ± 0.07)	(1.07 ± 0.05) $RSD_{n=5}=3.8\%$	2.749
4	(5.6 ± 0.6)	(5.4 ± 0.3) $RSD_{n=5}=3.9\%$	2.121

Fig. 4. Although results demonstrated that the calibration graph showed linearity up to 0.04 g L^{-1}, the resulting linear range was shown to be too narrow for practical purposes. However, a polynomial relation, ($y=a+bx+cx^2+dx^3$) similar to that described for other L-malic acid biosensors [3,5,7] was observed from 1.4×10^{-3} to 0.12 g L^{-1} and therefore used for analytical purposes (Fig. 4). Table S2 (in the Supporting information) summarizes the polynomial parameters obtained after fitting the experimental values. In addition, LOD and LQ values were calculated according to the same criteria mentioned in Section 3.1.3 (confidence intervals were calculated for $\alpha=0.05$).

3.2.2. *Determination of malic acid in wine reference materials with certified content of L-malic acid*

The semiautomatic FIA system was used to determine the L-malic acid content in two wine reference materials. Similarly to that occurring when the analysis was carried out by amperometry in stirred solutions, no matrix effects were observed and therefore, quantification could be accomplished by simple interpolation of the measured current from 25 (Reference 1) and 100 (Reference 4)−times diluted samples into the calibration plot constructed with L-malic acid standard solutions over the 0.03–0.12 g L^{-1} concentration range. The results shown in Table 3 show clearly as no statistically significant differences were found with the certified values for the two analyzed reference materials at the significance level of 95% (t_{exp} values shown in Table 3 were lower than the t_{tab} value of 2.262).

3.3. *L−malic acid determination at the automatic bioanalyzer*

A detailed description of the constructed automatic bionalyzer is provided in the Supporting Information. Due to the use of the new peristaltic pumps the bioanalyzer worked at a flow rate of 4.0 mL min^{-1}, slightly higher than that used in the semiautomatic system (3.0 mL min^{-1}). Under the experimental conditions

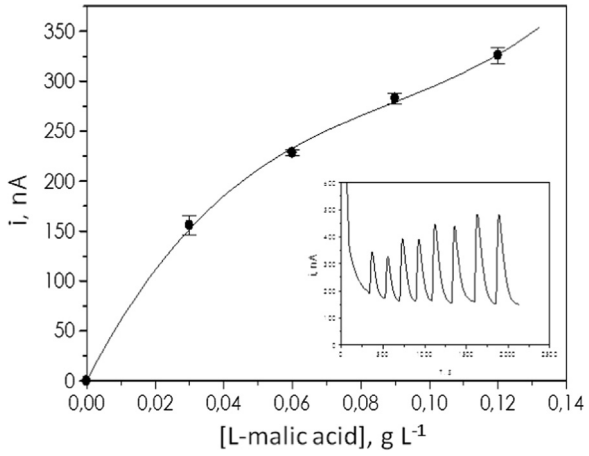

Fig. 4. Calibration plot obtained with the L-malic acid bienzyme biosensor and the semiautomatic FIA system. Inset: corresponding fiagrams. Supporting electrolyte, 0.05 mol L^{-1} phosphate buffer solution of pH 8.0 (containing 1.0 mmol L^{-1} NAD^+); q=3.0 mL min^{-1}; V_i=500 μL; E_{app}=+0.15 V (vs Ag/AgCl). Error bars estimated as triple of the standard deviation (n =3).

Table 3
Determination of the L-malic acid content (in g L^{-1}) in two wine reference materials using the L-malic acid bienzyme biosensor and the semiautomatic FIA system.

Reference standard	$[\text{L-malic acid}]_{certified}$	$[\text{L-malic acid}]_{found}$	t_{exp}
1	(1.5 ± 0.2)	(1.52 ± 0.06) $RSD_{n=10}=5.1\%$	0.822
4	(5.6 ± 0.6)	(5.6 ± 0.3) $RSD_{n=10}=6.9\%$	0.082

established for the automatic bioanalyzer, a polynomial relation was observed from 2.6×10^{-3} to 0.12 g L^{-1} L-malic acid with the parameters summarized in Table S3 (in the Supporting information).

These analytical characteristics are slightly worse than those achieved using the semiautomatic FIA system (Table S1) which can be attributed to the use of a higher flow rate and the low volume-cylindrical cell. Due to the design of this cell, the sample is slightly diluted before reaching the biosensor resulting in a slightly lower sensitivity than that achieved when working with the large volume wall-jet cell used in the semiautomatic system FIA (Fig. S13). However, it is important to note that despite this slight decrease, the sensitivity provided by the automatic bioanalyzer is still

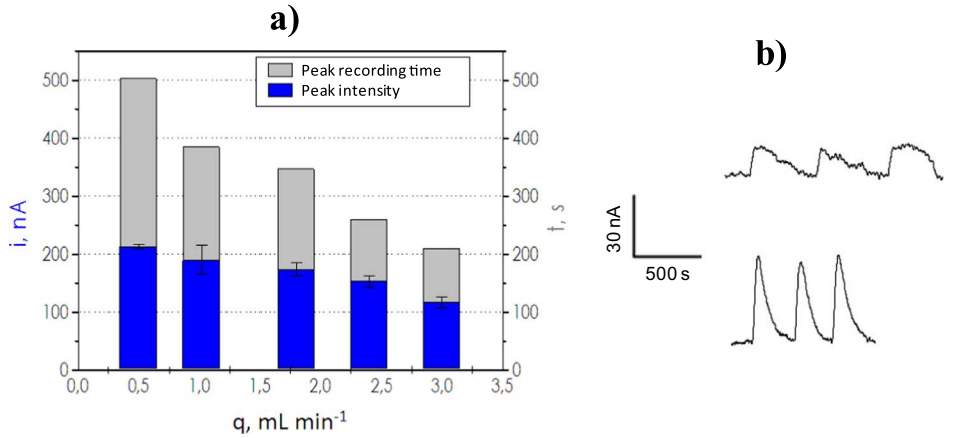

Fig. 3. Dependence of the measured peak current (blue bars) and the response width at the base peak (grey bars) obtained with the L-malic acid bienzyme biosensor as amperometric detector in the semiautomatic FIA system with the flow rate upon 500 μL injection of a 0.08 g L^{-1} L-malic acid solution (a). Amperometric signals recorded by injecting 100 (upper fiagram) and 500 (bottom fiagram) μL of a 4.0×10^{-3} g L^{-1} L-malic acid standard solution (q =3.0 mL min^{-1}) b). Carrier solution: phosphate buffer 0.05 mol L^{-1} pH 8.0 containing 1 mmol L^{-1} NAD^+; E_{ap}=+0.15 V (vs Ag/AgCl). Error bars estimated as triple of the standard deviation (n=3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

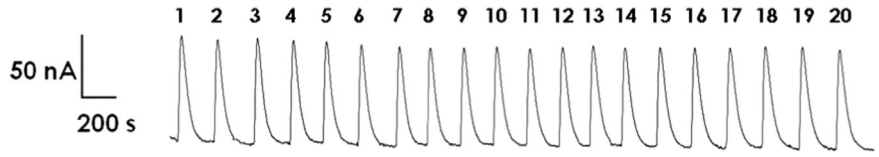

Fig. 5. Successive amperometric responses measured for 0.06 g L^{-1} L-malic acid using the automatic bioanalyzer by means of entries: 1 (peaks 1–5), 2 (peaks 6–10), 3 (peaks 11–15) and 4 (peaks 16–20). Carrier solution: phosphate buffer 0.05 mol L^{-1} pH 8.0 containing 1 mmol L^{-1} NAD^+; flow rate: 3.0 mL min^{-1}; sample volume: 500 μL; E_{ap}=0.15 V (vs Ag/AgCl).

Table 4.
Determination of L-malic acid (in g L^{-1}) in wine reference materials using the developed automatic bioanalyzer.

Reference standard	$[\text{L-malic acid}]_{certified}$	$[\text{L-malic acid}]_{found}$	t_{exp}
1	(1.5 ± 0.2)	(1.5 ± 0.1) $RSD_{n=10}$=9.2%	0.704
2	(1.02 ± 0.07)	(1.08 ± 0.07) $RSD_{n=10}$=8.9%	1.964
3	(0.96 ± 0.04)	(0.97 ± 0.04) $RSD_{n=10}$=5.7%	0.573
4	(5.6 ± 0.6)	(5.63 ± 0.08) $RSD_{n=10}$=2.1%	0.806

enough to perform the determination in wine reference materials as it will be shown below.

Fig. 5 shows 20 successive amperometric measurements of 0.06 g L^{-1} L-malic acid. The signals were obtained using the sample selector in order to test its functioning and verify whether the reproducibility of the measurements was not affected by the channel used to introduce the standards/samples. Therefore, five replicates were introduced for each of the four channels. Peak current RSD values of 2.2%, 1.4%, 1.4% and 1.8% were obtained for signals recorded for each of the four input selectors, respectively, while the RSD value for the whole 20 measurements was 2.9%. These results demonstrated the good reproducibility of the amperometric measurements provided by the automatic bioanalyzer, independently of the channel used for sample introduction.

Response time and sample frequency provided by the bioanalyzer was also evaluated. The response time, defined as the time taken by the system for the registration of a peak intensity data is a parameter that will depend on the settings made from the "software" and therefore modifiable according to analysis requirements. Using parameters specified above, the average response time was 3.5 min, resulting in a sampling frequency of 17 samples per hour.

The lifetime of the L-malic acid biosensor when employed as sensor detection system in the automatic bioanalyzer was checked by monitoring daily the responses obtained for 0.06 g L^{-1} malic acid. Under the conditions used, the biosensor loses 40% of its initial sensitivity after 10 days of use and it remained operational (requiring of course daily calibration) for 17 days, period during which the biosensor analyzed 70 samples.

3.3.1. Determination in wine reference materials with the automatic bioanalyzer

The potentiality of the constructed automatic bioanalyzer was demonstrated by determining L-malic acid in four wine reference materials with certified content of the target analyte. As expected, no matrix effect was also observed using the automatic analyzer and, therefore, the analysis was carried out by interpolation of the measured peak current in the diluted samples (materials 1, 2 and 3, 25 times and material 4, 100 times) in a calibration plot constructed with L-malic acid standards in the 0.03–0.12 g L^{-1} concentration range. The obtained results are summarized in Table 4 samples.

As it can be seen, the statistical comparison (with t_{exp} values $< t_{tab}$=2.262) demonstrates that there were no significant differences between the determined and certified values for the reference materials indicating the suitability of the automatic bioanalyzer for the determination of L-malic acid in wines.

4. Conclusions

In this work a new automatic bioanalyzer for L-malic acid using as detector a novel integrated amperometric biosensor has been developed for the first time. The amperometric biosensor demonstrated its usefulness working both in stirred solutions as well as a detector in a semiautomatic FIA system and in the new automatic bioanalyzer. The proposed methodologies have demonstrated very attractive analytical and operational characteristics including successful applicability to analyze wine reference materials with inherent advantages of simplicity, sensitivity and assay time. The malic acid automatic bioanalyzer developed has highly promising performance characteristics, such as amenability to field-based usage with a minimal training requirement, with regards to its potential for commercialization as an affordable and useful analytical tool for routine wine quality control. Moreover, since significant increases in L-malic acid concentration have been shown to serve as a primary indicator of fruit maturity, the developed bioanalyzer could be also potentially used to determine the ripeness and hence `self-life´of horticultural produces in an objective way.

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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.2016.05.050.

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