DISPOSABLE ELECTROCHEMICAL BIOSENSOR BASED ON SCREEN-PRINTING TECHNOLOGY FOR MALIC ACID DETERMINATION IN WINE

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Abstract – This work describes the fabrication of a disposable electrochemical biosensor based on screen-printing technology for malic acid detection in wine. The new biosensor was constructed using ferricyanide as electrochemical mediator and the Malate Quinone Oxidoreductase (MQO) enzyme. It showed great analytical performance in terms of reproducibility (RSD 4%; n=5), sensitivity (37µA/g/l), detection limit (0,33g/l) and stability on time (1 month at RT). Moreover, the disposable biosensor was successfully applied for the determination of malic acid in wines.

Keywords: screen-printing, biosensor, malic acid, Malate Quinone Oxidoreductase, wine.

1. INTRODUCTION

The quality of wine depends on several factors, such as the concentration and nature of the organic acids, with an emphasis on tartaric, malic and lactic acids. In particular, malic acid is fundamental to provide the best wine flavour, aroma and biological stability. If there is not enough, the wine will taste "flat," and will be more susceptible to spoilage. If there is too much, the wine will taste "green," or "sour." Thus, it is important for the winemaker to control the amount of malic acid present and to assess the correct progress of the malolactic fermentation [1].

A number of methods have been described in literature to determine and quantify malic acid in wine, such as fluorescence [2], chromatography [3] and enzymatic reaction [4]. However, most of these methods, are time consuming, tedious, and require of expensive laboratory equipment and/or skilled personnel, and therefore, cannot fulfil the demand of rapid screening that industry needs. Hence, a new generation of cost-effective, user-friendly and portable methods is required for "in situ" malic acid determination.

In this context, electrochemical biosensors based on screen-printing technology, are presented as a promising alternative to the previously mentioned methods, since they combine the inherent advantages of the electrochemical sensing (good detection limits, portable readers, instrumentation cost, robustness, etc.) with the screen-printing technology, one of the most promising approaches towards the simple, rapid and inexpensive production of sensors and biosensors. Biosensors based on screen printed electrodes (SPEs), have led to new possibilities in the detection and quantification of a wide range of molecules, becoming an emergent technology in biomedical [5], environmental [6] and food analysis [7]. Moreover, they have been successfully employed in the development of analytical methods that respond to the growing need to perform rapid "in situ" analyses [8].

In recent years, there have been reported several electrochemical biosensors, based on NAD^+-dependent Malate Dehydrogenase enzyme (MDH), $NADP^+$-dependent malic enzyme (ME) or Malate Quinone Oxidoreductase (MQO) enzyme for the determination of malic acid in wines [9]. However, work should be done in order to obtain good stability, high sensitivity and high selectivity to move from the lab to the real field.

In this contribution, a stable, reproducible, selective and sensitive disposable electrochemical biosensor based on screen-printing technology for malic acid quantification in wine is reported.

2. EXPERIMENTAL

2.1. Reagents and solutions

Bovine serum albumin (BSA), mucin, L-malic acid, D-glucose, D-fructose, citric acid, ascorbic acid and L-Lactic acid were purchased from Sigma-

Aldrich (Madrid, Spain). Malate Quinone Oxidoreductase (MQO) enzyme was produced at BIOLAN. Wine samples with certified content of malic acid were supplied from the Centre Oenologique de Bourgogne (Beaune, France). The L-malic acid spectrophotometric test for the specific measurement and analysis of malic acid in beverages and food products was purchased from Megazyme (Wicklow, Ireland).

2.2. Materials and instruments

All the electrochemical experiments were performed with a PGSTAT 128N potentiostat-galvanostat from Autolab (KM Utrecht, The Netherlands), using the software package NOVA 1.9 or the portable potentiostat developed by BIOLAN (Fig. 1).

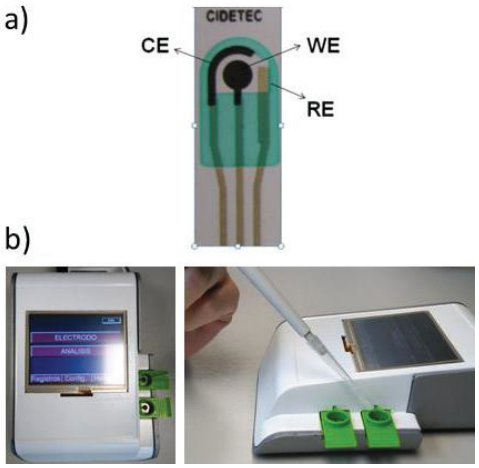

Fig. 1. The screen-printed electrodes produced at IK4-CIDETEC, consisting of a carbon working electrode (WE), a carbon counter electrode (CE) and an Ag/AgCl pseudoreference electrode (RE) (a) and the portable potentiostat developed by BIOLAN (b).

The disposable screen-printed electrodes (SPEs) consisting of a carbon working electrode, a carbon counter electrode and a silver/silver chloride (Ag/AgCl) pseudoreference electrode shown in Fig. 1 were produced at IK4-CIDETEC, using a Thieme 110E screen-printing machine from Thieme GmbH&Co (Teningen,Germany), an UV tabletop dryer Aktiprint T/A 40-2 from Technigraf (Hessen, Germany) and an oven UNE 200 from Memmert (Winsconsin,USA).

2.3. Preparation of the Malic Acid biosensor

The malic acid biosensor was constructed by modifying the WE of the SPEs with ferricyanide and MQO. Firstly, 1.5µl of 10mM ferricyanide in etanol:0.1M phosphate buffer (PB), pH 7 (1:1) were deposited and dried at RT. Then 1.5µl of etanol:water was uniformly spread on the WE and 3µl of 0.2U/µl of MQO in PB, pH 7 containing 1% of BSA:Mucin (1:1) were added and left drying at RT.

The fabricated biosensors were then stored with silica gel at RT until use.

2.4. Electrochemical assays

The amperometric measurements were performed by covering the three-electrode system with 60µl of the corresponding solution and by applying +100mV vs. Ag/AgCl pseudoreference electrode during 60s. The current value recorded at 60s was then used for constructing the calibration curve. For each measurement one biosensor was used and each point of the calibration curve was repeated 5 times.

2.5. Real sample measurements

All the samples were diluted with 0.1M PB, pH 7 before performing the amperometric measurements. The red wines were diluted 1:4 and rosé and white wines 1:14. In order to remove phenol compounds, the red wines were treated with activated carbon. Malic acid concentration was then calculated by interpolating the current value registered at 60s into the calibration curve constructed using standard solutions of malic acid (Fig.2).

3. RESULTS AND DISCUSSION

In order to obtain the best analytical properties in terms of sensitivity, detection limit, linearity, response time and storage stability, different parameters concerning the fabrication and characterization of the malic acid biosensors (such as the amount of ferricyanide, amount of enzyme, working potential, etc.) were optimized. Fig. 2 shows the calibration curve obtained for the optimized prototype using standard solutions of malic acid. The developed biosensor showed good reproducibility (RSD 4%; n=5), high sensitivity (37µA/g/l) and good detection limit (0,32g/l; calculated as the concentration corresponding to three times the standard deviation of the lowest point of the calibration curve).

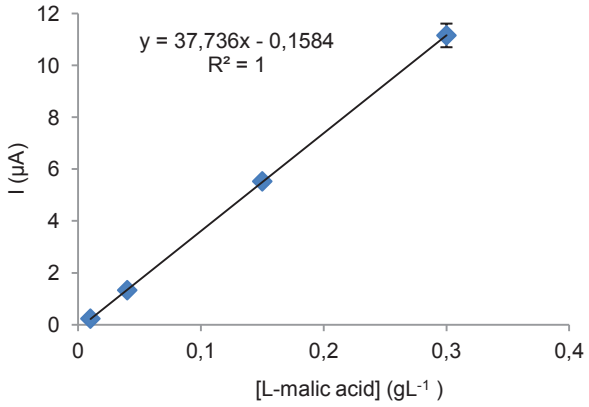

Fig. 2. Calibration curve of malic acid obtained with the developed biosensors. Each point represents the current value recorded at 60 seconds (Error bars; n=5). E_{app} = +100mV vs. Ag/AgCl pseudo reference electrode.

Moreover, the long-term stability of the biosensors was also evaluated. For this purpose, a batch of biosensors was fabricated and stored at RT. Then, calibration curves in the 0.01 – 0.3 gL^{-1} malic acid range were recorded every 7 days over a period of one month. Fig. 3 shows the results obtained, where each point represents the slope values recorded and upper and lower control limits are 10% of the slope value obtained the first day. As it can be seen, the slope remains almost constant after 1 month at RT.

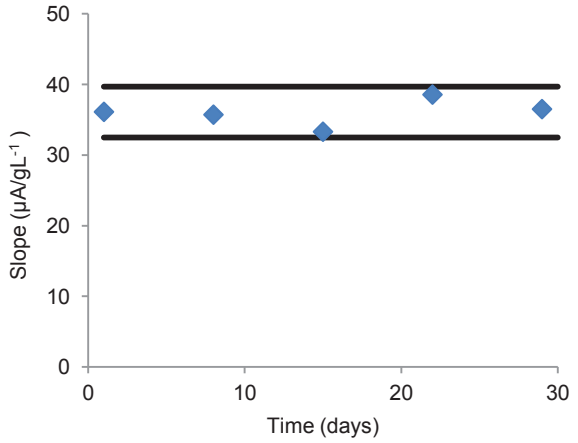

Fig. 3. Long-term stability chart obtained for the malic acid biosensors stored at RT. Upper and lower control limits are 10% of the slope value obtained the first day. E_{app} = +100mV vs. Ag/AgCl pseudo reference electrode.

The selectivity of the developed biosensor towards malic acid detection was also evaluated in the presence of some compounds commonly found in wine. Standard solutions with and without malic acid, containing at least twice the maximum concentration values reported for each compound in wine, were prepared and analysed: D-glucose (2.4 gL^{-1}), D-fructose (2.4 gL^{-1}), citric acid (2 gL^{-1}), ascorbic acid (0.03 gL^{-1}) and L-Lactic acid (6.5 gL^{-1}). Fig. 4 shows the percentage of signal obtained for a solution containing 0 and 0.02 gL^{-1} of malic acid with and without each interfering compound. In both cases, in the absence and presence of malic acid, the signals remained almost constant (change <10%) in the presence of the studied interfering compounds. Hence, it can be concluded that the developed biosensor is selective towards malic acid in the presence of other compounds commonly found in wine.

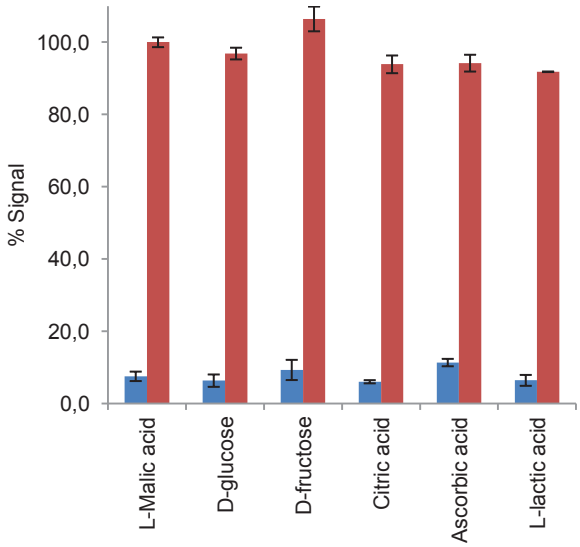

Fig. 4. Effect of the interfering compounds in the malic acid value obtained at the malic acid biosensors. Percentage signals are related to 0 (blue) and 0.02 gL^{-1} (red) malic acid in 0,1M PB, pH=7. (Error bars; n=3)

Finally, the applicability of the developed biosensor for the quantification of malic acid in wine was evaluated by testing 4 real wines with certified content of malic acid (2 red, 1 rosé and 1 white wine) and 4 commercial wines (Cune and Coto red wines; and Campo Viejo and Yela white wines). The obtained values were compared to those obtained with the commercially available spectrophotometric kit and to the reference values reported by the Centre Oenologique de Bourgogne. As shown in Table 1, the results obtained with the developed biosensor are comparable to those reported by the Centre Oenologique de Bourgogne and to those obtained with the Megazyme kit. Hence, it can be concluded that the disposable malic acid biosensor can be successfully applied for determining malic acid in wine.

Table 1. Malic acid values (mean value ± standard deviation; n=3) obtained for different wine samples analysed using the disposable malic acid biosensor (this work) and the Megazyme Kit.

Real Sample	Malic acid concentration (gL^{-1})		
	COB*	This work	MEGAZYME KIT
CEB ref.1 red wine	1.3±0.1	1.35±0.01	1.08±0.04
CEB ref.2 red wine	4.7±0.3	4.44±0.28	4.32±0.32
CEB ref.3 rosé wine	2.1±0.2	1.88±0.07	1.47±0.01
CEB ref.4 white wine	0.07±0.04	0.12±0.01	0.57±0.04
Cune red wine	---	0.04±0.01	0.02±0.02
Coto red wine	---	0.19±0.02	0.19±0.01
Yela white wine	---	2.29±0.09	2.11±0.21
Campo viejo white wine	---	1.13±0.01	1.06±0.03

*COB: Centre Oenologique de Bourgogne.

4. CONCLUSIONS

A disposable electrochemical biosensor based on screen-printing technology has been developed. The developed biosensor showed good reproducibility (RSD 4%, n=5), high sensitivity (37µA/g/l) and good detection limit (0,33g/l). Moreover it is stable for one month stored at RT and it has been successfully applied for the determination of malic acid in wines with a certified content of malic acid and in 4 commercial wines. Further work will be focused in evaluating the long-term stability at higher storage temperatures and over a longer period of time. Moreover the developed prototypes will be tested in real field, in particular, in different wineries located in Spain and Chile.

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