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An Amperometric Biosensor for the Determination of Lactic Acid During Malolactic Fermentation

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A lactate oxidase amperometric biosensor was developed and optimized for the malolactic fermentation monitoring during winemaking process.
Lactate oxidase enzyme was immobilized on prussian blue modified screen-printed carbon electrode in order to reduce the electrochemical interferences due to the high content of electroactive compounds abundant in wine and must, such as polyphenols and ascorbic acid.
The lactate oxidase biosensor developed showed high sensitivity (852 $\mu A\ M^{-1}$) and a detection limit for lactic acid of 0.005 mM (0.45 mg L^{-1}) The operational stability and the life time of the biosensors were also evaluated equal to 8 h and 30 days respectively.
Finally the biosensor in flow injection system was used for lactic acid analysis during malolactic fermentation of a red wine and the results were compared with those registered by ion chromatography with good agreement with two sets of data.

1. Introduction

During winemaking process, malolactic fermentation (MLF) is an important step to improve wine quality. MLF is a secondary fermentation that transforms the dicarboxylic L-malic acid in the monocarboxylic L-lactic acid, by lactic acid bacteria (LAB). This transformation causes acid reduction, flavour modification and also contributes to microbiological stability of red wine (Ribereau-Gayon et al., 2006).
The use of a fast and cheap analytical device (Flores et al., 2013) able to measure the change in MLF progress, such as screen-printed carbon amperometric biosensor, represents a key point for quality wines production because an uncontrolled MLF can causes a risk of wine spoilage and off-flavours development.
The amperometric biosensor for the monitoring of MLF takes advantage of the specific reaction between lactate oxidase enzyme (LOX) and L-lactic acid, that is the only isomeric form of lactic acid produced by malolactic enzymes. The enzymatic reaction between LOX and L-lactic acid produces H_2O_2 detected at 0.7 V vs. Ag/AgCl on electrodes surface. At high potential some electroactive compounds, such as polyphenols and ascorbic acid, may interfere during the hydrogen peroxide measurement giving mistakes in the results. For this reason electrochemical mediators able to detect hydrogen peroxide at low potential are required. Some authors overcome interference problem, related to the application of LOX biosensors in the analysis of food samples, using polyaniline-co-fluoroaniline films (Suman et al., 2005) or combining polysulfone multiwalled nanotubes with ferrocene (Perez and Fabregas, 2012). Others studies used chitosan membrane with ferrocyanide (Monosik et al., 2012) or polyvinylimidazole-Os (Li et al., 2013) as electrochemical mediators and redox polymer, respectively.
The use of prussian blue (PB) or ferric ferrocyanide is widespread in amperometric biosensor application, due to it's low cost, high stability at certain conditions, ease of electrode surface modification and no substrate saturation (Ricci and Palleschi, 2005). The main application of PB derives from its catalytic function in the

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electrochemical reduction of hydrogen peroxide at low potentials, avoiding or greatly reducing the effect of the most common electrochemical interfering substances.
This paper was aimed to the developing of a cheap and handy amperometric LOX biosensor able to detect the progress of MLF in red wines.
For this purpose, PB was deposited on screen-printed carbon electrode working surface, while the LOX was entrapped on tetraethyl orthosilicate (TEOS) –polyvinyl alcohol (PVA) silica-sol gel matrix and immobilized on PB-modified screen-printed carbon electrode (PB-modified SPCE).
The LOX biosensor was characterized in terms of sensitivity, linear range, limit of detection (LOD), operational stability and lifetime. Finally, the biosensor was applied to measure L-lactic acid change in Tintilia red wine undergone to MLF by means of *Oenococcus oeni* starter culture.

2. Materials and methods

2.1 Reagents

Lactate oxidase from *Pediococcus sp*. (37.6 U mg^{-1} solid), lactic acid, hydrogen peroxide (30 % H_2O_2 solution), tetraethyl orthosilicate (TEOS), ferric chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$), hydrolysed polyvinyl alcohol (Mw 13,000–23,000), Nafion®, potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic monohydrate (NaH_2PO_4-H_2O) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich.

2.2 Screen-printed carbon electrode preparation

Sensors based on a three-electrode (working/auxiliary/reference) layout were produced in three steps, as described in Albanese et al. (2011).

2.3 Prussian blue deposition

The deposition of PB was conducted on screen-printed carbon electrode surface after cleaning treatment of 6 min at 1.7 V vs. Ag/AgCl in a 0.05 M buffer phosphate solution (0.1 M KCl, pH 6.8). Chemical deposition was carried out by dropping 5 µL of a 1:1 ratio solutions of 0.1 M $K_3Fe(CN)_6$ in 0.01 M HCl and 0.1 M $FeCl_3$ in 0.01 M HCl (Ricci et al., 2003) onto the working electrode area. After 10 min, the PB excess was washed with a 0.01 M HCl and placed for 1 h in heater at 100 °C.

2.4 Lactate oxidase entrapment on PB-modified screen-printed carbon electrode

Lactate oxidase was immobilized on PB-modified SPCEs surface according to Albanese et al. (2014). PVA solution 5% (w/v) was heated at 85 °C for 1.5 h stirring periodically. Then, the PVA solution was frozen at −18 °C for at least 3 h and thawing at 6 °C for 5 h. The silica TEOS hybrid gel was prepared by mixing 1 mL TEOS and 0.5 mL of 15 % ethanol in 0.01 M HCl solution. The mixture was sonicated for 1.5 h until a homogenous solution was obtained.
TEOS-PVA silica sol-gel was made mixing 20 µL of PVA 5 % and 90 µL of TEOS sol. Then, 18 µL of TEOS-PVA solution was mixed with 5 µL of LOX diluted in buffer solution (0.2 mg μL^{-1}) and few microliters was dropped on the PB-modified electrode working surface. Finally 1 % Nafion solution was dropped on TEOS-PVA-LOX membrane. LOX biosensor was stored at 4 °C before use.

2.5 Electrochemical measurements

All the electrochemical experiments were carried out by Metrohm potentiostat/galvanostat (Autolab B. V.). The amperometric measurements were performed in flow injection analysis (FIA) as described in Albanese et al. (2010). The biosensor was placed in a handmade electrochemical flow cell, while a potential of 0.0 V vs. Ag/AgCl was applied. Carrier solution (0.05 M phosphate buffer, 0.1 M KCl, pH 6.8) from a reservoir was pumped with a peristaltic pump (Miniplus 3, Gilson) at flow rate of 0.5 mL min^{-1}. Samples were injected in flow injection analysis (FIA) system by a valve (sample injection valve; Omnifit) equipped with a sample loop of 500 µL.

2.6 Monitoring of malolactic fermentation by LOX biosensor

Tintilia red wine at the end of alcoholic fermentation was provided from a winery in Molise region (Italy). Tintilia wine was divided in three batches (0.5 L) and submitted to pasteurization treatment (75 °C for 15 s). Malolactic fermentation was induced by O. *oeni* MBR® starter (Lallemand) according to the following procedure: O. *oeni* was rehydrated in distilled water at 25 °C for 15 min and added to wine (20 mg L^{-1}) for a final concentration of about 10^6 cfu mL^{-1}. The wine, was placed in a thermostat at 22 °C and monitored at regular interval time by LOX biosensor. The lactic acid biosensor measurements were compared with those registered by ion chromatography according to Albanese et al. (2007).

3. Results and discussion

3.1 Prussian blue deposition on screen-printed carbon electrode

The capability of PB as electrochemical mediator is showed in Figure 1. The voltammogram showed the typical PB redox peaks corresponding to the reversible redox interconversion of PB into its reduced form, the prussian white (PW), which occurs when a stable PB layer is deposited on the working electrode surface.

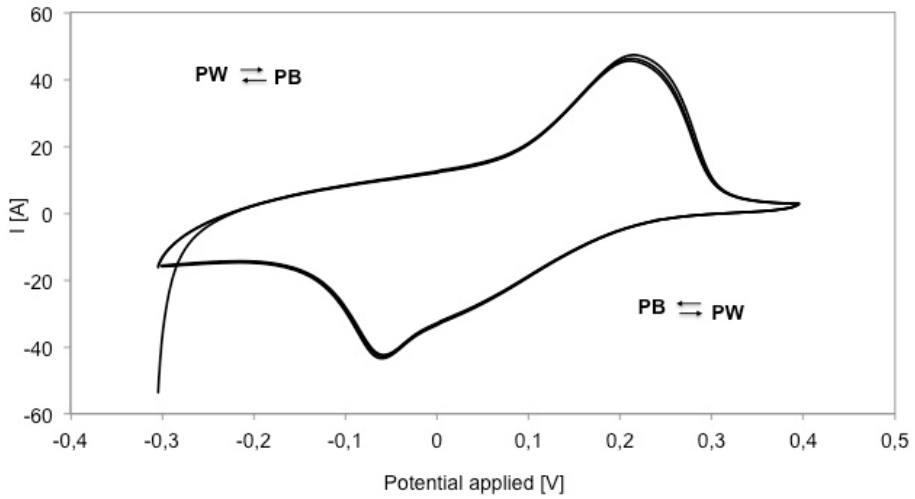

Figure 1: Cyclic voltammetry from -0.3 V to 0.4 V vs. Ag/AgCl of PB-modified screen-printed carbon electrode in 0.05 M phosphate buffer, 0.1 M KCl, pH 6.8.

The presence of PW gives the possibility to catalyse the H_2O_2 reduction at 0.0 V vs. Ag/AgCl during chronoamperometric measurements.
In order to highlight the advantages of using the PB as electrochemical mediator, towards the detection of H_2O_2, a comparison of the analytical parameters between naked and PB-modified screen-printed carbon electrodes was carried out. The data, reported in Table 1, showed the best performances of PB-modified SPCEs with a wider linear range, a lower LOD and a higher sensitivity value when compared with naked ones.

Table1: analytical parameters of PB-modified SPCE at 0.0 V vs. Ag/AgCl and naked SPCE at 0.7 V vs. Ag/AgCl.

	PB-modified SPCE 0.0 V vs. Ag/AgCl	**Naked SPCE 0.7 V vs. Ag/AgCl**
Linear range (μM)	0.005-5,000	0.5-5,000
LOD[1] (μM)	0.005	0.5
R.S.D. % (n = 5)[2]	4.22	1.04
Sensitivity (μA M^{-1})	4,896.35 ± 379.35	1,764.7 ± 134.44
R^2	0.99	0.99

[1] Limit of detection, defined as the hydrogen peroxide concentration that yields a signal-to-noise (S/N) ratio =3.

[2] defined as the % ratio between standard deviation and average of five consecutive injections of 0.1 mM H_2O_2 standard solution.

3.2 Analytical performance of LOX biosensor

The effectiveness of TEOS-PVA sol-gel entrapment for the immobilization of LOX on PB-modified electrodes surface was evaluated on five LOX biosensors by the injections of lactate standard solutions at different concentrations (Figure 2). The analytical biosensors performance showed a wide linear range (0.005-1 mM; 0.45-90.08 mg L^{-1}), a LOD of 0.005 mM, high sensitivity (852.20 ± 60.86 μA M^{-1}) and a time response variable between 1.5 and 3 min for 0.005 and 1 mM of lactic acid, respectively. If we consider that the amount of L-lactic acid in wine samples ranging from few mg L^{-1} to about 4 g L^{-1} before and after MLF respectively, the analytical performances of LOX biosensors showed the capability of these devices to detect and monitor L-lactic acid variation during winemaking process.

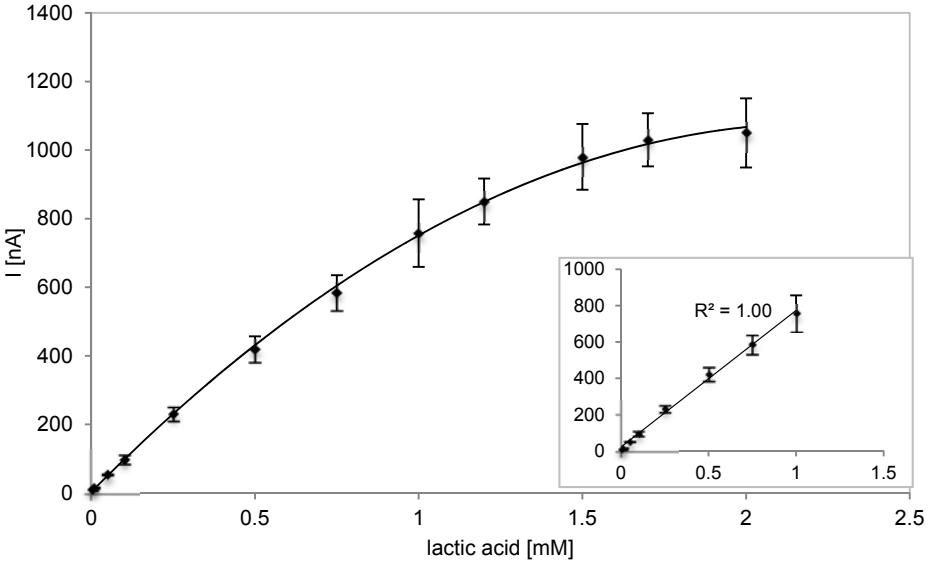

Figure 2: LOX biosensor calibration curves as average of five different biosensors. Inset: linear range of a LOX biosensors.

3.3 Operational stability and lifetime of LOX biosensor

Operational stability is an important parameter for the evaluation of LOX biosensor performance because describes the stability of the biosensor during routine analysis, such as the monitoring of MLF in wine. To asses the capability of lactate biosensor for routine analysis, 0.05 mM lactate standard solutions were injected in FIA system for 8 h, at interval time of 10 min. Through the comparison of current response at the beginning and at the end of the test period, no significant response decrease was observed.

After use, the biosensors were stored in their electrochemical cell in presence of phosphate buffer at 4°C and characterized by calibration curve every week. Under these conditions the lifetime (t_{L50}), defined as the storage time necessary for the sensitivity within the linear range decrease by a factor of 50 %, was 33 days.

4. Malolactic fermentation monitoring by LOX biosensor

Some of the most important analytical parameters of Tintilia wine before and after MLF were analyzed and reported in Table 2. As expected the enzymatic transformation of malic in lactic acid by MLF induce a decrease of total acidity and pH increase. The evolution of malolactic fermentation in Tintilia wine was monitored by LOX biosensor. The wine samples were properly diluted in 0.05 M phosphate buffer solution (0.1 M KCl, pH 6.8), filtered and injected in FIA system for the analysis. During the first two days, no significative differences in lactic acid content was observed, probably due to the lag period of O. *oeni* starter culture, necessary to their adaptation in wine matrix (Figure 3). After this period the increasing in lactic acid content up to 9 days of MLF was observed, without changes for the remaining fermentation days. Thus MLF was stopped after 11 days, with a lactic and malic acid content equal to 3.3 g L^{-1} and 0.42 g L^{-1}, respectively.

Table 2: analytical parameters of Tintilia red wine before and after MLF.

	Tintilia wine before MLF	**Tintilia wine after MLF**
Alcohol (%vol)	13.01	13.25
pH	3.78 ± 0.23	3.95 ± 0.16
Total Acidity (g of H_2SO_4 L^{-1})	7.60 ± 0.38	5.70 ± 0.25
Malic acid (g L^{-1})	3.52 ± 0.21	0.42 ± 0.02

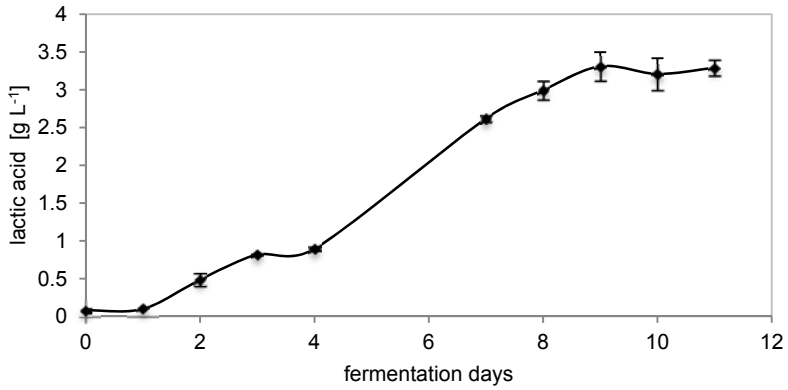

Figure 3: MLF monitoring of Tintilia red wine by LOX biosensor

Lactic acid results by LOX biosensors were compared with those obtained by ion chromatography (Table 3). A regular difference of 0.3 g L^{-1} in lactic acid between the two set of data, during the MLF, was observed. This difference may be due to the presence of D-lactic acid in wine samples that is detected only by ion chromatography. D-lactic acid in wine samples is due to heterolactic bacteria which convert sugars, that have not been totally transformed by alcoholic fermentation, in acetic acid and D-lactic acid. This alteration doesn't cause change in quality parameters of wine up to 0.4 g L^{-1} of D-lactic acid (Ribereau-Gayon et al., 2006).

Table 3: lactic acid amount in wine during MLF monitoring, by LOX biosensor and Ion Chromatography.

Fermentation days	LOX biosensor lactic acid [g L^{-1}]	Ion chromatography lactic acid [g L^{-1}]
0	0.08 ± 0.02	0.40 ± 0.05
1	0.10 ± 0.03	0.54 ± 0.10
2	0.48 ± 0.07	0.78 ± 0.04
3	0.82 ± 0.00	1.12 ± 0.09
4	0.89 ± 0.02	1.19 ± 0.14
7	2.61 ± 0.03	2.72 ± 0.22
8	2.99 ± 0.12	3.19 ± 0.18
9	3.31 ± 0.18	3.52 ± 0.07
10	3.21 ± 0.20	3.52 ± 0.23
11	3.29 ± 0.10	3.60 ± 0.15

5. Conclusions

The analytical performances and the short response times showed by LOX/PB-modified screen-printed biosensor highlight its capability for the analyses of L-lactic acid in wine samples. Moreover the biosensor lifetime of 30 days, underlines its use as easy and cheap analytical device for the monitoring of long malolactic fermentation, which can occur during winemaking process.

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