



Strategies to develop malic acid biosensors based on malate quinone oxidoreductase (MQO)

Bogdan Bucur^{a,b}, Elena Mallat^a, Ana-Maria Gurban^{a,b}, Yana Gocheva^c,
Carine Velasco^c, Jean-Louis Marty^a, Thierry Noguer^{a,*}

^a *Université de Perpignan, BIOMEM, 52, Av. Paul Alduy, 66860 Perpignan Cedex, France*

^b *University of Bucharest, Faculty of Chemistry, Sos. Panduri, No. 90-92, 70326 Bucharest, Romania*

^c *GTP Technology, rue Pierre et Marie Curie, BP28262, 31682 Labège Cedex, France*

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Abstract

An amperometric biosensor based on malate quinone oxidoreductase (MQO) was developed for monitoring of the malolactic fermentation of wines. Screen-printed electrodes coupled with appropriate mediators were used as transducers for this novel biosensor. MQO was immobilized by physical entrapment in a photo-cross-linkable poly(vinyl alcohol) polymer (PVA-SbQ) on the surface of the working electrode. Several electrochemical mediators were studied in order to lower the applied potential and minimise the matrix effects. Among them, 2,6-dichlorophenol indophenol (DPIP) and phenazine methosulfate (PMS) were chosen for further development. The working conditions (mediator concentration, applied potential and pH) were optimised for both DPIP and PMS. Detection limits for both types of biosensors were of 5 μM malic acid. Sensitivities obtained for the linear part of the calibration curve were 0.85 and 1.7 mA/M for the biosensors based on DPIP and PMS, respectively. Interferences due to non-specific oxidations were shown to be negligible when using PMS as mediator.

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1. Introduction

From earliest development, wine has had a special place in our customs, diet and social gatherings. 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 an alcoholic fermentation conducted by yeast and a secondary fermentation performed by lactic acid bacteria, called malolactic fermentation (Alexandre et al., 2004). During malolactic fermentation the L-malic acid is converted to lactic acid and CO₂ by the lactic bacteria *Leuconostoc sp.* and *Lactobacillus sp.* (Du Plessis et al., 2002). Malolactic fermentation has an important influence on the final taste of the wine and, depending of the wine type, it should be either avoided, controlled or even encouraged (de Revel et al., 1999). Malolactic

fermentation can be controlled by the use of starter cultures and, therefore, the determination of L-malic acid during the wine fermentation is necessary to allow the winemaker to take the proper decisions (Maicas et al., 2000).

The monitoring of malolactic fermentation is usually done by measuring the total acidity and the volatile acidity of wine (Kinkee, 1991), by means of chromatographic methods (Palma and Barroso, 2002) or by electrophoresis (Castineira et al., 2000). These methods are not adapted to the competences and financial constraints of small winemakers, for whom cheap and smart devices like biosensors represent an attractive alternative. Biosensors are easy-to-handle devices able to detect selectively and quantitatively a specific compound in real time and in situ.

In literature, mono- and bi-enzymatic biosensors have been described for the determination of malic acid in wines. Most of them are based on NAD⁺-dependent dehydrogenases. Malate dehydrogenase (MDH, EC 1.1.1.37) has been shown to be an appropriate enzyme for the specific detection of L-malic acid. However, due to the unfavourable equilibrium of the enzymatic reaction, MDH-based biosensors require high NAD⁺ concen-

* Corresponding author. Tel.: +33 468662255; fax: +33 468662223.
E-mail address: noguer@univ-perp.fr (T. Noguer).

trations to shift the reaction equilibrium towards the product side. The NAD^+ may be added free in the measuring media or immobilized on the transducer (Gorton and Dominguez, 2002). Another option to shift the equilibrium is to use more complex bi-enzymatic systems involving glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) (Möllering, 1985). A selective determination of L-malate in wine was also done using a bi-enzymatic biosensor based on the coupling of L-MDH and diaphorase (Katrlik et al., 1999). The transducer consists in a graphite solid binding matrix including the coenzyme NAD^+ . The enzymes were placed onto the transducer surface and covered by a dialysis membrane for reducing interferences. Hexacyanoferrate (III) was used as mediator and the optimum value of the polarization potential for L-malate biosensor was +300 mV versus SCE. Non-specific responses were observed due to the oxidation of easily oxidizable components of red wines like phenolic acids, flavonoids and other polyphenols. Another bi-enzymatic sensor was described based on MDH and NADH oxidase using an oxygen electrode to monitor oxygen consumption (Mizutani et al., 1991). This sensor enables determination of L-malic acid with a detection limit of 5 μM .

The NADP^+ -dependent malic enzyme (ME, EC 1.1.1.40) has also been used to develop mono-enzymatic amperometric biosensors for the detection of L-malic acid in red wines (Esti et al., 2004). The enzyme was immobilized in a reactor, while NADP^+ and the electrochemical mediator phenazine methosulphate (PMS) were added in standard solutions or diluted wine samples (dilution factor 1:100). The working principle of the biosensor is based on the reduction of molecular oxygen to H_2O_2 , by NADPH in the presence of PMS, followed by the oxidation of the H_2O_2 at the surface of the Pt electrode polarised at +650 mV versus Ag/AgCl. The detection limit achieved with this biosensor was 3 μM and the response time was 3.5 min. The effect of the red wine matrix on biosensor performance was studied by spiking wine samples after malolactic fermentation with known amounts of the L-malic acid. A recovery between 93 and 100% and a relative standard deviation of less than 4% was observed.

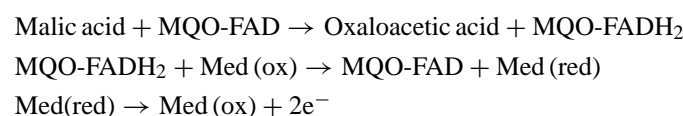
Despite their interesting characteristics, the most important problem concerning the previous mono- or bi-enzymatic biosensors is that all of them are based on malate dehydrogenase or malic enzyme, which are respectively NAD^+ and NADP^+ -dependent. The stable immobilization of the enzymes, mediators and cofactors is still challenging and work should be done in order to obtain satisfactory stability, sensitivity and selectivity for detection in real samples and commercial applications.

This paper presents a completely different approach to develop a biosensor for malic acid using the membrane-associated malate quinone oxidoreductase (MQO, EC 1.1.99.16). MQO is a flavin adenine dinucleotide (FAD) enzyme able to catalyse the oxidation of L-malate to oxaloacetate in the presence of electron donors. The main advantage of this enzyme is the fact that it catalyses the irreversible conversion of malate without requiring any soluble coenzyme like NAD^+ . It is mostly studied in *Corynebacterium glutamicum*, but its existence was proven in several bacteria (Molenaar et al., 1998).

The MQO is known to interact with electron donors like: 2,6-dichlorophenol indoplenol (DPIP), 3(4,5-dimethyl thiazolyl 1-2) 2,5-diphenyl tetrazolium bromide, menadione (Vitamin K), cytochrome C (Asano and Brodie, 1963), phenazine methosulfate (PMS), ferricyanide (Ohsima and Tanaka, 1993) and ubiquinones (Bott and Niebis, 2004). An important issue to take into account in the development of the amperometric biosensors is the choice of a suitable electrochemical mediator that ensures the proper electronic transfer and at the same time avoids interferences due to matrix effects. There are numerous organic and inorganic electrochemical compounds that may be used as mediators, the choice being made in function of the particularity of the developed system and the imposed working conditions.

In this study, screen-printed electrodes have been used as transducers due to their overall performances, low cost and the possibility to be mass-produced. Several electrochemical mediators have been tested either in solution or immobilized on the electrode surface, as the mediated process may be homogenous or heterogeneous (Chaubey and Malhotra, 2002).

The working principle of our biosensors is the following:



To our knowledge, this is the first time that MQO is used as a biological element for the construction of a biosensor.

2. Materials and methods

2.1. Reagents

Malate quinone oxidoreductase (MQO, EC 1.1.99.16) with an activity of 3.5 IU/mL was provided by GTP Technology (Labège, France) and stored at 4 °C for up to 3 months. The enzymatic activity of MQO was measured by spectrophotometry using DPIP (Sigma, France) as electronic acceptor; DPIP reduced form was determined at $\lambda = 600 \text{ nm}$ ($\epsilon = 22000 \text{ L mol}^{-1} \text{ cm}^{-1}$).

The enzymes were immobilized by entrapment in a photocrosslinkable polyvinyl alcohol containing stilbazolium groups (PVA-SbQ), type SPP-S-13 (bio) (polymerization degree 1700) provided by Toyo Gosei Kogyo Co. (Japan).

Stock solutions of 0.1 M malic acid (Sigma, France), 10 mM DPIP (Merck, France), 0.4 mM benzoquinone (BQ) (Aldrich, France), 5 mM naphthoquinone sulfonic acid (NQSA) and 10 mM phenazine methosulfate (PMS) (Fluka, France), 10 mM 8-dimethylamino-2,3-benzophenoxazine (Meldola's blue (MB)), 5 mM Nile blue (NB) and 10 mM potassium hexacyanoferrate (Sigma, France) were prepared in distilled water. PMS and BQ stock solutions were kept in ice, protected from light and were stable only few hours.

Screen-printed electrodes were fabricated using the following pastes: Electrodag PE-410, 423SS, 6037SS (Acheson, UK), C2030408D3 (Gwent Electronic Materials, UK) and graphite Timrex T15 (Lonza, Switzerland). Clear, transparent PVC sheets

(200 mm × 100 mm × 0.5 mm) were used as electrode supports. 7,7,8,8-Tetracyanoquinodimethane (TCNQ) (Aldrich, France), ammonium tetrathiocyanodiammonochromate (Reinecke salt) (Sigma, France) and hydroxyethyl-cellulose (HEC) (Fluka, France) were used to prepare the chemically modified working electrodes. Two French wines were used as real samples: Rivesaltes Tuilé (red wine) and Comté Tolosan Moelleux (white wine).

The supporting electrolyte was a 0.1 M phosphate buffer solution (PBS) containing 0.1 M KCl and adjusted to pH 7.1 or 7.8.

2.2. Apparatus

Spectrophotometric measurements of the enzymatic activity were performed using a Hewlett Packard diode array 8451A spectrophotometer. Amperometric measurements were carried out using a 641VA potentiostat (Metrohm, Switzerland), connected to a BD40 X-t recorder (Kipp & Zonen, The Netherlands). Cyclic voltammetric studies were carried out using an Autolab PGSTAT12 electrochemical analyser (Eco-Chemie, The Netherlands). A Minispin centrifuge (Eppendorf, Germany) with Nanosep 10 K Omega filtration microtubes (Pall Company, USA) were used for enzyme desalting.

2.3. Preparation of the screen-printed transducers

Screen-printed electrodes (SPEs) were produced in our laboratory using a DEK 248 printing machine according to a previously described procedure (Andreescu et al., 2002), but in a three-electrode configuration. The working electrode (WE) was a 4 mm graphite disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line surrounding the WE and the Ag/AgCl pseudoreference electrode was a 5 mm × 1.5 mm straight line close to the WE. The WE were modified with electrochemical mediators using: (1) a water-based mixture of HEC with graphite-TCNQ according to Andreescu et al. (2002); (2) an aqueous mixture of HEC, graphite powder (Timrex T15) and Meldola's blue complexed with Reinecke salt, following the procedure from Avramescu et al. (2001); (3) a cobalt-phtalocyanine-modified polymeric ink based on the approach developed by Abass and Hart (1997) or (4) Prussian blue by slightly modifying a previously described protocol (Li et al., 2004).

2.4. Enzyme immobilization

The enzyme was immobilized directly on the WE surface by entrapment in PVA-SbQ using a previously reported procedure slightly modified (Andreescu et al., 2002). Fifty microliters of MQO were separated from ammonium sulfate and stabilizers by five successive ultrafiltrations at 10,000 rpm for 15 min. The first centrifugation allowed removing of enzyme storage buffer. The subsequent two centrifugations were carried out after addition of 100 μL of 0.1 M PBS while for the last ones 100 μL of distilled water were added. If the liquid did not completely pass through during the 15 min centrifugation, then another centrifugation step was performed before the addition of the subsequent rinsing solution. Finally, the desalted enzyme was collected by

rinsing the Nanosep 10 K Omega filtration microtube three times with 20 μL of distilled water. This 60 μL desalted enzyme solution was mixed with 60 μL of PVA-SbQ and homogenised by vortex mixing. A complete removal of ammonium sulphate from the enzyme solution and an homogeneous mixing with PVA-SbQ conditioned the good operational stability of biosensors. Then, 2 μL of the mixture were carefully spread on the WE surface (directly or after modification with the electrochemical mediator). The electrodes were exposed to neon light for 4 h at 4 °C to allow the entrapment of the enzymes by photopolymerization. The electrodes were then kept for 24 h in a dessicator at 4 °C and stored at this temperature for up to 1 month.

2.5. Detection procedure

Amperometric measurements were performed in a glass cell containing 5 mL of PBS solution magnetically stirred. A fixed potential was then applied versus the screen-printed Ag/AgCl pseudoreference electrode, depending on the electrochemical mediator used. When the mediator could not be incorporated into the electrode material, an aliquot of the mediator stock solution was injected in the cell. After current stabilization (baseline signal), standard solutions of malic acid or wine samples were injected. As a result of substrate addition, the current increased up to a plateau corresponding to the steady state response. The analytical signal was measured as the difference between the current intensity recorded for the baseline and for the steady state. The cell was washed with distilled water between measurements. Interferences were estimated by repeating the measurements under identical conditions, but using a SPE without enzyme.

3. Results and discussion

3.1. Choice of the electrochemical mediator

Several redox mediators were studied in the aim of optimising the biosensor performance in terms of sensitivity and interference minimization. Mediators (organic and inorganic) are capable of accelerating heterogeneous electron transfer, for molecules that are reasonably electroactive. The gain in detection limit and selectivity in comparison to a normal electrode may be substantial (Nagels and Staes, 2001). In oxidase-based biosensors, mediators are low molecular weight redox couples, which shuttle electrons from the redox centre of the enzyme to the electrode surface. During the catalytic reaction, the mediator first reacts with the reduced enzyme and then diffuses to the electrode surface to undergo rapid electron transfer. The rate of production of the reduced mediator is measured amperometrically by oxidation at the electrode (Chaubey and Malhotra, 2002). The use of redox mediators for the construction of the oxidase-based biosensors has been shown to be an effective approach to lower the applied potential and therefore to minimise the oxidation of the numerous species present in the sample matrix. However, an electrochemical mediator will not allow to completely eliminate interferences, due to the fact that it may also mediate the reduction/oxidation of the sample matrix (Chen et al., 2001). Therefore, the ideal electrochemical medi-

ator must shuttle electrons between the WE and the enzyme at a high rate and show only a limited interaction with other chemical species from the sample matrix. Other than the nature of the electrochemical mediator, the overall performance of the biosensors is influenced by the mediator concentration, their use free in solution or immobilized on the WE, the applied potential and the working pH. In this work, the working potential for each mediator was chosen by cyclic voltammetry and confirmed by amperometry testing the biosensor response at different potentials.

Several electrochemical mediators were investigated in order to construct an appropriate transducer. Nile blue, MB, NQSA, potassium hexacyanoferrate, BQ, DPIP, PMS were tested free in the electrolyte solution, while Prussian blue, TCNQ, cobalt (II) phthalocyanine and Meldola's blue precipitated with Reinecke salt (MBRS) were incorporated in the WE prior to enzyme immobilization. All tests were performed with the enzyme immobilized in a PVA matrix on the WE.

Preliminary tests for the choice of the electrochemical mediators consisted in the evaluation of the acquirable analytical signal using biosensors and the level of interferences using enzyme-free electrodes. The analytical signal was determined using 1 mM malic acid. This substrate concentration corresponds to the beginning of the plateau of the calibration curve and represents the maximum analytical signal that may be obtained. The interferences were evaluated by injecting 50 μL of red wine in the electrochemical cell containing 5 mL of PBS.

DPIP was already used for the development of glucose oxidase biosensors (Amine et al., 1993). As MQO is also a FAD enzyme and its activity can be spectrometrically estimated using a protocol based on DPIP, some efforts have been made towards the application of this mediator to the construction of the malic acid sensor. Some preliminary tests were carried out leading to significant analytical responses when working at +50 mV versus Ag/AgCl and using 0.2 mM DPIP. Under these conditions, MQO biosensors provided an important analytical signal (350 nA), higher than the interferences (90 nA for the red wine). Further studies concerning the optimization of the main parameters affecting the analytical response using this mediator are shown in detail in next section.

PMS was subsequently proposed as an alternative electrochemical mediator, because of its reported successful performance for the fabrication of biosensors based on FAD oxidases (Wang et al., 2001). Amperometric measurements carried out at -50 mV versus Ag/AgCl and using 0.3 mM PMS produced high current intensities, with a magnitude similar with the one obtained using DPIP and showing smaller interference levels. Further studies concerning the use of PMS are presented in the next section.

Benzoquinone (BQ) was used at a concentration of 40 μM . Although a potential of +50 mV versus Ag/AgCl is normally enough to oxidize the hydroquinone (reduced BQ), no analytical response was obtained with the MQO biosensors under these conditions and a potential of +400 mV was needed to measure an oxidation current. Under these conditions, the signal intensity was 90% of that obtained using DPIP. However, at this potential, the interferences produced by red wine were higher than

the sensor response to 1 mM malic acid. The current produced by interferences was substantial smaller at +50 mV, but at this potential biosensors were unoperational. In view of all these results, the BQ was considered to be inappropriate for the development of malic acid biosensors.

Screen-printed electrodes modified with TCNQ were first tested at a potential of +100 mV versus Ag/AgCl either using 50 μM cysteamine or 1 mM acetylthiocholine after their modification with acetylcholinesterase. The transducers presented appropriate analytical characteristics under these conditions, but the MQO-based biosensors showed a negligible current intensity, equivalent to only 5% of the one obtained with DPIP. This signal was considered too small to make a successful analytical device for practical approaches. The SPEs modified with cobalt (II) phthalocyanine were tested under the same conditions as those modified with TCNQ. These sensors did not give any analytical signal when tested at +100 mV or even +400 mV versus Ag/AgCl.

Potassium hexacyanoferrate was tested at a potential of +350 mV versus Ag/AgCl with a concentration of 0.1 mM (final in the electrochemical cell). This electrochemical mediator was shown to allow exchange of electrons with MQO, the analytical signal being 40% of that obtained with DPIP under the same conditions. However, the interfering current measured upon injection of 50 μL of red wine was 400 nA, which is substantially higher than the analytical signal obtained with 1 mM malic acid. Due to this high level of interference, potassium hexacyanoferrate was considered to be unsuitable for the determination of malate in real samples. The same behaviour was observed using 0.1 mM naphthoquinone sulfonic acid at a potential of 0 mV versus Ag/AgCl.

Meldola's blue was tested also free in solution at a potential of +10 mV versus Ag/AgCl and at a final concentration of 0.1 mM. Under these conditions, the magnitude of the analytical signal of MQO-based biosensors was small, only 15% of that achieved using DPIP. This tendency was confirmed when using electrodes modified with precipitated Meldola's blue (MBRS), which did not produce any analytical signal, probably because of the distance between the enzyme active site and the incorporated mediator. The overall performance of Meldola's blue-modified sensors were thus considered unsatisfactory to continue further studies.

Prussian blue-modified electrodes were obtained by applying a potential of +200 mV for 4 min in a freshly prepared aqueous solution of FeCl_3 1 mM and $\text{K}_3[\text{Fe}(\text{CN})_6]$ 1 mM. The transducers were then thoroughly rinsed with distilled water and dried overnight in a desiccator under slight vacuum at room temperature. They were amperometrically tested at a potential of +10 mV versus Ag/AgCl by injecting H_2O_2 to a final concentration of 9 μM or after their further modification with glucose oxidase at the same potential using 0.1 mM glucose as substrate. Under these conditions they showed a satisfactory operational stability (up to 10 successive measurements) even at pH 7, but it was necessary to avoid the presence of high concentrations of Na^+ to prevent Prussian blue decomposition. The transducers were stable when the buffer contained K^+ instead of Na^+ , but Prussian blue did not interact with MQO to produce an analyt-

Table 1
Performances of the electrochemical mediators tested

Mediator	Form used	Working potential (mV)	Analytical signal (nA)	Interferences by red wine (nA)
DPIP	Free in solution (0.2 mM)	+50	350	90
PMS	Free in solution (0.3 mM)	−50	300	−15
BQ	Free in solution (40 μM)	+50	No signal	
BQ	Free in solution (40 μM)	+400	320	1100
TCNQ	Immobilized on WE	+100	Negligible	
Co (II) phthalocyanine	Immobilized on WE	+100	No signal	
Co (II) phthalocyanine	Immobilized on WE	+400	No signal	
Potassium hexacyanoferrate	Free in solution (0.1 mM)	+350	140	400
MB	Free in solution (0.1 mM)	+10	Small signal	
MB	Immobilized on WE	+10	No signal	
PB	Immobilized on WE	+10	No signal	
Nile blue	Free in solution (0.1 mM)	−150	No signal	

ical signal. The same negative results were achieved using Nile blue 0.1 mM as mediator at −150 mV versus Ag/AgCl. The main results of this mediator investigation are summarized in Table 1.

3.2. Optimization of detection parameters

Among all electrochemical mediators tested, only DPIP and PMS were chosen for further MQO-based biosensors development, due to their ability to transfer electrons from MQO and their relatively low non-specific interactions with the sample matrix. The use DPIP was already described for the mediated oxidation of NADH (Prieto-Simon and Fabregas, 2004), for the development of glucose oxidase based biosensors (Amine et al., 1993) or for the construction of a lactate biosensor (Hirano et al., 2001). The PMS was previously used for the construction of a biosensor for dimethyl sulfoxide (Abo et al., 2003), a glucose oxidase-based biosensor (Wang et al., 2001) and dehydrogenase based biosensors (Curulli et al., 1997).

In this work, these two mediators were used in soluble forms. The concentration of mediator, the applied potential as well as the working pH were optimised.

The concentration of the used mediator has a significant influence on the response and, for a constant enzyme activity, an increase in mediator concentration or its efficiency results in increases in both sensitivity and dynamic range (Gooding et al., 2000). Thus, the typical behaviour for a mediator-based sensor shows an increase of the sensitivity and dynamic range with the mediator concentration (when the response is limited by the enzyme-mediator kinetics) followed by a stabilization at high mediator concentration (when the response is limited by enzyme-substrate kinetics) (Liu et al., 2000). The optimization of DPIP and PMS concentration was performed by successive injections of the electrochemical mediator in the electrochemical cell and measuring the induced current variations. This study was performed with MQO biosensors at a potential of +100 mV versus Ag/AgCl, using 1 mM malic acid at pH 7.1. As expected, an increase of the mediator concentration induced an enhancement of the analytical response, until a plateau was reached. The lowest mediator concentration of the plateau was considered to be the optimum one, as it provided the maximum signal. As

shown in Fig. 1, the optimum concentration of DPIP and PMS were 0.2 and 0.3 mM, respectively.

The most appropriate working pH for a biosensor is dependent on the enzyme activity, but also on the influence of the pH on the performances of the mediator (Chaubey and Malhotra, 2002). The choice of the most appropriate working potential was accomplished by comparing the analytical signals of the biosensors with the level of interference at different potentials. Triplicate measurements of the analytical signal were carried out using 1 mM malic acid in PBS pH 7.1 containing either 0.2 mM DPIP or 0.3 mM PMS. The interferences due to easily oxidizable compounds present in wine samples were studied using either red wine or gallic acid, which is often used as polyphenolic model compound. The signal due to interfering compounds was estimated, under the same measuring conditions, with an enzyme-free electrode upon addition of 50 μL of red wine or 5 mM gallic acid in the measuring cell containing 5 mL PBS. These non-specific responses were expressed relatively to the analytical response of the sensors to 1 mM malic acid.

The studied voltage ranged from −10 to 100 mV versus Ag/AgCl for DPIP and from −100 to 50 mV versus Ag/AgCl for PMS. When the potential was shifted to more cathodic values, a decrease of the analytical signal and the interfering current was observed for both electrochemical mediators. The optimum potential was chosen as a compromise between the magnitude

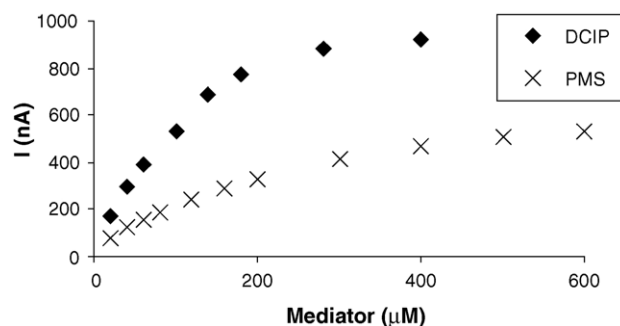


Fig. 1. Influence of the electrochemical mediator concentration on the magnitude of the analytical signal. Experimental conditions: working potential +100 mV vs. Ag/AgCl pseudoreference, substrate: 1 mM malic acid in 0.1 M PBS pH 7.1.

Table 2
Effect of the working potential on the interferences level observed using red wine and gallic acid

Potential (mV vs. Ag/AgCl)	DPIP						PMS					
	Malic acid (1 mM)		Red wine		Gallic acid		Malic acid (1 mM)		Red wine		Gallic acid	
	AS (nA)	Relative AS	IS (nA)	RI	IS (nA)	RI	AS (nA)	Relative AS	IS (nA)	RI	IS (nA)	RI
100	417 ± 52	1	105 ± 15	0.25	355 ± 48	0.85	–	–	–	–	–	–
50	405 ± 63	0.97	100 ± 9	0.25	345 ± 34	0.85	490 ± 81	1	25 ± 5	0.05	55 ± 13	0.11
10	342 ± 41	0.82	95 ± 10	0.28	337 ± 42	0.99	460 ± 88	0.94	15 ± 5	0.03	14 ± 13	0.03
–10	205 ± 26	0.49	75 ± 9	0.37	300 ± 40	1.46	400 ± 72	0.82	0 ± 3	0	7 ± 15	0.02
–50	–	–	–	–	–	–	315 ± 65	0.64	–10 ± 7	–0.03	–17 ± 12	–0.05
–100	–	–	–	–	–	–	275 ± 57	0.56	–10 ± 7	–0.04	3 ± 15	0.00

AS, analytical signal; relative AS, ratio between the analytical signal of the biosensor obtained at the studied potential and the current measured with the biosensor at +100 mV vs. Ag/AgCl (DPIP) or +50 mV vs. Ag/AgCl (PMS); IS, interfering signal; RI, relative interference = interference current/analytical signal. Average of three independent measurements.

of the analytical signal and the relative percentage of the interferences. From the experimental results presented in Table 2, the most appropriate potentials were considered to be +50 mV and –10 mV versus Ag/AgCl for DPIP and PMS, respectively. In the case of DPIP, the relative interferences were very high whatever the potential used, while for PMS the interference level was much reduced.

The choice of the working pH was carried out by measuring the analytical signal corresponding to 1 mM malic acid in PBS solutions adjusted to different pH values. The working conditions were those previously optimised (0.2 mM DPIP at +50 mV versus Ag/AgCl and 0.3 mM PMS at +10 mV versus Ag/AgCl). Using a single biosensor, only few successive measurements were performed. An eventual enzyme denaturation was checked at the end of the measurement by performing a new determination at pH 7.1. In order to reduce the errors produced by the differences between the analytical signals of different biosensors, data interpretation was made by reporting the measured current value at each pH to the one obtained at pH 7.1 with the same biosensor. The investigated pH range for DPIP was 6.7–8.7, as it was not possible to work at pH lower than 6.7. The maximum analytical signal using DPIP was obtained at pH 7.1. In the case of PMS, the investigated pH range was 6.2–8.7. The pH-related variations of the analytical signal were shown to be more important for this electrochemical mediator, the optimum response for PMS being observed at pH 7.8 (Fig. 2).

3.3. Biosensors characterization

The biosensors presented a satisfactory operational stability, as they allowed at least 10 successive determinations. The operational stability was checked for every lot of biosensors.

The reproducibility of the DPIP-based biosensors was tested by measuring the response to 1 mM malic acid using 0.2 mM DPIP at +50 mV versus Ag/AgCl. The average of the amperometric signal using the same biosensor for successive substrate injections was 351 ± 12 nA ($n=9$, R.S.D. = 3.3%). Reproducibility assays using 6 biosensors of the same lot showed a R.S.D. of 14% (361 ± 52 nA). The reproducibility between biosensors produced in different batches was 26% (386 ± 99 nA).

The reproducibility of PMS-based biosensors was comparable to the DPIP-based ones. It was studied using 0.3 mM PMS, 1 mM malic acid and a working potential of –10 mV versus Ag/AgCl. The average response of the same biosensor was 407 ± 25 nA ($n=8$, R.S.D. = 6.2%). Concerning PMS, an important source of errors was due to the poor stability of this mediator. The PMS stock solutions have to be kept in ice and used only during a few hours. A change of colour of PMS stock solution from yellow to green was an indication of mediator degradation.

The calibrations for malic acid were carried out by successive substrate additions. Under the optimised working conditions, a limit of detection of $5 \mu\text{M}$ malic acid and a plateau due to substrate saturation around 1 mM malic acid were achieved using DPIP as mediator. The calibration curve of MQO biosensors was linear from 5 to $250 \mu\text{M}$ of malic acid ($I(\text{nA}) = 853 \times C(\text{mM}) + 8.8$; $n=8$, $R^2 = 0.9912$), but the dynamic range was substantially larger since a polynomial relation was observed from 5 to $750 \mu\text{M}$ malic acid ($I(\text{nA}) = -678 \times C^2(\text{mM}^2) + 933 \times C(\text{mM}) + 10$; $n=13$, $R^2 = 0.9932$). The apparent Michaelis constant $K_M^{\text{app}} = 0.3$ mM was calculated using Lineweaver–Burk representation ($1/I(\text{nA}) = 0.0008 \times (1/C(\text{mM})) + 0.0019$; $n=19$, $R^2 = 0.9989$).

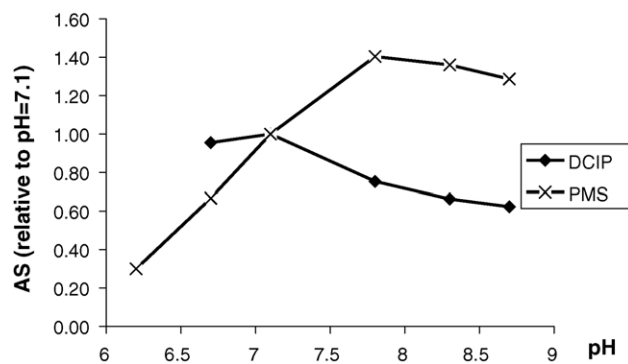


Fig. 2. Influence of pH on the relative magnitude of analytical signal (compared to response at pH 7.1). Experimental conditions: 0.2 mM DPIP, applied potential +50 mV vs. Ag/AgCl, or 0.3 mM PMS, applied potential –10 mV vs. Ag/AgCl, substrate: 1 mM malic acid in 0.1 M PBS.

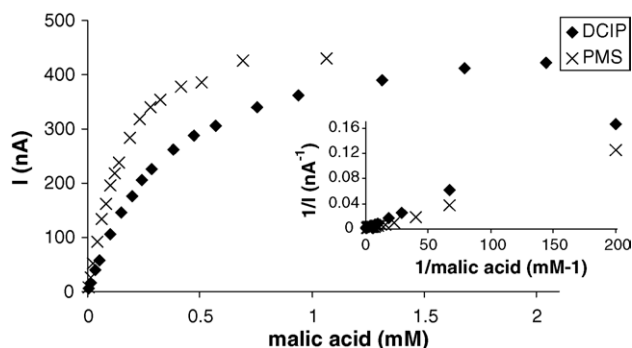


Fig. 3. Calibration graphs obtained for MQO biosensors using DPIP (0.2 mM, applied potential 50 mV vs. Ag/AgCl, pH 7.1) and PMS (0.3 mM, applied potential -10 mV vs. Ag/AgCl, pH 7.8). Inserted graph shows Lineweaver–Burk representation used to calculate apparent K_m .

The MQO biosensors based on PMS showed also a detection limit of $5 \mu\text{M}$ malic acid, but saturation was achieved at lower substrate concentrations than using DPIP. The calibration graph showed linearity for malic acid concentrations ranging from 5 to $150 \mu\text{M}$ ($I(\text{nA}) = 1740 \times C(\text{mM}) + 9.1$; $n = 9$, $R^2 = 0.9862$), whereas, the polynomial dynamic was ranging from 5 to $420 \mu\text{M}$ ($I(\text{nA}) = -2737 \times C^2(\text{mM}^2) + 1997 \times C(\text{mM}) + 7.7$; $n = 14$, $R^2 = 0.9946$). Lineweaver–Burk representation allowed to calculate the apparent Michaelis constant $K_M^{\text{app}} = 0.3 \text{ mM}$ ($1/I(\text{nA}) = 0.0004 \times (1/C(\text{mM})) + 0.0014$; $n = 15$, $R^2 = 0.9931$) (Fig. 3). The sensitivity of the biosensors, calculated from the linear part of the calibration curve, was 0.85 and 1.7 mA/M using, respectively DPIP and PMS as mediator.

The response time of the biosensors based on DPIP or PMS was 5 min , longer than when using BQ or potassium hexacyanoferrate-based sensors, which showed response time of $2\text{--}3 \text{ min}$. This is probably due to differences in the diffusion of the different mediators through the PVA-SbQ matrix. Using DPIP as mediator, longer times were needed for baseline stabilization, due to the tendency of DPIP to get adsorbed on the WE surface.

3.4. Real sample analysis

Red and white wines were analysed using both PMS and DPIP-based biosensors (Table 3). The samples were analysed directly or after enrichment with 10 mM malic acid. The analysis was performed by injecting $50 \mu\text{L}$ of sample solution in the cell ($1:100$ dilution with buffer). Differential measurements were carried out to remove the effect of interfering compounds.

Table 3
Analysis of wines using MQO-biosensors based on DPIP or PMS. Average of triplicate measurements

	DPIP (mM)	Recovery	PMS (mM)	Recovery
White wine	9.0 ± 2.7		9.6 ± 3.1	
White wine +10 mM malic acid	22.2 ± 6.8	132%	18.5 ± 5.5	89%
Red wine	4.1 ± 1.1		3.5 ± 0.9	
Red wine +10 mM malic acid	15.6 ± 4.9	115%	11.5 ± 3.3	80%

For that purpose, non-specific current was measured with an enzyme-free SPE and the obtained current values were subtracted from the analytical signals obtained with MQO biosensors. This procedure was used for both PMS and DPIP-based sensors, even if interferences were shown to be negligible using PMS. The results obtained for unspiked wine with both DPIP and PMS are in good agreement. The recovery percentages of enriched samples were between 80 and 132% , respectively (Table 3).

4. Conclusions

This work highlights the possibility to develop MQO-based amperometric biosensors for the determination of malic acid in wine. This enzyme presents a promising alternative for the development of NAD-independent malic acid sensors. Several electrochemical mediators have been tested, only DPIP, PMS, BQ and $\text{K}_3[\text{Fe}(\text{CN})_6]$ allowing to obtain a significant analytical signal. Among them, DPIP and PMS showed suitable electrochemical properties and could be applied for the malate biosensor development. The electrochemical mediator concentration, working pH values and applied potential were optimised. The DPIP-based biosensors possess the advantages of high stability, good reproducibility and wide dynamic range. Their main drawback consists in the important influence of the sample matrix on the analytical response and, furthermore, the fact that the DPIP has the tendency to be adsorbed on the surface of the WE. On the other hand, the PMS-based biosensors showed less influences due to interfering compounds, but they showed a lower dynamic range and suffered from the poor stability of PMS.

Further work will be focused in research or synthesis of new electrochemical mediators, able to shuttle the electrons from the enzyme to the WE. The ideal mediator should be stable, interact at a minimum extent with the redox components of wine, and should be incorporated in the WE to construct reagentless biosensors.

To our knowledge, this is the first attempt to construct a malic acid biosensor based on MQO. This paper demonstrates the possibility to produce this type of biosensor, however, a successful analytical device requires further improvements concerning the performance of the electrochemical mediator. The MQO presents the potential to produce alternative biosensors for malic acid, but adequate transducers and electrochemical mediators have still to be found.

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