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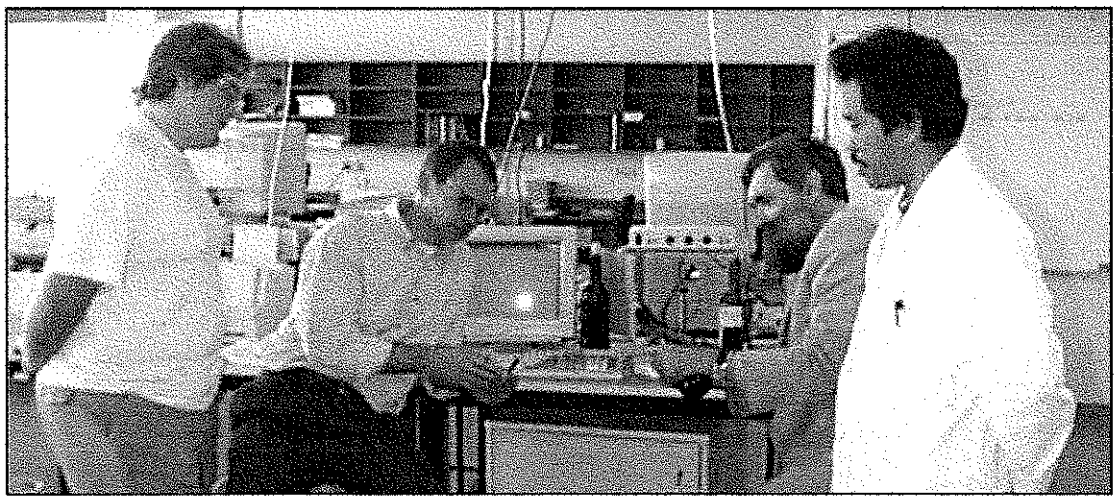
An Enzyme Biosensor for the Analysis of Malic Acid in Wine



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **UNSW 99/1**

Principal Investigator: **Dr. J. Justin Gooding**

Research Organisation: **The University of New South Wales**

Date: **25 September 2000**

Summary

Project Title: An enzyme biosensor for the analysis of malic acid in wine

Project no: UNSW 99/1

Supervisor: Dr. J. Justin Gooding
Staff: Mr. M. Situmorang, Mr. J. Ashmore

This project was a one year pilot project to assess the viability of developing an electrochemical enzyme biosensor for analysing malic acid in wine. If such an electrochemical biosensors was found to be viable the specific objectives it was required to fulfill were:

1) To develop an electrochemical biosensor for monitoring malic acid concentration in wine
2) To make the analyses cheap and accurate without requiring specialist knowledge, training or equipment
3) To utilise a protocol for the fabrication of this malic acid biosensor which is not only compatible with bulk manufacturing but is also generic to other enzymes so that biosensors for other analytes can easily be developed.

The expected outcomes of this project were
1) A prototype malic acid biosensor to allow the assessment of whether commercialisation of such a device is viable.
2) A prototype biosensor which has the capability of operating as a research instrument, thus allowing the monitoring of L-malic acid levels in wine in real time.
3) A generic methodology for the fabrication of biosensors which can successfully operate in wine so as to allow biosensors for other analytes of interest to the wine industry to be more easily developed.

The expected outcomes above were all met by developing a single enzyme electrode with a similar mode of operation to a pH electrode. In this system the enzyme malic dehydrogenase (EC 1.1.1.37) was immobilized over a Tungsten solid state pH electrode. The enzyme was immobilized over the electrode by covalent attachment to a polymer, polytyramine, which is electrochemically deposited over the Tungsten pH electrode. The resultant enzyme electrode was able to be fabricated with a high degree of reproducibility (less than 5% variability between electrodes), was very stable (no loss in response after three months storage), had a detection limit of 5mg/l mM and a linear range between 100 and 350 mg/l. These performance characteristics are compatible with the monitoring of malic acid in wine and outstanding for an enzyme electrode. The drawback of the enzyme electrode developed was it was also susceptible to interference from variation in solution pH. However, an effective measurement protocol should overcome this problem.

The malic acid enzyme electrode developed has highly promising performance characteristics with regards to its potential for commercialization. Of particular benefit is that it measures the concentration of malic acid in the same way as a pH electrode. Therefore, this sensor could be manufactured to be compatible with a standard pH meter, thus obviating the need for wineries to purchase expensive measurement equipment. This project was however only a pilot project and extensive research and development is required for commercialization of the prototype

biosensor. Whether commercialization is viable very much depends on size of the market for such a device as commercialization can prove expensive. It is recommended that evaluation of the industry need for such a device is conducted before further research support is dedicated to this initially promising sensor.

Background

The importance of malo-lactic acid fermentation in the evolution of some wines makes the quantitative analysis of L-malic acid highly desirable. However, analysis in a complex media such as wine is problematical because of the need to separate the analyte of interest from the rest of the sample. The instrumentation required to perform such separations is too expensive for the average wine laboratory and hence a cheap and easy method of performing L-malic acid determination, and similar such analyses, is required. Test kits marketed by companies such as as *Boehringer Mannheim* exploit the specificity of enzymes (L-malate dehydrogenase in this case) to obviate the need for separation steps. However, the UV transduction in these kits is prone to error due to the colour of many wines. Incorporation of electrochemical transduction with the appropriate enzyme to detect the L-malic acid would give a superior method of developing a cheap assay for L-malate. This coupling of electrochemistry with enzyme detection can be achieved by immobilising the enzyme onto the surface of an electrode and electrochemically monitoring a product of the enzyme reaction. The immobilisation of an enzyme onto an electrode to produce an analytical device is the basis of a biosensor.

Objectives

This project was a one year pilot project to assess the viability of developing an electrochemical biosensor for malic acid. If such an electrochemical biosensors was found to be viable the specific objectives it was required to fulfill were:

4) To develop an electrochemical biosensor for monitoring malic acid concentration in wine
5) To make the analyses cheap and accurate without requiring specialist knowledge, training or equipment
6) To utilise a protocol for the fabrication of this malic acid biosensor which is not only compatible with bulk manufacturing but is also generic to other enzymes so that biosensors for other analytes can easily be developed.

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4) A prototype malic acid biosensor to allow the assessment of whether commercialisation of such a device is viable.
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6) A generic methodology for the fabrication of biosensors which can successfully operate in wine so as to allow biosensors for other analytes of interest to the wine industry to be more easily developed.

Introductory Technical Information

Most Common Current Method of L-malic acid analysis – Enzyme Kits

The Analytical Services of the Australian Wine Research Institute use a simple enzyme analysis kit for Malic acid, and for several other wine analytes. These enzyme kits exploit the incredible specificity that some biological molecules have for their substrate. The test kit for L-malic acid marketed by Boehringer Mannheim (now part of Roche) is a classic example of how these test kits operate. The test combination contains the enzyme L-malic dehydrogenase (L-MDH) which selectively

converts L-malate to oxaloacetate without interference from other similar species in complex solutions such as D-malate or L-lactate. During this reaction the enzyme cofactor (nicotinamide dinucleotide, NAD^+) is reduced to NADH. The NADH produced can be monitored spectroscopically as it has a characteristic absorbance at 340nm. As the amount of NADH produced is related to the concentration of L-malate in solution, the monitoring of the absorbance produced in the enzyme assay is used to determine the amount of malic acid in the wine. The ability of L-MDH to specifically react with L-malate obviates the need for complex separation steps and hence limits the equipment requirements to a simple spectrophotometer for measuring the absorbance.

Despite their elegant simplicity, there are a number of limitations to these test kits. The main problem is the measurement of absorbance is a highly coloured media, such as red wine, suffers from error due to the wine absorbing light as well as the NADH. The result is an over estimation of the amount of analyte L-malate. This problem was identified by AWRI (Gishen *et al*, 1998) and strategies, which further complicate the analysis, based on preparing blanks or filtering the wine with polyvinylpolypyrrolidone (PVPP), were developed to circumvent this problem. However, with the exception of using colourless samples, that use of absorbance based methods of determining analyte concentrations in wine will always be complicated by the wines colour.

The use of PVPP to remove the colour of the wine and eliminate the creep problems is a viable approach for a dedicated analytical laboratory. However, the current malic acid measurement method is not viable for many wineries (hence the offering of such a service by AWRI) because:

1) the need for an instrument for the measurement of absorbance may be beyond the financial resources of many smaller wineries

2) the analysis requires a number of processing steps which therefore requires a highly trained analytical chemist to perform the analyses with acceptable precision.

A Possible Solution

In the spectroscopic enzyme assay for the detection of malic acid, the enzyme provides the detection part of the assay and the spectroscopic monitoring of the NADH produced is the transduction. The problem of creep and the expense of the equipment are all related to the transduction aspect of the assay. An alternative method of transduction which can overcome both these shortcomings is electrochemical. The combination of an enzyme to specifically detect an analyte and an electrode to transduce the extent of enzyme reaction is the basis of the most successful form of biosensor, an enzyme electrode. The classical example of such a device is the glucose monitors used by diabetics for measuring their blood glucose levels. In these devices the enzyme glucose oxidase is immobilised over an electrode by entrapment in a polymer membrane. By placing a drop of blood onto the device, the enzyme reacts specifically with glucose and in the process produces a species that can be detected at the electrode. Hence a low cost analysis can be conducted by untrained personnel without any sample preparation. An ideal method of analysis!

This concept of immobilising an enzyme over an electrode to form an analytical device can be generalised to any analyte provided there is an enzyme for the particular analyte. An additional requirement for enzyme electrodes is that one of the species involved in the enzyme reaction must b able to be detected at the electrode. If an enzyme exists but it does not produce a product that is electrochemically active, then it is possible to immobilise multiple enzymes which operate cooperatively so as to allow electrochemical transduction. Such a strategy is necessary with an L-malate enzyme electrode for two reasons. First, the mechanism of the reaction between L-malate and L-malate dehydrogenase are such that product

must be removed to avoid inhibition of the enzyme reaction and second, none of the products are easily detectable at normal metal electrodes.

So in a malic acid enzyme electrode an enzyme which reacts specifically with malic acid is required. The enzyme must produce a molecule which is detectable at the electrode. If this is not achievable with one enzyme then the malic acid detecting enzyme must be integrated with a second enzyme to produce the electrochemically active molecule. The enzyme(s) are then immobilized over the electrode by attaching them to a polymer film deposited on the electrode surface. The electrode can either be an amperometric electrode where the detected molecule from the enzyme reaction is oxidised when the appropriate voltage is applied to the electrode. Hence the current at the electrode is related back to the concentration of malic acid in the wine Alternatively, the electrode could be a potentiometric electrode where a change in concentration of the detected molecule causes a change in electrode potential. Amperometric electrodes are usually the preferred mode of operation for enzyme electrodes because they are more sensitive and less prone to interferences.

Enzyme Electrodes for L-Malate

There have been a few of attempts to develop L-malate enzyme electrodes (Mizutani *et al*, 1991; Karyakin *et al*, 1994; Palleschi *et al*, 1994; Messia *et al*, 1996; Gajovic *et al* 1997), all of which have utilise one of the two common forms of L-malate dehydrogenase, one from chicken liver (EC 1.1.1.40) and the other from porcine heart (EC 1.1.1.37) linked with other enzymes. The two successful enzyme combinations are shown below in schemes 1 and 2. In both schemes the second enzyme produces hydrogen peroxide as a product. It is this hydrogen peroxide which is the electroactive species required for transduction. The hydrogen peroxide is oxidized at the electrode, and the resultant current is related to the amount of malic acid in the wine. Most of these enzyme electrodes appear to have worked adequately for a given electrode, having detection limits of the order of 0.07mg/l and a linear range up to approximately 150mg/l. This performance compares favourably with the Boehringer-Mannheim test kits where the detection limit is quoted as 0.5mg/l and linear range to 350mg/l (Boehringer 1999). Furthermore, of these published devices, the enzyme electrodes used to analyse real samples of wine gave similar results to the

Scheme 1

$$\text{L-Malate} + \text{NADP}^+ \xrightarrow[\text{(EC 1.1.1.40)}]{\text{L-MDH}} \text{Pyruvate} + \text{NADPH} + \text{CO}_2$$

$$\text{Pyruvate} + \text{Phosphate} + \text{O}_2 \xrightarrow[\text{(EC 1.2.3.3)}]{\text{Pyruvate Oxidase}} \text{Acetylphosphate} + \text{CO}_2 + \text{H}_2\text{O}_2$$

Scheme 2

$$\text{L-Malate} + \text{NAD}^+ \xrightarrow[\text{(EC 1.1.1.37)}]{\text{L-MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+$$

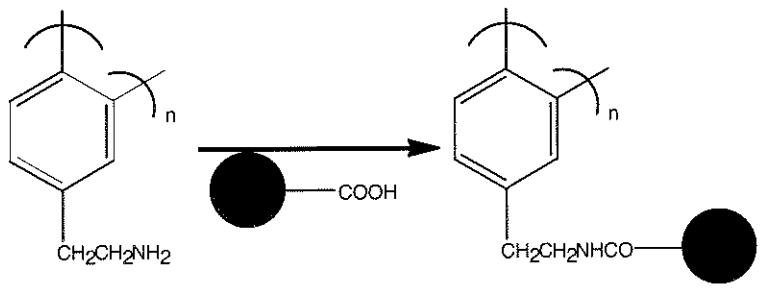
$$\text{NADH} + \text{H}^+ + \text{O}_2 \xrightarrow{\text{NADH Oxidase}} \text{NAD}^+ + \text{H}_2\text{O}_2$$

enzyme test kits.

The absence of any of these devices on the market was believed to be likely as a result of problems which have plagued all biosensors, namely irreproducibility between devices, interfering compounds reacting directly at the electrode and poor long-term stability. *These problems are related to having a poor system of immobilizing the enzymes.*

Research Methodology

Our previous research has been dedicated to solving the problems of irreproducibility and interferences in enzyme electrodes. The culmination of this research is the fabrication of the enzyme layer using the electrochemically deposited polymer, polytyramine, shown in figure 1. The essential features of polytyramine for immobilising enzymes are that the enzyme can readily be attached to the polymer backbone and the amount of polymer deposited onto the electrode surface can be very precisely controlled. By immobilising enzymes using this method we have produced enzyme electrodes for a variety of analytes (including glucose, lactate and sulphite) which **in all cases** have reproducibility superior to any published research or commercial devices (Situmorang *et al*, 1998; Situmorang *et al* 1999). Furthermore, the biosensors have also been shown to effectively screen out interferants in biological matrices (Situmorang *et al*, 2000). Thus, unlike many other studies, *this project utilized an effective method of fabricating the enzyme layer and concentrated on developing the assay with the specific requirements of sensing in wine.*



Polytyramine

Figure 1: Schematic representing the covalent attachment of enzymes to free amines on the polytyramine backbone. The covalent attachment maintains the enzyme within the polymer membrane, thus aiding the reproducibility and stability of the enzyme electrode.

The target method of fabricating the L-malate biosensor involved electrodeposition of a polytyramine film on a platinum electrode surface in the presence of the enzymes involved in the detection of L-malate. By depositing the polymer film in the presence of the enzymes, the enzymes become entrapped throughout the polymer film. Reagents are then added to allow a covalent bond to be formed between the enzymes and the polymer backbone as shown in figure 1. This coupling procedure involved placing the electrode into a solution of 0.015 M 1-ethyl-3(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.03 M N-hydroxysuccinimide (NHS), pH 5.5, for one hour at room temperature. It is necessary to prevent the enzymes leaching out of the polymer membrane, a problem associated with many previous enzyme electrodes for malic acid. The primary focus was the enzymes shown in scheme 1. In scheme 1 it is clear that besides the enzymes and L-malate, the cofactor $NADP^+$ is also required for malate dehydrogenase to operate and phosphate and oxygen are required for the pyruvate oxidase.

Part of the development philosophy during this project will be to allow bulk manufacturing requirements to dominate research decisions so as to ensure that any device developed can actually be commercialised. Targetting the sensor to the needs of the wine industry is vital to this process. The planned strategy for this pilot project is to demonstrate we can make the basic sensor using polytyramine in the first year.

The key features of the target method are

A. the reproducible method of fabricating the enzyme layer using polytyramine
B. covalent attachment of the enzymes to the polytyramine to provide enhanced stability
C. the specific detection of the malic acid using L-malic dehydrogenase (scheme 1)
D. the use of the second enzyme, pyruvate oxidase, to remove a product from the first enzyme reaction (hence driving the equilibrium towards the products) and to produce hydrogen peroxide for amperometric transduction (via oxidation at the platinum electrode at 0.65V versus Ag/AgCl).

One of the key aspects of this project relates to the stability of the enzymes and their ability to coexist in the same environment. Therefore, the process of determining the viability of the proposed enzyme system (scheme 1) are:

1) check each enzyme is compatible with the conditions for fabricating the polymer layer
2) verify the two enzymes can be co-immobilised within the polytyramine layer
3) make sure the subsequent covalent attachment of the enzymes does not affect their activity.

If the enzyme system can be reliably immobilized with retained activity the enzyme electrode will be

4) calibrated in ideal malate solutions to determine electrode performance properties
5) the performance characteristics will be optimized via variation of fabrication parameters
6) test calibrated enzyme electrodes in real wine samples.

Detailed Results

Schemes 1 and 2 outline two possible two-enzyme combinations which will allow an amperometric enzyme electrode to be fabricated for the detection of malic acid in wine. Scheme 2 was discarded as a viable option for a commercial biosensor because of the low activity, high expense and difficulty in supply of NADH oxidase. Therefore, scheme 1 where malate dehydrogenase (EC 1.1.1.40) was combined with pyruvate oxidase (EC 1.2.3.3).

Immobilisation of Pyruvate oxidase (PyOx)

According to the scientific literature, pyruvate oxidase has successfully been immobilised by itself in a number of studies (Arai, 1999, Mascini, 1987, Peguin, 1989). These studies however, give no information regarding the stability of the resultant sensor. An important aspect of this project was being able to successfully immobilise pyruvate throughout the polytyramine matrix and then covalently attach is to the polymer using a cross-linking reagent.

The procedure we developed previously (Situmorang, 1999a, Situmorang 1999b) for the immobilisation of a variety of other enzymes was investigated as a method of immobilising pyruvate oxidase. In brief this procedure involves electrochemically depositing the polymer layer in the presence of the enzyme in a phosphate buffer solution at pH 8. In this way the enzyme is entrapped throughout the polymer matrix. The enzyme is then covalently attached to the polymer by soaking a solution of 0.015

M 1-ethyl-3(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.03 M N-hydroxysuccinimide (NHS), pH 5.5, for one hour at room temperature. The covalent attachment prevents the enzyme leaching out of the polymer membrane during use. Unfortunately, pyruvate oxidase was found to be a particularly fragile enzyme and all enzyme activity was lost during this procedure. Therefore, pyruvate oxidase was incompatible with the standard method of immobilizing throughout a polytyramine membrane.

A variety of other immobilization procedures were attempted with little success (see table 1). The one successful method of immobilizing he enzyme shown in table 1 involves electrodepositing the polytyramine and then cross-linking the enzyme to the surface of the polymer using EDC under humid conditions. The response of this enzyme electrode to pyruvate is shown in figure 2. The excellent performance characteristics are shown in table 2. The selectivity of the pyruvate enzyme electrode was investigated and found to only respond to pyruvate and not to potential interferences such as malate.

Table 1: Enzyme-immobilisation procedures to attach the enzymes onto the surface of polytyramine and throughout polytyramine film.

Immobilisation method	Enzyme	Crosslinking agents	Results
On the surface of polytyramine	PyOx or ME	EDC/NHS or Glutaraldehyde	-
	PyOx and ME	EDC/NHS or Glutaraldehyde	-
Throughout the polytyramine	PyOx or ME	EDC/NHS	-
	PyOx and ME	EDC/NHS	-
On the surface of polytyramine at humid condition	PyOx or ME	EDC or Glutaraldehyde	+
	PyOx and ME	EDC or Glutaraldehyde	-
Onto a self-assembled monolayer	PyOx or ME	EDC/NHS	-

- Unsuccessful + Successful

Table 2: Performance characteristics of enzyme electrodes where either pyruvate oxidase or malic dehydrogenase (EC 1.1.1.40) were immobilized onto the surface of an electrodeposited polytyramine layer

	PyOx electrode	ME Electrode PyOx in solution
Linear range /mM	0.1 – 3.0	0.5 – 2.5
Detection limit /mM	0.05	0.01
Sensitivity / $\mu A\ mM^{-1}$	0.42	0.272
Reproducibility	3.9 – 9.8%	-

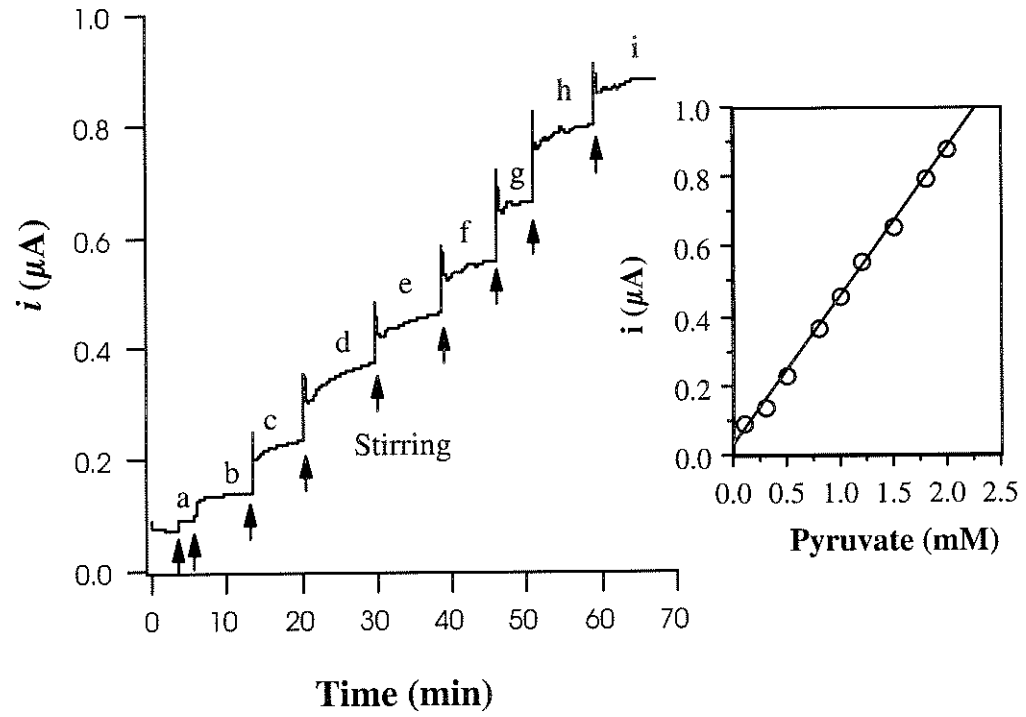


Figure 2: Typical current-time response for enzyme electrode for successive injection of: (a) 0.1, (b) 0.3, (c) 0.5, (d) 0.8, (e) 1.0, (f) 1.2, (g) 1.5, (h) 1.8, and (i) 2.0 mM pyruvate in trisma buffer solution (0.01 M, pH 7.24) containing 0.04 M KH_2PO_4 and cofactors (2 μM TPP, 2 μM FAD and 1 mM Mg^{2+}). The Insert is the calibration curve of the corresponding concentration of the pyruvate.

Immobilisation of Malic Dehydrogenase (ME) EC 1.1.1.40

Prior to co-immobilising the ME with the pyruvate oxidase, the ability to immobilise ME successfully was investigated. The ME was immobilised using the same procedure as for the PyOx. If PyOx was present in solution, the Malate enzyme electrode responded to malic acid with good performance characteristics, Table 2.

Co-immobilisation of PyOx and ME

As a method was found by which both enzymes could be successfully immobilised with retained activity, the next step in the evolution of the enzyme electrode was to co-immobilise both enzymes in the one step. Unfortunately, when co-immobilised, the result was loss of activity of both enzymes. The loss of activity of both enzymes was confirmed by spiking active enzyme into solution and checking for a response to Malic acid or pyruvate. Personal communication with two world experts on enzyme electrodes, Palleschi in Italy, who has published extensively on malic acid enzyme electrodes, and Turner in England, who is currently trying to develop such a device, revealed that they too found such incompatibility between these two enzymes.

The incompatibility of the two enzymes and the difficulty in using PyOX meant a decision was made to divert from the original strategy of developing an amperometric enzyme electrode to a single enzyme strategy where the electrode would operate potentiometrically.

Single Enzyme-potentiometric strategy

Potentiometric transduction is familiar to all wineries through a pH electrode. This new strategy involved the second form of L-malic dehydrogenase (EC 1.1.1.37) shown in scheme 2. In the first reaction sequence in scheme 2, it can be seen that the reaction of L-MDH with malate in the presence of NAD^+ release an acidic proton, H^+, into solution. The increase in concentration of H^+ adjacent to the electrode can be detected with a pH electrode. Therefore, the second approach investigated to develop an enzyme electrode for malate involved immobilising L-MDH throughout a polytyramine membrane over a pH sensitive electrode.

The pH electrode

A standard glass pH electrode cannot be used for this purpose as to deposit the polytyramine layer requires a metal electrode. Therefore, a tungsten electrode for measuring changes in pH was used. Tungsten has been reported previously to be able to measure changes in H+ concentration due to an enzyme reaction (Situmorang 1999c). Tungsten was found to be linearly sensitive to H+ in the potential range of 5.2 – 11.4 with a non- ideal slope of 29mV. The slope and pH range where the response of the tungsten electrodes to H+ when coated with polytyramine remain unchanged. This is an exceedingly positive result as it shows the polytyramine does not influence the pH detecting ability of the electrode. Therefore, modification of a tungsten electrode with L-MDH modified polytyramine should be able to detect malate due to the protons produced in the enzyme reaction.

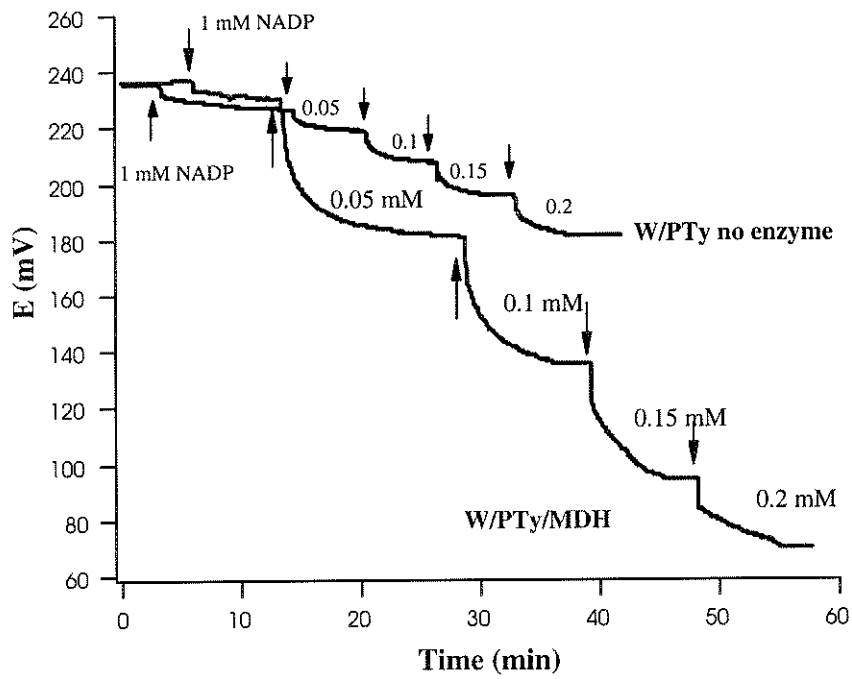


Figure 3: Response of the MDH modified electrode (W/Pty/MDH) with successive additions of malic acid and the response of the same electrode without MDH immobilised (W/Pty no enzyme). The measurement were performed in a background solution of 1mM NADP in 0.01 M Trisma buffer, at pH 8.0.

Immobilisation of L-MDH (EC 1.1.1.37)

The L-MDH was immobilised during the electrodeposition of the polytyramine. The enzyme was then covalently attached to the polymer backbone using the EDC/NHS coupling reagents as described above for pyruvate oxidase and previously for glucose oxidase, sulfite oxidase, lactate oxidase and amino acid oxidase (Situmorang, 1999a; Situmorang, 1999b). The immobilised enzyme was shown to retain its activity during the immobilisation procedure as shown by a change in the potential of the tungsten pH electrode in the presence of the enzyme cofactor NAD^+ upon the addition of malic acid. If the enzyme was not present, or if NAD^+ was not added, the tungsten electrode still gave a response to the addition of malic acid but the change in potential was far less significant. The response of the electrode in the absence of the enzyme is due malic acid being a weak acid and therefore increasing its concentration results in a change of pH.

The change in potential with successive additions of malic acid for the L-MDH enzyme electrode and an electrode modified with polytyramine but not enzyme is shown in figure 3. The calibration curves for these two different electrodes are shown in figure 4. As can be seen, there is a much large change in potential when the enzyme is present indicating the enzyme is converting the malate to oxaloacetate and in the process a proton is release which changes the pH. The difference in signal between the electrode with and without enzyme can then be used to determine the malic acid concentration of a wine.

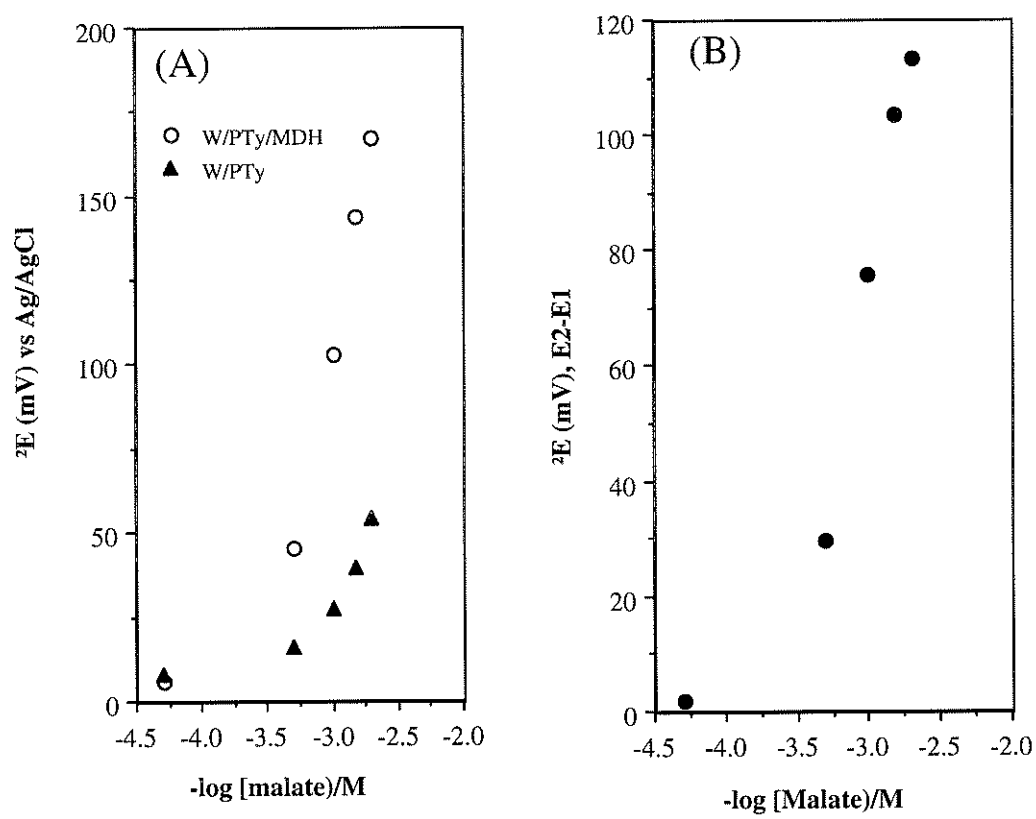


Figure 4: Calibration curves for the response of tungsten electrodes to malate. (A) The response of the MDH-polytyramine modified electrode relative to the same electrode modified with polytyramine alone and no enzyme. (B) The difference in response between the two curves in figure (A).

Performance Characteristics

The plots in figures 3 and 4 show that L-MDH can be immobilised in a polytyramine matrix with retained activity. Thus a potentiometric malate enzyme electrode has been successfully developed. The next stage after the demonstration of a working system is to characterise the enzyme electrodes performance and investigate how to tune the response of the sensors. In the short time period of this project the reproducibility, the stability of the enzyme electrode and the influence different fabrication parameters have on the response of the enzyme electrode were investigated.

The influence of the amount of enzyme-modified polymer deposited on the electrode (represented in the number of growth cycles of the polymer) on the performance of the enzyme electrode is illustrated in figure 5 and summarised in table 3. The figure and table show that the thicker the enzyme layer over the electrode the greater the sensitivity of the resultant enzyme electrode but the slower the response time. The variation in response with enzyme layer thickness provides the power to tune the response of the manufactured sensor depending on the requirements of a given application.

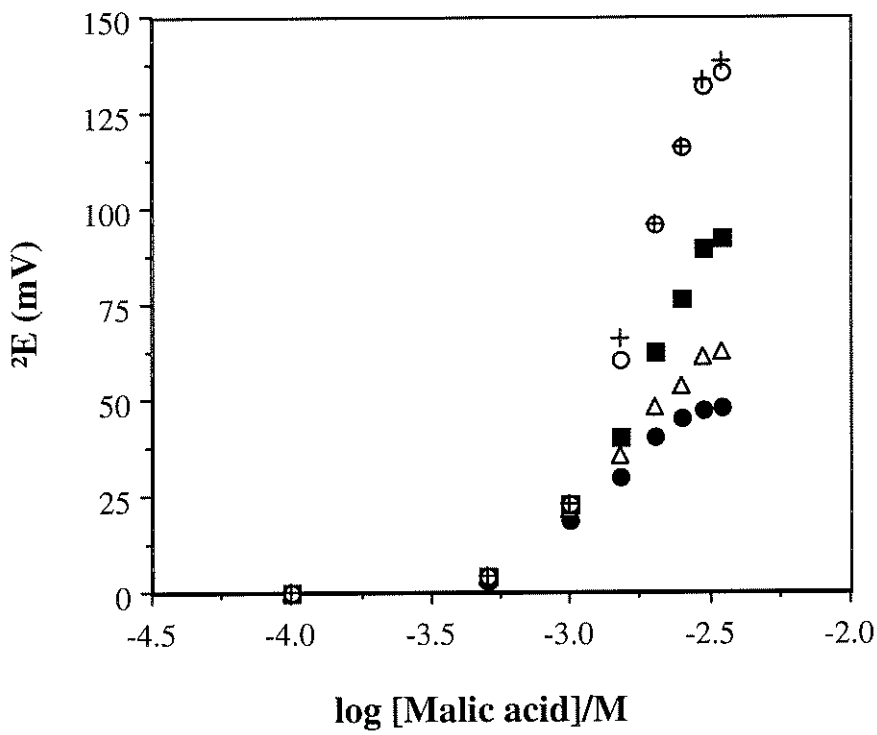


Figure 5: Calibration plots for malic acid biosensors as a function of the number of voltammetric cycles for which the polytyramine films were grown on tungsten electrode at 0.5 V s^{-1} *vs* Ag/AgCl between –0.2 V and +0.5 V in 0.1 M tyramine solution containing 500 units ml^{-1} MDH in methanol-phosphate buffer (1:3). Electropolymerization were conducted at (●) one cycle, (Δ) 3 cycles, (■) 5 cycles, (O) 10 cycles and (+) 15 cycles.

Table 3: The influence of the amount of enzyme modified polymer on the performance characteristics of the malate potentiometric enzyme electrode.

Electrodeposition sweep cycles	Polytyramine (mmol cm^{-2})[a]	Sensitivity (slope, mV M^{-1})	Response time (minutes)[b]
1	0.053	35.60	5.2
3	0.111	44.57	6.4
5	0.150	64.28	8.3
10	0.224	96.55	9.0
15	0.283	97.93	11.2

For the electrode fabricated using 10 cycles, the detection limit was 0.05mM (5.5 mg /l) and the calibration curve was linear between 1 and 3 mM (100 – 350 mg/l). The inferior detection limit and linear range compared with some previously reported enzyme electrode reflects the potentiometric rather than amperometric transduction. The performance of the potentiometric electrode however does appear to be satisfactory for the analysis if malic acid in wine. Repeated fabrication of the enzyme electrode on different days produced a device with a relative standard deviation of less than 5% across the entire concentration range. This is outstanding reproducibility makes the approach highly promising with regards to bulk manufacture and such low variability could allow devices to be calibrated before shipment to the end-user. The long-term stability of malic acid was assessed by storing several enzyme electrodes dry at 4 °C. The enzyme electrodes showed negligible loss of sensitivity compared with a one day old device after three months of storage. Such stability is a very promising result as it has important consequences with regards to the storage of a commercial enzyme electrode.

Discussion of Results

The primary objectives of this one year pilot project was to assess the viability of developing an electrochemical biosensors for malic acid. The original target enzyme systems were those shown in schemes 1 and 2. These dual enzyme systems were chosen as in both cases the one of the end products of the enzyme reaction was hydrogen peroxide which could be oxidized at the electrode to produce a current. The advantages of amperometric enzyme electrodes, over potentiometric enzyme electrodes is the ability to discriminate between small changes in concentration (sensitivity) and selectivity of the electrodes response. Hence the target of an amperometric system for this pilot project.

In the case of an enzyme electrode for malic acid, the drawback of the amperometric system is the need for two enzymes to obtain a product that can be detected at the electrode. This drawback is emphasized in the detailed results presented above for scheme 1 where malic dehydrogenase (ME) and pyruvate oxidase (PyOx) are combined. In many ways, this enzyme system demonstrates one of the problems of many enzyme electrodes, the delicate nature of some biological molecules. Although both enzymes could be immobilized with retained activity this could only be achieved under very special conditions which really weren't compatible with a commercial manufacturing operation. The fact that the two enzymes could not be immobilized together, because the enzymes inactivated each other, *made the amperometric two*

enzymes systems unviable. Hence the change in direction towards a single enzyme system with potentiometric transduction.

The potentiometric electrode that all wineries are familiar with is the pH electrode. In this electrode the concentration of H^+ ions adjacent to the electrode influences the potential of the pH electrode relative to a reference electrode. With the malate potentiometric electrode developed in this project, one of the products of the enzyme reaction between malate dehydrogense (EC 1.1.1.37, L-MDH) is H^+ ions. Hence immobilising L-MDH over a pH sensitive electrode enables the determination of the concentration of malic acid in a solution. As shown in figures 3 and 4 this is successfully achieved by immobilising the enzyme within a polytyramine membrane over a tungsten electrode.

The advantages of this approach are

1) Only one enzyme is employed making the system simpler
2) The advantages of polytyramine with regards to reproducibility can still be employed.
3) Most wineries already possess a pH electrode and therefore apart from the sensor no specialist equipment would be required on the part of the wineries.

The disadvantages are

1) potentiometric transduction is less sensitive to small changes in analyte concentration than amperometric sensors
2) The sensor is sensitive to interferences from changes in wine pH.

Apart from the advantages outlined above the particularly positive aspect of the potentiometric malate enzyme electrode developed are firstly the fact that a solid state potentiometric electrode is used which is compatible with manufacturing a device and secondly that the enzyme was easy to immobilize and was relatively stable. Secondly, the excellent reproducibility of less than 5% variability between electrodes. This reproducibility overcomes one of the common problems of enzyme electrodes. Such reproducibility has important implications with regards to commercialization as it could mean only a single electrode in a batch need be calibrated and the rest of the electrodes could be shipped with calibration information. This would only be possible of course if there is no loss in response with time. Here the excellent storage stability of the devices become all important. In the case of the prototype no loss in response after three months storage in a fridge is very promising. The major drawback of this potentiometric sensor is the fact that the sensor also responds to other forms of acid. However, either appropriate measurement protocols or use of a blank recording electrode should overcome this drawback.

It is important to emphasize that in the single year of the project the majority of the research efforts were directed towards the amperometric system and the potentiometric system was only successfully developed at the end of the project. Therefore the results presented here are only preliminary data and further evaluation is required. Despite this the system looks very positive and the potential for it to be integrated with pH meters already located in winery laboratories is exceedingly attractive. At this stage the type of research that needs to be done includes

1) Developing a research laboratory protocol for dropping the electrode into a sample already containing malic acid. Such a protocol would include determining whether the sample should first be buffered, whether it should be diluted and whether a blank measurement needs to be taken prior to the malic acid measurement.

2) using the electrode to measure the malic acid content in real wine sample.
3) Validating this measurement using the *Boehringer-Mannheim* malic acid enzyme kits
4) Investigate long term storage stability of the enzyme electrode
5) Develop method of isolating the required enzyme cofactor, NADH, within the enzyme layer such that it does not require the user to add.
6) Identify methods of fabricating each component which is compatible with manufacturing a real sensor
7) Develop an end user measurement protocol and validate this protocol and the final enzyme electrode.

Although this pilot project has come to the end of its term steps 1) to 3) above are still being explored at UNSW by a completing Ph.D. and honours students. Steps 4) to 7) are only viable if the preliminary results presented are considered promising enough by the GWRDC and wine industry for the final target of a malic acid enzyme electrode to be pursued. The commercial viability of the final device very much depends on the volumes that are expected to be required. A visit to the Analytical Services Department of the Australian Wine Research Institute indicated that they perform a few analyses for a winery each year. However, the chief investigator was also contacted via email by one winery about whether a malic acid sensor was already available indicating keen interest in some sectors of the wine industry.

Implications and Recommendations

The primary objective of this pilot project to ascertain the viability of an enzyme electrode for the measurement of malic acid in wine was successfully achieved. The potentiometric system developed in the latter phase of this project certainly shows that there is considerable promise in such an approach.

Of the objectives and expected outcome stated in the original project proposal and the top of this report, the first objective and all three outcomes were successfully achieved. The successful achievement of objectives 2) and 3) will rely on a further research commitment by the research organization and the GWRDC. The implications of successfully developing an enzyme electrode for malic acid are it will provide wineries with the capability to perform quick analyses of malic acids in wines and musts relatively cheaply.

It is important to emphasize that was has been achieved thus far is the easy part of the fabricating of a sensor for wine. The original cautious approach for funding, requesting only a small amount of funding for a single year, was employed because of the difficulties in commercializing enzyme electrodes. This strategy therefore allows the GWRDC to evaluate the initial research effort and decide whether to make a greater commitment to the development of a malic acid enzyme electrode.

The recommendations of the researchers are:
1) the size of the market for a malic acid enzyme electrode should be identified by the GWRDC.
2) Considering the potential of the device developed in the original pilot project if the market is considered sufficiently large for a commercial device to viable a suitable consortium comprising the researchers and appropriate wine industry figures should be invited to plan a larger scale project.

Intellectual Property

No patent has so far been applied for this research.

Enzyme electrodes is a reasonable heavily patented field and therefore few broadly encompassing patent opportunities still exist. Of the components used in the above project, the use of tungsten electrodes as pH electrodes and their integration with enzymes, the use of polytyramine membranes for the immobilization of enzymes and the use of malic dehydrogenase (EC 1.1.1.37) for a potentiometric enzyme electrode are already in the public domain. However, the combination of these components has not previously been made public are therefore a patent opportunity may exist in the process of combining the three.

Technical Summary

A malic acid enzyme electrode has been developed which is capable of analyzing the concentration of malic acid in solutions such as wine. The enzyme electrode is composed of

1) a tungsten electrode which is sensitive to the concentration of H^+ ions adjacent to its surface (a pH electrode)
2) a membrane of electrodeposited polytyramine
3) incorporated within the membrane was covalently attached malic dehydrogenase (EC1.1.1.37) which provides the electrode with selectivity to malic acid

The enzyme reacts with the malic acid according to the following mechanism

$$\text{L-Malate} + NAD^+ \xrightarrow[(EC\ 1.1.1.37)]{\text{L-MDH}} \text{Oxaloacetate} + NADH + H^+$$

Therefore, when the electrode is placed into a solution containing malic acid, H^+ ions are produced and the electrode gives a response proportional to the concentration of malic acid. The integration of these components represents a new methodology for the measurement of malic acid.

This enzyme electrode could be made compatible with a pH meter already available within a winery, is reasonable stable, can measure malic acid in the concentration range of 1-3 mM and can be fabricated with less than 5% variability between electrodes.

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