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# Reagentless enzyme electrode for malate based on modified polymeric membranes

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**Abstract**

A series of polymeric membranes have been employed as outer barriers in an amperometric malate dehydrogenase (MDH)/diaphorase (DI) or MDH/DI/$NAD^+$/mediator enzyme electrode for the determination of malate in undiluted neutral or acidic media. MDH/DI were physically entrapped with $NAD^+$ and mediator in a mixed ester cellulose membrane. Outer membranes such as non-anionic surfactant-modified cellulose acetate/Tween-80 and unplasticised spin coated PVC/polycarbonate (PC) resin in conjunction with an ascorbate oxidase (AOD) layer were utilised. Mechanical strength, thickness studies and diffusional properties of the membranes were investigated. Hexacyanoferrate(III), 2,4-dichlorophenolindophenol (DCPI) and naphthoquinone (NQ) were tested as mediators for enzymatically produced NADH using cyclic voltammetry. Analytical utility of the sensors is demonstrated. 

*Keywords:* Biosensors; Malate; Membranes; Reagentless measurements

## 1. Introduction

Citrus fruits are the most important tree fruit crop in the world, with production far exceeding that of all deciduous tree fruits (apples, pears etc.) [1]. Organic acids, mainly citric and malic, with traces of others, constitute ca. 10% of the total soluble solids of a non-acid fruit. The current testing procedures used within the fruit industry are: *juice content*, *total soluble solids* (°Brix) and *total acid content* [2]. The above tests leave a lot to be desired in terms of objectively measuring taste. Individual acids may vary in taste, although probably to a lesser degree.

Malic acid is widely distributed in small amounts in many natural food products. It is the predominant acid in many fruits and vegetables and the second highest in concentration in citrus fruits, various berries, figs, beans and tomatoes [3]. There is a need to develop selective methods based on existing biosensor technology to determine malic acid, which is a taste determining constituent of fruits and fruit juices. An available non-instrumental AOAC [4] method is time consuming, tedious and therefore not suitable for routine analysis. Whilst instrumental methods based on ion chromatography with a quartz crystal detector [5], gas chromatography [6], infra-red mass spectroscopy (IR-MS) [7], capillary

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isotachophoresis [8], liquid chromatography [9,10] and sequential injection-Fourier transform infra-red (FTIR) spectrometry [11] have been reported, and are suitable for routine analysis, they do not provide the analytical simplification of biosensors.

The enzymatic pathway exploited for the determination of malate is

$$\text{malate} + \text{NAD}^{+} \overset{\text{MDH}}{\leftrightarrow} \text{oxaloacetate} + \text{NADH} + \text{H}^{+} \quad (1)$$

$$\text{NADH} + \text{H}^{+} + \text{Mox} \overset{\text{DI}}{\leftrightarrow} \text{NAD}^{+} + \text{Mred} \quad (2)$$

$$\text{L-ascorbate} + \tfrac{1}{2}\text{O}_2 \overset{\text{AOD}}{\rightarrow} \text{dihydroascorbate} + \text{H}_2\text{O} \quad (3)$$

where MDH is malate dehydrogenase; Mox/Mred are the oxidised/reduced forms of the mediator; DI is diaphorase and AOD is ascorbate oxidase.

Many enzymatic methods have been developed using soluble [12,13] or immobilised enzyme(s) [14–18] combining reaction schemes (1) and (2) [14,15,18]; or using MDH and aspartate aminotransferase [12], or using malic enzyme (EC 1.1.1.40) and pyruvate oxidase [16,17]. An amperometric enzyme electrode based on MDH and NADH oxidase [19] and a fermentation control method based on immobilised *lactobacillus casei* cells [20] have also been reported. A bi-enzyme electrode for malate based on co-immobilised malic enzyme and salicylate hydroxylase was recently reported by Gajovic et al. [21].

The majority of the proposed methods have good analytical characteristics and are suitable for routine analysis, but are not applicable to undiluted sample matrices, for example fruit juices, in which the malate concentration exceeds the enzyme $K_m$ (200 μM for both malate and $NAD^+$) [22] by two or more orders of magnitude (>5 mM malate) and where the pH is acidic (<4) [3]. Additionally, the high polarising voltage for amperometric NADH detection, typically +650 mV versus Ag/AgCl, leads to serious interference from electro-oxidisable species, especially in citrus fruits where ascorbate up to 3 mM may be present.

The present work explores the feasibility of special membrane structures with the required properties of malate permeability, retention of co-factor and mediator, response linearity over the environmental concentration range and protection of enzymes from low pH deactivation. An AOD outer layer has been utilised for the effective elimination of ascorbic acid interference.

## 2. Experimental

### 2.1. Apparatus

All measurements were performed using an amperometric cell supplied by Rank Bros. (Bottisham, Cambs, UK), consisting of a platinum anode (1.6 mm diameter) and an annular silver pseudo-reference electrode, with an overlying Perspex sample well incorporated, enabling direct magnetic stirring to be employed over the electrodes. Polarisation of the working electrode was performed using an 'in house' potentiostat (Department of Chemistry Workshop, University of Newcastle upon Tyne, UK) of +/−1.2 V range, giving a current display in nA and an output to a strip chart recorder (Goertz–Matrawatt, Vienna).

Cyclic voltammetry experiments were carried out in a voltammetric cell (VC-2, Bioanalytical System, W. Lafayette, IN) using a computer controlled potensiostat [14]. A Fourier transform infra-red (FTIR) spectrometer (model 550, Nicolet Instrument, Warwick, UK) was used for the determination of membrane thickness [23].

### 2.2. Diffusion chamber studies

Diffusion coefficients of glucose, ascorbate, malate and citrate were determined using an in-house diffusion chamber consisting of two chambers with stirrers, separated by a membrane through which analyte could pass from one chamber to the other [24]. A membrane, ca. 4 cm in diameter, was placed between the chambers and solutions of glucose (1.0 mol $l^{-1}$), ascorbic acid (1.0 mmol $l^{-1}$), malic acid (0.1 mol $l^{-1}$) or citric acid (0.1 mol $l^{-1}$) were placed (each one separately) in one chamber. Samples were withdrawn from the second chamber at intervals for analysis to determine transmembrane fluxes. Stirring at ca. 600 rpm avoided a significant unstirred layer. Glucose and ascorbic acid were determined directly by anodic oxidation (Rank Bros. Electrochemical Processor, model 1000). Malic and citric acids were determined conductimetrically

with a conductivity bridge (WPA CMD 400) and cell (WPA CDC 80).

### 2.3. Chemicals

MDH (EC 1.1.1.37) from pig heart, 50% in glycerol, 1200 U $mg^{-1}$ was supplied by Boehringer (Mannheim, Germany). AOD (EC 1.10.3.3) from *Curcubita* sp., 538 U $mg^{-1}$, was obtained from Biozyme (Poole, UK). DI (EC 1.8.1.4) from *Clostridium kluyveri*, 5.8 U $mg^{-1}$ solid, $NAD^+$, NADH, L-malic acid (disodium salt), ascorbic acid, albumin (Bovine, Fraction V) and Pluronic F-68, were obtained from Sigma (Poole, UK). Cellulose acetate (39.7% acetyl content), PC resin (secondary standard), Tween-80, Span-85 (sorbitan trioleate-85) dichlorophenolindophenol (DCPI) and naphthoquinone (NQ) were obtained from Aldrich (Gillingham, Dorset, UK). Potassium hexacyanoferrate(III), glutaraldehyde (25% v/v solution, EM grade), aluminium oxide (particle size 0.3 μm), and poly(vinyl chloride) (MW 200 000 Da) were from BDH (Poole, UK). Tetrahexyl ammoniumbenzene (THAB) was purchased from Fluka (Deisenhofen, Germany).

A 50 mM phosphate buffer solution, pH 7.8, containing 51.3 mmol $l^{-1}$ NaCl and 2.1 mmol $l^{-1}$ EDTA was prepared in purified water (Milli-Q, Millipore). All solutions were made up with this buffer and the pH was adjusted, if necessary, to the desired values with HCl or NaOH.

### 2.4. Membranes

Cuprophan dialysis membranes used as support membranes in the enzyme laminates were obtained from a haemodialysis cartridge (Gambro, Sweden). High protein-binding membranes (HA, mixed esters of cellulose acetate and cellulose nitrate, 0.45 μm pore size), were purchased from Millipore.

#### 2.4.1. Enzyme laminate fabrication

A composite solution of MDH (208 U $ml^{-1}$), DI (8.7 U $ml^{-1}$) and albumin (0.1 g $ml^{-1}$) was prepared in 0.1 M phosphate buffer, pH 7.8. 10 μl of MDH/DI–albumin solution and 5 μl of glutaraldehyde (5%, v/v) in buffer were mixed rapidly and placed on a 1 $cm^2$ portion of dialysis membrane.

For the AOD layer, a composite solution of 10 mg of AOD (5380 U) and 100 mg of albumin in 10 μl of 0.1 M phosphate buffer, pH 7.8, and 5 μl of 5% glutaraldehyde solution was pipetted onto a 1 $cm^2$ dialysis membrane. In both cases, a further 1 $cm^2$ portion of dialysis membrane was placed on the top and glass plates were used to compress the enzyme film so that it was evenly distributed between the membranes.

#### 2.4.2. Physical adsorption onto nitrocellulose membranes

Physical adsorption of MDH (208 U $ml^{-1}$) and DI (8.7 U $ml^{-1}$) was carried out by pipetting the composite enzyme solution onto nitrocellulose membranes, which were allowed to air-dry for 60 min. Any unattached protein was removed by washing the membranes with buffer. In the 'reagentless' configuration, in addition to the enzymatic solution, 5 mM $NAD^+$ and 0.25 mM hexacyanoferrate(III) or DCPI in buffer solution were also pipetted.

#### 2.4.3. Solvent cast membranes

Modified cellulose acetate membranes were cast from 5 ml of 5% (w/v) cellulose acetate and 1–10% (v/v) Tween-80 non-anionic surfactant (density = 1 g $ml^{-1}$), or 5% (v/v) Span-85, or 5% (v/v) THAB, or 5% (v/v) Pluronic F-68 in acetone. Membrane casting was carried out following one of two strategies; solvent casting into a covered Petri dish and in a controlled casting chamber.

#### 2.4.4. Spin coating

PVC/polycarbonate resin (PVC/PC) polymer composites were cast from a solution of 2.4% PVC (w/v) and 2.4% (v/v) PC resin in tetrahydrofuran (THF), spin coated under conditions of rapid rotation (250 pm). Excess of polymer solution was spun off, leaving a thin film of polymer composite and minimal residual solvent to evaporate, thus minimising the time required for membrane formation [25].

### 2.5. Electrode assembly

Before use, electrodes were polished with aluminium oxide slurry, washed thoroughly with purified water and covered with a small volume of buffer and membrane(s). High protein binding membranes bearing MDH/DI or MDH/DI/NAD/hexacyanoferrate(III),

Table 1
Mechanical properties of membranes

| Membrane | Mechanical strength and flexibility | Suitable for use within the amperometric cell |
|---|---|---|
| Cuprophan dialysis | good | yes |
| Cellulose acetate | poor[a] | yes (internal membrane) |
| Cellulose acetate/surfactant | good | yes |
| Plasticised PVC | good[b] | yes (if supported) |
| Unplasticised PVC | good | yes |
| PVC/PC | good | yes |

[a] Too brittle for reliable use as external membrane but suitable as internal membrane.
[b] Difficult to handle and required support on another membrane for use in cell.

additional dialysis, surfactant modified cellulose acetate, or PVC/PC membranes were superimposed. The baseline current in buffer (<5 nA) was attained before measurement. All measurements were carried out at room temperature.

## 3. Results and discussion

### *3.1. Membrane evaluation — diffusion chamber studies*

Applied membranes were evaluated in physical terms and by their diffusional properties. The mechanical handling strength of each membrane was initially assessed in order to determine its suitability for use within the amperometric test cell. Membranes that were excessively brittle or too easily damaged during handling and testing were rejected. Brittleness was considered a problem if membranes cracked or were irreversibly distorted on folding, and fragility was also a problem if transfer from one solid surface to another led to partial tearing. Mechanical properties of the membranes are described in Table 1.

An FTIR spectrometer was used for the determination of membrane thickness [23]. The distance between successive interference fringes on the IR spectrum of the membrane was determined. The advantages of this method over the conventional micrometer method were that it is a non-contact method, therefore causing minimal damage, and that it proved to be more accurate for thin membranes (<10 μm). Calculated values of membrane thickness are given in Table 2.

The values of the diffusion coefficients, $D$ ($cm^2\ s^{-1}$), were calculated from Fick's First Law, the integrated Eq. (4). The results as determined by this method are given in Table 3:

Table 2
Membrane thickness measurements (μm)

| Membrane | By micrometer | Weighing[a] | IR |
|---|---|---|---|
| Cellulose acetate | 120 | 122 | 123 |
| Cuprophan dialysis | 13 | – | 14 |

[a] Calculated by weighing a known area of known density.

Table 3
Diffusion coefficients ($D$)

| Membrane | $D$ ($cm^2\ s^{-1}$) | | |
|---|---|---|---|
| | Cuprophan | Cellulose acetate | Unplasticised PVC |
| Glucose | $1.8 \times 10^{-9}$ | $4.3 \times 10^{-11}$ | $1.2 \times 10^{-10}$ |
| Ascorbate | $3.8 \times 10^{-9}$ | $0.5 \times 10^{-11}$ | $1.4 \times 10^{-11}$ |
| Malic acid | $3.2 \times 10^{-9}$ | $4.3 \times 10^{-11}$ | $1.5 \times 10^{-11}$ |
| Citric acid | $4.1 \times 10^{-9}$ | – | $4.5 \times 10^{-11}$ |

$$D = \frac{V_1 V_2 l}{10 t A (V_1 + V_2)} \log \frac{n_i V_2}{n_i V_2 - n_2 (V_1 + V_2)} \qquad (4)$$

where $V_1$ = volume of the analyte chamber (l), $V_2$ = volume of the purified water chamber (l), $l$ = thickness of membrane (m), $A$ = area of the membrane ($m^2$), $n_i$ = moles of analyte in chamber 1 at $t = 0$, $n_2$ = moles of analyte in chamber 2 at time $t$ and $t$ = time of diffusion (s).

### *3.2. Malate biosensor*

For developing a reagentless biosensor for malate, outer membranes had to possess particular characteristics to overcome various problems associated with the retention/immobilisation of $NAD^+$/mediator

species, sufficient permeability to malate, extension of response linearity over environmental concentration ranges and protection of immobilised MDH/DI from low pH inactivation. Inner membrane characteristics comprised pH independent ascorbate rejection with minimum attenuation of response. The use of a dual-purpose linearising/ascorbate rejecting outer membrane [26] is not feasible for the malate sensor owing to the difficulty of achieving selectivity for the analyte over the somewhat similar ascorbate molecule. Therefore, an outer ascorbate eliminating enzyme layer was employed.

### 3.2.1. Malate chemical pathway investigations

At neutral to low pH, the equilibrium of MDH reaction (Eq. (1)) is towards malate and NADH formation is not favoured. Many previous workers have used high pH (>9) to enhance NADH formation, especially in flow systems under kinetically limited conditions [14], but it was found in the present work that the use of an efficient electron transfer mediator and/or the enzyme DI, shifted equilibrium to oxaloacetate formation, for the first time allowing NADH production in neutral/acidic samples. In addition to this, the reaction catalysed by MDH is affected by oxaloacetate concentration, owing to the reverse reaction (Eq. (1)) in the presence of NADH. As indicated in the literature, the effect of oxaloacetic acid is already significant at a level of 0.26 mmol $l^{-1}$ and thus approaches utilising aspartate aminotransferase have been reported [12]. The $K_m$ value for the reverse reaction is 34 μmol $l^{-1}$, at 0.12 mmol $l^{-1}$ NADH [27]. Alternatively, in a more economic way, this problem can be overcome using an efficient mediator, which, as shown in Eq. (2), keeps the NADH concentration at a low level.

Initial experiments with MDH, DI, $NAD^+$ and DCPI in solution showed good malate sensitivity and demonstrated the importance of DI for enhancement of signal size. MDH and DI were subsequently co-immobilised between dialysis membranes (in the absence of glutaraldehyde) and when supplied with optimum concentrations of $NAD^+$ (4.5 mmol $l^{-1}$) and DCPI (0.25 mmol $l^{-1}$), acceptable malate sensitivity was achieved with response linearity up to 1–2 mM malate. The basic malate system was found to be very sensitive to pH with a pH decrease from 7.8 to 6.4 causing a 44% decrease in signal (Fig. 1). The immobilised MDH/DI laminate was also relatively unstable

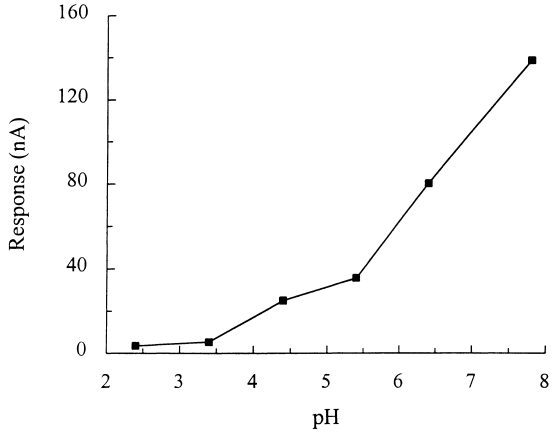


Fig. 1. Effect of pH on malate sensing membrane: 208 U $ml^{-1}$ MDH and 8.7 U $ml^{-1}$ DI co-immobilised into dialysis laminate; 5 mM $NAD^+$ and 0.25 mM DCPI in solution; 10 mM malate. 50 mV vs. Ag/AgCl.

with a 50% signal decrease after 7 h of use (probably due to enzyme leaching from the edge of the membrane as well as some inactivation of the enzyme), and finally, only 10% of the initial activity remained when the enzyme laminate was kept overnight at 4°C.

High concentrations of ascorbate in citrus fruit also caused problems for the malate enzyme electrode. Although direct oxidative interference due to ascorbate was decreased at the lower anodic potentials applied (+50 mV versus Ag/AgCl), DCPI was found to mediate ascorbate oxidation [28]. It was thus essential to prevent ascorbate from reaching the mediator. Similarities in molecular size and p$K_a$ between malate and ascorbate necessitated the use of an AOD outer layer, which almost entirely removed the problem, as 98–99% of the ascorbate was oxidised (Fig. 2).

### 3.2.2. Mediators

As indicated above, use of DI and an efficient mediator such as hexacyanoferrate(III), DCPI or NQ has important advantages; however some drawbacks are introduced as mediators may facilitate charge transfer between possible interferents and the electrode, thus increasing the interference problem, and they may also leach from the system, thus producing a progressively diminishing response to analyte. Extensive electrochemical studies of proposed mediators using cyclic voltammetry have been published previously [14].

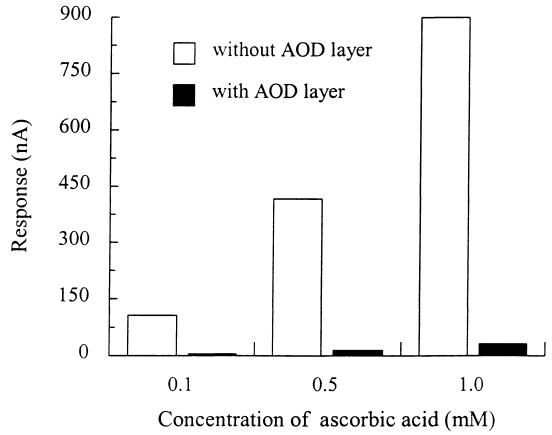


Fig. 2. Removal of the interference of ascorbate in a malate sensing system by an AOD outer layer.

The electron transfer mediators DCPI, hexacyanoferrate(III) and NQ were compared in solution and as co-immobilised forms. Hexacyanoferrate(III) and NQ gave large responses (at +300 mV versus Ag/AgCl); they were more evenly dispersed in the immobilised form and their reaction with ascorbate is not so kinetically favored as in case of DCPI ($k = 5.6 \times 10^4\ \mathrm{mol^{-1}\ s^{-1}}$) [29].

It was thought that NQ may have been more readily immobilised than hexacyanoferrate(III) and a parallel study involved the use of this mediator with co-immobilisation of MDH, DI and $NAD^+$ between dialysis membranes. Using NQ in solution, a response of 10–200 nA for 0–10 mM malate was produced. However, attempted immobilisation of NQ together with the MDH/DI/$NAD^+$ resulted in no response to malate. Further studies were therefore carried out using hexacyanoferrate(III).

### 3.3. Enzyme immobilisation strategies

#### 3.3.1. Enzyme cross-linking

MDH and DI co-immobilised by glutaraldehyde cross-linking with albumin showed low activity, even with the additional cofactor ($NAD^+$) and mediator (DCPI or hexacyanoferrate(III)) supplied in solution. Glutaraldehyde appeared to significantly deactivate MDH (much more so than DI), and thus, alternative immobilisation strategies were investigated.

#### 3.3.2. Immobilisation on membranes

High protein-binding membranes (HA, high protein affinity) were investigated. MDH/DI co-immobilised on nitrocellulose membranes showed good response to malate and were stable with respect to time/exposure number, keeping more than 90% of its initial activity after 3 h, with the $NAD^+$/hexacyanoferrate(III) supplied in solution. With the $NAD^+$/hexacyanoferrate(III) 'immobilised' along with the enzymes and an outer dialysis membrane, the system rapidly lost activity with time due to co-factor/mediator leaching (addition of 0.5 mM hexacyanoferrate(III) and 2 mM $NAD^+$ solutions to the cell resulted in retrieval of the initial response).

A range of possible cofactor-retaining/substrate-permeable outer membranes was then tested [9,10]. A Tween-80 modified cellulose acetate (5%CA + 5% Tween-80) membrane was found to be effective in terms of retaining the immobilised cofactor and mediator as well as allowing the substrate to reach the immobilised enzymes (Fig. 3). Membrane permeability to malate and to $NAD^+$/hexacyanoferrate(III) could be varied by changing the Tween content (1–10% v/v), as shown in Fig. 4. A low concentration of the surfactant in the composite polymer solution diminishes the diffusion of malate (low response) and does not allow hexacyanoferrate(III) to reach the DI (addition of $NAD^+$/hexacyanoferrate(III) mixture). Higher diffusion rate of malate (high initial response)

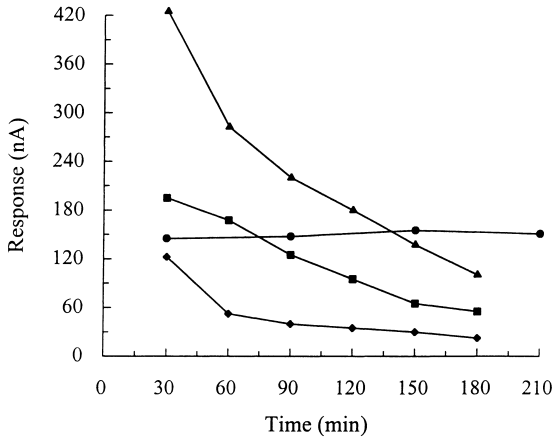


Fig. 3. Stability effect of different surfactant-modified cellulose acetate outer membranes; 10 mM malate. 50 mV vs. Ag/AgCl, pH 7.8; (●) 5% CA/5% Tween-80; (▲) 5% CA/5% THAB; (◆) 5% CA/3% Pluronic F-68; (■) 5% CA/5% SPAN-85.

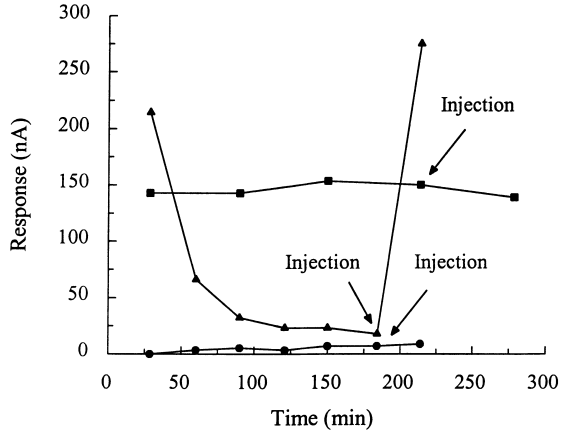


Fig. 4. Stability effect of different concentrations of Tween-80 in a surfactant-modified cellulose acetate outer membrane; 208 U ml$^{-1}$ MDH, 8.7 U ml$^{-1}$ DI, 2 mM $NAD^+$ and 0.5 mM ferricyanide co-immobilised on high protein affinity membrane; 10 mM malate. 300 mV vs. Ag/AgCl, pH 7.8. Injection: 0.5 mM hexacyanoferrate(III) and 2 mM $NAD^+$; (●) 5% CA/1% Tween-80; (▲) 5% CA/10% Tween-80; (■) 5% CA/5% Tween-80.

and hexacyanoferrate(III) (increasing the response after the addition of hexacyanoferrate(III) and $NAD^+$) could be achieved by using a higher concentration of Tween-80 in the composite polymer. However, retention of $NAD^+$/hexacyanoferrate(III) is not possible and the membrane loses its activity rapidly. A reagentless malate sensor was produced with an outer CA/Tween-80 membrane and gave an approximately linear calibration up to 10 mM malate. However, rapid enzyme deactivation (similar to that of the dialysis laminate, Fig. 1) occurred, when determining malate at low pH.

The diffusion studies were limited to the unmodified membranes, and show diffusion coefficients that are two to three orders of magnitude lower than those for bulk solution transport for Cuprophan and four to five orders of magnitude lower for cellulose acetate and PVC. The latter low diffusion would preclude satisfactory measurement of malate, and the satisfactory plasticiser/surfactant would have had the important effect of raising these, probably to an order similar to that of Cuprophan, whilst limiting transport, partly by restricting partitioning into the plasticised membranes of the physically retained cofactors, thus reducing transmembrane transport to the low levels likely with the unmodified membranes.

### 3.3.3. Spin-coated membranes

An HA membrane bearing MDH/DI/$NAD^+$ and hexacyanoferrate(III) in conjunction with a 2.4% PVC/2.4% PC outer membrane was also tested for the construction of a reagentless malate sensor. By using this configuration, a linear calibration graph up to 30 mM malate at pH 7.8 was constructed (Fig. 5A). The sensor showed adequate stability, retaining almost 80% of its initial activity after 3 h of continuous exposure to 5 mM malate. In addition to promoting good stability, PVC/PC membranes were found to protect the enzymes/co-factor/mediator system from pH inactivation.

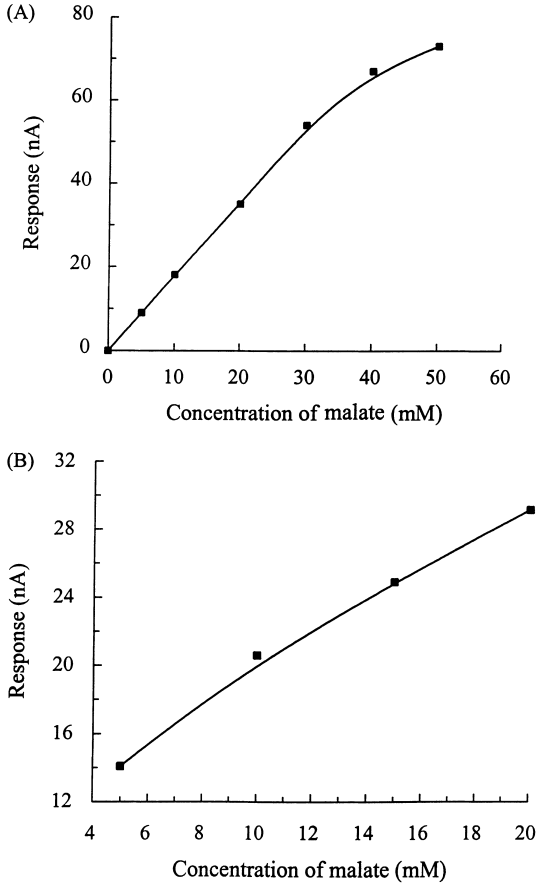


Fig. 5. (A) Calibration graph using a 2.4% PVC/PC outer membrane in pH 7.8; (B) calibration graph using a 2.4% PVC/PC outer membrane with AOD layer in (phosphate buffer) pH 3.3; 208 U ml$^{-1}$ MDH, 8.7 U ml$^{-1}$ DI, 2 mM $NAD^+$ and 0.5 mM hexacyanoferrate(III) co-immobilised on a high protein affinity membrane; 50 mV vs. Ag/AgCl.

The sensor allowed measurement of malate in acidic medium (phosphate buffer, pH 3.3) similar to that of undiluted orange juice, resulting in usable calibration over the range 5–20 mmol $l^{-1}$, which is within the range found in undiluted samples (Fig. 5B). The lifetime of the sensor at pH 3.3 is shorter than that at pH 7.8, but is sufficient for calibration and testing for over 1 h. Comparing the response values for 5 and 10 mM malate solutions (Fig. 5A and B), it is clear that the PVC/PC membrane protects the sensor from low pH inactivation. In the absence of a PVC/PC membrane, an immediate decrease in the response by 95–98% is observed.

These characteristics and the minimal ascorbate interference resulting from the use of the AOD layer (>98% rejection) produce a sensor which, to our knowledge, is the first valid reagentless malic acid sensor that would be suitable for use in unmodified citrus juice.

The systematic membrane optimisation provides for single use enzyme electrodes that are now ready for testing in specific fruit juices, but which may yet require minor adjustment based on requirements for individual types of fruit juices. Importantly, a new approach to the reagentless function of a cofactor-dependent electrode has been devised.

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