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Potentiometric Determination of L-Malate Using Ion-Selective Electrode in Flow Injection Analysis System

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Abstract

A potentiometric biosensor employing a CO_3^{2-} ion-selective electrode (ISE) and malic enzyme immobilization in a flow injection analysis (FIA) system was constructed. Analytical parameters were optimized for L-malate determination. The CO_3^{2-}-ISE-FIA system was composed of a pump, an injector, a malic enzyme (EC 1.1.1.40) reactor, a CO_3^{2-} ion-selective electrode, a pH/mV meter and a recorder. Cofactor NADP was also injected with substrate for the enzyme reaction into the system. Optimized analytical parameters for L-malate determination in the CO_3^{2-}-ISE-FIA system were as follows: flow rate, 14.5 ml/hr; sample injection volume, 100 μl; enzyme loading in the reactor, 20 units; length of the enzyme reactor, 7 cm; tubing length from the enzyme reactor to the detector as a geometric factor in FIA, 15 cm. The response time for measuring the entire L-malate concentration range (10^{-2}~10^{-5} mol/L; 4 injections) was <15 minutes. In this CO_3^{2-}-ISE-FIA system, the potential differences due to the formation of CO_3^{2-} by the reaction of malic enzyme on L-malate were correlated to L-malate concentration in the range of 10^{-2}~10^{-5} mol/L; the detection limit was 10^{-5} mol/L. This potentiometric CO_3^{2-}-ISE-FIA system was found to be useful for L-malate measurement.

Key words: biosensor, L-malate, ion-selective electrode, flow injection analysis system

INTRODUCTION

The quantity and nature of organic acids are essential in determining the quality of fruits and their products, and how they affect taste and flavor. L-Malate, particularly abundant in apples and grapes, is the second most common acid in fruits after citric acid, and it is also the main component in wines (1). The interest in L-malate measurement in fruits and wines is widespread since L-malate influences our perception of the sourness in these fruits and wines, and thus affects the taste of fruit and wine products (2-4). Furthermore, rapid, accurate and selective measurement of L-malate is very important for the quality control of fruits and their products (5).

Biosensor techniques can provide some of the most rapid and selective methods for the measurement of organic compounds and furthermore a combination of a biosensor and a flow injection analysis (FIA) method serves as a suitable technique for measurement (4,6). Also, the coupling of enzymatic reactions with electrochemical monitoring is popular at present in order to measure the substrate (7). Through an enzyme reaction, the sensor device gives a relatively high selectivity and is convenient for measuring a variety of organic compounds (8). Ion selective electrodes (ISEs) are also relatively simple electrochemical devices that can be used for the direct measurement of ions in complex samples (9-12). There are several distinct advantages in using ISE detection, including simple and low cost instrumentation requirements, rapid analysis time, measurement of only free ionic activity and, above all, little or no pretreatment of the sample (13). The separation of ions can alter an electrical potential, which is the principle of ISE detection (14). In the present study, both malic enzyme (EC 1.1.1.40) immobilization and CO_3^{2-} ISE were employed to measure L-malate in the FIA system.

Most studies of malate determination, for which an enzyme reactor was used, have so far been made using malate dehydrogenase (MDH, EC 1.1.1.37) (2, 15-17). However, the reaction which was catalyzed by MDH at equilibrium favors the substrates (L-malate and NAD^+) rather than the products (oxaloacetate and NADH) and thus, is not proper for the exact determination of malate (2,5). In this study, we used malic enzyme (EC1.1.1.40) and cofactor $NADP^+$ for L-malate determination. L-Malate can be oxidatively decarboxylated by malic enzyme (EC1.1.1.40). Consequently, it produces carbonate ions (CO_3^{2-}) according to the following equations:

$$\text{L-Malate}+NADP^+ \xrightarrow{\text{(malic enzyme)}} \text{pyruvate}+NADPH+CO_2+H^+$$

$$CO_2+H_2O \longrightarrow HCO_3^-+H^+ \longrightarrow CO_3^{2-}+H^++H^+$$

Therefore, the measurement of the potential differences due to the production of CO_3^{2-} by the malic enzyme reaction can be correlated to the amount of substrate present in the samples and thus can be used for the determination of L-malate in the CO_3^{2-}-ISE-FIA system. The resulting CO_3^{2-} can be measured by the ion CO_3^{2-} ISE, which is equipped with a membrane that responds to CO_3^{2-} with specific selectivity in FIA system.

This useful CO_3^{2-}-ISE-FIA system for L-malate determina-

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tion has two important implications when compared to the other already estabilished malate biosensors; The first is the use of the malic enzyme reaction which favors the products, and thus can measure L-malate concentration accurately; and the second is the use of the ISE which responds to CO_3^{2-} with specific selectivity. In a previous study (18), the variables for the optimized CO_3^{2-} selectivity of the ISE, such as buffers, plasticizers and polymers for a CO_3^{2-} ion- selective-membrane (ISM), were already evaluated. In the present study, for the optimum determination of L-malate, several parameters which influence the CO_3^{2-} selectivity in FIA, such as flow rate, sample injection volume, enzyme activity, enzyme reactor length, geometric factor in FIA, etc., were investigated in the CO_3^{2-}-ISE-FIA system. Once all parameters had been optimized, L-malate was determined.

MATERIALS AND METHODS

Manufacturing of an ISE and setting up the CO_3^{2-}-ISE-FIA system

A CO_3^{2-}-ISE was manufactured in a modified method of Meyerhoff and Kovach (13) and Meyerhoff et al. (19). The composition of the CO_3^{2-}-ISM casting solution and the method for construction of CO_3^{2-}-ISE and CO_3^{2-}-ISE-FIA system were described in detail in a previous paper (18). The commercial Philips electrode body (IS-561, Glasbläserei Möller, Switzerland) was used for CO_3^{2-}-ISE. L-Malic acid (L-hydrobutanedioic acid, monosodium salt, Sigma) and β-nicotinamide adenine dinucleotide phosphate (β-NADP, Sigma) were used for the substrate and coenzyme, respectively. A 0.01 mol/L KCl (Fluka, Switzerland) solution and 1 mmol/L phosphate buffer (pH 7.4) which was adjusted with 0.1 mol/L NaOH, were used as the internal filling solution of the electrode and the carrier buffer, respectively.

Malic enzyme immobilization was carried out by the modified Chemnitius and Schmid's method (2) which was also described in detail in a previous paper (18). Briefly, malic enzyme was covalently immobilized in controlled pore glass beads (200~400 mesh; mean pore diameter, 75 Å, Sigma) with glutaraldehyde. The enzyme-immobilized glass beads were then packed into teflon tubing (i.d. 1.2 mm). This tubing was defined as the enzyme reactor and was used in the FIA system. Tygon tubing (i.d., 0.89 mm) was connected to the entire flow system. When not in use, the enzyme reactor was stored at 4°C in a 1 mmol/L phosphate buffer, pH 7.4. The enzyme coupling efficiency was 95.4% by protein measurement (20).

The CO_3^{2-}-ISE-FIA system for L-malate determination consisted of a peristaltic pump (IPC-N-8-IV 34; Isamatec SA, Switzerland), a syringe loading sample injector (Model 7725I, Rheodyne, USA) equipped with a sample injection loop, an malic enzyme reactor, a single junction reference electrode (Model 90-01, Orion Research Inc., USA) and a CO_3^{2-}-ISE with a flow-through cell, a pH/mV meter (Mettler Delta 350, Mettler-Toledo Ltd, England) as a signal detector, and a chart recorder (Kipp & Zonen, Netherlands). Both a reference electrode and a CO_3^{2-}-ISE were connected to a pH/mV meter.

Optimizing the parameters and L-malate determination in the CO_3^{2-}-ISE-FIA system

Once L-malate/NADP was injected into the buffer stream, L-malate/NADP reacted with malic enzyme in the reactor, and then, the produced CO_3^{2-} was selective for the CO_3^{2-}-ISE and monitored by a detector. The potential signals were obtained from L-malate/NADP standard solutions to the stream of background electrolyte (1 mmol/L phosphate buffer, pH 7.4). The potentiometric response of the working CO_3^{2-}-ISE was measured relative to a single junction Ag/AgCl reference electrode at room temperature (25°C) with L-malate standard solutions. Data was obtained by plotting the potential difference, which was measured from the baseline to potential peak, versus log concentration of each injected L-malate standard solution.

Variations in electrochemical properties of the CO_3^{2-}-ISE-FIA system, such as flow rate, sample injection volume, amount of enzyme loading, enzyme reactor length, tubing length from the reactor to the detector as a geometric factor in the manifold, were tested for optimization for L-malate determination in the CO_3^{2-}-ISE-FIA system. The stability of the enzyme reactor and the response time in the CO_3^{2-}-ISE-FIA system were also evaluated. The standard solution of L-malate/NADP was used to determine the relationship between the substrate concentration and the potential difference. L-Malate determination was carried out in the optimized CO_3^{2-}-ISE-FIA system.

RESULTS AND DISCUSSION

Optimization of the CO_3^{2-}-ISE-FIA system

Effect of flow rate

The effect of the buffer flow rate in CO_3^{2-}-ISE-FIA was tested for the optimal CO_3^{2-} detection since varying the flow rate could affect the enzymatic reactions or CO_3^{2-} selectivity. Optimization of the pH level of the carrier buffer had already been established as 1 mM phosphate buffer (pH 7.4) (18). Fig. 1 shows the effect of increasing the flow rate from 14.5 to 32.5 ml/hr on the sensor reponse potential. The data showed that the faster the flow rate, the lower the response for CO_3^{2-}. The potential showed the lowest responses at the highest flow rate (32.5 ml/hr) and the highest responses at the lowest flow rate (14.5 ml/hr). As the flow rate increased, the signal for L-malate decreased since the residence time in the enzyme reactor might have been shorter, thus making lower responses at the highest flow rate. The time required from the beginning of potential peak to the reversion of the peak also decreased as the flow rate increased. Fourteen point five milliliter per hour was selected as the optimum flow rate.

Effect of sample injection volume

The effect of sample injection volumes ranging between 50

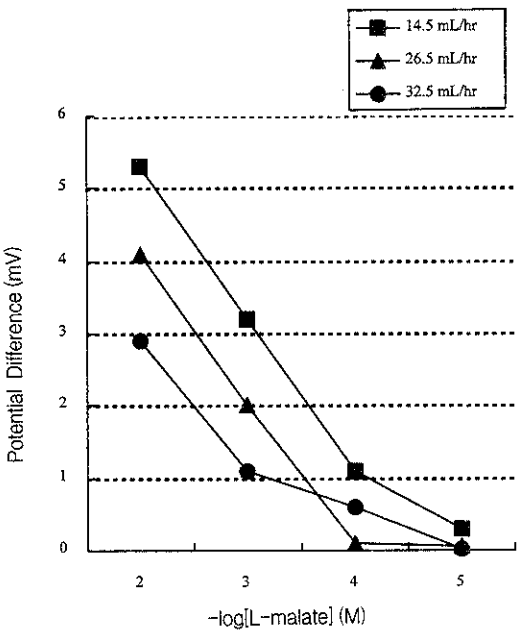

Fig. 1. Effect of various flow rate for L-malate determination in the CO_3^{2-}-ISE-FIA system. The potential difference which was produced by different L-malate concentration, was measured from the baseline to the potential peak. The analytical conditions were as followed: carrier buffer, 1 mM phosphate, pH 7.4; injection volume, 100 μl; malic enzyme units in the enzyme reactor, 20 units; length of the enzyme reactor, 7 cm. The measurements were carried out at 25°C.

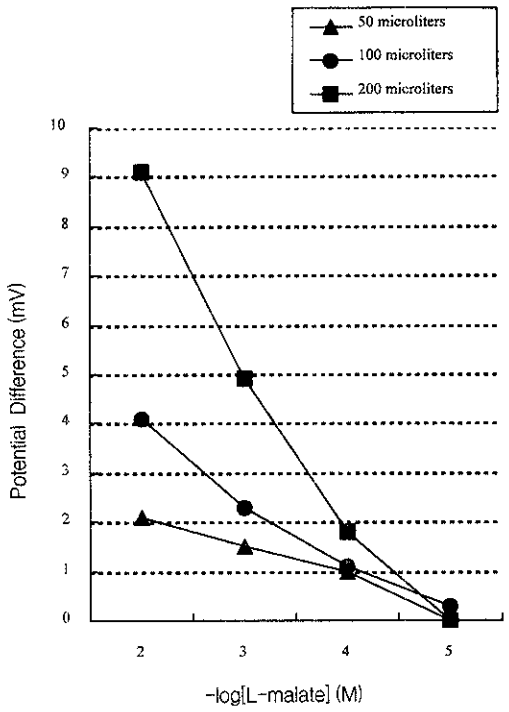

Fig. 2. Effect of various injection volume for L-malate determination in CO_3^{2-}-ISE-FIA system. Description for the potential difference and the conditions for L-malate determination were the same as in Fig. 1 except using a flow rate of 14.5 ml/hr and varying injection volumes.

and 200 μl was investigated. The analytical signal rose with an increase of the injection volume (Fig. 2). Two hundred microliters of sample injection showed the highest sensitivity. However, the broad and less prominent peak, appeared due to the large injection volume (200 μl). Therefore, a sample injection volume of 100 μl seemed to be a good compromise between sensitivity and sample throughput, and was used for all future testing.

Effect of enzyme loading

The enzyme loading test was used to estimate the enzyme content necessary to obtain maximum sensitivity. The various contents of malic enzyme under the same length of enzyme reactor using the same amount of the glass beads were tested (Fig. 3). The best signal was shown with 20 units. Twenty enzyme units showed better response in the entire L-malate concentration range than other two enzyme units (40 and 60 units) did. The largest enzyme unit (60 units) showed the lowest response, which was not expected. Forty enzyme units were selected for optimization.

Effect of the enzyme reactor length

With the confined enzyme units, different enzyme reactor lengths were also tested. In Fig. 4, the most sensitive response to L-malate was achieved in an enzyme reactor length of 7 cm. The potential response to the substrate was not sufficient in the shortest enzyme reactor (6 cm) nor in the longest one (8 cm). The longer the enzyme reactor was, the longer the re-

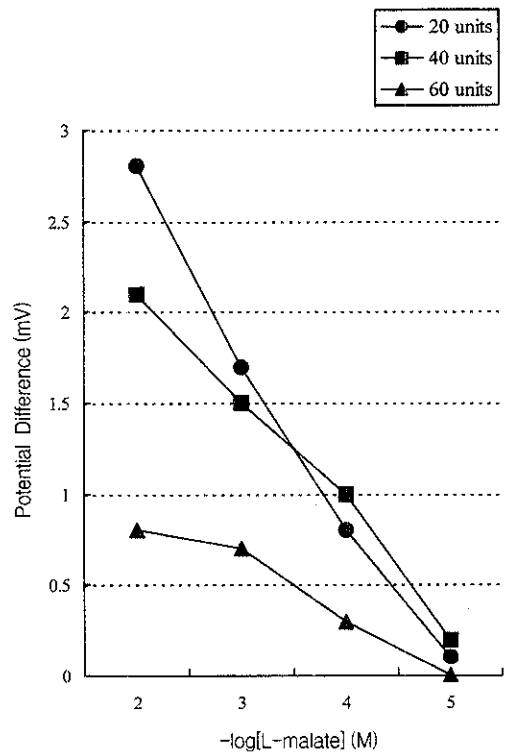

Fig. 3. Effect of various enzyme amounts in the reactor for L-malate determination in CO_3^{2-}-ISE-FIA system. Description for the potential difference and the conditions for L-malate determination were the same as in Fig. 1 except using the flow rate of 14.5 ml/hr and varing enzyme units in the reactor.

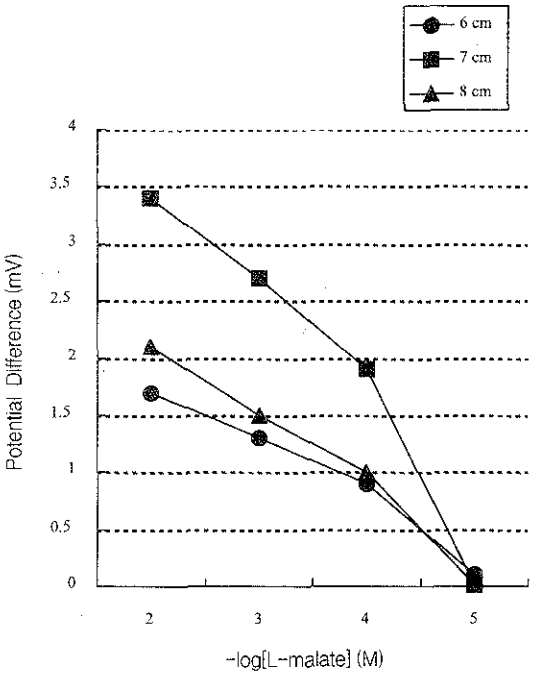

Fig. 4. Effect of various length of the enzyme reactor for L-malate determination in CO_3^{2-}-ISE-FIA system. Description for the potential difference and the conditions for L-malate determination were the same as in Fig. 1 except using the flow rate of 14.5 ml/hr and varying lengths of the enzyme reactor.

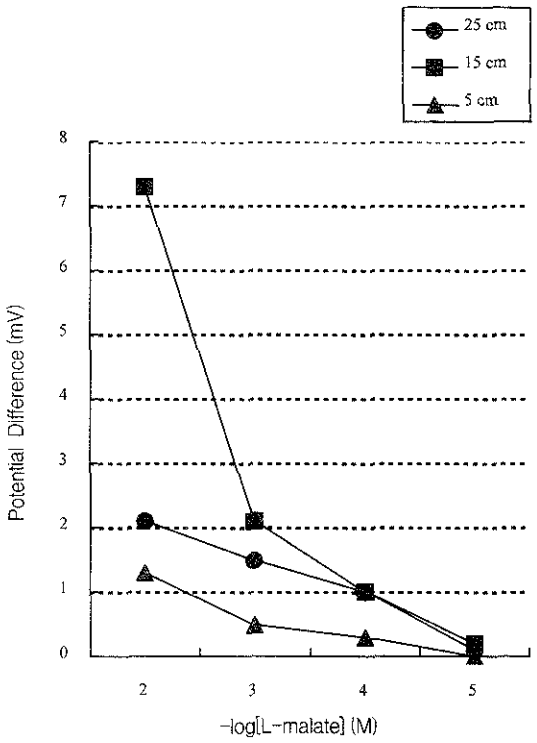

Fig. 5. Effect of various tubing lengths in CO_3^{2-}-ISE-FIA system for L-malate determination. The tubing length from the enzyme reactor to the detector was considered as geometric factor. Description for the potential difference and the conditions for L-malate determination were the same as in Fig. 1 except using the flow rate of 14.5 ml/hr.

sponse time might be, which probably made the less sensitive response and, vice versa, in the shorter reactor. For further tests, an enzyme reactor length of 7 cm was selected due to its high sensitivity.

Effect of geometric factor in FIA

A study of the dimensions of the tubing, which was used for connecting the CO_3^{2-}-ISE-FIA system, was performed. The length of the tubing from the enzyme reactor to the detector was varied in order to obtain a good peak of potential difference. Variation in tubing length influenced the analytical signals which were obtained for the L-malate. The response to the substrate was not sensitive when using the longest tubing (25 cm), because produced CO_3^{2-} might be lost on passing through the reactor to the detector which resulted in the decreased signals (Fig. 5). Also, the response of the shortest tubing length (5 cm) from the enzyme reactor to the detector showed the least sensitivity for insufficient potential production. The optimum length for the connection tubing from the enzyme reactor to the detector was 15 cm.

We also found that measurement of L-malate by using a malic enzyme reactor in this CO_3^{2-}-ISE-FIA was possible for almost two months with acceptable sensitivity when used almost daily at an optimal pH level of 7.4. Compared to batch analysis, the high stability of the CO_3^{2-}-ISE-FIA sensor is presumably due to the stable and appropriate enzyme immobilization and the use of flow injection analysis; in batch analysis, the working electrode is usually immersed in the sample solution for analysis, which then makes the immobilized enzyme be more easily detached in the sample solution.

L-Malate determination using the CO_3^{2-}-ISE-FIA system

From the above experiments, the following optima were established: flow rate, 14.5 ml/hr; sample injection volume, 100 μl; enzyme units in the enzyme reactor, 20 units; enzyme reactor length, 7 cm and length of reaction tubing from the enzyme reactor to the detector, 15 cm. Under these optimum conditions, routine measurements of L-malate standard solutions were performed at 25°C using 1 mM phosphate buffer (pH 7.4). The selected temperature was appropriate for the enzyme reaction and the best signals were produced. The response time for measuring the entire L-malate concentration range (10^{-2}~10^{-5} mol/L; four injections per range) in the CO_3^{2-}-ISE-FIA system was less than 15 minutes. The sampling frequency of standard solutions was about 15 injections/hr.

Under these optimum conditions, L-malate concentration showed a good correlation with the produced potentials. Fig. 6 shows the L-malate calibration curve and r^2 is also given. The potential differences were linearly related to the L-malate concentrations in the range of 10^{-2}~10^{-5} mol/L. The minimum detection level was 10^{-5} mol/L L-malate since changes were barely discernible below this limit. The least-squares method used to generate a calibration curve from the data of Fig. 6, gave a y-intercept of 10.6 and a slope of -1.22. The correlation

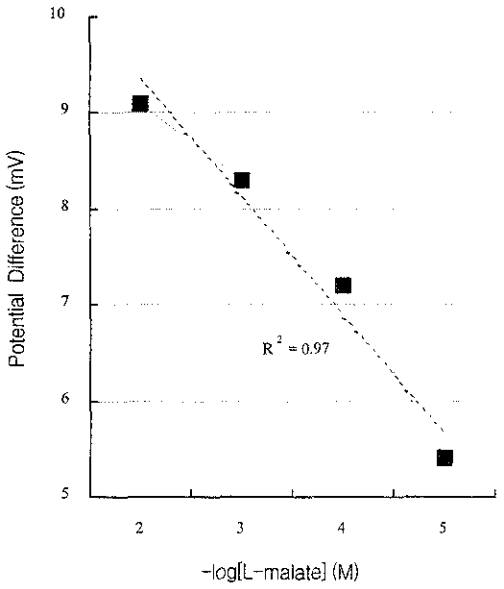

Fig. 6. Calibration curve of L-malate in optimized CO_3^{2-}-ISE-FIA system. Each value is a mean for three replicates. Correlation coefficient (r^2) with 4 points was 0.97.

coefficient, r^2, was 0.97.

According to Mollering (21), soluble malic enzyme revealed a non-linear behavior as the sensor, and Gajovic et al. (22) also reported that the calibration curve with L-malate was not linear, but partially logarithmic at a range of L-malate 10^{-3}~10^{-1} mol/L. In the present study, however using the CO_3^{2-}-ISE-FIA biosensor system for L-malate determination, the potential response to L-malate revealed a linear correlation with the substrate in the range of 10^{-2}~10^{-5} mol/L.

In summary, the variables for potential responses in the CO_3^{2-}-ISE-FIA biosensor system were optimized. The performance of the CO_3^{2-}-ISE-FIA system under the optimized conditions showed a good correlation between the potential responses and the amount of L-malate. The potentiometric ion selectivity of this system has been proven to be suitable for L-malate measurement in the range of 10^{-5}~10^{-2} mol/L. The suitability of this CO_3^{2-}-ISE-FIA system for practical analysis of L-malate in fruits or their products needs to be studied further.

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REFERENCES

1. Reineccius, G. : Flavoring materials contributing to taste. In *"Source book of flavors"* Chapman & Hall, New York, p. 638 (1994)
2. Chemnitius, G. C. and Schmid, R. D. : L-Malate determination in wines and fruit juices by flow injection analysis; Adaptation of a coupled dehydrogenase/transferase system. *Anal. Letters*, **22**, 2897 (1989)
3. Kanellis, A. K. and Soubelakis-Angelakis, K. A. : *Biochemistry of fruit ripening*. Chapman & Hall, New York, p. 208 (1993)
4. Matsumoto, K., Higuchi, S. and Tsukatani, T. : Measurement of L-malate using immobilized enzyme reactors: comparison of result obtained with four different enzymatic systems. *Biosci. Biotech. Biochem.*, **60**, 847 (1996)
5. Mizutani, F., Yabuki, S. and Asai, M. : L-Malate-sensing electrode based on malate dehydrogenase and NADH oxidase. *Anal. Chim. Acta*, **245**, 145 (1991)
6. Deshpande, S. S. and Rocco, R. M. : Biosensors and their potential use in food quality control. *Food Technology*, June, 146 (1994)
7. Scheller, F. and Schubert, F. : Monoenzyme sensors. In *"Biosensors"* Techniques and instrumentation and analytical chemistry, Elsevier, New York, Vol. 11, p. 25 (1992)
8. Blaedel, W. J. and Engstrom, R. C. : Reagentless enzyme electrodes for ethanol, lactate, and malate. *Anal. Chem.*, **52**, 1691 (1980)
9. Dinten, B., Spichiger, U. E., Chaniotakis, N., Gehrig, B. R., Morf, W. and Simon, W. : Lifetime of neutral-carrier-based liquid membranes in aqueous samples and blood and the lipophilicity of membrane components. *Anal. Chem.*, **63**, 596 (1991)
10. Pranitis, D. M., Telting-Diaz, M. and Meyerhoff, M. : Potentiometric ion-, gas-, and bio-selective membrane electrodes. *Critical Reviews in Analytical Chemistry*, **23**, 163 (1992)
11. Yim, H. S., Kibbey, C. E., Ma, S. C., Kliza, D. M., Liu, D., Park, S. B., Torre, C. E. and Meyerhoff, M. E. : Polymer membrane-based ion-, gas- and bio-selective potentiometric sensors. *Biosensors & Bioelectronics*, **8**, 1 (1993)
12. Cha, M. J. Shin, J. H., Oh, B. K., Kim, C. Y., Cha, G. S., Shin, D. S. and Kim, B. : Asymmetric cellulose acetate membrane-base carbonate- and chloride-selective electrodes. *Anal. Chim. Acta*, **315**, 311 (1995)
13. Meyerhoff, M. E. and Kovach, P. M. : An ion-selective electrode/flow-injection analysis experiment. *J. Chem. Education*, **60**, 766 (1983)
14. Craggs, A., Moody, G. J. and Thomas, J. D. R. : PVC matris membrane ion-selective electrodes. *J. Chem. Ed.*, **51**, 541 (1974)
15. Almuiabed, A. M. and Townshend, A. : Flow-injection determination of malate with immobilized malate dehydrogenase. *Anal. Chim. Acta*, **221**, 337 (1989)
16. Yoshioka, S., Ukeda, H., Matsumoto, K. and Osajima, Y. : Simultaneous flow injection analysis of L-lactate and L-malate in wine based on the use of enzyme reactors. *Electroanalysis*, **4**, 545 (1992)
17. Kiba, N., Inagaki, J. and Furusawa, M. : Chemiluminometric flow-injection method for determination of free L-malate in wine with co-immobilized malate dehydrogenase/NADH oxidase. *Talanta*, **42**, 1751 (1995)
18. Kwun, I. S. and Kim, M. : Potentiometric biosensor for detection of L-malate and D-isocitrate employing CO_3^{2-}-selective electrode and enzyme immobilization in flow injection analysis. *J. Food Sci. Nutr.*, **3**, 36 (1998)
19. Meyerhoff, M. E., Pretsch, E., Welti, D. H. and Simon, W. : Role of trifluoroacetophenone solvents and quaternary ammonium salts in carbonate-selective liquid membrane electrodes. *Anal. Chem.*, **59**, 144 (1987)
20. Bradford, M. M. : A rapid and sensitive method for the quantization of microgram quantities protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **72**, 248 (1976)
21. Mollering, H. : *Methods of enzymatic analysis*. 3rd ed., Berlag Chemie, Weinheim, Vol. VI, p. 39 (1984)
22. Gajovic, N., Warsinke, A. and Scheller, F. W. : A novel multienzyme electrode for the determination of citrate. *J. Chem. Tech. Biotechnol.*, **63**, 337 (1995)

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