

Potentiometric Biosensor for Detection of L-Malate and D-Isocitrate Employing CO₃²⁻-Selective Electrode and Enzyme Immobilization in Flow Injection Analysis

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Abstract

Ion-selective electrodes (ISEs) are simple electrochemical devices for the direct measurement of ions in the samples. A novel potentiometric biosensor for the determination of L-malate or D-isocitrate has been developed by using CO₃²⁻-selective electrode and enzyme immobilization in flow injection analysis (FIA) system. The CO₃²⁻-ISE-FIA system was composed of a pump, an injector, a malic enzyme or isocitric dehydrogenase enzyme reactor, a CO₃²⁻-ISE, a pH/mV meter, and an integrator. The various factors, such as buffer capacity, types of plasticizer and polymer, were optimized for the CO₃²⁻ selectivity. In this novel CO₃²⁻-ISE-FIA system, the potential difference due to the amount of CO₃²⁻ produced from each enzyme reaction was proportional to the amount of L-malate or D-isocitrate.

Key words: L-malate, D-isocitrate, flow injection analysis, ion-selective electrode, enzyme immobilization

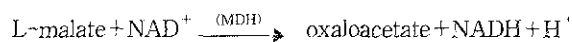
INTRODUCTION

Among the various organic acids in fruits and its products, L-malate and D-isocitrate are the main components which affect the flavor of the fruits. Especially, the amount of L-malate, which is abundant in apple, grape, and wine, changes during the maturation of the fruits or the fermentation of wine(1). Since L-malate concentration decreases continually during storage due to decomposition by microorganisms, L-malate determination can be used for the estimation of shelf-life(2). D-isocitrate can be also formed as a by-product of the fermentative production of citric acid(3). To qualify the good status of fruits and its products, it is helpful to develop the biosensor to measure even the small amount of L-malate or D-isocitrate in the foods.

FIA is the analytical method which involves the injection of a sample directly into a flowing reagent stream which carries the sample to a suitable analytical detector. With the use of accurate injector connecting tubing, small volume flow-through detectors, and consistent flow rates, an FIA system offers rapid assay time and high precision, and even requires small volume (<200 μ l) of sample(4-6). Polymer membrane-type ISEs are also relatively simple

electrochemical probes that can be used for the direct measurement of ions in complex samples(7,8). The separation of ions would give changes in electrical potential, which is the principle of the ion selective membrane (ISM) electrodes. An appropriate lipophilic ionophore is dissolved in an organic solution and a membrane is prepared by evaporating the solution. The membrane is placed between the sample solution and an internal reference solution and the produced membrane potential can be the indicator of the activity of analyte ions in the sample(9).

So far, most of studies for malate determination for which enzyme reactor was used, have been made on the development of such sensors on which malate dehydrogenase (MDH, EC1.1.1.37), which catalyzes the dehydrogenation of L-malate by NAD⁺, was immobilized:

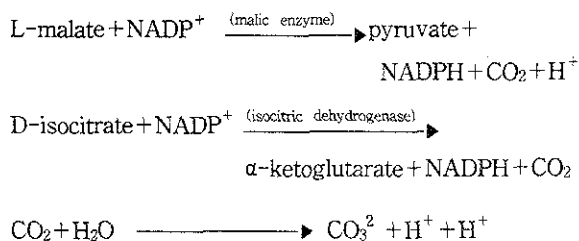


The consumed malic acid was measured by the fluorescence(2), the chemiluminescence(10), the spectrophotometry(11-13), or by using an oxygen probe for the formed NADH(14,15). For the determination of isocitrate and citrate, an oxygen electrode using the coimmobilized horseradish peroxidase and D-isocitrate

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dehydrogenase(16) and a modified H_2O_2 electrode using the reaction of citrate lyase were used, respectively(17).

In the present study, the measurement of carbonate ions(CO_3^{2-}) using a CO_3^{2-} -ISE equipped with CO_3^{2-} -ISM which contains the CO_3^{2-} ion carrier that responds to CO_3^{2-} with the specific selectivity(18) was used for the determination of L-malate or D-isocitrate according to the following equations. L-Malate and D-isocitrate are oxidatively decarboxylated by malic enzyme(EC1.1.1.40) or isocitric dehydrogenase(EC1.1.1.42) to produce carbonate ions(CO_3^{2-}), respectively.



Therefore, the measurement of CO_3^{2-} by the potential difference caused from CO_3^{2-} production by the malic enzyme or isocitric dehydrogenase can be correlated with the amount of substrates and can be used for the determination of L-malate or D-isocitrate in the CO_3^{2-} -ISE-FIA system. For the optimized detection of L-malate or D-isocitrate, experimental parameters for the CO_3^{2-} -ISM, such as the working buffer capacity(pH), the plasticizer, and the polymer were optimized.

MATERIALS AND METHODS

Reagents

All of solutions were made with distilled water. For the enzyme reactor, NADP-dependent malic enzyme (from chicken liver) and isocitric dehydrogenase(from porcine heart), glutaraldehyde and N-(2-aminoethyl)-3-amino propyl glass were purchased from Sigma Chemical Co.(USA). For the preparation of the polymer CO_3^{2-} -ISM, carbonate ionophore IV(trifluoroacetyl- ρ -butylbenzene, TFABB), tridodecylmethylammonium chloride(TDMACl), bis(2-ethylhexyl) sebacate(dioctyl sebacate, DOS), polyvinylchloride(PVC) and tetrahydrofuran(THF) were purchased from Fluka Chemika(Switzerland). A carrier buffer (1 mM phosphate buffer, pH 7.4) which was used in the CO_3^{2-} -ISE-FIA system, was made with K_2HPO_4 and KH_2PO_4 both from Fluka BioChemica(Switzerland). KCl (0.1 M) and $\text{Na}_2\text{CO}_3(10^{-5} \sim 10^{-1}\text{M})$ were used as electrolyte

and standard solutions, respectively. L-Malic acid (L-hydrobutanedioic acid, monosodium salt), three-Ds (+)isocitric acid(Ls[4]isocitric acid, monopotassium salt) and β -nicotinamide adenine dinucleotide phosphate(β -NADP) were purchased from Sigma Chemical Co.

Preparation of CO_3^{2-} -ISM and construction of CO_3^{2-} -ISE

A CO_3^{2-} -selective polymer membrane for a flow-through CO_3^{2-} -ISE was cast using the conventional method for ISE membrane preparation(19,20). The casting solution of ISE membrane was made by dissolving TFABB(6.3 mg) and TDMACl(3.1mg) as the ion transfer agents, DOS (92.6mg) as a plasticizer, and PVC(55mg) as a polymer into THF(2ml). The casting solution was mixed thoroughly and pipetted into the glass cutter(i.d. 2.5cm) which was put on the glass board. The casting solution was allowed overnight for evaporating THF, resulting in a plasticized PVC polymer CO_3^{2-} -ISM. A flow-through CO_3^{2-} -ISE was constructed by a modified method of Meyerhoff and Kovach(4) and Meyerhoff et al.(21). Small disk(i.d., 7 mm) of polymer membrane was cut and then mounted into the tip of the Philips electrode body(IS-561, Glasblaserei Möller, Switzerland). KCl solution(10^{-2} M) was used as the internal filling solution for CO_3^{2-} -ISE. The electrode was soaked in the same 10^{-2} M KCl solution for a few hours prior to use. The home-made flow-through ISE cell jacket was covered at the sensing part of the CO_3^{2-} -ISE. This assembly is shown in Fig. 1.

Enzyme immobilization

A modified Chemnitz and Schmid's method(2) was used for enzyme immobilization. N-(2-Aminoethyl)-3-amino-propyl glass(protein sequencing reagent, 0.2g) was treated with 2.5% glutaraldehyde(5ml) in 0.1 M phosphate buffer(pH 7.4) for 2 hr at 22°C ; this treatment made the peptide bonds between amino group(NH_2) of the long chain glass beads and the aldehyde group (-CHO) of glutaraldehyde. After treatment, the glass beads were washed with the deionized-distilled water to remove the unreacted glutaraldehyde. Malic enzyme (20units) dissolved in 0.1M phosphate buffer(pH 7.4, 5ml) was added to the treated glass beads to react for another 2 hours in the refrigerator at 4°C ; At this time, the cross-linkage bond between another aldehyde group of glutaraldehyde and amino group of enzyme was made. The enzyme-supported glass was washed with 0.1 M

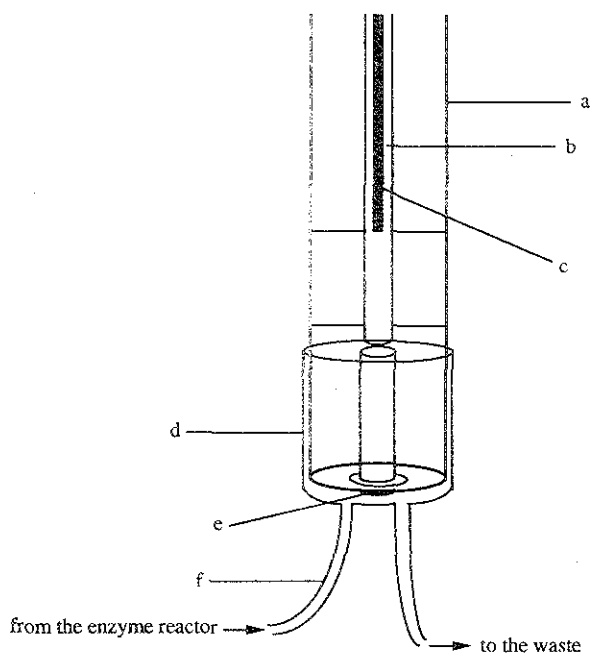


Fig. 1. Flow-through cell type of CO_3^{2-} -selective electrode(ISE) assembly.

a. Philips commercial electrode body, b. Internal filling solution(10^{-2} M KCl), c. Ag/AgCl reference electrode, d. Flow-through cell jacket, e. CO_3^{2-} -selective membrane, f. Tygon tubing.

phosphate buffer(pH, 7.4) after treatment and then packed into the teflon tubing(i.d. 2.0mm, length 10cm). The coupling efficiency of the enzyme, which was 95.4 %, was measured by the Bradford method(22). The enzyme reactor was filled with the buffer and stored at 4°C .

CO_3^{2-} -ISE-FIA system

A schematic diagram of the CO_3^{2-} -ISE-FIA system for measurement of L-malate or D-isocitrate is shown in Fig. 2. The system consisted of the carrier buffer, the peristaltic pump(IPC-N-8-IV 34; Ismatec SA, Switzerland), the syringe loading sample injector(Model 7725I, Rheodyne, USA) equipped with a 100 μl sample injection loop, the enzyme reactor, the CO_3^{2-} -ISE covered with a flow-through cell jacket, a single junction reference electrode (Model 90-01, Orion Research Inc., USA), the pH/mV meter(Mettler Delta 350, Mettler-Toledo Ltd, England) and the integrater(BD 40, Kipp & Zonen, Holland). A Tygon tubing(i.d. 0.89mm) was used to connect the whole flow system. As carrier buffer, 1 mM phosphate buffer was used for both malic enzyme and isocitric dehydrogenase reactions. The enzyme reactor was maintained at 22°C . A few drops of 0.1 M KCl solution was added to the distilled water reservoir. To eliminate

pulsating noise in the electrodes and in the streaming potentials which was associated with the flow of solution across the surface of ISM, the system was grounded in any place when it needed(4). The sample solution(10^{-1} ~ 10^{-4} M Na_2CO_3 standards, 10^{-1} ~ 10^{-3} M malate/NADP, or 10^{-1} ~ 10^{-5} M isocitrate/NADP) was injected into a carrier stream through the injector. The flow rate was 15.9ml/hr or 26.4ml/hr. Potentiometric measurements were made by connecting the CO_3^{2-} -ISE and the reference electrode to pH/mV meter and the response of CO_3^{2-} -ISE was measured as a relative potential compared to a single junction Ag/AgCl reference electrode. After enzyme reaction, the formed CO_3^{2-} was selective to the CO_3^{2-} -ISE and then monitored at the detector, a pH/mV meter. Data was obtained by plotting the changed potential from the baseline or the potential differences from the baseline to the peak height versus log concentration of the sample. Several parameters for CO_3^{2-} -selectivity, such as the carrier buffer, a plasticizer, and a polymer, were tested. For the optimal selectivity of the CO_3^{2-} -ISM, three buffer solutions(1 mM Tris/HCl, pH 7.4; 1 mM phosphate buffer, pH 7.4; 1 mM Tris/ H_2SO_4 , pH 8.7), two plasticizers(*o*-NPOE and DOS), and two polymers(PVC and polyurethane) were tested, respectively.

For the optimization of plasticizer and buffer for CO_3^{2-} -selectivity of CO_3^{2-} -ISM, both the reference electrode and CO_3^{2-} -ISE were connected to the pH/mV meter and, at the same time, were immersed in 50ml of 1 mM phosphate buffer solution(pH 7.4). Standard solutions were added into the buffer solution with the sequential concentrations(10^{-5} ~ 10^{-1} M Na_2CO_3) while stirring the buffer solution with a magnetic bar and the data was acquired from the four experiments. For the optimization of polymer for CO_3^{2-} -selectivity and the determination

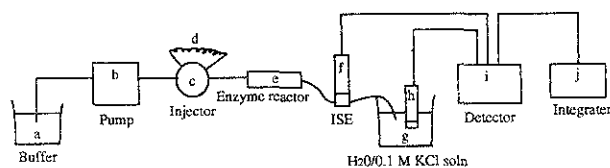


Fig. 2. Scheme of CO_3^{2-} -selective electrode(ISE) in FIA system for determination of L-malate or D-isocitrate.

a. Carrier buffer, 1 mM phosphate buffer, pH 7.4, b. Peristaltic pump, c. Sample injector, d. Coiled injection loop, e. Malic enzyme or isocitric dehydrogenase reactor, f. Flow-through CO_3^{2-} -selective electrode, g. $\text{H}_2\text{O}/0.1$ M KCl solution, h. Ag/AgCl reference electrode, i. Digital pH/mV meter, j. Integrater

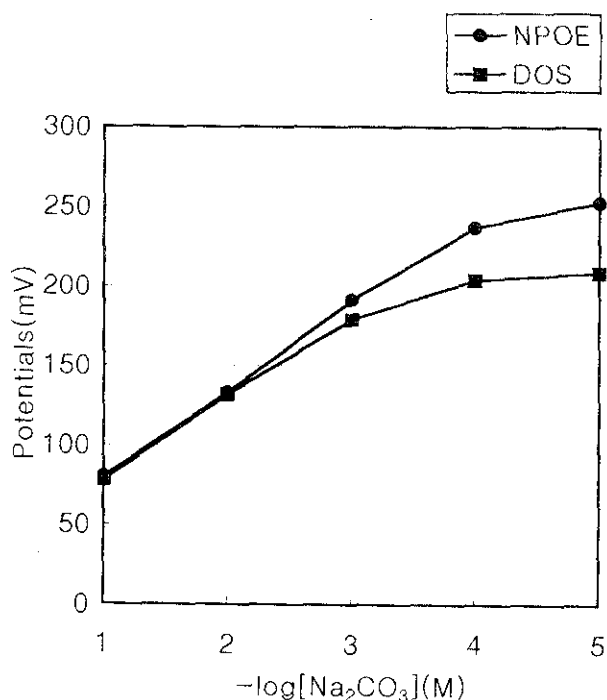


Fig. 3. Effect of the different plasticizer on the CO_3^{2-} selectivity in ISM.

Calibration curve was obtained by measurement of the potential differences from the baseline to the changed potentials by the CO_3^{2-} -ISE. Each value is a mean for four replicates. Conditions: 1 mM Tris/HCl buffer (pH 7.6) containing 0.1 M KCl at 22°C; stirring rate, 100 rpm; *o*-NPOE (*o*-nitrophenyloctylether), DOS (dioctyl sebacate).

of L-malate and D-isocitrate, standard solutions and substrate/NADP were tested in the CO_3^{2-} -ISE-FIA system. The data was acquired from five experiments.

RESULTS AND DISCUSSION

Optimizing the factors for CO_3^{2-} detection

Effect of plasticizer in ISM

Fig. 3 shows the effect of the plasticizer in ISM for the CO_3^{2-} selectivity in the standard sample solution and the data represents as the mean of four replicates. The addition of plasticizer can make the membrane behave like a liquid and thus the properties of the membrane electrode are very similar to those of the wet membranes (9). *o*-NPOE-containing ISM electrode showed higher CO_3^{2-} -selectivity than DOS-containing ISM electrode in lower concentration of standard solution (10^{-3} ~ 10^{-5} M Na_2CO_3), but they didn't show any differences in higher concentration (10^{-2} ~ 10^{-1} M Na_2CO_3). This result might

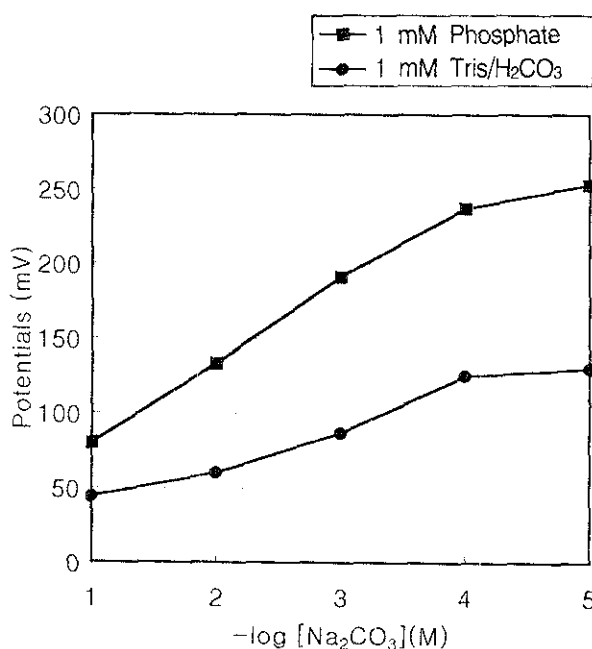


Fig. 4. Effect of the different buffer solutions on the CO_3^{2-} selectivity.

Calibration curve was obtained by measurement of the potential differences from the baseline to the changed potentials which was corresponded to each concentration of Na_2CO_3 standard solution by the CO_3^{2-} ISE. Each value is a mean for four replicates. Conditions: stirring rate, 100 rpm; at 22°C.

be due to higher dielectric constant of *o*-NPOE than that of the DOS which means *o*-NPOE-containing ISM electrode would show the less electrical resistance in the carrier buffer than DOS-containing ISM electrode would show in the carrier buffer. DOS-containing ISM still showed high correlation ($r=-0.956$; 5 points) between the Na_2CO_3 concentration and the produced potential with the wide range of the potential changes (ca. Δ mV, 131 mV from 10^{-5} ~ 10^{-1} M), although it didn't show as much high as the correlation of *o*-NPOE-containing ISM ($r=-0.984$; 5 points) did. Therefore, DOS would be considered as proper plasticizer for usage because *o*-NPOE-containing ISM showed darker yellow color and stiffer texture than DOS-containing ISM did.

Effect of buffer

The pH variance of the buffer is important for both the production of CO_3^{2-} and enzyme reaction. The optimal pH range for the reaction of the malic enzyme or the isocitric dehydrogenase is about pH 7.4, while the optimal level for the CO_3^{2-} production is pH 8.7. There was no differences on the CO_3^{2-} production between 1 mM Tris/HCl and 1 mM phosphate buffer both of which were

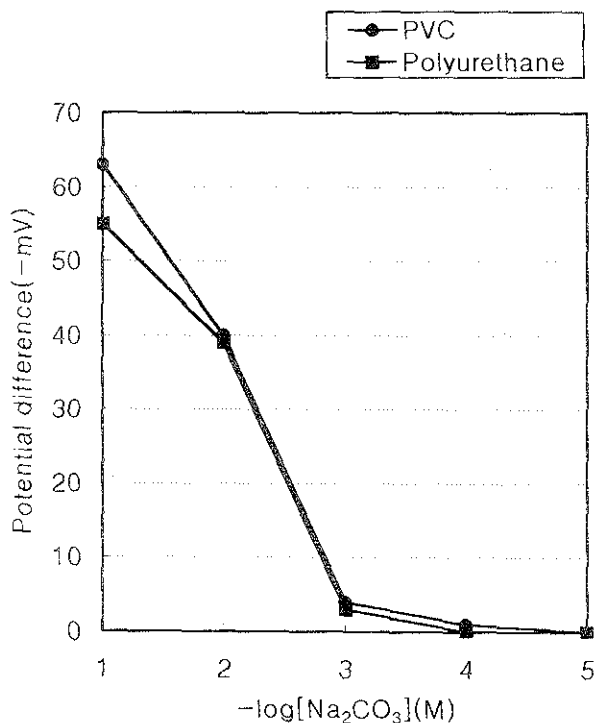


Fig. 5. Effect of the polymer in ISM on the CO_3^{2-} -ISE-FIA.

Potential difference was measured from the baseline to the potential peak which was caused by CO_3^{2-} production. Each value is a mean for five replicates. Conditions for CO_3^{2-} -ISE-FIA: carrier buffer, 1 mM phosphate buffer, pH 7.4; flow rate, 26.4 ml/hr; injection volume, 100 μl ; at 22°C.

within the same pH range (data not shown). Since Cl^- in 1 mM Tris/HCl could increase the buffer capacity, which means 1 mM Tris/HCl buffer might decrease the detection limit for the measurement of the produced CO_3^{2-} , and 1 mM phosphate buffer would be better than 1 mM Tris/HCl was. As shown in Fig. 4, the detection limit of 1 mM phosphate buffer was better than that of 1 mM Tris/ H_2SO_4 . Hence the optimal carrier buffer for the CO_3^{2-} detection was 1 mM phosphate buffer.

Effect of polymer in ISM

The polymer matrices can enable the covalent attachment of ion transfer agents and thus can affect the CO_3^{2-} selectivity of the CO_3^{2-} ionophore (TFABB) and the anion carrier (TDMACl). The effect of two polymers (PVC and polyurethane) in the CO_3^{2-} -ISM was tested. Even though CO_3^{2-} selectivity of two different polymers in the CO_3^{2-} -ISM was not much different at low standard concentration (10^{-3} – 10^{-5} M Na_2CO_3), however, the PVC-containing ISM showed higher CO_3^{2-} selectivity

than the polyurethane-containing ISM did at high concentration of standard solution (10^{-2} – 10^{-1} M Na_2CO_3) (Fig. 5). Polyurethane was not easily dissolved in the membrane casting solution and the polyurethane-containing ISM was tinted light-brown color and even less flexible than the PVC-containing ISM. PVC was considered for the proper polymer for the maximal CO_3^{2-} selectivity.

L-Malate and D-isocitrate measurement by using the CO_3^{2-} -ISE-FIA system

Under the above optimized CO_3^{2-} -ISE-FIA system for CO_3^{2-} selectivity, the determination of CO_3^{2-} with the standard solutions (10^{-4} – 10^{-1} M Na_2CO_3) showed that the calibration curve was linear over the range 10^{-4} – 10^{-1} M Na_2CO_3 with a regression coefficient (4 points) of -0.952 . The detection limit was 10^{-4} M Na_2CO_3 .

The relationship between the CO_3^{2-} production by the malic enzyme reaction and the concentration of L-malate/NADP in CO_3^{2-} -ISE-FIA system is shown in Fig. 6. The results showed a linear response within the range of 10^{-1} – 10^{-3} M L-malate/NADP. The selectivity of CO_3^{2-} which responded to the various concentrations of L-malate/NADP was lower than that of the various

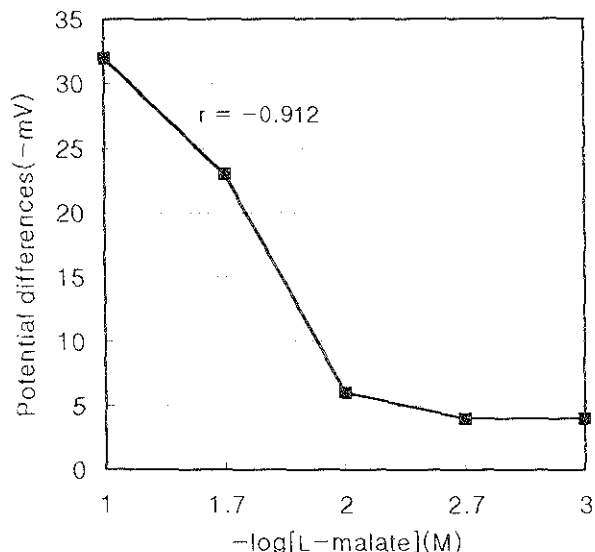


Fig. 6. Calibration curve of L-malate in CO_3^{2-} -ISE-FIA.

Potential difference from the baseline to the peak height by the CO_3^{2-} production due to the malic enzyme reaction was measured for each concentration of L-malate. NADP was injected with each of the same concentration of L-malate. Each value is a mean for five replicates. Conditions: carrier buffer, 1 mM phosphate buffer, pH 7.4; injection volume, 100 microliter; flow rate, 15.9 mL/hr; at 22°C.

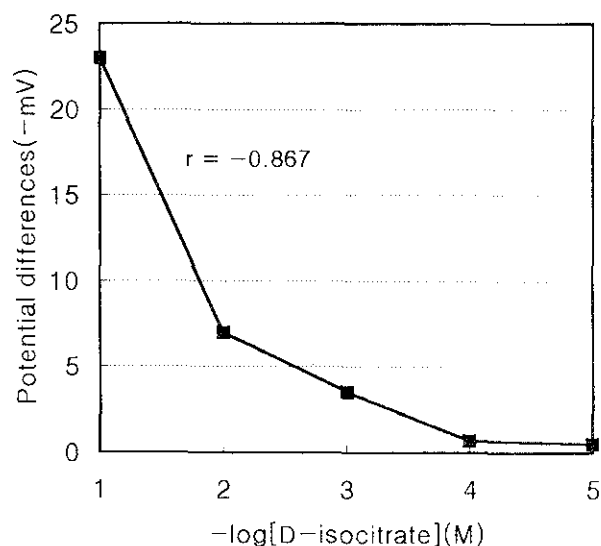


Fig. 7. Calibration curve of D-isocitrate in CO_3^{2-} -ISE-FIA.

Each value is a mean for five replicates. Conditions for CO_3^{2-} -ISE-FIA analysis were the same as in Fig. 6.

concentrations of Na_2CO_3 standard solution. This results might be due to less CO_3^{2-} production by the enzyme reactions that convert L-malate/NADP to pyruvate, NADPH and then finally CO_3^{2-} owing to resistance to the enzyme reactor along with the flow or the enzyme reaction to the substrate.

The calibration curve for the CO_3^{2-} production by isocitric dehydrogenase to the concentration of isocitrate/NADP in CO_3^{2-} -ISE-FIA system is shown in Fig. 7. A linear response was shown within the range of 10^{-1} ~ 10^{-4} M isocitrate/NADP, but any remarkable CO_3^{2-} selectivity was not found below 10^{-4} M isocitrate/NADP. While the detection of L-malate showed a linear regression ($r=-0.912$) between 10^{-1} ~ 10^{-3} M L-malate(5 points)(Fig. 6), isocitrate determination showed a correlation($r=-0.867$)(5 points) in a wider D-isocitrate concentration (10^{-1} ~ 10^{-5} M)(Fig. 7). Values in Fig. 6 and 7 are means \pm SD($n=5$).

This potentiometric CO_3^{2-} -ISE-FIA system using the immobilized-malic enzyme or isocitric dehydrogenase can be considered as the unique analytical device for the measurement of L-malate or D-isocitrate.

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