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# Determining citrate in fruit juices using a biosensor with citrate lyase and oxaloacetate decarboxylase in a flow injection analysis system

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**Abstract**

A biosensor system was constructed for the determination of citrate concentration in foods. The citrate biosensor system consisted of a sample injector, peristaltic pump, enzyme reactor, $CO_3^2$-ion selective electrode (ISE), reference electrode, detector and recorder. Citrate lyase and oxaloacetate decarboxylase were immobilized for the enzyme reactor. The carbonate ions produced through the enzyme reactions of citrate were potentiometrically detected by an ISE. The optimum conditions for the biosensor system were investigated. A linear relationship between the potential difference and logarithmic citrate concentration was obtained in the range of $10^{-1}$–$10^{-4}$ M. There was little interference effect observed regarding most sugars and organic acids on the citrate biosensor system. Citrate concentrations in fruit juices were determined by the biosensor and GC. There was no significant difference between the two analytical methods. Therefore, this citrate biosensor was considered to be a useful method for the determination of citrate concentration in fruit juices.


*Keywords:* Biosensor; Citrate; Flow injection analysis

## 1. Introduction

Citric acid is a predominant organic acid of citrus fruits and is a common parameter used in evaluating the quality of agricultural products and food control points in the food process (Gary, 1992). Since citrus-based beverages are among the most widely consumed beverages in the world, the determination of citrate is very important regarding quality control of the citrus products. Citrate determination has been commonly performed by gas chromatography, high performance liquid chromatography, or enzymatic method (Flores, Kline, & Johnson, 1970; Macrae, 1982; McNair & Bonelli, 1969; Molnar-Perl & Pinter-Szakacs, 1986). The use of chromatographic methods requires a complicated pretreatment procedure, expensive equipment, and long analysis times. An enzymatic method is specific for a desired analyte but is relatively expensive due to the difficulty of enzyme recycling. Recently, a great deal of attention has been devoted in developing biosensors for food analysis and process control. Biosensors have the advantages of high specificity, rapid response, reliability and a simple sample pretreatment procedure (Scott, 1997).

The classical enzymatic method for the determination of citrate used citrate lyase (CL), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH). $NAD^+$, a cofactor, was reduced to NADH after reaction with citrate, and NADH concentration was monitored spectrophotometrically at 340 nm (Moellering & Gruber, 1966). Plantá, Lázaro, Puchades, and Maquieira (1993) spectrophotometrically monitored NADH using a reversed-flow injection method to determine citrate concentration. They used two enzymes, CL and MDH, and immobilized MDH. A linear range was obtained between 1 and 20 mg/dm$^3$. This method, however, was expensive because CL was not immobilized and could not be reused.

A biosensor approach for determining citrate has been described by Hasebe, Hikima, and Yoshida (1990). This biosensor was composed of CL and oxaloacetate decarboxylase (OD). The citrate was converted to pyruvate, which

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was determined by differential pulse polarography. In addition, the simultaneous quantitation of citrate and isocitrate was tried by flow-injection analysis (FIA) using the enzyme reactor (Matsumoto & Tsukatani, 1996). Citrate was quantitated with a co-immobilized CL and OD reactor and an immobilized pyruvate oxidase (PO) as the upstream and downstream reactors, respectively. Hydrogen peroxide produced by the CL-OD and PO reactors was monitored amperometrically. In their study, however, oxaloacetate and pyruvate as the intermediates were produced through the enzyme reactions and these intermediates disturbed the measurement of the citrate. In addition, thiamin pyrophosphate (TPP) and flavin adenine dinucleotide (FAD) were also needed to drive the reactions as cofactors.

In this study, we constructed a biosensor system using an enzyme reactor with the immobilized CL and OD using a FIA to efficiently determine citrate concentration. Immobilization enables the enzymes to be used repeatedly and an increase in the stability of the enzymes. The FIA system can provide some advantages such as short analysis times, small sample volume, high reproducibility and easy online monitoring (Kim & Kwun, 1998). The citrate biosensor in this study is based on the following reactions:

$$\text{Citrate} \xrightarrow{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate}$$

$$\text{Oxaloacetate} \xrightarrow{\text{oxaloacetate decarboxylase}} \text{pyruvate} + CO_2$$

Carbon dioxide produced by the reactions is converted into carbonate ions under alkalic conditions. The carbonated ions could be monitored potentiometrically with a carbonate selective electrode.

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

We optimized the parameters that affect the biosensor sensitivity, quantitatively analyzed the citrate concentrations of some foods using the citrate biosensor, and then these concentrations were compared to the levels measured by a GC reference method.

## 2. Materials and methods

### 2.1. Reagents and apparatus

For the preparation of the $CO_3^{2-}$-selective membrane, tridodecylmethylammonium chloride (TDMACl), dioctyl adipate (DOA), poly(vinylchloride) (PVC), and tetrahydrofuran (THF) were purchased from Fluka Chemie (Buchs, Switzerland) and trifluoroacetyl-*p*-decylbenzene (TFADB) was obtained from Trans World Chemicals (Rocksville, MD, USA).

For the preparation of the enzyme reactor, citrate lyase (EC 4.1.3.6, from *Enterobacter aerogenes*) and oxaloacetate decarboxylase (EC 4.1.1.3, from *Pseudomonas* sp.) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Fluka Chemie, respectively. Aminopropyl glass (mean pore diameter 500 Å, 200–400 mesh controlled pore glass) and glutaraldehyde (25% v/v) were bought from Sigma Chemical Co. to immobilize the enzymes.

The pH of 0.1 M tris[hydroxymethyl]-aminomethane (TRIZMA BASE, Sigma Chemical Co.), a carrier buffer, was adjusted with $H_2SO_4$, and NaOH. The standard solution of citrate was prepared from citric acid (anhydrous). The buffer solution and standard solution were prepared with deionized water (18 MΩ) and all other reagents used were of analytical-reagent grade.

For the GC analysis, glutaric acid was obtained from Acros Organics (NJ, USA) and lead acetate was obtained from Duksan Pharmaceutical Co. (Kyonggido, Korea). Pyridine (Aldrich Chemical Co., Milwaukee, USA), hexamethyl-disilazane (Sigma Chemical Co.) and trimethylchlorosilane (Junsei Chemical Co., Japan) were used to prepare the silylating reagent. A GC (DS 6200, Donam, Korea) was used to analyze the citrate concentration of sample.

A peristaltic pump (IPC-N-8-IV 34, Ismatec, Glattbrugg-Zurich, Switzerland), an injector with a sample injector loop (Rheodyne, Cotati, CA, USA), a single junction reference electrode (Model 90-01, Orion Research Inc., Boston, MA, USA), a working electrode (Philips IS-561, Glasblaserei Moler, Switzerland), a pH/mV meter (Delta 350, Mettler, England) and a chart recorder (Kipp and Zonen, Delft, Netherlands) were assembled for the citrate biosensor system.

### 2.2. Preparation of $CO_3^{2-}$-ion selective electrode (ISE)

First, a $CO_3^{2-}$-ion selective membrane (ISM) was prepared and then this membrane was inserted into a working electrode to make a $CO_3^{2-}$-ISE. A $CO_3^{2-}$-ISM was made of the following composition of materials: 8.3 mg of TFADB, 2 mg of TDMACl, 66 mg of PVC and 100 μl of DOA. The components were thoroughly dissolved in 900 μl of THF. This solution was poured into a small glass ring (diameter 22 mm) on a glass plate and dried for a day at room temperature. After the solvent evaporated, circular membrane pieces (diameter 7 mm) were stamped out and the membrane was mounted in a working electrode body. A flow-through ISE jacket was manufactured for a FIA system and it was put on the end part of the working electrode. A solution mixture containing 0.1 M $NaH_2PO_4$, 0.1 M $Na_2HPO_4$ and 0.01 M NaCl was used as the internal filling electrolyte.

### 2.3. Preparation of enzyme reactor

Various amounts of aminopropyl glasses were treated with 10 ml of 2.5% glutaraldehyde dissolved in a 50 mM phosphate buffer (pH 7.5) and were shaken for 2 h at room temperature. The glass beads were washed with deionized-distilled water and a 50 mM phosphate buffer in turn in order to remove the unreacted glutaraldehyde. The glass beads were then kept in a 50 mM phosphate buffer for 30 min at 4 °C. Citrate lyase and oxaloacetate

decarboxylase were dissolved in a 50 mM phosphate buffer and the enzyme solution was allowed to react with the treated glass beads for 12 h at 4 °C. The coupling efficiency of the enzyme solution was calculated using Bradford's method (1976). The enzyme-coated glass beads were washed with a 50 mM phosphate buffer and packed into a Teflon tubing (diameter, 0.89 mm). The enzyme reactor was filled with the buffer (0.1 M Tris–$H_2SO_4$, pH 7.5) and stored at 4 °C when not in use.

### 2.4. Construction of citrate biosensor system

The citrate biosensor was constructed with the prepared $CO_3^{2-}$-ISE and enzyme reactor in a FIA system. A schematic diagram of the FIA-citrate sensor system is shown in Fig. 1.

The buffer consisted of carrier buffer 1 (0.1 M Tris–$H_2SO_4$, variable pH) and carrier buffer 2 (0.6 M Tris–$H_2SO_4$, pH 11.0) because of different optimum pH levels for enzyme activity and carbonate production. Carrier buffer 1 was transported to the enzyme reactor by a peristaltic pump and it was mixed with carrier buffer 2 in a mixing coil. The mixed solution was transported to the $CO_3^{2-}$-ISE. The $CO_3^{2-}$-ISE detected the carbonate ions and the potentials produced were measured in a pH/mV meter.

### 2.5. Optimization of the citrate biosensor system

The experimental parameters of the system were optimized. This included the method of enzyme immobilization, the amount of enzyme, amount of glass beads, the pH of the carrier buffer and the flow rate of the carrier buffer.

### 2.6. Interference test of sugars and organic acids

The interference effects of sugars and organic acids on the citrate biosensor system were investigated. Glucose, sucrose, fructose, malate, ascorbate, tartrate, and pyruvate (5 mM) were added to the 10 mM citrate solution. Their potential differences were measured and compared with the results for the 10 mM citrate solution.

### 2.7. Determination of citrate in fruit juices

Apple juice (Haitai beverage Co., Ltd, Korea), carrot juice (Bum Yang Food, Korea) and tomato juice (Bum Yang Food, Korea) were purchased from a local market and strawberry juice was prepared from strawberries using a juicer (Alona Electro Co., LTD, Korea). Each kind of fruit juice was centrifuged at 2000 rpm for 15 min, diluted 10-fold with a buffer (0.1 M Tris–$H_2SO_4$, pH 7.5) and the citrate concentration was determined using the biosensor system. The citrate concentration of the fruit juice was measured with four replicates.

The citrate concentrations in the fruit juices were also determined according to Flores et al.'s method using a GC (Flores et al., 1970). The operating conditions of the GC were as follows: column, a glass column (10% SE-30 on Chrom-M-Aqw), 1.83 m (length) × 6.35 mm (diameter); detector, flame ionization detector; initial column temperature, 100 °C; holding time, 15 min; programmed rate, 5 °C/min; final column temperature, 240 °C; injector temperature, 240 °C; detector temperature, 260 °C; carrier gas, $N_2$; flow rate of nitrogen, 30 ml/min. The GC analysis was conducted with four replicates.

## 3. Results and discussion

### 3.1. Optimization of the citrate biosensor

#### 3.1.1. Immobilizing method

Sensitivity and specificity are the most important features of a biosensor, determined by biochemical properties of the biorecognition elements (enzymes) and performance of the transducer (Kuan & Guilbault, 1987; Sethi, 1994). In this study, enzymes were immobilized on glass beads by cross linking with glutaraldehyde. For the preparation of an enzyme reactor, two types of immobilizing methods were investigated. In one method, each of the CL and OD was separately immobilized and packed in turn in a reactor tubing. In the other method, the CL and OD were co-immobilized on the glass beads and packed in a reactor tubing. The two immobilization methods showed a positive correlation between the potential difference and citrate concentration with regression coefficients of co-immobilization and separate-immobilization of 0.99 and 0.98, respectively (Fig. 2). The responses, however, obtained from the co-immobilization method were higher than those from the separate-immobilization method. This result was probably due to the inactivation of the CL by oxaloacetate produced during the reaction (Srere, Brigitte, & Brooks, 1972). Oxaloacetate must be converted to

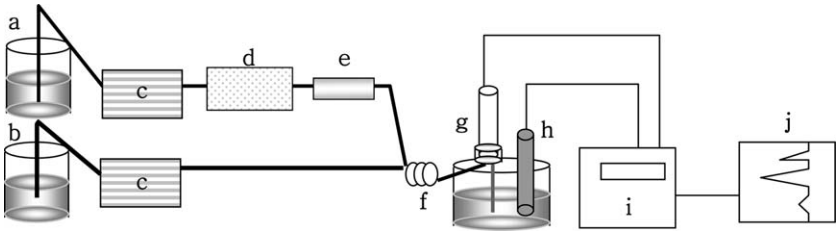


Fig. 1. Scheme of the $CO_3^{2-}$-selective FIA system for citrate determination. (a) Carrier buffer 1; (b) carrier buffer 2; (c) peristaltic pump; (d) sample injector; (e) enzyme reactor; (f) mixing coil; (g) $CO_3^{2-}$-selective electrode; (h) reference electrode; (i) detector (pH/mV meter); (j) recorder.

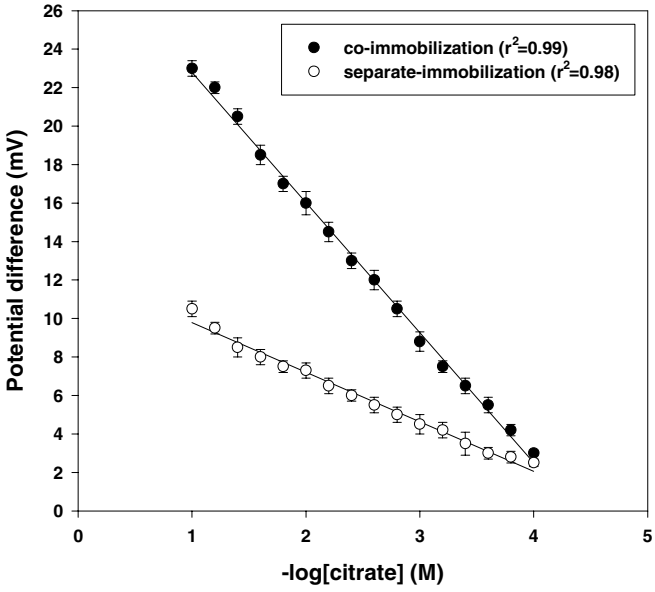


Fig. 2. Effect of the enzyme immobilization methods on the citrate biosensor. Each point is a mean for four replicates. Conditions: carrier buffer 1, 0.1 M Tris–$H_2SO_4$, pH 7.5; carrier buffer 2, 0.6 M of Tris–$H_2SO_4$, pH 11.0; CL, 10 U and OD, 60 U; glass beads, 0.3 g; flow rate, 18 mL/h.

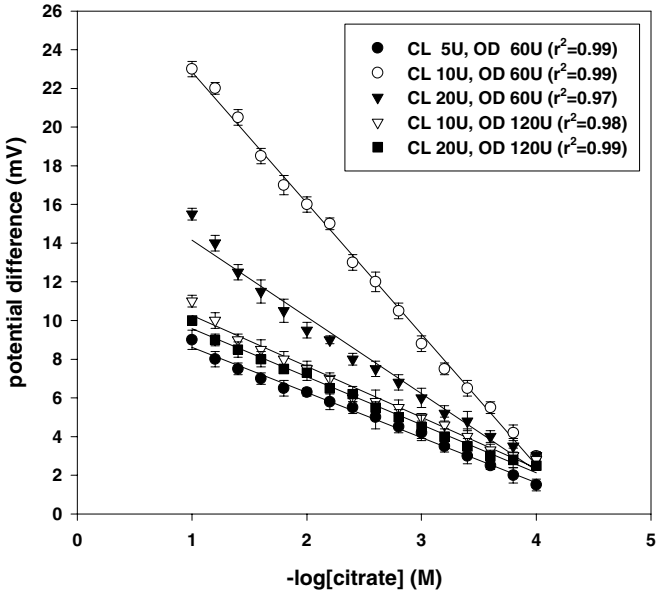


Fig. 3. Effect of the amount of enzymes on the citrate biosensor. Each point is a mean for four replicates. Conditions: carrier buffer 1, 0.1 M Tris–$H_2SO_4$, pH 7.5; carrier buffer 2, 0.6 M Tris–$H_2SO_4$, pH 11.0; glass beads, 0.3 g; flow rate, 18 mL/h.

pyruvate rapidly by the co-immobilized CL and OD (Matsumoto & Tsukatani, 1996). In the separate-immobilization method, it might take a time for oxaloacetate to be converted to pyruvate because CL and OD were separately immobilized on the glass beads and the glass beads were packed into the reactor tubing in sequence. Therefore, oxaloacetate could be accumulated, which could possibly interfere with the action of the CL. It could make the driving force of the reaction be weaker and the responses would also be lower. Whereas, in the co-immobilization method, oxaloacetate produced by the CL could be immediately converted to pyruvate by the OD. Therefore, the co-immobilization method was thought to be more desirable for this biosensor.

### 3.1.2. Enzyme amounts

Optimization of an enzyme reactor is essential in using enzymes economically. The enzyme amounts and ratio necessary for the maximum sensitivity of the biosensor system were investigated. When 60 U of OD were used, 5, 10 or 20 U of CL were added and when 120 U of OD were used, 10 or 20 U of CL were used. As the use of less amounts of OD than 60 U showed the very low response in the preliminary experiment, over 60 OD units were used in this study. As shown in Fig. 3, at 10 CL units and 60 OD units the maximum potential difference and the highest regression coefficient were observed ($r^2 = 0.99$). Therefore, this combination of the enzymes was considered to be the best condition for the performance of the reactions. Five units of CL seemed to be insufficient in performing the reaction. In addition, the use of excess enzyme amounts does not increase the response and leads to wasted enzyme (Gajovic, Warsinke, & Scheller, 1995). The responses for the use of 120 U of the OD were a little bit lower than those for the use of 60 U in this study. It might be due to the insufficient amounts of glass beads on loading the 20 CL units and 120 OD units. Moreover, only one kind of enzyme could be excessively loaded on the glass beads in this case. It could make the response to be lower.

### 3.1.3. Glass bead amounts

The optimum amount of glass bead support material needed in order to maintain high enzyme activity and response was studied. Different amounts of glass beads (0.25, 0.3 and 0.33 g) were employed for enzyme (CL 10 U and OD 60 U) immobilization. When 0.3 g of glass beads was used, the highest potential difference and linearity were obtained (Fig. 4). The enzyme coupling efficiencies for 0.25, 0.3 and 0.33 g of glass beads measured by Bradford's method were 0.85%, 0.98% and 0.98%, respectively. Therefore, it was thought that 0.25 g of glass beads was not enough to load the enzymes and 0.3 g of glass beads was enough to do so. When 0.33 g of glass beads were used, the enzyme-coupling efficiency was also good. The use of 0.33 g of glass beads, however, showed a lowering in the potential difference. It was possibly due to the fact that the larger amount of glass beads could make the barrier against the transport of carrier stream, lead to retardation of the enzyme reaction, and increase the diffusion time and peak broadening (Bilitewski & Rohm, 1997). As a result, the amount of 0.3 g of glass beads was selected for the optimum enzyme immobilization.

### 3.1.4. Carrier buffer pH

The pH is a major external factor that influences the sensitivity of the biosensor. The optimum pH of the free

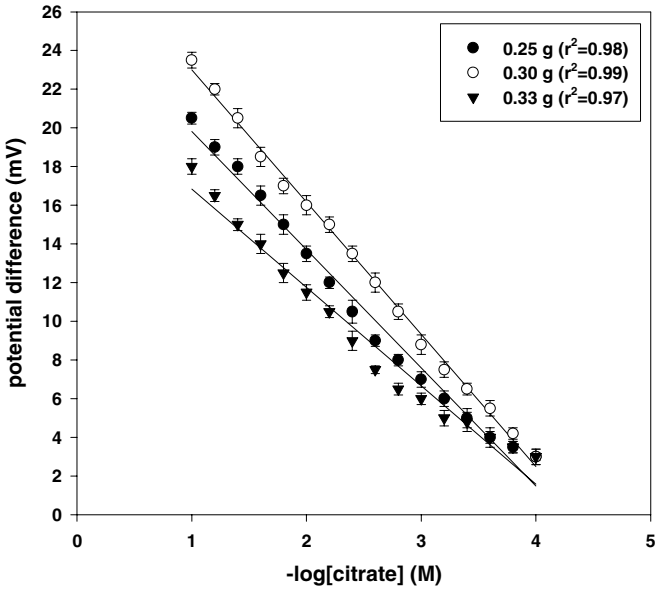


Fig. 4. Effect of the amount of glass beads used for the enzyme immobilization on the citrate biosensor. Each point is a mean for four replicates. Conditions: carrier buffer 1, 0.1 M Tris–$H_2SO_4$, pH 7.5; carrier buffer 2, 0.6 M Tris–$H_2SO_4$, pH 11.0; CL, 10 U and OD, 60 U; flow rate, 18 mL/h.

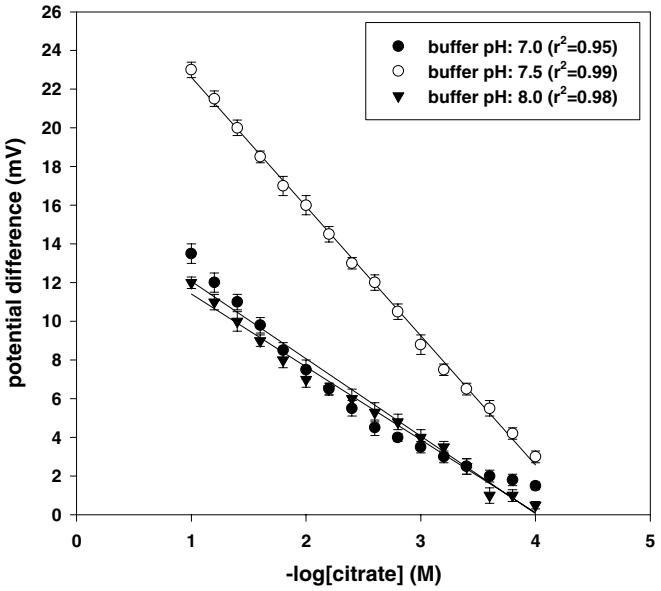


Fig. 5. Effect of the pH of carrier buffer 1 on the citrate biosensor. Each point is a mean for four replicates. Conditions: carrier buffer 2, 0.6 M Tris–$H_2SO_4$, pH 11.0; CL, 10 U and OD, 60 U; glass beads, 0.3 g; flow rate, 18 mL/h.

(non-immobilized) CL and OD were about 6.8–7.5 and 7.5–8.0, respectively (Matsumoto & Tsukatani, 1996; Singh & Srere, 1971), but the optimum pH for the carbonate ion production is 8.4–8.8 (Shin, Sakong, Nam, & Cha, 1996). Therefore, to optimize both enzyme reactions and carbonate ion production, carrier buffers were divided into two buffers with different pHs. Carrier buffer 2 was fixed with 0.6 M Tris–$H_2SO_4$ (pH 11.0) and the pH effect on the response was investigated by changing the pH of carrier buffer 1. Carrier buffer 1 (0.1 M Tris–$H_2SO_4$, variable pH) passed through the enzyme reactor and was then mixed with carrier buffer 2 in a mixing coil in order to increase the pH for carbonate production.

To determine the optimum pH for the responses involving the two enzymes, carrier buffers with pH levels of 7.0, 7.5 and 8.0 were tested. Fig. 5 shows that the carrier buffer with pH 7.5 yielded the maximum response and regression coefficient ($r^2 = 0.99$). The pH of the mixture solution of carrier buffers 1 and 2 that passed through the ISE became pH 8.6, which was the optimum level for carbonate productivity.

### 3.1.5. Flow rate

The dispersion can be manipulated by the injection sample volume, the tube length, and flow rates. Among these factors, the flow rate strongly influences the response sensitivity and the time of base-line reversion (Matsumoto, Ishida, & Nomura, 1984). The time of base-line reversion is defined as the duration from the sample injection to the base-line reversion of the signal.

The effect of different flow rates on the sensitivity was studied by changing the flow rate of carrier buffer 1 from 10.4 to 13.6, 18.0 and 22.2 mL/h. The most desirable flow rate was 18.0 mL/h as shown in Fig. 6. This flow rate showed the highest response and regression coefficient ($r^2 = 0.99$). Decreasing the flow rate leads to a high diffusion of substrate and then the response peak is lowered (Bilitewski & Rohm, 1997). Therefore, flow rate of 10.4 and 13.6 mL/h showed a lower response than the flow rate of 18.0 mL/h. In addition, a slower flow rate required more

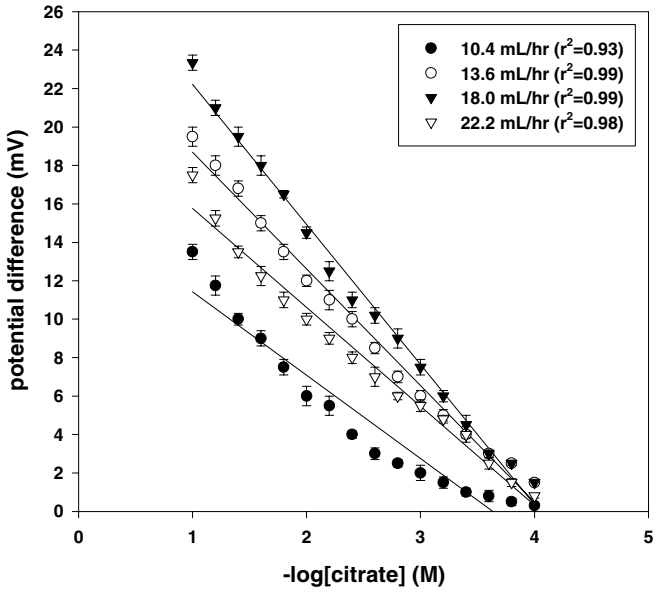


Fig. 6. Effect of the flow rate of carrier buffer 1 on the citrate biosensor. Each point is a mean for four replicates. Conditions: carrier buffer 1, 0.1 M Tris–$H_2SO_4$, pH 7.5; carrier buffer 2, 0.6 M Tris–$H_2SO_4$, pH 11.0; CL, 10 U and OD, 60 U; glass beads, 0.3 g.

measuring time than a faster flow rate. The flow rate of 22.2 mL/h, however, showed a lower response than 18.0 or 13.6 mL/hr. It was explained by the fact that a very fast flow rate could cause a short reaction time of the substrate with enzymes and the product amounts would be small. Therefore, it is regarded that the desirable flow rate should not cause excess substrate dilution along with providing enough time for the reaction of a substrate with enzymes.

In summary, the following optimum parameters were chosen for the operation of the citrate biosensor in the FIA system: co-immobilization of CL 10 U and OD 60 units on 0.3 g glass beads, carrier buffer 1, pH 7.5 at a flow rate of 18.0 mL/h. A calibration curve was obtained using the citrate standard solution under these optimum conditions of the biosensor. A linear relationship of $10^{-1}$ and $10^{-4}$ M was observed with a regression coefficient of 0.9997, between the potential difference and logarithmic citrate concentration (Fig. 7).

### 3.2. Interference test

Fruits and vegetables contain various sugars and organic acids. Therefore, the interference of sugars and organic acids on a citrate biosensor system was examined with commonly occurring sugars (glucose, sucrose and fructose) and organic acids (malate, ascorbate, tartrate and pyruvate).

As shown in Table 1, the effect of their interference was less than 5%. Therefore, the citrate biosensor was expected to be useful in determining the citrate levels of concentration of foods containing sugars and organic acids.

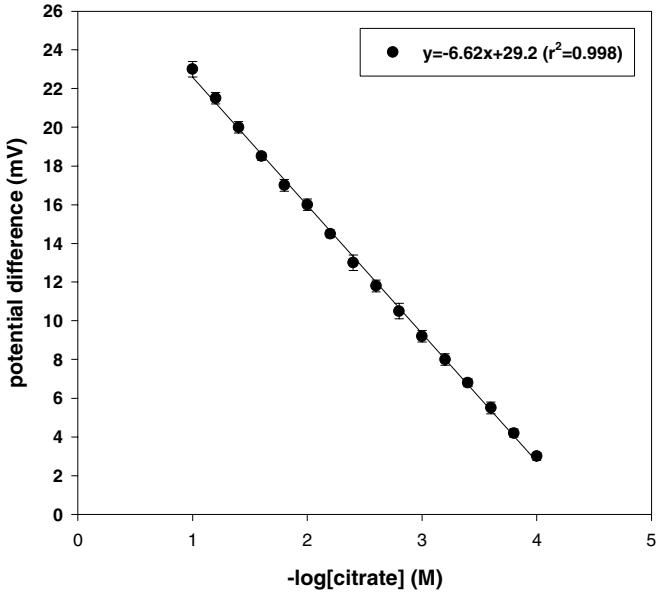


Fig. 7. Calibration curve of citrate solution in the citrate biosensor under the optimum conditions. Each point is a mean for four replicates. Conditions: carrier buffer 1, 0.1 M Tris–$H_2SO_4$, pH 7.5; carrier buffer 2, 0.6 M Tris–$H_2SO_4$, pH 11.0; CL, 10 U and OD, 60 U; glass beads, 0.3 g; flow rate, 18 mL/h.

Table 1
The effect of interference of various sugars and organic acids on the citrate biosensor system

| Additive[a] | Concentration (mM) | Response (%) |
|---|---|---|
| Blank (citrate) | 10 | 100 |
| Glucose | 5 | 100 |
| Sucrose | 5 | 100 |
| Fructose | 5 | 100 |
| Malate | 5 | 103.1 |
| Ascorbate | 5 | 96.2 |
| Pyruvate | 5 | 103.1 |
| Tartrate | 5 | 104.0 |

[a] Each additive was added to the 10 mM citrate solution.

Table 2
Comparison of citrate levels of concentration of fruit juices determined by the biosensor and gas chromatography

| Juice | Citrate concentration (M)[a] | | Difference (%) | Significance |
|---|---|---|---|---|
| | Biosensor | GC | | |
| Carrot | $0.019 \pm 0.001$ | $0.019 \pm 0.003$ | 0 | NS[b] |
| Strawberry | $0.017 \pm 0.002$ | $0.016 \pm 0.002$ | 5.9 | NS |
| Tomato | $0.022 \pm 0.001$ | $0.020 \pm 0.004$ | 9.10 | NS |
| Apple | $0.021 \pm 0.002$ | $0.018 \pm 0.005$ | 14.3 | NS |

[a] Each value is a mean of four replicates.
[b] NS, not significant ($p < 0.05$).

### 3.3. Application of fruit juice samples

The biosensor system was applied to determine citrate concentrations in apple juice, carrot juice, tomato juice and strawberry juice under optimum conditions. After the pretreatment of the juice, 100 μl of the sample was injected into the biosensor and the citrate concentration was analyzed. The range of citrate levels in the fruit juices was from $1.7 \times 10^{-2}$ to $2.1 \times 10^{-2}$ M (Table 2). These values belonged to the linear range of the calibration curve and showed good reproducibility.

The citrate levels in the fruit juices were also measured by a GC and compared to the results obtained from the biosensor (Table 2). There were no significant differences between the two analytical methods ($p > 0.05$). There was a required analysis time of 20 min per sample when the biosensor was used, whereas the GC method required about 2 h. Additionally, the biosensor method needed about 20 min but the GC method required about 3 h in the sample preparation. These results suggest that citrate determination with the biosensor system is fast, accurate and reliable, and is therefore a suitable alternative to conventional analysis methods.

## 4. Conclusions

A potentiometric biosensor for citrate analysis was developed using a $CO_3^{2-}$-selective electrode and enzyme immobilization in a FIA. The citrate biosensor showed a linear correlation ($r^2 = 0.9997$) between the potential difference and logarithmic citrate concentration in the range of

$10^{-1}$–$10^{-4}$ M citrate. The interference effect of major sugars and organic acids on the sensor system was less than 5%. There was no significant difference in the citrate concentrations of fruit juices obtained by the biosensor and GC. Therefore, the citrate sensor developed in this study can be considered a fast, useful and reliable system in determining the citrate concentrations in fruit juices.

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