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# Flow-injection fluorometric quantification of pyruvate using co-immobilized pyruvate decarboxylase and aldehyde dehydrogenase reactor: Application to measurement of acetate, citrate and L-lactate

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**Abstract**

A flow-injection system for the quantification of pyruvate based on the coupled reactions of pyruvate decarboxylase (PDC) and aldehyde dehydrogenase (AlDH) was conceived and optimized. A co-immobilized PDC and AlDH reactor was introduced into the flow line. Sample and reagent ($NAD^+$) were injected into the flow line by an open sandwich method and the increase of NADH produced by the immobilized-enzyme reactor was monitored fluorometrically at 455 nm (excitation at 340 nm). Linear relationships between the responses and concentrations of pyruvate were observed in the ranges of $2.0 \times 10^{-5}$ to $1.5 \times 10^{-3}$ M at the flow rate of 1.0 ml $min^{-1}$ and $5.0 \times 10^{-6}$ to $1.0 \times 10^{-3}$ M at the flow rate of 0.5 ml $min^{-1}$. The relative standard deviation for 10 successive injections was 0.95% at the 1.0 mM level. This FIA system for pyruvate was applied to the measurement of acetate, citrate and L-lactate.


*Keywords:* Flow-injection analysis; Immobilized-enzyme reactor; Fluorometry; Pyruvate; Pyruvate decarboxylase; Aldehyde dehydrogenase

## 1. Introduction

Recently, pyruvate has been quantified by chemiluminescent methods based on the derivatizations using chemical reagents [1,2]. However, these methods are deficient in terms of substrate specificity. High specificity and rapidity are required for the analysis in the field of biochemistry. Enzymatic quantification is a suitable method to meet this demand. The combination of a biosensor and flow injection analysis (FIA) constitutes a powerful technique for the quantification of pyruvate in consideration of rapidity and specificity [3,4].

Enzymatic methods for the spectrophotometric quantification of pyruvate in food and biological fluids have been developed previously [5–7]. In addition, FIA for pyruvate using the enzymatic reaction has been reported [8,9]. These conventional methods are based on the reaction catalyzed by L-lactate dehydrogenase (L-LDH). The resulting consumption of NADH, measured spectrophotometrically, is proportional to the concentration of pyruvate present in the sample:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{L-LDH}}{\rightleftarrows} \text{L-lactate} + \text{NAD}^+ \quad (1)$$

However, NADH is a reactant in reaction (1) and it must be present in excess of the pyruvate to force the equilibrium towards the lactate side. The concentration of NADH should not be too high, otherwise the background increases. Usually a low background is desirable for a high sensitivity setting to allow detection of a low concentration of the substrate.

Thus, we propose a coupled reaction of pyruvate decarboxylase (PDC) and aldehyde dehydrogenase (AlDH) as follows:

$$\text{pyruvate} \xrightarrow{\text{PDC}} \text{acetaldehyde} + \text{CO}_2 \quad (2)$$

$$\text{acetaldehyde} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{AlDH}} \text{acetate} + \text{NADH} + \text{H}^+ \quad (3)$$

The principle of this method is the quantification of pyruvate using a co-immobilized PDC and AlDH reactor, with the

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NADH produced being monitored fluorometrically at 455 nm (excitation at 340 nm).

L-LDH has also been used to quantify other organic acids in FIA systems using the immobilized enzyme reactor. In the FIA systems for acetate reported previously [10,11], the pyruvate produced by the reaction of acetate kinase (AK) and pyruvate kinase (PK) is finally reduced by L-LDH with NADH.

$$\text{acetate} + \text{ATP} \overset{\text{AK}}{\rightleftarrows} \text{acetylphosphate} + \text{ADP} \quad (4)$$

$$\text{ADP} + \text{phosphoenolpyruvate} \overset{\text{PK}}{\rightleftarrows} \text{pyruvate} + \text{ATP} \quad (5)$$

The resulting consumption of NADH, measured spectrophotometrically or electrochemically, is proportional to the concentration of acetate in the sample. However, the resulting system shows low sensitivity because the concentration of NADH cannot be too high. Thus, we set the co-immobilized AK–PK reactor immediately in front of the co-immobilized PDC–AlDH reactor and then tried to quantify acetate. It is thought that the proposed method using the multi-enzyme system shows a high sensitivity because the concentration of $NAD^+$ can be high.

An FIA system for citrate using a free citrate lyase (CL) and an immobilized L-malate dehydrogenase (L-MDH) reactor has been reported [12]. The oxaloacetate produced by the CL reaction is finally reduced by L-MDH with NADH in this system. The resulting consumption of NADH, measured spectrophotometrically, is proportional to the concentration of citrate in the sample. However, the concentration of NADH cannot be too high because a low background is desirable to be able to detect a low concentration of the substrate. Thus, we tried introducing a co-immobilized CL-oxaloacetate decarboxylase (ODC) as an upstream reactor into the FIA system for the measurement of citrate. This method for the quantification of citrate is based on the following reactions:

$$\text{citrate} \overset{\text{CL}}{\rightleftarrows} \text{oxalacetate} + \text{acetate} \quad (6)$$

$$\text{oxaloacetate} \overset{\text{ODC}}{\rightleftarrows} \text{pyruvate} + \text{CO}_2 \quad (7)$$

The pyruvate produced by the reaction of CL and ODC is finally converted to acetate by the co-immobilized PDC–AlDH reactor in the presence of $NAD^+$. It is thought that the proposed method using the multi-enzyme system shows a high sensitivity because the concentration of $NAD^+$ can be high.

FIA systems for L-lactate using an immobilized L-LDH reactor have been reported [13,14]. However, the L-LDH reaction must be carried out under strongly alkaline conditions to force the equilibrium towards the pyruvate side. Thus, the stability of an immobilized L-LDH reactor may be poor. In the quantification of L-lactate, an immobilized L-lactate oxidase (LOD) reactor was set immediately in front of the co-immobilized PDC–AlDH reactor. It is thought that the proposed FIA using the multi-enzyme system can be accomplished in the neutral pH region, and then L-LOD, PDC and AlDH have activities high enough to be useful. This method for the quantification of L-lactate is based on the following reaction:

$$\text{L-lactate} + \text{O}_2 \overset{\text{L-LOD}}{\longrightarrow} \text{pyruvate} + \text{H}_2\text{O}_2 \quad (8)$$

Thus, this FIA system for pyruvate was applied to the measurement of acetate, citrate or L-lactate.

## 2. Experimental

### 2.1. Reagents

Acetate kinase (AK, EC 2.7.2.1, from *E. coli*), aldehyde dehydrogenase (AlDH, EC 1.2.1.5, from yeast) and pyruvate kinase (PK, EC 2.7.1.40, from rabbit muscle) were obtained from Roche (Basel, Switzerland). Citrate lyase (CL, EC 4.1.3.6, from *Klebsiella pnueumoniae*), oxaloacetate decarboxylase (ODC, EC 4.1.1.3, from *Pseudomonas* sp.), pyruvate decarboxylase (PDC, EC 4.1.1.1, from baker's yeast) and aminopropyl-controlled pore glass (APCPG, 80–120 mesh, mean pore diameter 700 Å) were bought from Sigma (St. Louis, MO, USA). L-Lactate oxidase (L-LOD, EC number not available, from *Pediococcus* sp.) was obtained from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). ATP and $NAD^+$ were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

### 2.2. Preparation of immobilized-enzyme reactor

APCPG was used as the support for the enzyme immobilization. PDC (50 U) and AlDH (50 U) were co-immobilized on APCPG as reported previously [15]. AK (250 U) and PK (400 U) were co-immobilized as an upstream reactor for the measurement of acetate. CL (25 U) and ODC (100 U) were co-immobilized for the measurement of citrate. L-LOD (100 U) was immobilized for the measurement of L-lactate.

APCPG (dry weight 0.12 g) was mixed with 0.2 M carbonate buffer (pH 10) containing 5% glutaraldehyde for 2 h at 20 °C while shaking. The support thus treated was placed in 1 ml of 0.1 M phosphate buffer (pH 7.0, coupling solution) containing the enzymes, and allowed to react for about 18 h at 5 °C. After the coupling reaction, the support was washed thoroughly with distilled water and 0.1 M phosphate buffer containing 0.5 M NaCl (washing solution) and coupling solution in turn. The residual functionalities on the enzyme-immobilized support were then blocked with 2 ml of 15 mg $ml^{-1}$ glycine solution by standing for 2 h at 20 °C. The enzyme-immobilized support (0.22 g wet weight) was packed into a glass column (2 mm i.d. × 10 cm), and the immobilized-enzyme reactor was stored in 50 mM glycylglycine buffer (pH 7.5) containing 20 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP) and 0.1 mM dithiothreitol (DTT) at 5 °C until use.

### 2.3. Flow system

A schematic diagram of the FIA system for the quantification of pyruvate is shown in Fig. 1(A). The carrier solution in the reservoir was propelled by a micro-tube pump (MP-

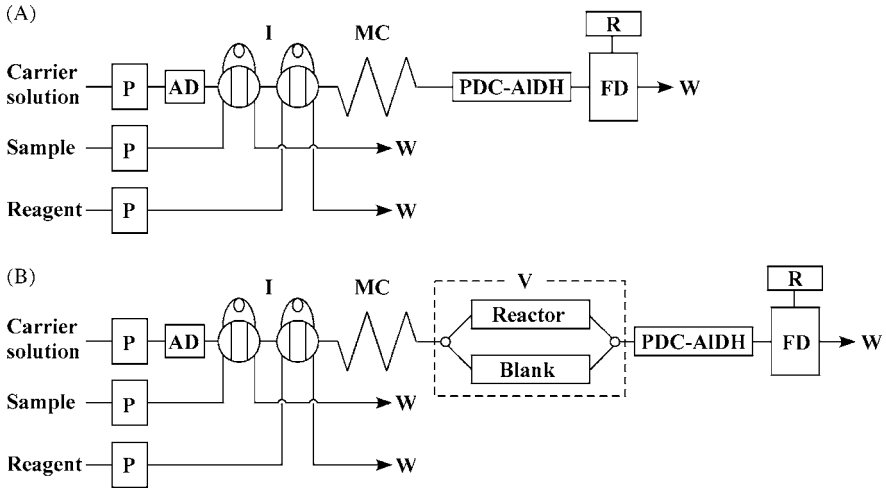


Fig. 1. Schematic diagram of the flow injection system for the quantification of organic acids. (A) FIA system for pyruvate. (B) FIA system for acetate, citrate or L-lactate. P: micro-tube pump (carrier solution, 0.5 ml $min^{-1}$); AD: air damper; I: injector (10-way switching valve); MC: mixing coil (50 cm); V: six-way switching valve; PDC–AlDH: co-immobilized PDC and AlDH reactor (2.0 mm i.d. × 10 cm); Reactor (2.0 mm i.d. × 10 cm), co-immobilized AK and PK reactor for the quantification of acetate, co-immobilized CL and ODC reactor for the quantification of citrate, immobilized L-LOD for the quantification of L-lactate; Blank: blank column; FD: fluorescence detector; R: recorder; W: waste. Inner diameter of the tube is 1.0 mm.

3, Tokyo Rikakikai, Tokyo, Japan) through an air damper, a sample injection valve (10-way switching valve, Select Pro, Alltech, Lexington, KY, USA), a mixing coil (50 cm), and the co-immobilized PDC–AlDH reactor, then transported to a spectrofluorometer (Scanning Fluorescence Detector 470, Waters, Milford, MA, USA) with a flow-through cell connected to a recorder (FBR-251A, TOA, Tokyo, Japan) and finally to a waste tank. The sample flow system consisted of another micro-tube pump connected to a sample injection valve (10-way switching valve) equipped with sample loops. The sample and the reagent ($NAD^+$) were injected by an open sandwich method in order to save coenzymes. In this injection mode, sample (50 μl) and reagent (50 μl) were injected into zones next to each other, as shown in our previous paper [16,17]. Then, the sample and reagent were transported to the immobilized-enzyme reactor while being mixed. The fluorescence intensity was measured at an excitation wavelength of 340 nm and emission wavelength of 455 nm with a spectrofluorometer. The flow system was operated at 25 °C.

In the measurement of acetate, citrate or L-lactate, the immobilized enzyme reactor for the quantification of each substrate and a blank column were set in parallel before the co-immobilized PDC–AlDH reactor, as shown in Fig. 1(B). The main flow line was divided into two flow lines by a six-way switching valve (MPV-6, GL Science, Tokyo). The six-way switching valve was switched to the upstream enzyme reactor for analyte signal intake or to a blank column for a blank value intake. Therefore, the signal of a blank column corresponds to the total fluorescence of the sample itself and of the response for pyruvate and aldehydes in the sample. Especially, ATP, which is injected with a sample, would give a relatively high blank signal. The peak height corresponded to substrate concentration in a sample might be calculated by subtracting the signal of the blank column from the signal of the immobilized-enzyme reactor. The blank column was the same size as the enzyme reactor and contained only APCPG as a support. As the upstream enzyme reactor, a co-immobilized AK–PK reactor, co-immobilized CL-ODC reactor or immobilized L-LOD reactor was used for the measurement of acetate, citrate or L-lactate, respectively. A mixture of 5.0 mM $NAD^+$, 3.0 mM ATP and 2.5 mM phosphoenolpyruvate was injected as the reagent for the measurement of acetate. In the measurement of citrate and L-lactate, 5.0 mM $NAD^+$ alone was used as a reagent.

## 3. Results and discussion

### 3.1. Optimization of FIA system for pyruvate

In order to obtain the maximum sensitivity in FIA system and minimize reagent cost for routine work, the effect of concentrations of $NAD^+$ in a reagent on the FIA response to pyruvate at the 1.0 mM level was studied (data not shown). Acetaldehyde produced from pyruvate by the PDC reaction is oxidized to acetate in the presence of $NAD^+$ by AlDH. The $NAD^+$ solution was injected as a reagent into the flow line by the open sandwich method for economy [16,17]. The response increased with increasing concentration up to 5.0 mM. Thus, $NAD^+$ was used at the concentration of 5.0 mM for economy.

In order to determine the optimal concentration of components in the carrier solution, the effect of concentrations of $Mg^{2+}$, TPP and DTT on the FIA response was investigated. The enzymes used here require $Mg^{2+}$ as an activator. We studied the effect of the concentration of $Mg^{2+}$ on the response to pyruvate at the 1.0 mM level. The response increased with increasing $Mg^{2+}$ concentration and became almost constant above 20 mM (data not shown). Thus, the $Mg^{2+}$ concentration of 20 mM was selected for the carrier solution. PDC is a ternary complex of the apoenzyme with TPP and $Mg^{2+}$, which catalyses the decarboxylation of pyruvate to acetaldehyde [18]. Therefore, it is essential to add TPP and $Mg^{2+}$ to the carrier solution to catalyze the

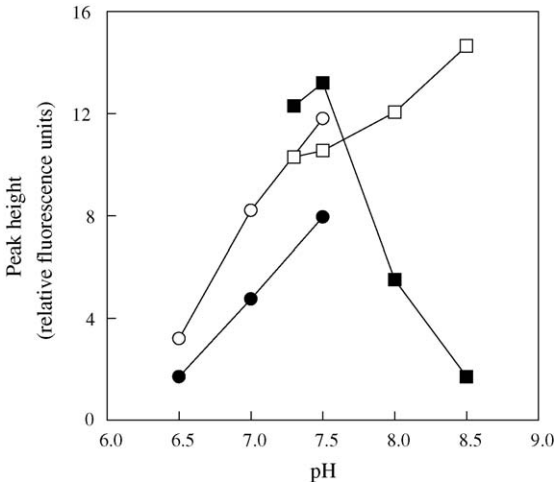


Fig. 2. Effect of pH on the responses to pyruvate and acetaldehyde. 0.1 M Bis–Tris buffer (pH 6.5–7.5): (●), pyruvate; (○), acetaldehyde. 0.1 M glycylglycine buffer (pH 7.3–8.5): (■), pyruvate; (□), acetaldehyde.

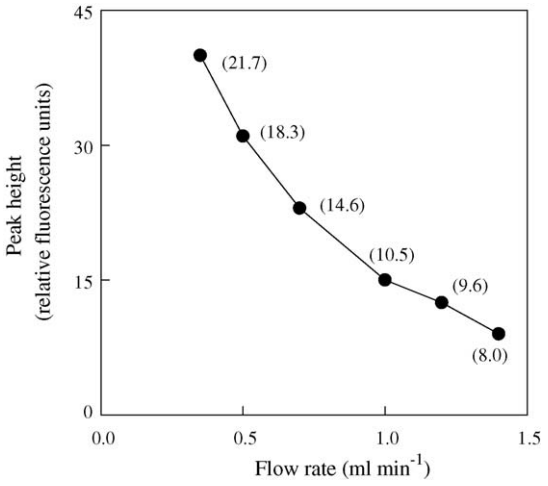


Fig. 3. Effect of flow rate on the response to pyruvate. The value in the parenthesis indicates the conversion efficiency (%) of pyruvate to acetaldehyde by the co-immobilized PDC–AlDH reactor at each flow rate.

reaction. The response to pyruvate is constant above the TPP concentration of 0.1 mM. Thus, TPP was used in the carrier solution at the concentration of 0.5 mM because the holoenzyme is fully reconstituted by the addition of large amounts of TPP and $Mg^{2+}$ to the apoenzyme. In addition, AlDH requires DTT as a stabilizer [19]. Thus, DTT was added to the carrier solution at the final concentration of 1.0 mM.

The effect of pH value on the responses to pyruvate (1.0 mM) and acetaldehyde (0.1 mM) was studied using 50 mM Bis–Tris buffer (pH 6.5–7.5) and 50 mM glycylglycine buffer (pH 7.3–8.5) (Fig. 2). The response to pyruvate increased with increasing pH up to pH 7.5 and then decreased remarkably above that pH value. Immobilized AlDH activity increased with increasing pH. It is reported that the stability of AlDH decreases with increasing pH and a pH of 7.5 is chosen as a compromise between the activity and the stability of immobilized AlDH [19]. Therefore, 50 mM glycylglycine buffer (pH 7.5) was used as the carrier solution. On the basis of these results, we decided to use 50 mM glycylglycine buffer (pH 7.5) containing 20 mM $MgCl_2$, 0.5 mM TPP and 1.0 mM DTT as the carrier solution.

The effect of the flow rate on the response to pyruvate at the 1.0 mM level was investigated in the range of 0.35–1.40 ml $min^{-1}$. The responses to pyruvate decreased remarkably with increasing flow rate as shown in Fig. 3. The conversion efficiency of pyruvate to acetaldehyde by the immobilized-enzyme reactor was 18.3% and 10.5% at the flow rate of 0.5 ml $min^{-1}$ and 1.0 ml $min^{-1}$, respectively. The conversion efficiency of acetaldehyde to acetate was greater than 80% regardless of the flow rate. These results show that the response to pyruvate depends on the reaction rate of PDC.

### 3.2. Calibration, reproducibility, stability and interference

Standard mixtures containing pyruvate were measured at the flow rate of 1.0 ml $min^{-1}$ using the FIA system. FIA responses increased rapidly just after injection of the sample and returned to baseline within about 3.0 min, as shown in Fig. 4. The determination frequency was about 20 tests per hour. Linear relationships between the responses and concentrations of pyruvate were observed between $2.0 \times 10^{-5}$ and $1.5 \times 10^{-3}$ M at the flow rate of 1.0 ml $min^{-1}$ and $5.0 \times 10^{-6}$ and $1.0 \times 10^{-3}$ M at the flow rate of 0.5 ml $min^{-1}$, respectively, with correlation coefficients larger than 0.999 ($n = 8$).

The sensitivity is normally defined as the slope of the calibration curve, which is estimated as the change in relative fluorescence units (RFU) induced by a change in pyruvate con-

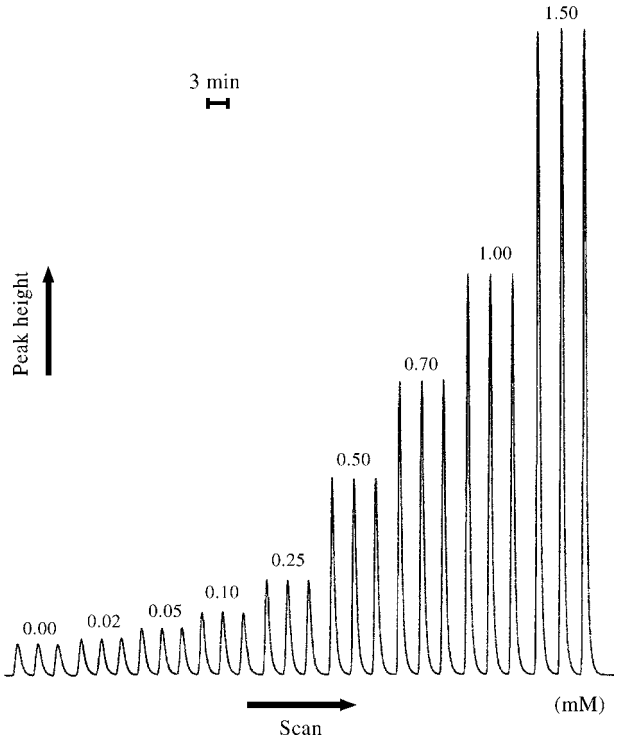


Fig. 4. Typical FIA peaks for the quantification of pyruvate. Conditions are as follows: carrier solution, 50 mM glycylglycine buffer (pH 7.5) containing 20 mM $MgCl_2$, 0.5 mM TPP and 1.0 mM DTT; reagent, 5.0 mM $NAD^+$; reactor, co-immobilized PDC–AlDH reactor; Flow rate, 1.0 ml $min^{-1}$.

Table 1
The measurement parameters of the resulting systems for acetate, citrate and L-lactate

| Analyte | Upstream reactor | Flow rate (ml min$^{-1}$) | Reagent | Linear range (M) |
|---|---|---|---|---|
| Acetate | AK–PK | 0.5 | 5.0 mM $NAD^+$ + 3.0 mM ATP+ 2.5 mM phosphoenolpyruvate | $5.0 \times 10^{-5}$ to $2.5 \times 10^{-3}$ |
| Citrate | CL-ODC | 1.0 | 5.0 mM $NAD^+$ | $2.0 \times 10^{-5}$ to $1.0 \times 10^{-3}$ |
| L-Lactate | L-LOD | 1.0 | 5.0 mM $NAD^+$ | $2.0 \times 10^{-5}$ to $1.5 \times 10^{-3}$ |

centration (mM). The sensitivity of the proposed FIA in the linear range was determined to be 13.37 RFU mM$^{-1}$. On the other hand, the conventional method using an immobilized L-LDH reactor showed the sensitivity of 2.94 RFU mM$^{-1}$. In the conventional method, pyruvate was measured using FIA system shown in Fig. 1(A) with an immobilized L-LDH reactor instead of the co-immobilized PDC–AlDH reactor. The NADH (0.1 mM) was injected as the reagent for the measurement of pyruvate and then the decrease of fluorescence was monitored. The proposed method showed better sensitivity than the conventional method.

It is reported that immobilized PDC showed poor reproducibility and a remarkable loss (more than 90%) of activity with repeated injections of pyruvate [20]. In the co-immobilized PDC–AlDH reactor prepared in our laboratory, the relative standard deviation for ten successive injections was 0.95% at the 1.0 mM level. In addition, the lifetime of the co-immobilized PDC–AlDH reactor was evaluated. The response to pyruvate by the enzyme reactor decreased gradually to 44.2% of the initial value when 347 assays had been performed in a month. The immobilized-enzyme reactor was stored in 50 mM glycylglycine buffer (pH 7.5) containing 20 mM $MgCl_2$, 0.5 mM TPP and 0.1 mM DTT at 5 °C. It is thought that the immobilization of PDC has advantages from the viewpoint of the reuse of enzymes.

The influence of various organic acids (acetate, citrate, formate, L-malate, L-lactate, oxalate, succinate, L-tartrate) on the quantification of pyruvate was investigated. The addition of these components (1.0 mM) had no affect on the quantification of pyruvate at the 1.0 mM level (data not shown).

### 3.3. Application to measurement of acetate, citrate and L-lactate

The FIA system for pyruvate was applied to the measurement of acetate, citrate or L-lactate. The measurement parameters of the resulting systems are shown in Table 1.

In the quantification of acetate, a co-immobilized AK–PK reactor was set immediately in front of the co-immobilized PDC–AlDH reactor, and a mixture of 5.0 mM $NAD^+$, 3.0 mM ATP and 2.5 mM phosphoenolpyruvate was injected as a reagent with a sample. The pyruvate produced by the reaction of AK and PK is measured with the co-immobilized PDC–AlDH reactor. Since the reaction of AK and PK was carried out in the range of pH 7.0–7.6 [10,11], 50 mM glycylglycine buffer (pH 7.5) was used as the carrier solution. A linear relationship between the responses and concentrations of acetate was observed in the range of $5.0 \times 10^{-5}$ to $2.5 \times 10^{-3}$ M at the flow rate of 0.5 ml min$^{-1}$. At this flow rate, the conversion efficiency of acetate to pyruvate by the co-immobilized AK–PK reactor was 17.5%. The equilibrium constant for reaction (4) is $8.6 \times 10^{-3}$, and the equilibrium lies very far in the direction of acetate formation in the neutral pH region [21]. Thus, the response to acetate depends on the reaction rates of AK in addition to PDC.

The sensitivity of the proposed FIA in the linear range was determined to be 5.73 RFU mM$^{-1}$. On the other hand, the conventional method using an immobilized L-LDH reactor showed the sensitivity of 0.88 RFU mM$^{-1}$. In the conventional method, acetate was measured using FIA system shown in Fig. 1(B) with an immobilized L-LDH reactor instead of the co-immobilized PDC–AlDH reactor. A mixture of 0.1 mM NADH, 3.0 mM ATP and 2.5 mM phosphoenolpyruvate was injected as the reagent for the measurement of acetate and then the decrease of fluorescence was monitored. The conventional FIA systems for acetate showed a low sensitivity because the concentration of NADH could not be too high. On the other hand, the proposed FIA system showed a high sensitivity because the concentration of $NAD^+$ can be high.

A co-immobilized CL-ODC reactor was used upstream for the measurement of citrate. It is reported that the optimum pH of CL reaction is 7.5 [22]. Therefore, 50 mM glycylglycine buffer (pH 7.5) was used as the carrier solution. A linear relationship between the responses and concentrations of citrate was observed in the ranges of $2.0 \times 10^{-5}$ to $1.0 \times 10^{-3}$ M at the flow rate of 1.0 ml min$^{-1}$. At this flow rate, the conversion efficiency of citrate to pyruvate by the co-immobilized CL-ODC reactor was 75.8%. Thus, the response to citrate depends mainly on the reaction rate of PDC. In the FIA system for citrate reported previously [12], the concentration of NADH could not be too high because a low background is desirable for a high sensitivity setting to be able to detect a low concentration of the substrate. The background can be low in the proposed FIA system since $NAD^+$, which hardly shows absorption at 340 nm, was used instead of NADH having a maximum absorbance at 340 nm.

For the quantification of L-lactate, an immobilized L-LOD reactor was set immediately in front of the co-immobilized PDC–AlDH reactor. Since the reaction of L-LOD was carried out in the neutral pH region [23,24], 50 mM glycylglycine buffer (pH 7.5) was used as the carrier solution. A linear relationship between the responses and concentrations of L-lactate was observed in the ranges of $2.0 \times 10^{-5}$ to $1.5 \times 10^{-3}$ M at the flow rate of 1.0 ml min$^{-1}$. At this flow rate, the conversion efficiency of acetate to pyruvate by the immobilized L-LOD reactor was 97.4%. Thus, the response to L-lactate depends mainly on the reaction rate of PDC. In the FIA systems for L-lactate reported previously [13,14], the L-LDH reaction must be carried out under strongly alkaline conditions to force the equilibrium towards the

pyruvate side. Thus, the stability of immobilized L-LDH may be poor. It is reported that the response decreases due to the instability of the immobilized L-LDH under higher pH [25]. The half-time of the immobilized L-LDH is eight days. Contrastingly, the proposed FIA could be accomplished in the neutral pH region. Under neutral pH, L-LOD, PDC and AlDH have activities high enough to be useful.

Thus, the FIA system using the immobilized PDC–AlDH reactor can provide measurement of acetate, citrate or L-lactate in addition to pyruvate.

## 4. Conclusions

A new FIA method for the quantification of pyruvate using an immobilized-enzyme reactor was developed. Pyruvate was quantified using a co-immobilized PDC and AlDH reactor. Compared to conventional methods based on the NADH consumption with L-LDH, this FIA method, which is based on the NADH increase, is more sensitive for the analysis of pyruvate. The proposed method was applied to the measurement of acetate, citrate and L-lactate, and linear relationships between the responses and concentrations of substrates were observed.

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