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Sequential fluorometric quantification of malic acid enantiomers by a single line flow-injection system using immobilized-enzyme reactors

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Abstract

A method for the sequential enantiomeric quantification of D-malate and L-malate by a single line flow-injection analysis was developed using immobilized-enzyme reactors and fluorescence detection. An immobilized D-malate dehydrogenase (D-MDH) reactor and an immobilized L-malate dehydrogenase (L-MDH) reactor were introduced into the flow line in series. Sample and coenzyme (NAD^+ or $NADP^+$) were injected into the flow line by an open sandwich method. D-Malate was selectively oxidized by D-MDH when NAD^+ was injected with a sample. When $NADP^+$ was injected with a sample, L-malate was oxidized only by L-MDH. NADH or NADPH produced by the immobilized-enzyme reactors was monitored fluorometrically at 455 nm (excitation at 340 nm). Linear relationships between the responses and concentrations of D-malate and L-malate were observed in the ranges of 1×10^{-6}–1×10^{-4} M and 1×10^{-6}–2×10^{-4} M, respectively. The relative standard deviations for ten successive injections were less than 2% at the 0.1 mM level. This analytical method was applied to the sequential quantification of D-malate and L-malate in fruit juices and soft drinks, and the results showed good agreement with those obtained using conventional method (F-kit method).

Keywords: Flow-injection analysis; Immobilized-enzyme reactor; D-Malate; L-Malate; Fruit juice; Fluorometry

1. Introduction

A racemic mixture of malic acid is approved for use as a food additive and is used mainly as an acidulant. Although food additives can be legally used in food products provided that they are declared on the label, the possibility of adulterative addition of synthetic DL-malic acid cannot be absolutely ignored in the production of fruit juices such as apple juice. Of the two enantiomeric forms of malic acid, L-malic acid occurs naturally. On the other hand, D-malic acid is found in appreciable concentration only in the metabolism of some micro-organisms. Therefore, the detection of D-malic acid shows the adulteration with synthetic DL-malic acid [1].

Malic acid enantiomers have been simultaneously quantified by high performance liquid chromatography (HPLC) with chiral stationary phase columns [2–4] and the ligand-exchange mode on a normal column [5–7]. A method for quantification of malic acid enantiomers in apple juice by HPLC has been reported [8], but it requires a specific post-column reaction and is time consuming. As is well known, food is a complex mixture of many compounds, the concentrations of which are continuously changing. Therefore, high specificity and rapidity are required for the analysis of food. 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 food components with rapidity and specificity [9,10].

In most enzymatic assays of L-malate, the L-malate dehydrogenase (L-MDH) [EC. 1.1.1.37] reaction is used. However, the equilibrium lies very far in the direction of L-malate formation in the neutral pH region. Therefore, it is necessary to shift it in favor of NADH formation by removing the

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reaction products; oxalacetate is trapped with hydrazine or by the glutamate oxalacetate transaminase reaction [11]. Since such trapping reactions are relatively slow, their use is not recommended for FIA [12]. Contrastively, the $NADP^+$-specific L-malate dehydrogenase [EC. 1.1.1.40] reaction proceeds in the direction of pyruvate production in the neutral pH region [13]. Thus, we decided to use $NADP^+$-specific L-MDH for the quantification of L-malate. The D-malate dehydrogenase (D-MDH) reaction also proceeds in the direction of pyruvate formation in the presence of NAD^+ in weakly alkaline conditions [14]. It is thought that both enzymes can be applied to FIA for the quantification of D-malate and L-malate.

We developed a method for the sequential quantification of malic acid enantiomers by a single line FIA using immobilized-enzyme reactors and fluorescence detection. An immobilized-D-MDH reactor and an immobilized-L-MDH reactor were introduced into the flow line in series. The principle of this method for the sequential quantification of D-malate and L-malate makes use of the immobilized-enzyme reactors in a single line, the NADH or NADPH produced being monitored fluorometrically. D-Malate was selectively oxidized by D-MDH when NAD^+ was injected with a sample. When $NADP^+$ was injected with a sample, L-malate was oxidized only by L-MDH. This method for the sequential quantification of malic acid enantiomers is based on the following reactions:

$$\text{D-malate} + NAD^+ \xrightarrow{\text{D-MDH}} \text{pyruvate} + CO_2 + NADH + H^+ \quad (1)$$

$$\text{L-malate} + NADP^+ \xrightarrow{\text{L-MDH}} \text{pyruvate} + CO_2 + NADPH + H^+ \quad (2)$$

2. Experimental

2.1. Reagents

L-Malate dehydrogenase (L-MDH, oxalacetate-decarboxylating, EC 1.1.1.40, from chicken liver) and aminopropyl-controlled pore glass (APCPG, 80–120 mesh, mean pore diameter 700 Å) were bought from Sigma (St. Louis, MO, USA). D-Malate dehydrogenase (D-MDH, EC 1.1.1.83, from *Escherichia coli*) was obtained from Roche (Basel, Switzerland). NAD^+ and $NADP^+$ were purchased from Oriental Yeast (Tokyo, Japan). 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (HEPES) was bought from Dojindo (Kumamoto, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Preparation of immobilized-enzyme reactor

APCPG (0.12 g of dry weight) was used as the support for the enzyme immobilization. L-MDH (8 U) or D-MDH (7 U) was immobilized on APCPG as reported previously [15]. The enzyme-immobilized support was packed into a glass column (2.0 mm i.d. × 10 cm). The enzyme immobilization was carried out in 0.1 M phosphate buffer (pH 7.0). The enzyme reactor was stored in the coupling solution at 5 °C until use.

2.3. Flow system

A schematic diagram of the FIA system for the sequential quantification of D-malate and L-malate is shown in Fig. 1. The carrier solution in the reservoir was propelled by a micro-tube pump (MP-3, Tokyo Rikakikai, Tokyo, Japan) through an air damper, a sample injection valve (10-way switching valve, Select Pro, Alltech, KY, USA), a mixing coil (50 cm), the immobilized-enzyme reactors, 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 sample injection valve (10-way switching valve) equipped with sample loops. The sample and the coenzyme (NAD^+ or $NADP^+$) were injected by an open sandwich method in order to save coenzymes. In this injection mode, sample (50 μl) and coenzyme (50 μl) were injected into zones next to each other [16,17] as shown in Fig. 2. Then, the sample and coenzyme were transported to the immobilized-enzyme reactors with being mixed. By a six-way switching valve (MPV-6, GL Science, Tokyo) in Fig. 1, the line for the coenzyme was switched between NAD^+ and $NADP^+$. Fluorescence intensity was measured at an excitation wavelength of 340 nm and emission wavelength of

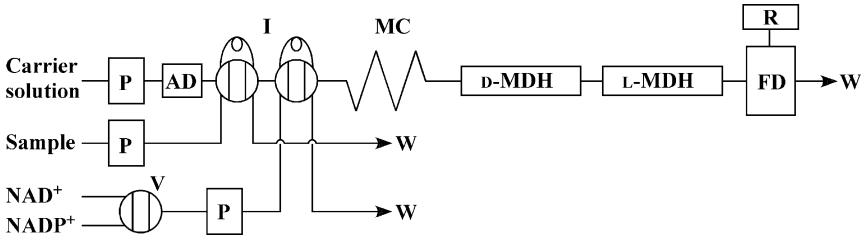

Fig. 1. Schematic diagram of the flow-injection system for the sequential quantification of D-malate and L-malate. P: micro-tube pump (carrier solution, 1.5 ml min^{-1}); AD: air damper; I: injector (10-way switching valve); MC: mixing coil (50 cm); D-MDH: immobilized D-MDH reactor (2.0 mm i.d. × 10 cm); L-MDH: immobilized L-MDH reactor (2.0 mm i.d. × 10 cm); V: switching valve (six-way switching valve); FD: fluorescence detector; R: recorder; W: waste. Inner diameter of tube is 1.0 mm.