Determination of Citrate Using Immobilized Citrate Lyase and Malate Dehydrogenase in a Flow System and Its Application to Analyze Citrate Content of Beverages

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The quantity of citrate was determined using apparatus comprised of a reactor with immobilized citrate lyase and malate dehydrogenase in the flow line. The decrease of β-nicotinamide adenine dinucleotide, reduced form by enzymatic reactions was spectrophotometrically detected as a negative peak. The maximum peak area was obtained at pH 7.6 when the pH of the carrier consisting of phosphate buffer ranged from 6.6 to 8.0. Various buffer types were also examined as carrier media at pH 7.6 and phosphate buffer showed the maximum peak area. When the carrier composed of phosphate buffer (0.1 M, pH 7.6) was used, the calibration curve for citrate was linear in the range 0.5–100 μM (r = 1.000). The detection limit (S/N = 3) was 0.2 μM. The relative standard deviation of the peak area at 10 μM was 2.2% (n = 7). This method was applied to analyze citrate in beverages, and citrate content determined by this method agreed with that determined by a commercially available test kit.

Key words —— citrate, citrate lyase, malate dehydrogenase, immobilized enzyme, flow injection analysis, beverage

INTRODUCTION

Citric acid is a key intermediate in the citric acid cycle. In food chemistry, citrate is used as an antibacterial substance, as an additive in fruit drinks and as an emulsifying agent. Various methods has been proposed for the determination of citric acid, including spectrophotometry,[1] spectrofluorometry,[2] gas chromatography,[3] high-performance liquid chromatography (HPLC)[4–6] and methods using soluble enzymes;[7–10] however, these methods are time-consuming and require complicated procedures.

We have utilized[11–13] immobilized enzymes in a flow system to determine the component of drinks. In these methods, enzymes can be used repeatedly. Owing to the specificity of enzymes, only one peak corresponding to a substrate is observed a short time after injection of a sample. Thus, many samples can be analyzed at a constant time.

In the present work, citrate was determined using a reactor containing citrate lyase (CL) and malate dehydrogenase (MDH). The enzymatic reactions utilized are shown below.

$$\text{citrate} \xrightarrow{\text{CL}} \text{oxalacetate} + \text{acetate}$$

$$\text{oxalacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$$

As citrate lyase is activated[7] by divalent ions, magnesium chloride is contained in the carrier. The decrease of β-nicotinamide adenine dinucleotide, reduced form (NADH) by enzymatic reactions was observed spectrophotometrically at 340 nm. The present method was applied to the analysis of citrate content in sports drinks and soft drinks.

MATERIAL AND METHODS

Materials —— CL and 25% aqueous solution of glutaraldehyde were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.), MDH and NADH were from Oriental Yeast Co., Ltd. (Tokyo, Japan), trisodium citrate dihydrate, triethanolamine hydrochloride and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) were from Wako Pure Chemical Industries Ltd. (Osaka, Japan), piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was from Dojindo Laboratories (Kumamoto, Japan) and aminopropyl glass (500 Å pore size, 200–400 mesh) was from CPG, Inc. (Lincoln Park, NJ, U.S.A.). F-kit is a test kit from Roche Diagnostics GmbH (Basel, Switzerland).

Enzyme Immobilization —— CL and MDH were co-immobilized as described below. The pH of the buffer for immobilization to activated aminopropyl glass differs from that in the previous studies.[11–13]

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To aminopropyl glass beads (0.4 g) in 3.6 ml of 0.1 M phosphate buffer (pH 10.0) was added 0.4 ml of a 25% aqueous solution of glutaraldehyde. The mixture was bubbled with N_2 gas for 1 hr at room temperature. After activated aminopropyl glass was filtered and washed with 100 ml water, 39 units of CL and 50 units of MDH were added with 3.2 ml of 0.05 M phosphate buffer (pH 7.0). The mixture was stirred with a shaker for 20 hr at 4°C, and shaken for a further 4 hr after bovine serum albumen was added to 1% (w/v). Enzyme-immobilized glass beads were filtered and washed with 100 ml of 0.05 M phosphate buffer (pH7.0) and then with 100 ml of water. They were stored in 3.2 ml of 0.05 M phosphate buffer (pH 7.0). In the immobilized enzymes, 5.4% and 15% of the activities of the initially applied enzymes were found for CL and MDH, respectively. The enzyme-immobilized glass beads thus prepared were packed into a stainless steel column (40 mm × 2 mm *i.d.*).

Apparatus —— The apparatus used in this study was shown in Fig. 1. The enzyme reactor (ER) containing immobilized enzymes was immersed in a water bath (WB) at 30°C. The carrier was 0.1 M phosphate buffer (pH 7.6) containing 0.1 mM NADH and 1 mM $MgCl_2$, and this was delivered at a rate of 0.4 ml/min by a Shimadzu LC-10AD pump (P, Shimadzu Cooperation, Kyoto, Japan). The decrease of NADH due to an enzymatic reaction was detected at 340 nm by a Hitachi L-4200H spectrophotometer (D, Hitachi, Ltd., Tokyo, Japan). The negative peak was converted to a positive peak, and this peak area was calculated by a data processor (DP), Hitachi D-2500. The sample injection volume was 50 μl.

Preparation of the Samples —— Three kind of sports drink and two kind of soft drink were used as samples for analysis. Soft drink-1 contains 1% lemon juice and soft drink-2 is a carbonated beverage containing 3% lemon juice. Ten milliliters of sports drinks and soft drinks were neutralized to pH 7–8 with sodium hydroxide solution, quantitatively transferred to a 20 ml volumetric flask, filled with water to the marked line and mixed (solution A). Soft drink-2 was degassed prior to neutralization and dilution. These solutions were appropriately diluted with the carrier and were subjected to analysis. Sports drink-1, -2 were 500 times diluted, and sports drink-3, soft drink-1, -2 were 200 times diluted with the carrier.

Determination of Citrate by F-kit —— Solution A described above was ten times diluted with water and this solution was used for analysis. Five minutes after mixing the sample with buffer containing NADH, MDH and lactate dehydrogenase, the absorbance (A_1) of the mixture was measured at 340 nm. Then, CL solution was added to the mixture. Absorbance (A_2) was measured 5 min after the addition of CL solution. The values of A_1 and A_2 were respectively calibrated with those for blank tests. The content of citrate was calculated from the difference of A_1 minus A_2 calibrated.

RESULTS AND DISCUSSION

Co-immobilization of CL and MDH to activated aminopropyl glass was first performed in the buffer of pH 6.0 according to previous studies;[11–13] however, CL was not immobilized at this pH. The reaction of enzymes with activated aminopropyl glass in the buffer of pH 7.0 resulted in the immobilization

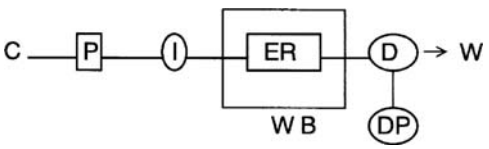

Fig. 1. Apparatus for the Determination of Citrate

C, carrier reservoir; P, pump; I, sample injector; ER, enzyme reactor; D, spectrophotometer; DP, data processor; WB, water bath; W, waste.

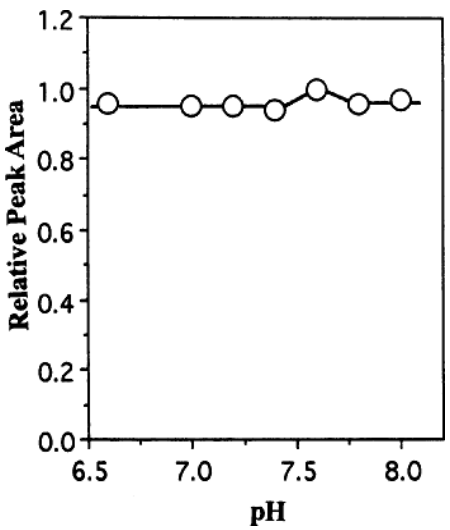

Fig. 2. Dependence of the Peak Area upon the Carrier pH

Ordinate indicates the peak area relative to that at pH 7.6. Values were obtained from the averages of triplicate determinations. Carrier contained 0.1 mM NADH and 1 mM $MgCl_2$. Concentration of citrate injected was 50 μM.

Table 1. Effects of Various Buffer Types as a Carrier Medium upon the Peak Area

Buffer (0.1 M, pH 7.6)	Relative Peak Area
Triethanolamine	0.92
Phosphate	1
PIPES	0.64
Tris	0.91

Values are peak areas relative to that obtained with phosphate buffer, and are the averages of triplicate determinations. Other conditions were as described in the legend of Fig. 2.

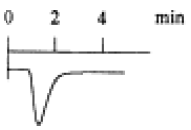

Fig. 3. Negative Peak Due to 10 μM Citrate

of CL and MDH at rates of 5.4% and 15% of the initially applied enzymes, respectively.

The decrease of NADH corresponding to citrate was observed as a negative peak. The negative peak was converted to a positive peak by a data processor, measuring the peak area. Figure 2 shows the dependence of the peak area upon the pH of the carrier comprised of phosphate buffer. Although the peaks in the pH range 6.6–8.0 gave comparable areas, the peak at pH 7.6 afforded the maximum area. Various buffer types were also examined as a carrier medium. Table 1 shows the effect of each buffer type (0.1 M, pH 7.6) as a carrier medium upon the peak area. Of the buffers, phosphate showed the maximum peak area. Thus, phosphate buffer (0.1 M, pH 7.6) was used as the carrier medium for the following experiments.

Figure 3 shows the negative peak, observed at 1.3 min under experimental conditions. The calibration curve obtained was linear (r = 1.000) in the citrate concentration range from 0.5 to 100 μM and the detection limit (S/N = 3) was 0.2 μM. This lower limit of detection is comparable with that observed in the study[9] where soluble CL and MDH were used, detecting the decrease of NADH fluorometrically. This value is larger than that (0.03 μM) observed in the analysis of D-malate[13] where NADH formed by immobilized enzymatic reactions was fluorometrically detected. The relative standard deviation of the peak area at 10 μM was 2.2% (n = 7). The sample containing citrate can be analyzed in 4 min with a single injection, in contrast to the method using soluble enzyme[10] which takes more than 10 min with a complicated procedure.

Table 2. Citrate Content of Beverages

Sample	Present method (mM)	F-kit method (mM)
Sports drink-1	14.3 ± 0.1	14.1
Sports drink-2	12.6 ± 0.1	12.3
Sports drink-3	2.33 ± 0.08	2.24
Soft drink-1	4.86 ± 0.09	4.62
Soft drink-2	6.32 ± 0.28	6.22

Values are the averages of triplicate determinations. The values indicated were obtained by doubling those for samples prepared as described in the Experimental section (solution A).

The present method was applied to analyze the citrate content of sports drinks or soft drinks. Table 2 lists the results of this analysis compared with those obtained by a commercially available test kit (F-kit), which showed good agreement. In the present study, samples applied were those which seemed to contain citrate in high concentration since the sensitivity of the present method was low. It would be interesting to apply the present method to various samples including those which contain citrate in low concentration.

The peak area of 20 μM citrate sample decreased to 76% of that initially detected after analyses of 60 samples, including standard samples for a calibration curve. This decrease of peak area would be due to the decreased activity of immobilized CL, since immobilized MDH had shown comparative stability, as reported in the previous study.[13] It was observed in the analysis of the samples 10 days after the first analysis. The enzyme reactor containing carrier was stored in the refrigerator (about 5°C) for the period between the first and the second analysis. The storage of the enzyme reactor in the medium other than carrier may improve the decrease of the enzyme activities. At present time, it can be said that the analysis within a short period may afford accurate results.

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