Determination of Carboxylesters by Indirect Photometric Ion Chromatography with an On-Line Enzyme Immobilized Column

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Carboxylesters, carboxylic and inorganic anions were determined separately by photometric ion chromatography with an enzyme immobilized column. In the system, a carboxylesterase immobilized column (4.6 mm i.d.×10 mm), which converted carboxylesters to carboxylic anions completely, was set between a sample injector and an analytical column. The other chromatographic conditions were as follows: an anion exchanger-type separation column (4.6 mm i.d.×250 mm); phthalate-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid eluent (pH 6.5); an oven of temperature 40°C; a flow rate of 1.0 ml/min; 280-nm UV detection; and an injection volume of 20 µl. Calibration curves for ethyl esters of acetate, propionate, butyrate and isovalerate were linear with the range 5×10^{-10} mol to 1×10^{-7} mol according to the peak-height method; their detection limits were 10^{-10} mol levels. The present method was applied for the determination of carboxylesters in practical samples.

Keywords Indirect photometric ion chromatography, enzyme immobilized column, carboxylester, carboxylesterase, carboxylic acid

Ion chromatography (IC)[1] has been widely used in the determination of ionic compounds. However neither non-ionic compounds, such as carboxylester do not give any signal, and large molecular ionic compounds, such as hyaluronic acid, have strong retentions of the column in IC. These two facts represent limitations on the application of IC. However, if such compounds are automatically converted to ionic compounds of small molecular size before being passed through a separation column, they can be determined sensitively by IC. In this respect, an enzyme immobilized column is a useful converter owing to its high, selective activity. Both conductivity detection (CD) and photometric detection (PD)[2] are popular methods in non-suppressed (single-column) IC. The authors have used PD–IC for the determination of organic as well as inorganic ions[3–7], since it can be performed on a conventional high performance liquid chromatography (HPLC) apparatus equipped with an ultraviolet (UV) absorbance detector. For the use of an enzyme whose optimum pH is neutral or alkaline PD-IC is more suitable than CD-IC, since its sensitivity for such weak acids as carboxylates and bicarbonate is low within the same pH region.[8] Accordingly, the authors constructed an on-line system with an enzyme immobilized column and conventional PD-IC. By using the enzyme immobilized column between a sample injector and a separation column, both non-ionic and large molecular ionic compounds could be converted to ionic compounds of small molecules which are separately determined by the following separation column and UV absorbance detector.

Although there have been reports concerning CD-IC using enzyme immobilized columns[9–11], they were off-line systems, enzyme immobilized columns were used under different conditions from those of eluents for CD-IC. The present on-line system is superior to the off-line system described above with regard to the following advantages: only one pumping system, common eluent conditions for both enzyme immobilized and separation columns, a small sample volume, and easy operation.

The purpose of this report is to describe the effectiveness of the proposed system regarding the determination of low molecular carboxylesters as sample analytes. Additionally, the influence of the enzyme immobilized column on chromatographic parameters is discussed, since this method is a new approach for using an enzyme immobilized column just before the separation column.

Experimental

Reagents

Phthalic acid and sodium salts of phthalate, benzoate, benzenesulfonate and 1,5-naphthalenedisulfonate were purchased from Wako Pure Chemicals (Osaka, Japan) and of reagent grade. Eluents were prepared by dissolving them in distilled deionized water. The pH

was adjusted with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–sodium hydroxide buffer. The eluent was treated with a Fuji FM-45 membrane filter (pore size of 0.45 μm) before use. Ethyl esters of acetate, propionate, butyrate and isovalerate of reagent grade were purchased from Wako Pure Chemicals. Their standard solutions were prepared by dissolving them supersonically into distilled-deionized water. Carboxylesterase (EC 3.1.1.1) solution (11 mg protein/ml) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All othter chemicals used were of reagent grade.

Apparatus and chromatographic conditions

The system consisted of a Shimadzu LC-6A pump (Kyoto, Japan); a Rheodyne 7125 injector (Cotati, CA, U.S.A.) with a loop of 20 μl; an enzyme immobilized column (4.6 mm i.d.×10 mm, stainless steel); a separation column (4.6 mm i.d.×250 mm, stainless steel) packed with MCI GEL SCA-03 (Mitsubishi Chemical, Tokyo, Japan) anion exchanger (styrene-divinylbenzene copolymer, anion exchange capacity of 0.03 meq./g) or MCI GEL SCA-02 anion exchanger (styrene-divinylbenzene copolymer, anion exchange capacity of 0.01 meg./g); a Shimadzu CTO-6AS column oven; a Shimadzu SPD-6AV spectrophotometric detector; and a Shimadzu C-R3A integrator. A schematic diagram of the system is shown in Fig. 1.

The typical eluents were 1.0×10^{-3} M phthalate–1.5×10^{-4} M HEPES (pH 6.5) for the MCI GEL SCA-03 column and 2.0×10^{-4} M phthalate–3.0×10^{-5} M HEPES (pH 6.5) for the MCI GEL SCA-02 column. Other conditions of the system were as follows: a flow rate of 1.0 ml/min; a 280-nm UV detection; and an oven temperature of 40°C. Nitrogen gas (purity over 99.99%) was purged into the eluent in order to remove any atmospheric carbon dioxide.[12] Upside-down chromatograms were recorded by changing the polarity.

Preparation of carboxylesterase (CE) immobilized column

N-Hydroxysuccinimide (NHS) method:[13] a CE solution (7.1 mg in 4 ml of 3.2 M ammonium sulfate) was dialyzed against 1000 ml of a 0.1 M 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer (pH 7.5) at 4°C overnight so as to prevent ammonium ions from interfering with the immobilization. One gram of Affi-prep 10 (*N*-hydroxysuccinimide ester-form resin of 40–60 μm diameter, Bio-Rad, Tokyo, Japan) was added to the dialyzed CE solution and the mixture was slowly stirred for 2 h at 4°C. After the supernatant was decanted, 7 ml of the MOPS buffer and 0.1 ml of 1 M ethanolamine were added successively to the resin and the mixture was slowly stirred for 1 h at 4°C. The resin was then filtered and washed with the MOPS buffer.

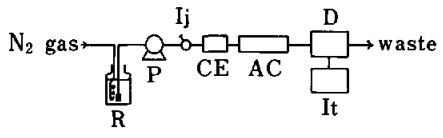

Fig. 1 Schematic diagram of the system. R, eluent reservoir; P, pump; Ij, injector; CE, carboxyl esterase immobilized column; AC, analytical column; D, UV absorbance detector; It, integrator.

Glutaraldehyde (GA) method:[14] The same CE solution as described above was dialyzed against 1000 ml of 0.1 M phosphate buffer (pH 8.0) at 4°C overnight. On the other hand, one gram of CPG-10 (controlled porosity glass beads of 125–177 μm diameter with a pore size of 120 Å, Electro-Nucleonics, NJ, U.S.A.) alkylated with 3-aminopropyltriethoxysilane was added to 5 ml of a 2.5% glutaraldehyde solution and the mixture was slowly stirred for 1 h at room temperature. After being filtered and washed with water, the beads were added to the dialyzed CE solution and the mixture was slowly stirred for 2.5 h at 4°C. These two steps were performed in a glove box filled with a nitrogen gas flow of 50 ml/min. The beads were filtered and washed with the phosphate buffer.

With CE immobilized supports made by the two methods, stainless-steel columns (4.6 mm i.d.) or Teflon tubings (2.0 mm i.d.) were packed using a slurry technique.

Assay of enzyme activity

The activity (unit) of the CE immobilized support (or intact CE) was determined as follows.[15] Fifty milliliters of the substrate solution (5.0×10^{-4} M 4-nitrophenyl acetate in 1.0×10^{-2} M phosphate buffer (pH 8.0)) in a flask was slowly stirred at 25°C. The solution was circulated through a Shimadzu SPD-6AV spectrophotometer at a flow rate of 1.0 ml/min. After the addition of CE immobilized supports (or intact CE) into the flask, the concentration of 4-nitrophenol was determined by monitoring the absorbance at 430 nm. On the other hand, 50 ml of ethyl butyrate (0 – 1.0×10^{-4} M)–1×10^{-2} M HEPES buffer (pH 6.5) was slowly stirred at 25°C. After the addition of CE immobilized supports (or intact CE), the concentration of butyrate was determined to be a hydrolyzed product by conventional PD-IC.[5] The Michaelis constant was calculated from the initial hydrolysis rate.

The relative activities (relative hydrolysis rate) of the CE immobilized column were measured as follows. Ethyl butyrate (1.0×10^{-3} M) dissolved in carrier solutions was pumped through the CE column at a flow rate of 1.0 ml/min. The concentration of butyrate in the eluate was determined by conventional PD-IC.[5]

Extraction of carboxylesters

Carboxylesters in strawberry essence, toothpaste and pineapple juice were extracted into water by nitrogen gas purging.[16] The extraction system consisted of a nitrogen gas bomb, a sample flask (pear-shaped glass, inner volume of 1000 ml) and a trapping bottle (gas-washing glass bottle) containing 100 ml of 2.0×10^{-4} M disodium phthalate as a trapping solution. The

trapping bottle was kept in an ice-water bath. Pineapple juice (100 ml), toothpaste (30 g dissolved in 100 ml of water) or strawberry essence (4 ml dissolved in 100 ml of water) was poured into a sample flask. After setting the sample flask in a water bath kept at 60 °C, nitrogen was purged into the sample solution at a flow rate of 100 ml/min for 1 h. An aliquot (20 µl) of the trapping solution containing carboxylesters was injected into the present system.

Results and Discussion

In the beginning the characteristics of CE immobilized by the two methods were compared. The amounts of immobilized CE were 1.90 mg of protein/g of support by the NHS method and 1.62 mg of protein/g of support by the GA method, respectively. The activity of the former (21 units/mg of protein) was higher han that of the latter (15 units/mg of protein) although intact CE had a higher activity (45 units/mg of protein), as shown in Table 1. Moreover, the enzyme activity of the former remained longer than that of the latter. Therefore, the NHS method was used for CE immobilization in the following experiments. Both the decrease in the activity and the increase in the Michaelis constant by immobilization might be ascribed to a conformational change in CE.

In the present on-line system, it is necessary to use an eluent which does not interfere with the enzyme reaction. The relative activities of the CE immobilized column were compared with such popular UV-absorbing eluents as benzoate, phthalate, benzenesulfonate and 1,5-naphthalenedisulfonate (Table 2).[1,17-20] The activities with these eluents were still more than 63% at 1×10^{-2} M, which was much higher than that usually used, although the activities slightly decreased upon increasing their concentrations. This result suggested that all of the eluents tested might be available for the present purpose. Phthalate was used as an eluent in the present system, since it has been shown to be the most effective eluent in the determination of low molecular-weight carboxylate anions which were hydrolyzed products with carboxylesterase.[5]

The pH is another important factor which affects enzyme activity. The activities of a CE immobilized column were compared with a 1.0×10^{-2} M phthalate eluent. The pH was adjusted to between 4.0 and 9.0 by the addition of a 1.0×10^{-2} M HEPES buffer. The activity increased with an increase of pH in the acidic region and reached a maximum at pH 7. The activity was maintained in a weak basic pH region (Table 3). An eluent of pH around 7 was also suitable for a sensitive determination owing to the following reason. The peak area of carboxylic acid increases as the result of an increase in the eluent pH in PD-IC since the ionic fraction of weak acid increases with an increase in the eluent pH.[8] Therefore, the eluent pH was adjusted to be 6.5 in the following experiments.

Table 1 Characteristics of immobilized carboxyl esterase

Immobilized		Intact
by NHS method	by GA method	
Immobilized amount		
1.90 mg protein/ g support	1.62 mg protein/ g support	
Enzyme activity		
21 unit/mg protein	15 unit/mg protein	45 unit/mg protein
Michaelis constant		
5.6 mM	1.0 mM	0.2 mM
Repeatability		
Yes	Yes	No
(Life time)		
(months)[a]	(days)[a]	

a. CE immobilized supports were packed in the stainless steel column of 4.6 mm i.d.×10 mm.

Table 2 Effect of eluent species on the activity of the CE-column[a]

Carrier solution	Concentration/ M	Relative activity[b]
Distilled-deionized water		1
Benzoate	10^{-4}	0.97
	10^{-3}	0.88
	10^{-2}	0.76
Phthalate	10^{-4}	0.95
	10^{-3}	0.90
	10^{-2}	0.70
Benzenesulfonate	10^{-2}	0.93
1,5-Naphthalenedisulfonate	10^{-2}	0.63

a. One ml of 1.0×10^{-3} M ethyl butyrate in each eluent was pumped into the CE-column (4.6 mm i.d.×10 mm) at a flow rate of 1.0 ml/min. An aliquot (20 µl) of the effluent was injected into the conventional PD-IC.[5]
b. Relative peak area of butyrate to that with distilled-deionized water as a carrier solution.

Table 3 Effect of pH of the phthalate eluent[a] on the activity of the CE-column

pH	Relative activity[b]
4	0
5	0.37
6	0.83
7	1
8	0.96
9	0.95

a. The concentrations of phthalate and HEPES were 1.0×10^{-2} M and 1.0×10^{-2} M, respectively.
b. Relative peak area of butyrate to that at pH 7. Other conditions were the same as in Table 2.

It is assumed that the activity of an enzyme immobilized column becomes higher and its lifetime becomes longer with an increase in the column volume. On the contrary, the number of theoretical plates decreases with an increase in the enzyme column volume. When 20 μl of ethyl butyrate of 10×10^{-3} M was injected into the CE immobilized column with the phthalate eluent at a flow rate of 1.0 ml/min, the column size of 2 mm i.d.×10 mm was quite sufficient to completely hydrolyze ethyl butyrate. A 4.6 mm i.d.×10 mm column was mainly used in the present system and its activity was still high enough to completely hydrolyze carboxylesters after successive use for 8 months.

From the results described above, the conditions of the present on-line system of PD-IC with the CE immobilized column were determined as shown in Fig. 1. The system has the following systematic advantages. First of all, it comprises a conventional one-pumping HPLC apparatus equipped with a UV detector. The present system requires a sample volume of less than 100 μl (the volume of the sample loop is 20 μl). This volume is much smaller than that of the off-line system, which required more than 1 ml.[9-11] The operation of the present system is easier than that of the off-line system, since the enzyme immobilized column of the latter system must be washed after every injection.

The present system was able to determine not only carboxylesters but also organic and inorganic anions simultaneously. Typical chromatograms were shown in Fig. 2 as the comparison of the system with and without CE-column. Both ethyl acetate and ethyl isovalerate were not detected without a CE immobilized column (Fig. 2A). While two ethyl carboxylates were observed respectively as peaks of acetate and isovalerate by the CE column (Fig. 2B). Carboxylate and inorganic anions were detected at their own retention times in both systems with the peak of water dip at the void volume.

Next, chromatographic parameters of the system with and without a CE immobilized column were compared in order to estimate the influence of an enzyme immobilized column positioned just before a separation column. Four ethyl carboxylates used as standards were detected separately, as shown in Fig. 3. Their apparent theoretical plate numbers were all within the range 2200 to 2600 with the CE immobilized column, as listed in Table 4. These numbers might be quite sufficient in practical analyses, though the CE immobilized column decreased the column efficiency by 7–25%. These percentages of the decrease ascribed to the dead volume might be made smaller by reducing the volume of the enzyme immobilized column.

It was interesting that the retention times of ethyl carboxylates were the same as those of the corresponding carboxylic acids (Table 5). This result suggested that the reaction time of CE and ethyl carboxylates was sufficiently short to be negligible and that the adsorp-

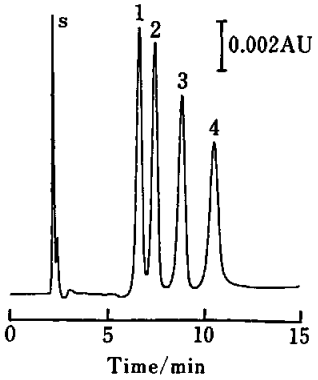

Fig. 3 Typical chromatogram of four ethyl carboxylates. Peaks: s, system peak; 1, ethyl acetate; 2, ethyl propionate; 3, ethyl butyrate; 4, ethyl isovalerate. Other conditions were the same as in Fig. 2.

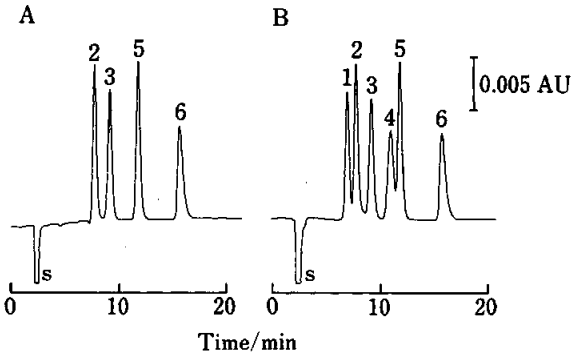

Fig. 2 Chromatograms of ethyl carboxylates, carboxylate anions and inorganic anions with and without the CE-column. A, without the CE-column; B, with the CE-column. Peaks: s, system peak; 1, ethyl acetate; 2, propionate; 3, butyrate; 4, ethyl isovalerate; 5, chloride; 6, nitrite. Sample amount, 1.0×10^{-8} mol each. Other conditions were described in the text.

Table 4 Apparent theoretical plate numbers for ethyl carboxylates and carboxylic acids with and without the CE-column

Injected compound	CE-column	
	with	without
Ethyl acetate	2400	N.D.[a]
Acetic acid	2100	2900
Ethyl propionate	2600	N.D.
Propionic acid	2400	3000
Ethyl butyrate	2400	N.D.
Butyric acid	2600	2800
Ethyl isovalerate	2200	N.D.
Isovaleric acid	2500	2700

a. Not detected.
See text for the chromatographic conditions.

Table 5 Retention times of ethyl carboxylates and carboxylic acids obtained with the CE-column plus separation column

Injected compound	Retention time/min
Ethyl acetate	6.8
Acetic acid	6.8
Ethyl propionate	7.6
Propionic acid	7.6
Ethyl butyrate	8.9
Butyric acid	8.9
Ethyl isovalerate	10.6
Isovaleric acid	10.6

See text for the chromatographic conditions.

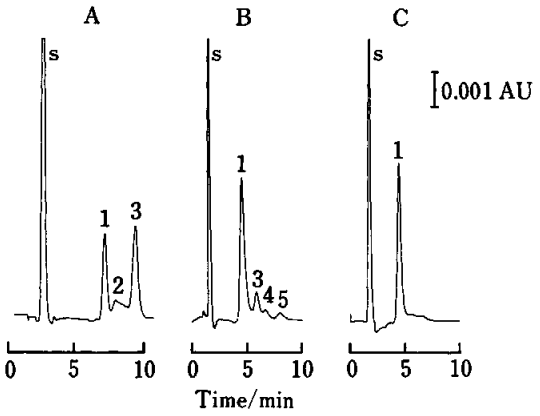

Fig. 4 Chromatograms of strawberry essence, toothpaste and pineapple juice. A, strawberry essence; B, toothpaste; C, pineapple juice. Separation column and eluent: A, MCI GEL SCA-03 (4.6 mm i.d.×250 mm), 1.0×10^{-3} M phthalate–1.5×10^{-4} M HEPES (pH 6.5); B, MCI GEL SCA-02 (4.6 mm i.d.×250 mm), 2.0×10^{-4} M phthalate–3.0×10^{-5} M HEPES (pH 6.5); C, the same as in B. Peaks: s, system peak; 1, acetate ester; 2, bicarbonate; 3, propionate ester; 4, butyrate ester; 5, isovalerate ester.

tion of esters to the resin was sufficiently small. The retentions of carboxylesters may be stronger than those of carboxylate anions by the use of hydrophobic resins.

The detection limits of the four ethyl carboxylates were in the range 3×10^{-10} mol (ethyl acetate) to 5×10^{-10} mol (ethyl isovalerate), since the signal-to-noise ratio was 3. The calibration curves were linear from 1×10^{-9} mol to 1×10^{-7} mol according to the peak-height method. The linearity was not restricted by the activity of the CE immobilized column but, rather, by the capacity of the separation column over 1×10^{-7} mol.

Carboxylesters in strawberry essence, toothpaste and pineapple juice were analyzed as applications of the present method after the extraction of esters into water. The extraction method with nitrogen gas purging has been used for the isolation of volatile compounds from several foods.[5] The recoveries of ethyl esters of acetate, propionate, butyrate and isovalerate by the method were all over 70%. Esters of acetate and propionate were detected in strawberry essence (Fig. 4A). Esters of acetate, butyrate and isovalerate were detected in toothpaste (Fig. 4B). Acetate ester was detected in pineapple juice (Fig. 4C). It should be emphasized that the technique of an enzyme immobilized column with PD-IC is applicable to other compounds. Such studies using other enzymes have been carried out; further details regarding these studies will be presented elsewhere.

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