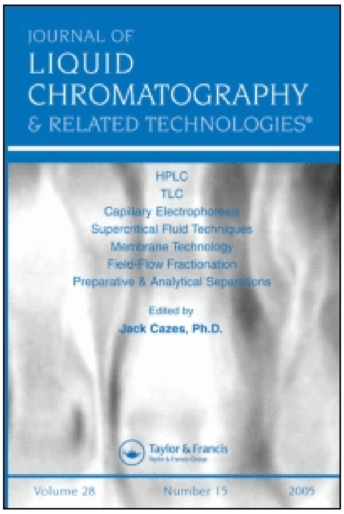

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Countercurrent Chromatographic Separation of Biotic Compounds with Extremely Hydrophilic Organic-Aqueous Two-Phase Solvent Systems and Organic-Aqueous Three-Phase Solvent Systems

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Abstract: Highly polar organic-aqueous two-phase and three-phase solvent systems were applied to the countercurrent chromatographic (CCC) separation of biotic compounds. The ethanol/2 M ammonium sulfate (3 : 5) system was used for protein separation between human serum albumin and lysozyme, and also for separations of sugars including D-(+)-glucose and L-(−)-fucose, D-(+)-xylose and α-L-rhamnose, and α-D-galacturonic acid, mannuronic acid lactone and D-(+)-glucoronolactone. When using the acetonitrile/1 M sodium chloride (5 : 4) system for applying water-soluble carboxylic acids, 2-naphthoic acid, *p*-methyl hippuric acid and *p*-amino hippuric acid were well separated with upper phase mobile, and maleic acid and fumaric acid were resolved with lower phase mobile.

An organic-aqueous three-phase solvent system composed of n-hexane/methyl *t*-butyl ether/acetonitrile/water (5 : 5 : 7.5 : 5) was also applied to the simultaneous CCC separation of fat-soluble vitamins and water-soluble vitamins. Using the middle phase as the stationary phase, the separation of thiamine hydrochloride and nicotinamide was achieved first with the lower mobile phase and then vitamin K1

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and K3 were eluted after the mobile phase was switched to the upper phase reversing the direction of elution.

The overall results demonstrated that both extremely hydrophilic organic-aqueous two-phase solvent systems and organic-aqueous three-phase solvent systems are useful for the CCC separations of polar compounds.

Keywords: Countercurrent chromatography, Separation, Organic-aqueous two-phase solvent system, Organic-aqueous three-phase solvent system, Protein, Sugar, Water-soluble carboxylic acid, Vitamin, Water-soluble vitamin, Fat-soluble vitamin

INTRODUCTION

Countercurrent chromatography (CCC) has been increasingly utilized for the separation and purification of various chemical compounds in both analytical and preparative levels.[1–4] The absence of solid support eliminates various complications such as loss of samples by adsorption and chemical degradation of compounds. Successful separation in CCC requires a proper choice of a two-phase solvent system that provides a suitable range of partition coefficients (K) for the target compounds.

One may conduct a literature search for the suitable solvent systems previously used for the similar compounds. If the nature of the compound is unknown or previous data for the similar compounds are not available, the search requires a time-consuming trial and error method. Oka et al., provided a simple test tube experiment for the selection of suitable organic-aqueous two-phase solvent system in a wide range of hydrophobicity by adjusting the volume ratios using n-hexane/ethyl acetate/1-butanol/methanol/water and chloroform/methanol/water systems.[5] Our previous studies also revealed that the polar two-phase solvent systems composed of methyl *t*-butyl ether/acetonitrile/water are available for the separation of water-soluble carboxylic acids.[6,7] Among these solvent systems described above, 1-butanol/water is most hydrophilic and has been applied to the separation of polar compounds such as sugars[8] and water-soluble vitamins.[9,10] However, more hydrophilic organic-aqueous two-phase solvent systems composed of ethanol or acetonitrile and inorganic salts may be possible to be used for the CCC separation of polar compounds.

While two-phase solvent systems are commonly used for CCC separations, three-phase solvent systems may also be useful for the separation of hydrophobic and hydrophilic compounds, due to a great difference in polarity between the upper and the lower phase sandwiching the middle phase.

This paper describes the CCC separation of biotic compounds with organic-aqueous two-phase solvent systems with high polarity and three-phase solvent systems using the middle phase as a stationary phase.

EXPERIMENTAL

Apparatus

The cross-axis coil planet centrifuge (X-axis CPC) employed in the present studies was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail.[11,12]

The type-J multilayer coil planet centrifuge (type-J multilayer CPC) was purchased from Hitachi Tokyo Electronics (presently Renesas Eastern Japan Semiconductors, Inc., Tokyo, Japan).

Preparation of Coiled Columns

The eccentric coil for the X-axis CPC was prepared by winding 1 mm ID and 2 mm OD Teflon tubing (Flon Kogyo Co., Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes, forming 20 units of left-handed coils connected in series. A set of these coil units was arranged symmetrically around the holder hub of 7.6 cm diameter in such a way that the axis of each coil unit is parallel to the axis of the holder. Two sets of coil assemblies were mounted on the rotary frame, one on each side, and serially connected with a flow tube. The total column capacity was 26.5 mL.

The multilayer coil for the type-J multilayer CPC was prepared by tightly winding a piece of 2.0 mm ID and 3.0 mm OD Teflon tubing around the holder hub of 9 cm in diameter, forming tight coiled layers between a pair of flanges spaced 5.6 cm apart. The total column capacity was 108 mL.

Reagents

The following test samples were purchased from Wako Pure Chemicals (Osaka, Japan): albumin (bovine), albumin (from human serum), D-(+)-glucose, galactose, D-(+)-mannose, D-(+)-xylose, L-(·)-fucose, D-ribose, L-arabinose, α-L-rhamnose, sucrose, lactose, D-(+)-glucuronolactone, α-D-galacturonic acid (monohydrate), N-acetyl-D-(+)-glucosamine, oxalic acid dihydrate, malonic acid, succinic acid, DL-malic acid, citric acid, tartaric acid, maleic acid, fumaric acid, hippuric acid, *p*-amino hippuric acid, phenaceturic acid, *o*-methyl hippuric acid, *m*-methyl hippuric acid, *p*-methyl hippuric acid, benzoic acid, 3-indoleacetic acid, 2-naphthoic acid, (+)-mandelic acid, (−)-mandelic acid, (±)-mandelic acid, vitamin A acetate, β-carotene, (±)-α-tocopherol acetate, (±)-α-tocopherol, calciferol, vitamin D3, vitamin K1, vitamin K3 (2-methyl-1,4-naphthoquinone), thiamine nitrate, thiamine hydrochloride, riboflavin sodium phosphate, riboflavin, L-(+)-ascorbic acid, pyridoxine hydrochloride, cyanocobalamin, nicotinamide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

(EDC). N-acetyl-D-galactosamine was obtained from Nacalai Tesque Inc. (Kyoto, Japan). 2-Nitrophenylhydrazine hydrochloride (ONPH) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Carbonic anhydrase, α-chymotrypsinogen A (from bovine pancreas), cytochrome C (from horse heart), γ-globulins (human), hemoglobin (human), lysozyme (from chicken egg white), myoglobin (from horse skeletal muscle), apo-transferrin (bovine), trypsin inhibitor (soybean), trypsinogen (from bovine pancreas), lactalbumin, D-glucuronic acid (sodium salt), and D-mannuronic acid lactone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems, Three-Phase Solvent Systems, and Sample Solution

Two sets of two-phase solvent systems were prepared: ethanol/aqueous 2 M ammonium sulfate (3 : 5) for separation of proteins and sugars and acetonitrile/aqueous 1 M sodium chloride (5 : 4), for water-soluble carboxylic acids. Three sets of three three-phase solvent systems were prepared for separation of vitamins: n-hexane/methyl *t*-butyl ether/acetonitrile/water (5 : 5 : 7.5 : 5), n-hexane/diethyl ether/acetonitrile/water (5 : 5 : 7.5 : 5) and n-hexane/acetonitrile/methyl acetate/water (7 : 5 : 5 : 5). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and each phase was separated after clear layers were formed.

The sample solutions for the X-axis CPC were prepared by dissolving each standard sample mixture in 0.5 mL of each phase of the two-phase solvent system used for separation, and those for the type-J multilayer CPC were prepared by dissolving the test samples in 1.0 mL of the middle layer of the three-phase solvent system.

Measurement of Partition Coefficients of Samples

Successful CCC separation highly depends upon the choice of the solvent system, which provides suitable partition coefficient (K) values for a set of analytes. In the present study, the K value of each standard sample was determined spectrophotometrically using a simple test tube experiment described by Oka et al.[5] as follows: Two milliliters of each phase of an equilibrated solvent system were delivered into a test tube to which about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of a desired solvent and the absorbance was measured at a desired wavelength using a spectrophotometer (model UV-1600, Shimadzu Corporation, Kyoto, Japan).

For the colorimetric detection, a 100 μL of each phase was separated and evaporated by a centrifugal evaporator (Model CVE-3100, Tokyo Rikakikai, Tokyo, Japan), and then redissolved in an aliquots of water. This solution was subjected to a specific color reaction, i.e., the phenol-sulfuric acid method[13] for neutral sugars, the carbazole method[14] for uronic acids, Morgan-Elson method[15] for N-acetyl amino sugars, and EDC-ONPH method[16] for water-soluble carboxylic acids.

The K value was obtained by dividing the absorbance value of the upper phase by that of the lower phase for two-phase solvent system. For the three-phase solvent systems, the following two K values were obtained: the absorbance in the upper phase divided by that of the middle phase, and the absorbance of the middle phase divided by that of the lower phase.

Separation Procedure

Unless otherwise specified, each separation was initiated by completely filling the column with the stationary phase, followed by injection of the sample solution into the column inlet. Then, the mobile phase was pumped into the column using a reciprocating pump (Model LC-10ADVP, Shimadzu

Table 1. Partition coefficients of various standard proteins in ethanol/aqueous 2 M ammonium sulfate (3 : 5) system

Protein	Partition coefficient ($K = CU/CL$)[a]
Bovine serum albumin	121
Human serum albumin	158
Carbonic anhydrase	36.0
α-Chymotrypsinogen A	27.7
Cytochrome C	15.0
γ-Globulins	6.0
Hemoglobin	42.9
Lysozyme	0.48
Myoglobin	46.0
apo-Transferrin	10.0
Trypsin inhibitor	99.5
Trypsinogen	18.2
Lactalbumin	2.4

[a] Partition coefficients of proteins were calculated from the absorbance of the upper phase (CU) divided by the lower phase (CL) obtained by the spectrophtometric method at UV280 nm.

Corporation), while the column was rotated at 800 rpm in the direction which gives the suitable head-tail elution mode. The effluent from the outlet of the column was collected in test tubes using a fraction collector (Model SF-160, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each collected fraction was diluted with aliquots of desirable solvent and the absorbance was measured using a spectrophotometer. The solutions for neutral sugars, uronic acids and water-soluble carboxylic acids were submitted to the specific colorimetric reaction described above. The absorbance was measured at 260 nm for water-soluble vitamins, 280 nm for proteins and fat-soluble vitamins, 490 nm for neutral sugars, and 530 nm for uronic acids and water-soluble carboxylic acids.

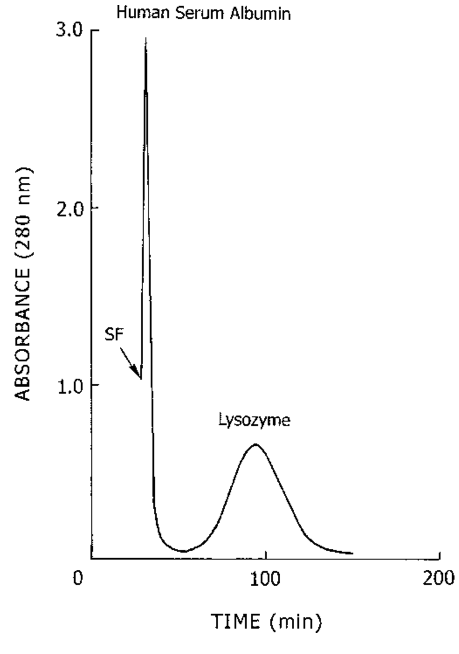

Figure 1. CCC Chromatogram of proteins obtained by the X-axis CPC using ethanol/aqueous 2 M ammonium sulfate (3 : 5) with the upper phase mobile. Experimental conditions: apparatus: X-axis CPC equipped with a pair of eccentric coil assemblies with 1 mm ID and a total capacity of 26.5 mL; sample: 5 mg each of human serum albumin and lysozyme; solvent system: ethanol/aqueous 2 M ammonium sulfate (3 : 5); mobile phase: upper phase; flow rate: 0.4 mL/min; revolution: 800 rpm. SF = solvent front.

RESULTS AND DISCUSSION

CCC Separation of Proteins, Sugars, and Water-Soluble Carboxylic Acids with Extremely Hydrophilic Organic-Aqueous Two-Phase Solvent Systems Using X-axis CPC

Among commonly used organic-aqueous solvent systems, 1-butanol/water is considered to be the most polar binary two-phase solvent system. However, more polar organic solvents, ethanol and acetonitrile for example, can form two layers when they are mixed with high concentrations of aqueous solutions of inorganic salts such as ammonium sulfate and sodium chloride. In the present study, we examined the applicability of two extremely polar two-phase solvent systems, ethanol/ammonium sulfate and acetonitrile/sodium chloride, to the CCC separation using the X-axis CPC. A set of

Table 2. Partition coefficients of various standard sugars in ethanol/aqueous 2 M ammonium sulfate (3 : 5) system

Sugar	Partition coefficient (K = CU/CL)[a]
Neutral sugar	
D-(+)-Glucose	0.40
Galactose	0.39
D-(+)-Mannose	0.40
D-(+)-Xylose	0.53
L-(−)-Fucose	0.62
D-Ribose	0.54
L-Arabinose	0.64
α-L-Rhamnose	0.97
Sucrose	0.47
Lactose	0.24
Uronic acid	
D-Glucuronic acid, sodium salt	0.26
D-(+)-Glucuronolactone	0.81
α-D-Galacturonic acid	0.18
Mannuronic acid lactone	0.47
N-acetyl amino sugar	
N-acetyl-D-(+)-glucosamine	0.01
N-acetyl-D-galactosamine	0.07

[a] Partition coefficients of neutral sugars, uronic acids and N-acetyl amino sugars were calculated from the absorbance of the upper phase (CU) divided by that of the lower phase (CL) obtained by the phenol-sulfuric acid method, the carbazole method and Morgan-Elson method, respectively.

studies on the composition of inorganic salts and volume ratios of these solvent systems revealed that ethanol/aqueous 2 M ammonium sulfate (3 : 5) and acetonitrile/1 M sodium chloride (5 : 4) formed two layers with almost equal volumes of each phase, and they were subsequently applied for the CCC separation of polar compounds such as proteins, sugars and water-soluble carboxylic acids.

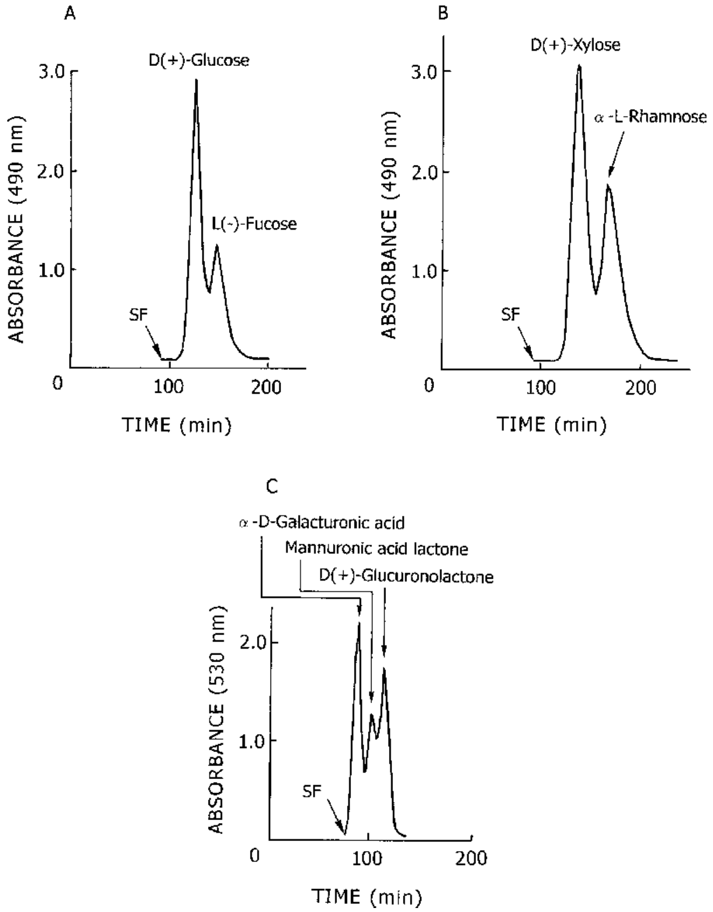

Figure 2. CCC Chromatograms of sugars obtained by the X-axis CPC using ethanol/aqueous 2 M ammonium sulfate (3 : 5) with the lower phase mobile. Experimental conditions: apparatus: X-axis CPC equipped with a pair of eccentric coil assemblies with 1 mm ID and a total capacity of 26.5 mL; sample: (A) 2.5 mg each of D-(+)-glucose and L-(−)-fucose; (B) 2.5 mg each of D-(+)-xylose and α-L-rhamnose; (C) 1.5 mg each of α-D-galacturonic acid, mannuronic acid lactone and D-(+)-glucuronolactone; solvent system: ethanol/aqueous 2 M ammonium sulfate (3 : 5); mobile phase: lower phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

Table 1 summarizes the K values of various standard proteins in ethanol/aqueous 2 M ammonium sulfate (3 : 5) using a simple test tube method. Most of proteins were partitioned in the upper phase except for lysozyme. Based on these results, the CCC separation between human serum albumin and lysozyme were sufficiently achieved with the upper mobile phase by the X-axis CPC as illustrated in Figure 1. The resolution between two peaks was 2.0 and the retention of the stationary phase, 57.7%.

The K values of various sugars including neutral sugars, uronic acids and N-acetyl amino sugars were similarly measured using the same solvent system as summarized in Table 2, where all sugars were partitioned more in the lower phase ($K < 1$). CCC separations of sugars using this solvent system are shown in Figure 2. D-(+)-Glucose and L-(−)-fucose

Table 3. Partition coefficients of various standard aromatic and aliphatic carboxylic acids in acetonitrile/aqueous 1 M sodium chloride (5 : 4) system

	Partition coefficient (K)[a]	
Sample	(CU/CL)	(CL/CU)
Aromatic carboxylic acid		
Hippuric acid	1.63	0.61
p-Amino hippuric acid	0.92	1.23
Phenaceturic acid	2.12	0.47
o-Methyl hippuric acid	2.13	0.47
m-Methyl hippuric acid	1.75	0.57
p-Methyl hippuric acid	1.02	0.57
Benzoic acid	3.67	0.27
3-Indoleacetic acid	3.18	0.31
2-Naphthoic acid	5.07	0.25
(+)-Mandelic acid	2.09	0.48
(−)-Mandelic acid	2.07	0.48
(±)-Mandelic acid	4.11	0.24
Aliphatic carboxylic acid		
Oxalic acid	0.11	9.38
Malonic acid	0.44	2.30
Succinic acid	0.77	1.30
DL-Malic acid	0.33	3.02
Citric acid	0.28	3.56
Tartaric acid	0.16	6.35
Maleic acid	0.38	2.66
Fumaric acid	0.74	1.35

[a] Partition coefficients of aliphatic carboxylic acids were calculated from the absorbance of the upper phase (CU) and that of the lower phase (CL) obtained by EDC-ONPH method.

(Figure 2A), and D-(+)-xylose and α-L-rhamnose (Figure 2B) were separated at the peak resolution of 0.7 and 0.9, respectively. The uronic acids including D-(+)-glucuronolactone, α-D-galacturonic acid and D-mannuronic acid lactone were partially separated from each other as illustrated in Figure 2C. The resolution between α-D-galacturonic acid and mannuronic acid lactone peaks was 0.8 and that between mannuronic acid lactone and D-(+)-glucronolactone was 0.5, while the retention of the stationary phase was 43.3%.

The partitioning of various carboxylic acids were further examined using the acetonitrile/aqueous 1 M sodium chloride (5 : 4) system. As summarized in Table 3, all aliphatic carboxylic acids partitioned mostly into the lower phase, while most of aromatic carboxylic acids were distributed in the upper phase. In the CCC separation, 2-naphthoic acid, *p*-methyl hippuric acid and *p*-amino hippuric acid were separated with the upper mobile phase as illustrated in Figure 3A. Using the lower phase as the mobile phase, maleic acid and fumaric acid were well resolved while the retention of the stationary phase was decreased to 16.1% (Figure 3B).

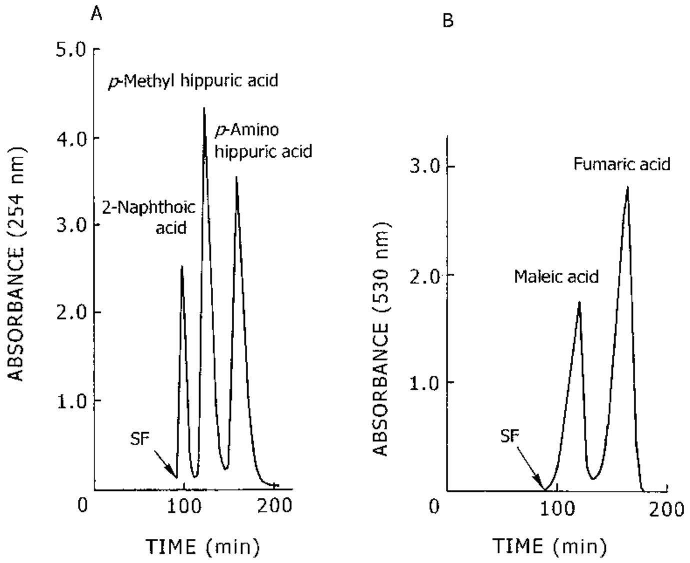

Figure 3. CCC Chromatograms of water-soluble carboxylic acids obtained by the X-axis CPC using the acetonitrile/aqueous 1 M sodium chloride (5 : 4) system. Experimental conditions: apparatus: X-axis CPC equipped with a pair of eccentric coil assemblies of 1 mm ID PTFE tubing; total column capacity: (A) 34.0 mL and (B) 26.5 mL; sample: (A) 2 mg each of 2-naphthoic acid, *p*-methyl hippuric acid and *p*-amino hippuric acid; (B) 2.5 mg each of maleic acid and fumaric acid; solvent system: acetonitrile/aqueous 1 M sodium chloride (5 : 4); mobile phase: (A) upper phase; (B) lower phase; flow rate: 0.2 mL/min; revolution: 800 rpm. SF = solvent front.

As described above, the overall results indicate that extremely hydrophilic organic-aqueous two-phase solvent systems composed of ethanol-ammonium sulfate, or acetonitrile-sodium chloride systems can be used for the CCC separation of polar compounds. Table 4 summarizes the analytical values obtained in the above CCC separations.

Simultaneous Separation of Water-Soluble and Fat-Soluble Vitamins with Organic-Aqueous Three-Phase Solvent System

Table 5 summarizes the experimental results of our studies on the three-phase formation in organic-aqueous solvent systems. The first experiment was carried

Table 4. Analytical values obtained from CCC separations with extremely hydrophilic organic-aqueous two-phase solvent systems

Compound	Partition coefficient[a]	Separation factor(α)	Resolution (Rs)	Stationary phase retention (%)
Protein (Fig. 1)				57.7
Human serum albumin	0.11	15.7	2.0	
Lysozyme	1.73			
Neutral sugar (Fig. 2A)				34.3
D-(+)-Glucose	0.76	1.58	0.7	
L-(−)-Fucose	1.20			
Neutral sugar (Fig. 2B)				31.9
D-(+)-Xylose	1.13	1.61	0.9	
α-L-Rhamnose	1.82			
Uronic acid (Fig. 2C)				43.3
α-D-Galacturonic acid	0.20	2.25	0.8	
Mannuronic acid lactone	0.45	1.42	0.5	
D-(+)-Glucurono-lactone	0.64			
Aromatic carboxylic acid (Fig. 3A)				27.1
2-Naphthoic acid	0.09	4.89	1.5	
p-Methyl hippuric acid	0.44	1.98	1.5	
p-Amino hippuric acid	0.87			
Aliphatic carboxylic acid (Fig. 3B)				16.1
Maleic acid	0.40	3.03	1.5	
Fumaric acid	1.21			

[a] Partition coefficients (K) were calculated according to the conventional formula: $K = (Vr - Vsf) / (Vc - Vsf)$, where Vr, Vc and Vsf indicate the retention volume of the solute peak, the column capacity and the retention volume of the solvent front (volume of the mobile phase in the column), respectively.

out by adding n-hexane or ethyl acetate to the polar two-phase solvent system composed of methyl *t*-butyl ether, 1-butanol, acetonitrile and water where no three phases occurred (Table 5A). Shibusawa et al., previously reported an n-hexane/acetonitrile/methyl acetate/water system forms three phases in which the upper and middle phases were applied to the CCC separation.[17]

Table 5. Three-phase formation by various kinds of organic-aqueous solvent systems

Added organic solvent					
Two-phase solvent system				Three-phase formation	
MBE	1-BuOH	MeCN	H_2O	n-hexane	EtOAc
A. Methyl *t*-butyl ether (MBE) /1-butanol/acetonitrile/water system					
1	0	0	1	×	×
4	0	1	5	×	×
6	0	3	8	×	×
2	0	2	3	×	×
4	2	3	8	×	×
2	2	1	5	×	×
n-hexane	MeCN	MeOAc	H_2O	Phase formation	Volume ratio (v/v) (Upper/Middle/Lower)
B. n-Hexane/acetonitrile/methyl acetate/water system					
5	1	5	5	Two	1.3/0/1
5	2	5	5	Two	1.4/0/1
5	3	5	5	Three	2/2/3
5	4	5	5	Three	1.8/2.4/3
5	5	5	5	Three	2/3/3
Solvent system				Phase formation	Volume ratio (v/v) (Upper/Middle/Lower)
C. n-Hexane/methyl *t*-butyl ether/acetonitrile/water system					
n-hexane	MBE	MeCN	H_2O		
1	1	1	1	Three	7/2/7
EtOAc	MBE	MeCN	H_2O		
1	1	1	1	×	—
n-hexane	MBE	EtOH	H_2O		
1	1	1	1	×	—
n-hexane	Et2O	MeCN	H_2O		
1	1	1	1	Three	8/1.5/8
n-hexane	Et2O	EtOH	H_2O		
1	1	1	1	×	—

×: No three-phase formed.

As shown in Table 5B, the solvent systems at the ratio of 5 : 3 : 5 : 5 to 5 : 5 : 5 : 5 formed three phases while that at the ratio of 5 : 2 : 5 : 5 made only two phases. This result indicates that the content of acetonitrile plays an important role in three-phase formation in this solvent system. Other solvent systems, including n-hexane/methyl *t*-butyl ether/acetonitrile/water and n-hexane/diethyl ether/acetonitrile/water also formed three phases with a small volume of the middle layer (Table 5C).

Table 6 summarizes the volume ratios of three-phase solvent systems obtained by varying the relative volume of acetonitrile. The equal volume ratio was attained at the solvent composition of (5 : 5 : 7.5 : 5) for n-hexane/methyl *t*-butyl ether/acetonitrile/water (Table 6A) and n-hexane/diethyl ether/acetonitrile/water (Table 6B), and (7 : 5 : 5 : 5) for n-hexane/acetonitrile/methyl acetate/water (Table 6C).

Table 7 summarizes the K values of vitamins in these three sets of three-phase solvent systems. Most of fat-soluble vitamins partitioned between the upper and the middle phases, but mainly in the upper phase, while all water-soluble vitamins predominantly partitioned in the lower phase.

Table 6. Volume ratio of three-phase solvent systems

A. n-Hexane/methyl t-butyl ether/acetonitrile/water system

n-hexane	MBE	MeCN	H_2O	Volume ratio (v/v) (Upper/Middle/Lower)
5	5	5	5	7/2/7
5	5	6	5	2/1/2
5	5	7	5	9/8/9
5	5	7.5	5	1/1/1

B. n-Hexane/diethyl ether/acetonitrile/water system

n-hexane	Et_2O	MeCN	H_2O	Volume ratio (v/v) (Upper/Middle/Lower)
5	5	7	5	9/8/9
5	5	7.5	5	1/1/1

C. n-Hexane/acetonitrile/methyl acetate/water system

n-hexane	MeCN	MeOAc	H_2O	Volume ratio (v/v) (Upper/Middle/Lower)
7	5	5	5	1/1/1

Figure 4 schematically illustrates the elution procedure to perform the simultaneous separation of fat-soluble vitamins and water-soluble vitamins using three-phase solvent systems. After filling the column with the middle phase as the stationary phase, the sample mixture of vitamins dissolved with about 1 mL of the middle phase is injected into the column through the sample port and pumped about 25 mL of the middle phase without rotating the column so that the sample is sandwiched between the middle stationary phases (Figure 4A). Then, the lower phase used as the first mobile phase is pumped into the column from the inner terminal after rotating the column at 800 rpm in the counterclockwise direction, while water-soluble vitamins

Table 7. Partition coefficient ratios of vitamins with three different kinds of three-phase organic-aqueous solvent systems

	Solvent system		
	n-hexane 5	n-hexane 5	n-hexane 7
	MBE 5	Et2O 5	MeCN 5
	MeCN 7.5	MeCN 7.5	MeOAc 5
	H_2O 5	H_2O 5	H_2O 5
Partition coefficient ratio	CU : CM : CL	CU : CM : CL	CU : CM : CL
Fat-soluble vitamin			
Vitamin A acetate	4 : 1 : 0.02	4 : 1 : 0.01	3 : 1 : 0.02
β-Carotene	6 : 1 : 0.09	5 : 1 : 0.05	3 : 1 : 0.04
$(\pm)$-α-Tocopherol acetate	22 : 1 : 0.06	21 : 1 : 0.03	19 : 1 : 0.08
$(\pm)$-α-Tocopherol	18 : 1 : 0.05	16 : 1 : 0.05	11 : 1 : 0.1
Calciferol	4 : 1 : 0.1	5 : 1 : 0.07	2 : 1 : 0.08
Vitamin D3	8 : 1 : 0.03	7 : 1 : 0	5 : 1 : 0.03
Vitamin K1	24 : 1 : 0.1	19 : 1 : 0.07	12 : 1 : 0.08
Vitamin K3	0.5 : 1 : 0.2	0.5 : 1 : 0.1	0.5 : 1 : 0.1
Water-soluble vitamin			
Thiamine nitrate	0.2 : 1 : 13	0.2 : 1 : 14	0.5 : 1 : 14
Thiamine hydrochloride	0.8 : 1 : 20	0.6 : 1 : 35	0.3 : 1 : 19
Riboflavin sodium phosphate	0.1 : 1 : 11	0.08 : 1 : 16	0.09 : 1 : 25
Riboflavin	0.1 : 1 : 4	0.03 : 1 : 5	0.02 : 1 : 4
Pyridoxine hydrochloride	0.8 : 1 : 5	0.5 : 1 : 3	0.1 : 1 : 14
Cyanocobalamin	0 : 1 : 125	0 : 1 : 312	0 : 1 : 200
L-(+)-Ascorbic acid	0.07 : 1 : 7	0.02 : 1 : 7	0.01 : 1 : 10
Nicotinamide	0.07 : 1 : 3	0.05 : 1 : 3	0.2 : 1 : 3

Partition coefficient ratios were calculated from the absorbance of the upper phase (CU), the middle phase (CM) and the lower phase (CL) obtained by the spectrophotometric method at UV 260 nm for water-soluble vitamins and 280 nm for fat-soluble vitamins. Abbreviations: MBE = methyl *t*-butyl ether; MeCN = acetonitrile; Et2O = diethyl ether; MeOAc = methyl acetate.

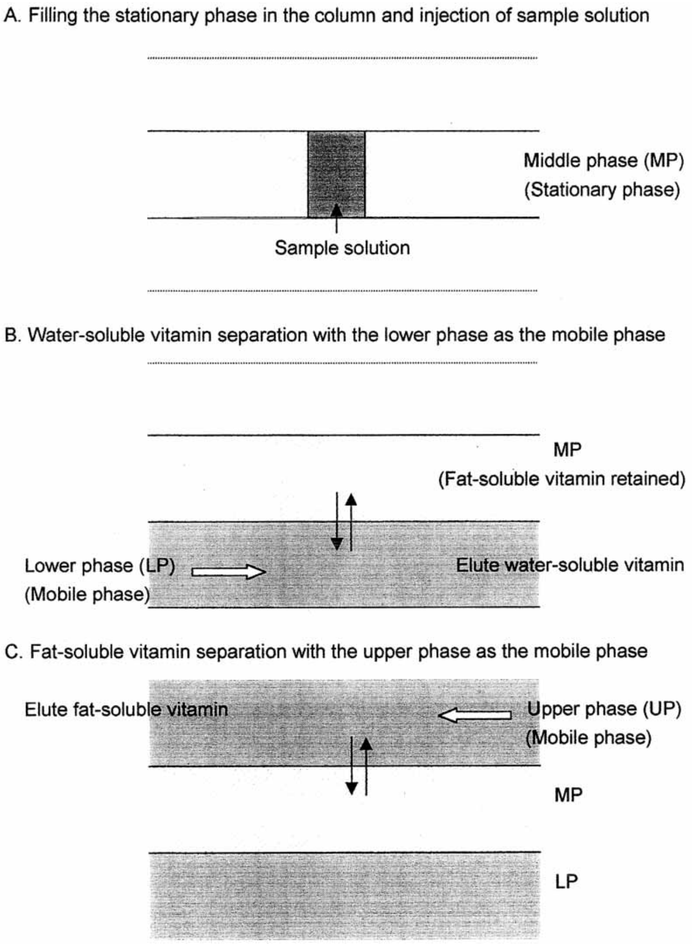

Figure 4. Schematic illustration of elution procedure for CCC separation using organic-aqueous three-phase solvent systems.

are separated and eluted with the lower mobile phase (Figure 4B). Then, the upper phase as the second mobile phase is pumped into the column by switching the inlet and outlet of the flow tube, where the upper mobile phase elutes from the outer terminal in the counterclockwise rotation. Then, fat-soluble vitamins are separated and eluted with the upper mobile phase (Figure 4C).

Figure 5 illustrates the CCC chromatogram obtained using the type-J multilayer CPC with a three-phase solvent system composed of n-hexane/methyl *t*-butyl ether/acetonitrile/water (5 : 5 : 7.5 : 5). Thiamine hydrochloride and nicotinamide are well separated with the lower mobile phase, and vitamin K1 and K3 are also separated with the upper mobile phase. The contents of

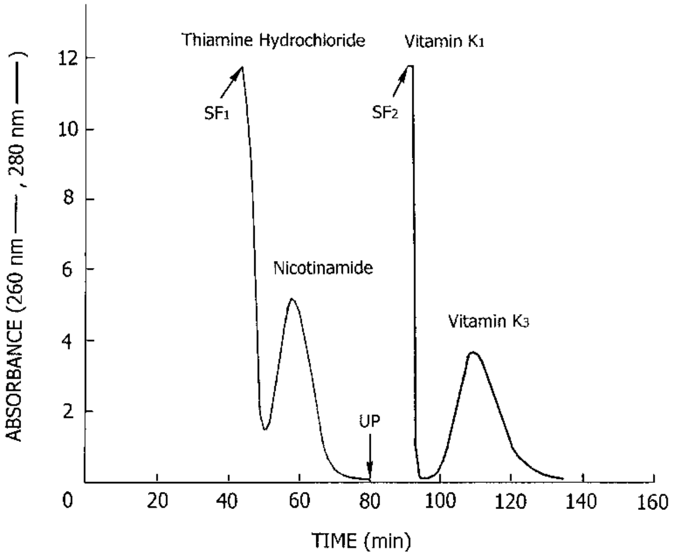

Figure 5. CCC Chromatograms of vitamins obtained by the type-J multilayer CPC using organic-aqueous three phase solvent system. Experimental conditions: apparatus: type-J multilayer CPC with a multilayer coil assembly with 2 mm ID and 108 mL capacity; sample: thiamine hydrochloride (2.5 mg), nicotinamide (2.5 mg), vitamin K1 (10 mg) and vitamin K3 (10 mg); solvent system: n-hexane/methyl *t*-butyl ether/acetonitrile/water (5 : 5 : 7.5 : 5); mobile phase: lower phase (during first 80 min) and upper phase (after 80 min); flow rate: 1 mL/min; revolution: 800 rpm. SF1 = solvent front for lower phase mobile, SF2 = solvent front for upper phase mobile.

each phase remained in the column after the separation was completed were 13.7% of the upper phase, 41.0% of the middle phase and 45.3% of the lower phase. These results demonstrate that the three-phase solvent system is useful for the simultaneous CCC separation of multiple compounds with a broad range of polarity.

CONCLUSION

Extremely hydrophilic organic-aqueous solvent systems were applied to the CCC separation of biotic compounds. The ethanol/aqueous 2 M ammonium sulfate (3 : 5) two-phase system was useful for the separation of proteins and sugars, and the acetonitrile/aqueous 1 M sodium chloride (5 : 4) system for the separation of water-soluble carboxylic acids.

CCC separations using organic-aqueous three-phase solvent systems were also demonstrated using a set of various vitamins as test samples. Using the middle phase as the stationary phase the water-soluble vitamins were separated with the lower mobile phase and then fat-soluble vitamins were eluted with the upper mobile phase by switching the mobile phase and reversing the direction of elution.

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