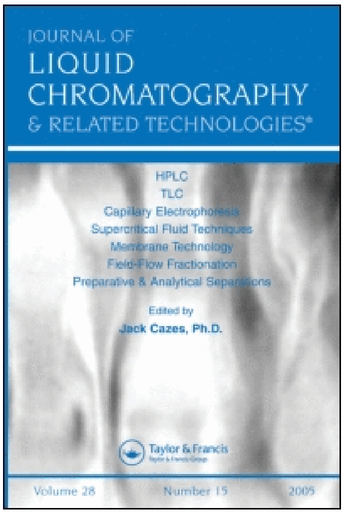

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COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF BIOTIC DICARBOXYLIC ACIDS WITH POLAR TWO-PHASE SOLVENT SYSTEMS USING CROSS-AXIS COIL PLANET CENTRIFUGE

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ABSTRACT

Various aliphatic dicarboxylic acids were separated by countercurrent chromatography using cross-axis coil planet centrifuge (cross-axis CPC) equipped with a pair of eccentric coil assemblies mounted in an off-center position. Partition coefficients of these samples were optimized by adjusting the volume ratio of a polar two-phase solvent system composed of methyl t-butyl ether/1-butanol/acetonitrile/aqueous 0.1% trifluoroacetic acid. Maleic acid and fumaric acid were separated at a volume ratio of 5:0:0:5

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by the lower aqueous phase mobile, while tartaric acid, succinic acid, and fumaric acid were well resolved by a more polar solvent system at a volume ratio of 2.9:0:2.9:4.3. Using the most polar binary solvent system composed of 1-butanol/water, oxalic acid, malonic acid, and succinic acid were resolved from each other by the lower aqueous phase mobile. The overall results of the present studies indicate that the cross-axis CPC is useful for the separation of various polar organic acids.

INTRODUCTION

Countercurrent chromatography (CCC) is a liquid-liquid separation technique relying on the partition of a solute between two immiscible solvents. The absence of solid support eliminates various complications, such as loss of samples by adsorption and chemical degradation of compounds. Among various CCC devices developed in the past, both type-J multilayer coil planet centrifuge and cross-axis coil planet centrifuge (cross-axis CPC) have proven most efficient for the separation of numerous natural and synthetic products.(1-3)

The cross-axis CPC produces a unique planetary motion, such that the column holder rotates about its horizontal axis while revolving around the vertical axis of the centrifuge.(4,5) This motion provides satisfactory retention of the stationary phase for viscous low-interfacial tension two-phase solvent systems, such as aqueous-aqueous polymer phase systems.

An improved model of the cross-axis CPC was constructed in our laboratory for performing CCC with aqueous two-phase solvent systems.(6-8) Our previous studies demonstrated, that the cross-axis CPC equipped with a pair of multilayer coils or eccentric coil assemblies in the off-center position was useful for the separation of proteins with polyethylene glycol–potassium phosphate solvent systems.(6-8) The apparatus is also useful for the separation of highly polar compounds such as sugars,(9) hippuric acid,and related compounds,(10) which require the use of polar two-phase solvent systems.

In the present paper, we report the separation of biotic dicarboxylic acids using the cross-axis CPC with eccentric coil assemblies.

EXPERIMENTAL

CCC Apparatus

The cross-axis CPC employed in the present study was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design of the apparatus was previously described in detail.(6-8)

Preparation of Separation Columns

A pair of eccentric coil assemblies was used in the present study. Each coil assembly was prepared by winding a 1 mm-ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) onto 7.6 cm long, 5 mm-OD nylon pipes forming a series of tight left-handed coils. A set of these coil units was symmetrically arranged 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. A pair of coil assemblies was mounted on the rotary frame, one on each side, and serially connected with the flow tube. The total column capacity is 26.5 mL.

Reagents

Standard samples including citric acid, fumaric acid, maleic acid, DL-malic acid, malonic acid, oxalic acid dihydrate, succinic acid, and tartaric acid, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), were purchased from Wako Pure Chemicals (Osaka, Japan). 2-Nitrophenylhydrazine hydrochloride (ONPH) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

A set of two-phase solvent systems was prepared from methyl t-butyl ether (MBE), 1-butanol, acetonitrile (AcN), and aqueous 0.1% trifluoroacetic acid (TFA) at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases separated after two clear layers were formed. Standard sample solutions for CCC separation were prepared by dissolving each sample mixture in 0.5 mL of each phase of the two-phase solvent system used for separation.

Measurement of Partition Coefficients of Standard Samples

Successful CCC separation highly depends on the choice of the two-phase solvent system, which provides suitable partition coefficient values for a set of analytes. In the present study, partition coefficient (K) of each standard sample was determined spectrophotometrically using a simple test tube experiment described by Oka et al.,(11) 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 two clear layers were formed, a 1 mL aliquot of each phase was evaporated and the residue was redissolved in 2 mL of distilled water. This solution was subjected to the special color reaction for polar carboxylic acids using 2-nitrophenylhydrazine hydrochloride (ONPH),(12) which produces acid hydrazides. The absorbance was measured using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan). The K value was obtained by dividing the absorbance value of the upper organic phase by that of the lower aqueous phase.

Separation Procedure

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 KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan), while the column was rotated at 800 rpm in a counterclockwise direction. The effluent from the outlet of the column was collected in test tubes (0.8 mL/tube) using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each fraction was evaporated to dryness and redissolved in an aliquot of distilled water. This solution was submitted to the specific color reaction for polar carboxylic acids as follows:(13) Two milliliters of the sample solution was mixed with 1 mL each of 0.01 M ONPH in 0.4 M HCl and 0.15 M EDC in 4% (v/v) pyridine. The solution was incubated at 40°C for 30 min, mixed with 1 mL of 1.5 M sodium hydroxide solution, and again incubated at 60°C for 15 min to maximize color development. The absorbance was measured at 530 nm using a spectrophotometer.

RESULTS AND DISCUSSION

Biotic dicarboxylic acids are extremely hydrophilic and require the specific color reaction for detection because these aliphatic acids contain no aryl group in each molecule (Fig. 1). In the present studies, the polar two-phase solvent systems composed of MBE/1-butanol/AcN/aqueous 0.1% TFA at various volume ratios were examined for suitable K values on a set of standard samples. TFA was added to the solvent system to protonate the carboxyl group, which increases hydrophobicity of the molecule.

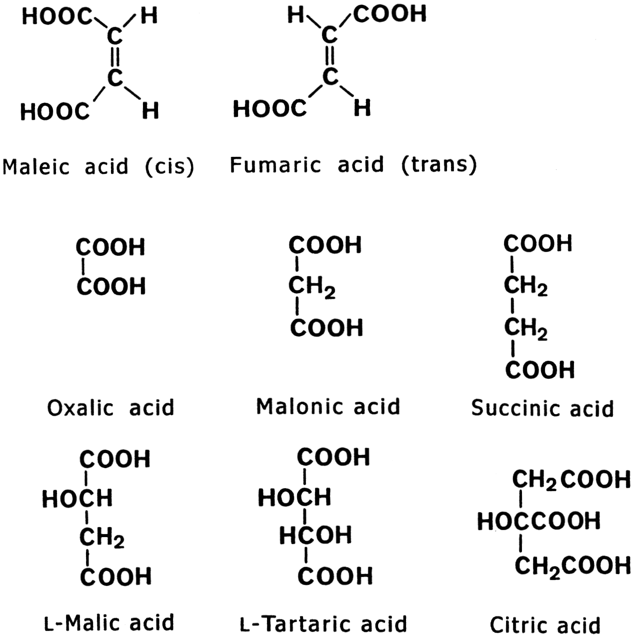

Figure 1. Chemical structures of standard samples used in the present studies.

Figure 2 illustrates the K values of standard samples in the above solvent systems. K values of the organic acids used in the present studies decreased as the hydrophobicity of the solvent system was increased, except for fumaric acid, which showed high K values regardless of the phase composition. These partition data may be useful for predicting the retention time of various organic acid samples.

Figure 3 illustrates the CCC chromatograms of dicarboxylic acids obtained with the above solvent system composed of MBE/1-butanol/AcN/aqueous 0.1% TFA. In Fig. 3A, maleic acid and fumaric acid are separated at a volume ratio of 5:0:0:5, which is used for the separation of aromatic acids, such as hippuric acid reported earlier.(13) Using a more polar solvent system at a volume ratio of 2.9:0:2.9:4.3, tartaric acid, succinic acid, and fumaric acid were well resolved by the lower aqueous mobile phase as shown in Fig. 3B.

Figure 4 illustrates a chromatogram of oxalic acid, malonic acid, and succinic acid, obtained using the most polar binary solvent system composed of 1-butanol/water. All components were well resolved from each other, and eluted 2.3 h, using the lower aqueous phase as a mobile phase.

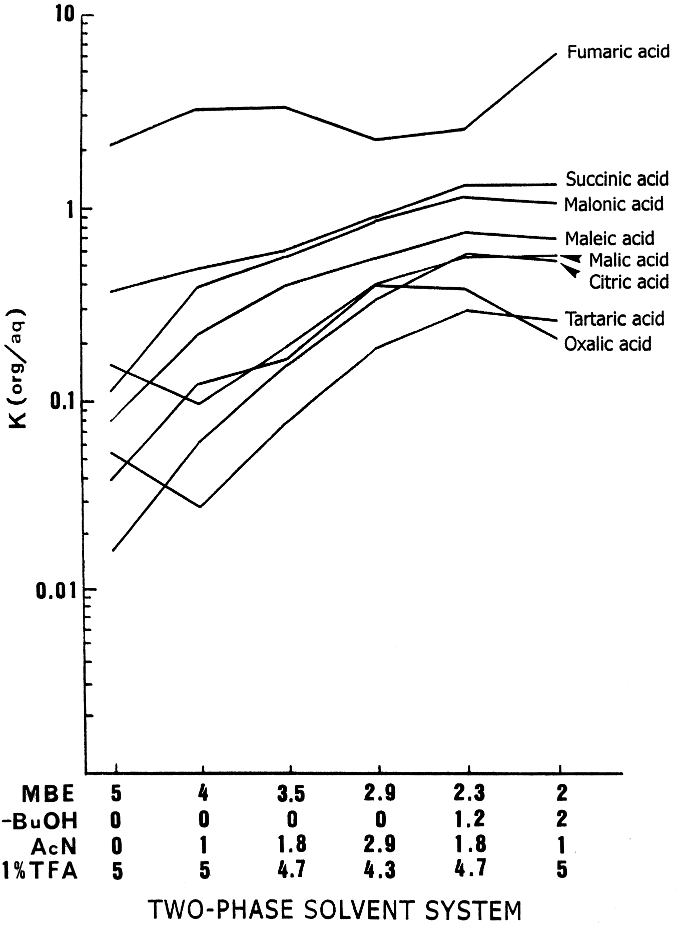

Figure 2. Partition coefficients (K = Corg/Caq) of polar organic acids in methyl t-butyl ether/1-butanol/acetonitrile/aqueous 0.1% trifluoroacetic acid system determined by the test tube experiments (see text). MBE = methyl t-butyl ether; 1-BuOH = 1-butanol; AcN = acetonitrile; TFA = trifluoroacetic acid.

Table 1 summarizes the analytical values obtained from the present CCC separation studies. The stationary phase retention obtained by the MBE/1-butanol/AcN/aqueous 0.1% TFA solvent systems using the cross-axis CPC with eccentric coil assemblies was 48.7%, which was a poor retention volume ratio compared with that obtained using the type-J multilayer coil planet centrifuge

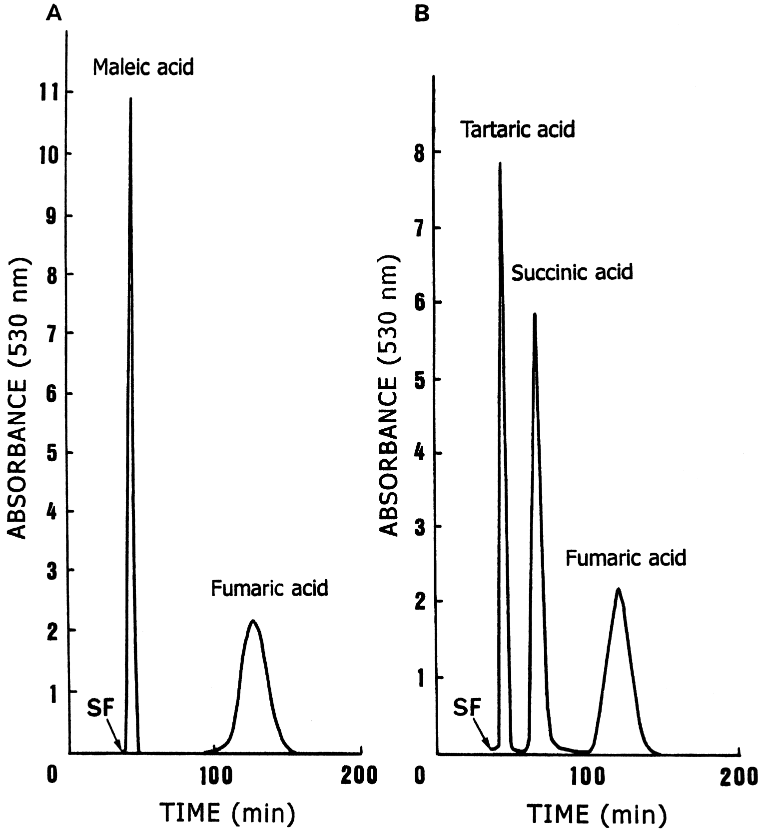

Figure 3. CCC separation of polar organic acids by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, 1mm ID and 26.5mL total column capacity; sample: (A) maleic acid (3 mg) and fumaric acid (3 mg); (B) tartaric acid (5 mg), succinic acid (5 mg) and fumaric acid (2.5 mg); solvent system: methyl t-butyl ether/1-butanol/acetonitrile/aqueous 0.1% trifluoroacetic acid (A)(5:0:0:5); (B)(2.9:0:2.9:4.3); mobile phase: lower phase; flow rate: 0.4 mL/min; revolution: 800 rpm. SF = solvent front.

(86%),(14) where ach separation was sufficiently achieved. However, as shown in Fig. 4, the cross-axis CPC can retain the most polar binary solvent system, such as 1-butanol/water, which gives poor retention in the separation column of the type-J multilayer coil planet centrifuge.

As shown in Table 1, most of the K values computed from the CCC chromatograms are substantially smaller than those obtained by the test tube experi-

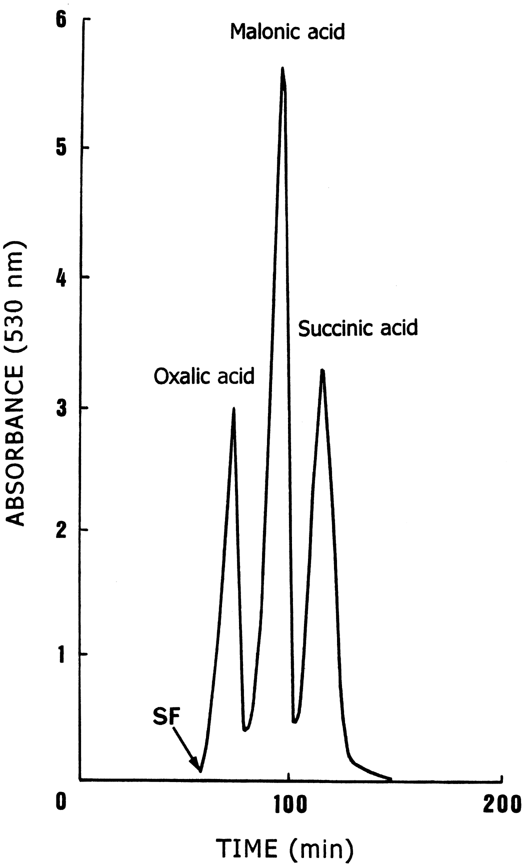

Figure 4**.** CCC separation of oxalic acid, malonic acid and succinic acid by cross-axis CPC. Experimental conditions: sample: 3 mg each; solvent system: 1-butanol/water; flow rate: 0.25 mL/min. For other experimental conditions, see the Fig. 3 caption. SF = solvent front.

ment. This discrepancy may be caused by some of the following factors: the sample mixture introduced into the column locally modified the two phase composition, which depleted the volume of the retained stationary phase; the system lost some amounts of the stationary phase by carryover after the solvent front had emerged; and increased column temperature during the run altered the phase composition and the K values of the solutes.

Table 1. Analytical Values Obtained from Present CCC Separation Studies

Organic Acid	Stationary Phase Retention (%)	Partition Coefficient*	Separation Factor (α)	Resolution (Rs)
I. Figure 3A (Solvent system: MBE/1-BuOH/AcN/aqueous 0.1%TFA = 5:0:0:5)				
	48.7			
Maleic Acid		0.31 (0.08)		
			9.39 (26.5)	4.0
Fumaric Acid		2.91 (2.12)		
II. Figure 3B (Solvent system: MBE/l-BuOH/AcN/aqueous 0.1%TFA = 2.9:0:2.9:4.3)				
	48.7			
Tartaric Acid		0.37 (0.19)		
			2.84 (4.89)	2.2
Succunic Acid		1.05 (0.93)		
			2.60 (2.53)	2.6
Fumaric Acid		2.73 (2.35)		
III. Figure 4 (Solvent system: 1-BuOH/H_2O)				
	47.2			
Oxalic Acid		0.36 (0.22)		
			2.22 (3.05)	1.4
Malonic Acid		0.80 (0.67)		
			1.50 (1.81)	1.1
Succinic Acid		1.20 (1.21)		

*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. The values in the parentheses were calculated from the experimental results obtained using the test tube method.

As described above, the overall results of the present studies indicate that various polar organic acids can be resolved by cross-axis CPC using a pair of eccentric coil assemblies mounted in the off-center position with polar two-phase solvent systems.

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