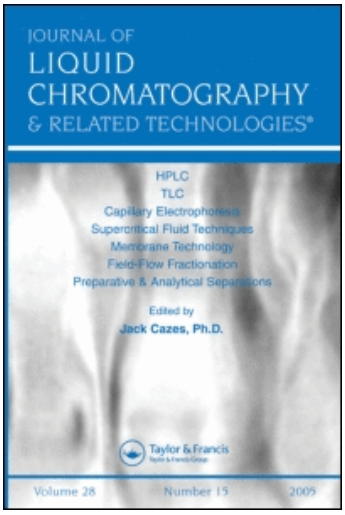

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COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF VITAMINS BY CROSS-AXIS COIL PLANET CENTRIFUGE WITH ECCENTRIC COIL ASSEMBLIES

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ABSTRACT

Vitamins were resolved by high-speed countercurrent chromatography using a cross-axis coil planet centrifuge equipped with eccentric coil assemblies. A two-phase solvent system composed of 1-butanol/ethanol/aqueous 0.15 M monobasic potassium phosphate (8:3:8) was used for the separation of water-soluble vitamins such as thiamine nitrate, pyridoxine hydrochloride, and nicotinamide, while 2,2,4-trimethyl pentane (isooctane)/methanol (1:1) was used for the separation of fat-soluble vitamins such as calciferol, vitamin A acetate, and (±)-α-tocopherol acetate.

Overall results of experiments revealed that the cross-axis coil planet centrifuge is useful for the separation of vitamins by selecting suitable two-phase solvent systems.

INTRODUCTION

Countercurrent chromatography (CCC) is essentially a form of liquid-liquid partition chromatography in which the stationary liquid phase is retained in the apparatus without use of a porous or adsorptive matrix. Since no supporting matrix is employed, the separation is not distorted by solute adsorption, and solute elution volumes can be accurately predicted from the partition coefficients in the solvent system and measurements of the stationary and mobile phase volumes. CCC has many advantages in the separation and purification of various natural and synthetic compounds.[1–3]

The majority of devices for CCC employ a coil of tubing that retains the stationary phase in more or less segmented compartments while the mobile phase is passed through it.

Among many CCC systems developed in the past, the cross-axis coil planet centrifuge (cross-axis CPC) has a unique mode of planetary motion such that the column holder rotates about its horizontal axis of the centrifuge.[4,5] The centrifugal force field produced by this planetary motion provides stable retention of the stationary phase even for aqueous-aqueous polymer phase systems with extremely low interfacial tension and high viscosity.

Recently, an improved model of the cross-axis CPC has been constructed in our laboratory for performing CCC with aqueous-aqueous polymer phase systems.[6–8] Our previous studies demonstrated that the cross-axis CPC equipped with either a multilayer coil or eccentric coil assembly in the off-center position of the column holder can be effectively applied for the separation of proteins[6–8] and sugars.[9]

This paper describes the separation of a series of vitamins by CCC using the cross-axis CPC.

EXPERIMENTAL

Apparatus

The cross-axis CPC employed in the present studies was constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The basic feature of the apparatus was previously described in detail[6-8] and a brief description is given here. The apparatus produces a synchronous planetary

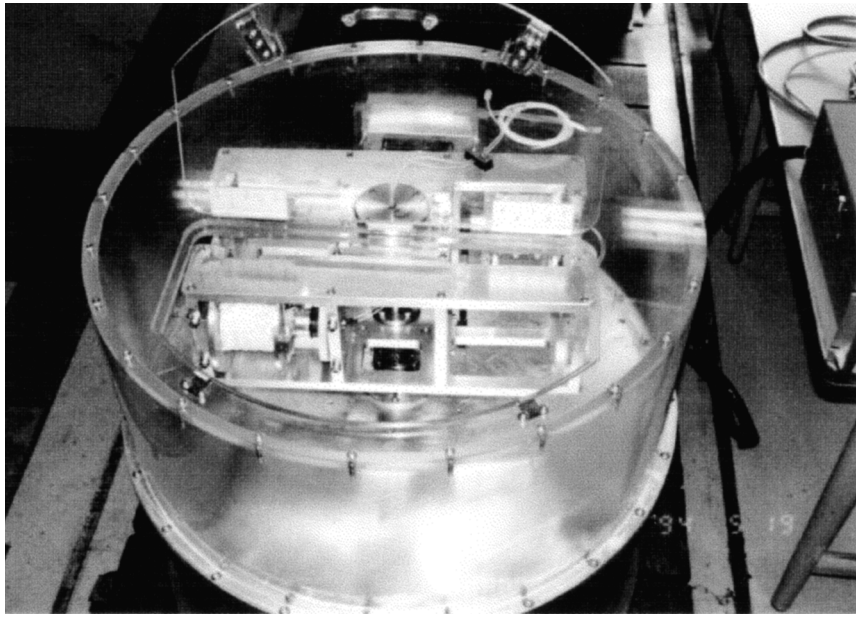

Figure 1. Photograph of the cross-axis CPC.

motion of the column holder which rotates about its horizontal axis and simultaneously revolves around the vertical axis of the apparatus at the same angular velocity. The column holder was mounted at an off-center position (X = 10 cm and L = 15 cm) which provides efficient mixing of the two phase solvent systems and stable retention of the stationary phase in the coiled column. Figure 1 shows a photograph of the cross-axis CPC used in the present studies.

Preparation of Coiled Columns

The separation columns used in the present study were a pair of eccentric coil assemblies. Each 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 20 units of serially connected left-handed coils. 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 the flow tube to obtain a total column capacity of 26.5 mL. Figure 2 illustrates the schematic drawing of the eccentric coil assembly used in the present studies.

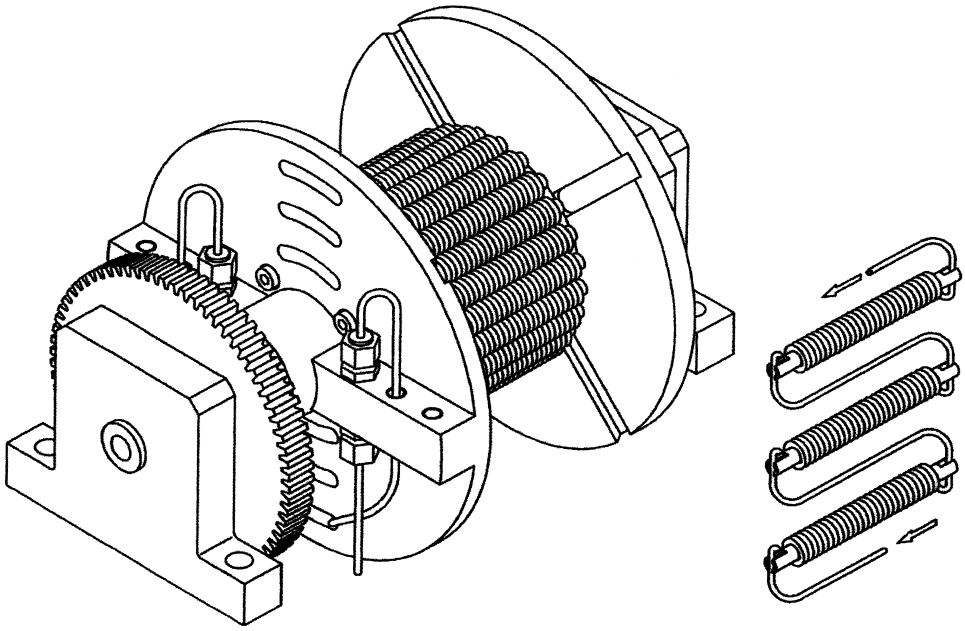

Figure 2. Schematic drawing of the eccentric coil assembly.

Reagents

Thiamine nitrate, thiamine hydrochloride, riboflavin, riboflavin sodium phosphate, pyridoxine hydrochloride, cyanocobalamin, L-ascorbic acid, nicotinamide, vitamin A acetate, calciferol, (±)-α-tocopherol acetate, vitamin K_1, and vitamin K_3 were purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were of reagent grade.

Preparation of Two-Phase Solvent Systems and Sample Solutions

According to the polarity of the analytes, several types of two-phase solvent systems were prepared for the CCC separation: 1-butanol/aqueous 0.15 M monobasic potassium phosphate (1:1); and 1-butanol/ethanol/aqueous 0.15 M monobasic potassium phosphate (8:3:8) for water-soluble vitamins; and 2,2,4-trimethyl pentane (isooctane)/methanol (1:1) for fat-soluble vitamins. Other solvent systems for the measurement of partition coefficients are shown in Table 1 and 2, described later.

Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated after two clear layers formed.

Table 1

Partition Coefficients of Water-Soluble Vitamins in 1-Butanol/Aqueous 0.15 M Monobasic Potassium Phosphate Two-Phase Solvent Systems*

1-Butanol	**1**	**4**	**8**
Ethanol	**0**	**1**	**3**
0.15 M KH_2PO_4	**1**	**4**	**8**
Thiamine Nitrate (M.W. 327.36)	0.03	0.05	0.11
Thiamine Hydrochloride (M.W. 327.27)	0.14	0.05	0.17
Riboflavin (M.W. 376.37)	0.54	0.83	1.08
Riboflavin Sodium Phosphate (M.W. 478.33)	0.11	0.18	0.34
Pyridoxine Hydrochloride (M.W. 205.64)	0.23	0.43	0.59
Cyanocobalamin (M.W. 1355.38)	0.04	0.12	0.24
L-Ascorbic Acid (M.W. 176.12)	0.07	0.11	0.15
Nicotinamide (M.W. 122.13)	1.84	1.41	1.34

* Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

Sample solutions were prepared by dissolving each vitamin mixture in 0.5 mL of each phase of the two-phase solvent system used for separation.

Measurement of Partition Coefficients of Vitamin Samples

The partition coefficient (K) of each vitamin was determined spectrophotometrically using a simple test tube procedure as follows: Two milliliters of each phase of the equilibrated two-phase 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 two clear layers formed, a 1 mL aliquot of each phase was diluted with 2 mL of methanol, except that the aqueous phase of water-soluble vitamins was diluted with distilled water. The absorbance was measured at 260 nm for water-soluble vitamins and 280 nm for fat-soluble vitamins, respectively, using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan). The partition

Table 2

Partition Coefficients of Fat-Soluble Vitamins in Four Different Two-Phase Solvent Systems*

	n-Hexane/ Aqueous 90% Acetonitrile (1:1)	n-Hexane/ Acetonitrile (1:1)	2,2,4-Trimethyl Pentane/ Methanol (1:1)	n-Hexane/ Ethyl Acetate/ Methanol/ Water (1:1:1:1)
Vitamin A Acetate	5.34	1.69	1.34	130
Calciferol (M.W. 396.66)	8.14	3.29	0.83	71.2
(±)-α-Tocopherol Acetate (M.W. 472.25)	48.5	10.1	3.11	62.6
Vitamin K_1 (M.W. 450.71)	61.6	5.79	3.92	9.23
Vitamin K_3 (M.W. 172.19)	0.31	0.22	0.35	2.40

* Partition coefficients were calculated from the absorbance of the upper phase divided by that of lower phase.

coefficient (K) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Separations of Vitamins

For each separation, the coil was completely filled with the upper stationary phase and the sample solution (ca. 1 mL) injected into the column inlet. Then, the lower mobile phase was pumped into the column at a flow rate of 0.4 mL/min 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 of water-soluble vitamins was diluted with 2 mL of distilled water and the absorbance was measured at 260 nm. To the fat-soluble vitamin

fraction, a 2 mL aliquot of methanol was added and the absorbance was determined at 280 nm.

RESULTS AND DISCUSSION

CCC Separation of Water-Soluble Vitamins

The partition coefficient values are used to select the optimal solvent system since it predicts the retention time of each component. Table 1 shows partition coefficients of various water-soluble vitamins in the 1-butanol/aqueous 0.15 M monobasic potassium phosphate system. Most of the water-soluble vitamins were partitioned almost unilaterally into the aqueous phase in this solvent system except that riboflavin and nicotinamide were distributed significantly into the organic phase (K = 0.54 – 1.84). Adding ethanol to the two-phase solvent system significantly increased the partition coefficients of riboflavin and pyridoxine hydrochloride.

Figure 3A illustrates the CCC separation of thiamine nitrate, riboflavin, and nicotinamide by the cross-axis CPC with 1-butanol/aqueous 0.15 M monobasic potassium phosphate (1:1) system. Riboflavin sodium phosphate is also resolved riboflavin with the same solvent system (Figure 3B). When a more polar solvent system of 1-butanol/ethanol/aqueous 0.15 M monobasic potassium phosphate (8:3:8) is used, thiamine nitrate, pyridoxine hydrochloride, and nicotinamide were well resolved with each other. Resolution between polar vitamins such as thiamine nitrate, L-ascorbic acid, and thiamine hydrochloride requires the use of more polar solvent systems and/or adding ion pair reagent to the solvent system in analogy with HPLC.

CCC Separation of Fat-Soluble Vitamins

In the present studies, four different kinds of solvent systems were tested for partition coefficients of fat-soluble vitamins. The results are summarized in Table 2. In the n-hexane/aqueous 90% acetonitrile (1:1) solvent system, most of the fat-soluble vitamins were partitioned almost unilaterally into the upper organic phase. Using a more hydrophobic non-aqueous solvent system composed of n-hexane/acetonitrile (1:1), vitamin A acetate and calciferol were significantly partitioned into the lower aqueous phase, whereas (±)-α-tocopherol acetate still remained in the upper organic phase. The most suitable solvent system examined for fat-soluble vitamins was composed of 2,2,4-trimethyl pentane (isooctane)/methanol.

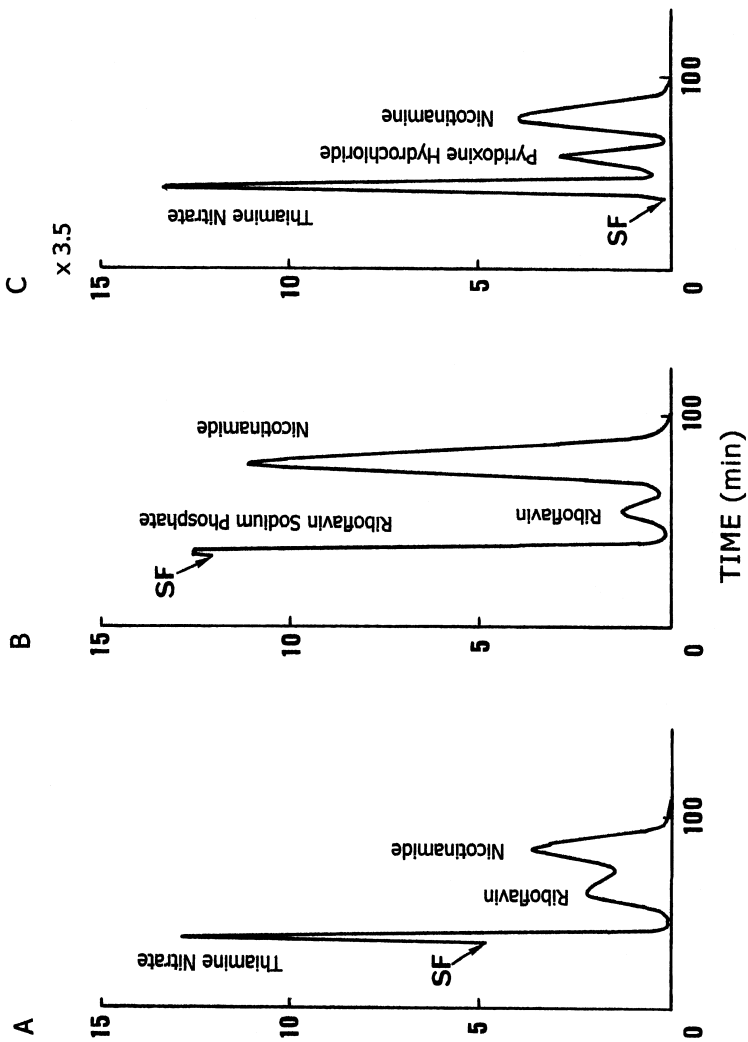

Figure 3. CCC separation of water-soluble vitamins by cross-axis CPC. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 26.5 mL capacity; sample: (A) thiamine nitrate (2.5 mg) + riboflavin (1.5 mg) + nicotinamide (2.5 mg), (B) riboflavin sodium phosphate (2.5 mg) + nicotinamide (2.5 mg) and (C) thiamine nitrate (2.8 mg) + pyridoxine hydrochloride (4.0 mg) + nicotinamide (3.0 mg); solvent system: (A),(B) 1-butanol/aqueous 0.15 M monobasic potassium phosphate (1:1) and (C) 1-butanol/ethanol/aqueous 0.15 M monobasic potassium phosphate (8:3:8); mobile phase: lower phase; flow rate: 0.4 mL/min; revolution: 800 rpm. SF = solvent front.

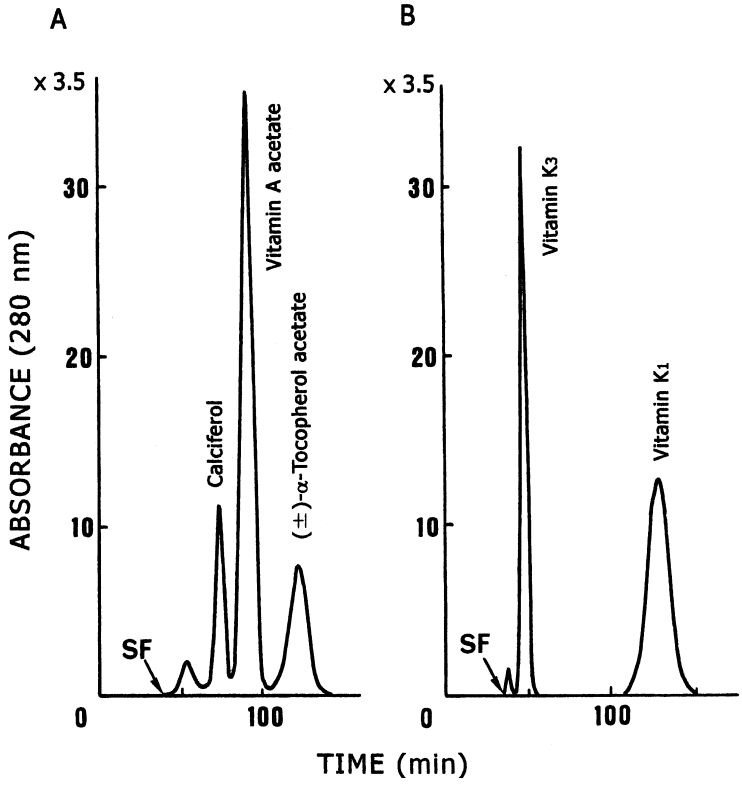

Figure 4. CCC separation of fat-soluble vitamins by cross-axis CPC. Experimental conditions: sample: (A) calciferol (3 mg) + vitamin A acetate (30 mg) + (±)-α-tocopherol acetate (40 mg) and (B) vitamin K_3 (3 mg) + vitamin K_1 (10 mg); solvent system: 2,2,4-trimethyl pentane/methanol (1:1); mobile phase: lower phase. For other experimental conditions, see the Fig. 3 caption. SF = solvent front.

Figure 4A illustrates the CCC separation of fat-soluble vitamins using the cross-axis CPC equipped with eccentric coil assemblies. Calciferol, vitamin A acetate, and (±)-α-tocopherol acetate were well resolved with each other and eluted within 2.5 h. Vitamin K_3 and K_1 were also completely resolved with the same solvent system as shown in Figure 4B.

CONCLUSION

The present study demonstrates that the separation of both water-soluble and fat-soluble vitamins may be performed by high-speed CCC using the cross-axis CPC equipped with eccentric coil assemblies.

Overall results indicate that the method provides suitable solvent systems according to the hydrophobicity of vitamins, i.e., a polar ternary solvent system of 1-butanol/ethanol/aqueous 0.15 M monobasic potassium phosphate (8:3:8) for the separation of several water-soluble vitamins and a nonaqueous solvent system of 2,2,4-trimethyl pentane (isooctane)/methanol for many fat-soluble vitamins. However, the separation of extremely polar vitamins such as thiamine nitrate, thiamine hydrochloride, L-ascorbic acid, and cyanocobalamin still requires a further search for suitable solvent systems. This may be accomplished by adding an ion-pairing reagent or exchanger to the solvent system.

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