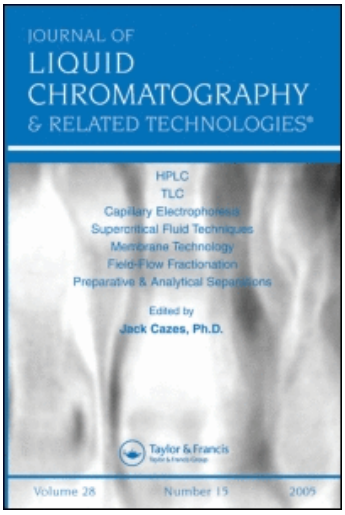

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Countercurrent Chromatographic Separation of Proteins by Cross-Axis Coil Planet Centrifuge: Choice of Polymer Phase Systems and Revolution Speed

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To cite this Article Shinomiya, K. , Kabasawa, Y. and Ito, Y.(1998) 'Countercurrent Chromatographic Separation of Proteins by Cross-Axis Coil Planet Centrifuge: Choice of Polymer Phase Systems and Revolution Speed', Journal of Liquid Chromatography & Related Technologies, 21: 11, 1727 — 1736

To link to this Article: DOI: 10.1080/10826079808001255

URL: http://dx.doi.org/10.1080/10826079808001255

J. LIQ. CHROM. & REL. TECHNOL., 21(11), 1727–1736 (1998)

COUNTERCURRENT CHROMATOGRAPHIC SEPARATION OF PROTEINS BY CROSS-AXIS COIL PLANET CENTRIFUGE: CHOICE OF POLYMER PHASE SYSTEMS AND REVOLUTION SPEED

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ABSTRACT

Countercurrent chromatographic separation of proteins by the cross-axis coil planet centrifuge was maximized by selecting the suitable polymer phase system and revolution speed. Polymer phase systems composed of polyethylene glycol (PEG) 1000 and several inorganic salts were examined to determine partition coefficient values (K) for various proteins. The overall results indicated that the polymer phase system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate yielded suitable K values for most proteins except for cytochrome C and apo-transferrin which may be separable with a solvent

system composed of 12.5% (w/w) PEG 1000 and 24% (w/w) potassium citrate. A series of experiments with the PEG 1000 - potassium phosphate system under various revolution speeds revealed that the best separation was achieved at 850 rpm. The above optimized conditions may be applied to separations of other protein samples.

INTRODUCTION

The cross-axis coil planet centrifuge (CPC) has been developed for performing countercurrent chromatography (CCC) using viscous, low interfacial tension solvent systems.[1-3] The apparatus undergoes a synchronous planetary motion in such a way that the column holder rotates about its horizontal axis and simultaneously revolves around the vertical axis of the centrifuge both at the same angular velocity. The centrifugal force field generated by this planetary motion ensures a good retention of the stationary phase even for viscous aqueous-aqueous polymer phase systems with extremely lower interfacial tension.[4] A series of previous experiments revealed that the ratio between the revolution radius (X) and the lateral deviation (L) of the column is an important parameter in retention of the stationary phase among various types of the cross-axis CPC including X,[5,6] XL,[7] XLL,[8] and XLLL.[9] These studies have shown that an increase in ratio L/X results in high retention of the stationary phase by reducing the phase mixing effect. Our previous studies indicated that versatile design of the cross-axis CPC, providing two positions (L/X = 1.5 and X = 0) for the column, was especially useful in the separation of proteins.[10,11] In our recent studies, the eccentric coil assemblies showed slightly higher efficiencies compared to those from the toroidal coil assemblies.[12]

The present paper describes the choice of suitable aqueous-aqueous polymer phase systems and the optimum revolution rates for maximizing the partition efficiencies in protein separation by the cross-axis CPC equipped with eccentric coil assemblies mounted in the off-center position.

EXPERIMENTAL

Apparatus

The cross-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[10,11] and so only brief description is given here. The apparatus produces a synchronous planetary motion of the column holder which rotates about its own horizontal axis and simultaneously revolves around the vertical axis of the centrifuge, at the same angular velocity. A pair of column holders was accommodated on the rotary frame at the off-center position with X = 10 cm and L = 15 cm (X is the distance between the holder axis and the central axis of the centrifuge and L, the deviation of the holder position from the mid portion of the rotary shaft).

The rotary frame of the centrifuge is encased in a durable circular steel plate measuring 6 mm thickness to avoid any serious accident during the operation.

Column Preparation

The separation columns used in the present studies were a pair of eccentric coil assemblies described earlier.[10,11] Each coil assembly was prepared by winding a single piece of 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo Co., Tokyo, Japan) onto 7.6 cm long, 5 mm OD nylon pipes forming 20 units of serially connected left-handed coils, which were then arranged around the holder with their axes parallel to the holder axis. A pair of identical coil assemblies was connected in series to obtain a total capacity of 26.5 mL.

Reagents

Polyethylene glycol (PEG) 1000 (M.W. 1000), cytochrome C (horse heart), myoglobin (horse skeletal muscle), ovalbumin (chicken egg), hemoglobin (human), trypsinogen (bovine pancreas), apo-transferrin (bovine), carbonic anhydrase (bovine erythrocytes), trypsin inhibitor (soybean), lactalbumin, α-chymotrypsinogen A (bovine pancreas), α-globulins (human) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Bovine serum albumin (BSA) was purchased from Wako Pure Chemicals, Osaka, Japan. All other chemicals were of reagent grade.

Preparation of Aqueous Two-Phase Solvent Systems and Sample Solutions

The solvent systems used in the present studies were prepared by dissolving PEG 1000 and inorganic salt each at a desired concentration in distilled water. Each solvent mixture was thoroughly equilibrated in a

separatory funnel at room temperature and the two phases separated after the two clear layers were formed. The sample solutions were prepared by dissolving each sample mixture in 1 mL of the two-phase mixture consisting of equal volumes of each phase.

Measurement of Partition Coefficient of Protein Samples

The partition coefficient of each protein was determined spectrophotometrically, using a simple test tube procedure. Two milliliters of each phase of the equilibrated two-phase solvent system was delivered into a test tube to which 1 mg of the sample was added.

The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan).

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

CCC Separations of Proteins

For each separation, the coiled column was completely filled with the PEG-rich upper stationary phase and the sample solution (ca. 1 mL) was injected into the column. Then, the salt-rich lower mobile phase was pumped into the column at 0.2 mL/min using a reciprocating pump (Model KHU-W-52H, Kyowa Seimitsu Co., Tokyo, Japan) while the column was rotated at a desired constant speed in a counterclockwise direction.

The effluent from the outlet of the column was collected in test tubes at 0.4 mL/tube using a fraction collector (Model SF-200, Advantec Co., Tokyo, Japan).

Analysis of CCC Fractions

Each fraction was diluted with 2.5 mL of distilled water and the absorbance was measured at 280 nm.

Table 1

Various Kinds of Two-Phase Solvent Systems Examined for Protein Separation

Salt	Aq. Two-Phase Solvent System	CCC Separation
Phosphates	I. PEG 1000 - Potassium Phosphate	Cyt C, Myo, OVA and Hem[10]
		Cyt C, Myo, and Trp[11]
		Cyt C, Myo, BSA, and CA
	II. PEG 1000 - Sodium Phosphate	
Citrates	III. PEG 1000 - Potassium Citrate	Cyt C and apo-T
	IV. PEG 1000 - Sodium Citrate	
Carbonates	V. PEG 1000 - Sodium Carbonate	
Sulfates	VI. PEG 1000 - Sodium Sulfate	
	VII. PEG 1000 - Magnesium Sulfate	
	VIII. PEG 1000 - Ammonium Sulfate	

Abbreviations: Cyt C = Cytochrome C; Myo = Myoglobin; OVA = Ovalbumin; Hem = Hemoglobin; Trp = Trypsinogen; BSA = Bovine serum albumin; CA = Carbonic anhydrase; apo-T = apo-Transferrin.

Evaluation of Partition Efficiency

The partition efficiencies of protein separations were computed from the chromatogram and expressed in terms of theoretical plate number (N) and peak resolution (Rs). Both values are based on an assumption that each peak represents the distribution of a single component.

RESULTS AND DISCUSSION

Choice of Aqueous Two-Phase Systems for Protein Separation by Cross-Axis CPC

In the past, various kinds of aqueous two-phase solvent systems have been developed by Albertsson et al.[4] Among those, the solvent systems composed of PEG-inorganic salt and PEG-dextran have been commonly used for the partition of biological samples. For the CCC separation of proteins, the PEG 1000 - potassium phosphate systems have been mainly used to evaluate the capability of the CCC apparatus. In addition, a few other polymer phase systems have been used for the protein separation by the cross-axis CPC.

The measurement of the partition coefficients of proteins is useful for predicting the peak resolution attained by CCC. Table 1 lists eight kinds of two-phase solvent systems examined in the present studies. The values of the partition coefficients of proteins obtained from PEG - potassium phosphate systems have been reported in our previous studies.[11] Among those solvent systems, 12.5% (w/w) PEG 1000 - 12.5% (w/w) dibasic potassium phosphate system gave the most suitable partition coefficient values for various protein samples.

The 12.5% (w/w) PEG 1000 - 24.0% (w/w) potassium citrate was useful for separating cytochrome C and apo-transferrin. Other solvent systems containing carbonates (Solvent V) and sulfates (Solvent VI - VIII) were not suitable for protein separation by the cross-axis CPC because proteins are partitioned almost unilaterally into either upper or lower phase of the solvent system. In general, proteins are partitioned in the lower phase of these solvent systems at a lower pH, and increasing the pH gradually shifts their partition toward the upper phase.

Our overall results indicated that, among all the solvent systems examined, 12.5% (w/w) PEG 1000 - 12.5% (w/w) dibasic potassium phosphate[12] is most useful for partitioning the proteins by the cross-axis CPC.

Optimization of Revolution Speed for Protein Separation by Cross-Axis CPC

As described above, a solvent system composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate is so far the best solvent system for protein separation by the cross-axis CPC. In our previous studies, CCC separations have been performed by using cytochrome C, myoglobin, and

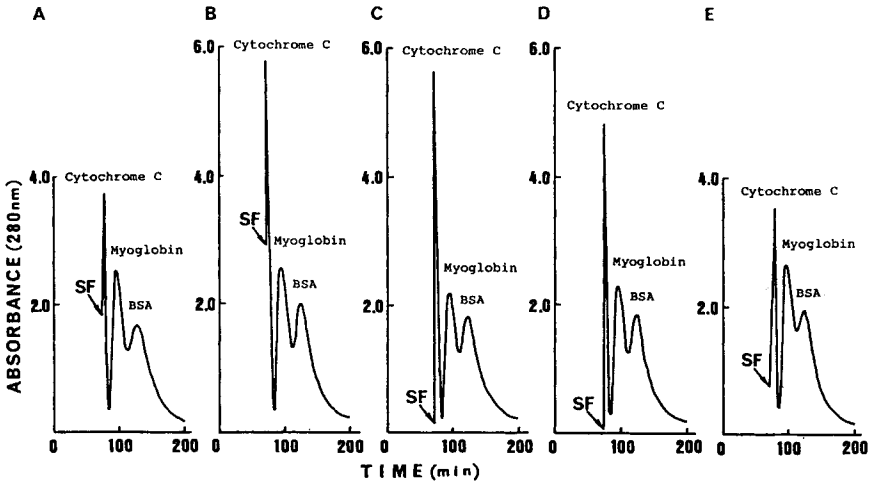

Figure 1. CCC separations of proteins by cross-axis CPC at various revolution speeds. Experimental conditions: apparatus: cross-axis CPC equipped with a pair of eccentric coil assemblies, 1 mm ID and 26.5 mL capacity; sample: cytochrome C (2.5 mg), myoglobin (8 mg) and BSA (30 mg); solvent system: 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate: mobile phase: lower phase; revolution: (A) 800 rpm, (B) 850 rpm, (C) 900 rpm, (D) 950 rpm and (E) 1000 rpm; flow rate: 0.2 mL/min. SF: solvent front.

Table 2

Peak Resolution of Three Proteins Obtained by Cross-Axis CPC Equipped with Eccentric Coil Assemblies

Revolution Speed (RPM)	Peak Resolution		Stationary Phase Retention (%)
	Cyt C/Myo	Myo/BSA	
800	0.95	0.68	34.4
850	1.13	0.79	34.0
900	1.11	0.75	33.6
950	1.07	0.75	35.8
1000	0.76	0.58	34.0

ovalbumin as a set of standard protein samples. However, it was found that the ovalbumin sample gave an abnormally broad peak probably due to the presence of denatured protein.[13] Therefore, the present studies were performed with a standard sample mixture of cytochrome C, myoglobin and BSA.

The revolution speed of the cross-axis CPC is one of the important parameters to determine the stationary phase retention in the column. Kosuge and Murayama described that the higher revolution produced better resolution in centrifugal partition chromatography[14] On the other hand, Shibusawa et al. reported that the revolution speed of 800 rpm gave the best retention of the stationary phase in a multilayer coil of XLL (cross-axis) CPC.[15] In the present studies, the effect of revolution speed on protein separation and stationary phase retention in the eccentric coil assemblies of the X-1.5L (cross-axis) CPC.

Figure 1 illustrates CCC separations of cytochrome C (2.5 mg), myoglobin (8 mg) and BSA (30 mg). These chromatograms were obtained by the cross-axis CPC operated at various revolution rates ranging from 800 rpm to 1000 rpm. The solvent system used was composed of 12.5% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate. Table 2 summarizes the analytical values calculated from these chromatograms. The best peak resolution was attained at the revolution speed 850 rpm, while the retention of the stationary phase remained almost constant (ca 35%) in all groups. In general, higher retention of the stationary phase improves peak resolution. The results in the present studies further indicates that the best peak resolution is attained at a critical revolution rate under a given level of stationary phase retention.

CONCLUSION

Optimum conditions for protein separation by the X-1.5L cross-axis CPC is determined. Among various PEG polymer phase systems examined, the 12.5% (w/w) PEG 1000 - 12.5% (w/w) dibasic potassium phosphate system produced suitable partition coefficient values for a variety of proteins. Using the above solvent system and a set of standard protein samples, a series of experiments was performed to investigate the effect of revolution speed and stationary phase retention in the eccentric coil assemblies mounted around the holder of the cross-axis CPC. The result indicated that the highest peak resolution was obtained at 850 rpm; either increasing or decreasing the revolution speed resulted in lower peak resolution, while the retention of the stationary phase remained almost constant (ca 35%) throughout the applied revolution speeds. The above optimized conditions may be applied to other proteins to maximize their peak resolution.

ACKNOWLEDGMENTS

The authors would like to thank Mr. Minoru Muto and his staffs for the improvement of our cross-axis CPC at the machining technology center of Nihon University, Chiba, Japan. The authors are also indebted to Mr. Makoto Kobayashi for his technical assistance.

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Received July 31, 1997
Accepted August 28, 1997
Manuscript 4529