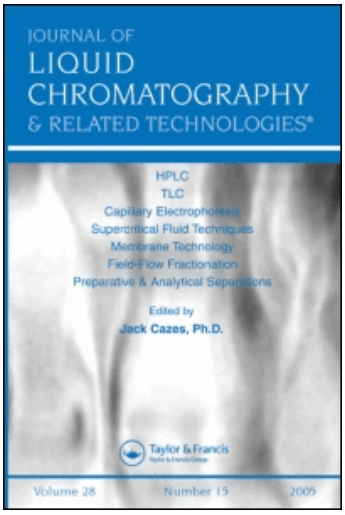


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# Countercurrent Chromatography of Proteins with Polyethylene Glycol-Dextran Polymer Phase Systems Using Type-XLLL Cross-Axis Coil Planet Centrifuge

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# COUNTERCURRENT CHROMATOGRAPHY OF PROTEINS WITH POLYETHYLENE GLYCOL-DEXTRAN POLYMER PHASE SYSTEMS USING TYPE-XLLL CROSS-AXIS COIL PLANET CENTRIFUGE

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## ABSTRACT

The performance of a new model of countercurrent chromatograph called the XLLL cross-axis synchronous coil planet centrifuge was evaluated for protein separation. The apparatus produced a satisfactory retention of the stationary phase for the polymer phase systems composed of dextran T500, polyethylene glycol 8000 and potassium phosphate buffers. The capability of the apparatus was demonstrated in separations of histones and serum proteins.

## INTRODUCTION

Various types of aqueous-aqueous polymer phase systems have been developed by Albertsson and his collaborators for the partitioning of biological macromolecules including proteins, nucleic

acids and polysaccharides, etc. [1]. Among those, dextran-polyethylene glycol (PEG) two-phase systems have been widely used for partitioning of a variety of biopolymers and cell particles. However, high viscosity and low interfacial tension of the two phases tend to delay the phase separation. Consequently, operation of the countercurrent distribution apparatus becomes tedious, requiring a long separation time.

Recently, the cross-axis coil planet centrifuge (X-axis CPC) system has been improved for performing countercurrent chromatography (CCC) with viscous polar solvent systems [2]. In the XLL CPC, the column holder is positioned laterally along the rotary shaft to generate a strong lateral force field which effectively stabilizes retention of the stationary phase for the polymer phase system composed of PEG 1000-potassium phosphate. The capability of the apparatus has been demonstrated in separations of cytochrome *c*, myoglobin, ovalbumin and hemoglobin [3]. However, the XLL CPC showed insufficient retention of the stationary phase for PEG-dextran polymer phase systems which possess higher viscosity and lower interfacial tension between the two phases compared with the PEG-phosphate phase system.

In order to improve retention of the PEG-dextran system, the design of the XLL CPC is modified in such a way that the revolution radius is reduced in half (3.8 cm) while keeping the position of the column holder is unchanged. This XLLL CPC can produce a greater lateral force field relative to the radial centrifugal force field at a given revolution speed. The performance of the apparatus was evaluated in terms of retention of the stationary phase to compare with that obtained by the XLL CPC. The capability of the apparatus is demonstrated in separations of histones and serum proteins with PEG-dextran polymer phase systems.

## EXPERIMENTAL

*Apparatus*

The basic design of the X-axis CPC has been described earlier [4]. The XLLL CPC holds a pair of horizontal rotary shafts

symmetrically, one on each side of the rotary frame, at a distance (R) of 3.8 cm from the central axis of the centrifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral position (L) 13.5 cm from the midpoint. The ratio L/R=3.5 represents a relative strength of the lateral force field which determines the stability of the stationary phase retention in the rotating column at a given radial centrifugal force field.

The synchronous planetary motion of each holder is established as follows: The stationary miter gear mounted on the centrifuge axis is interlocked to an identical gear on the horizontal countershaft radially mounted at the bottom on each side of the rotary frame through a pair of ball bearings. This gear arrangement produces a synchronous rotation of the countershaft on the rotating frame. This motion is further conveyed to the holder by coupling a pair of toothed pulleys, one mounted on the distal end of the countershaft and the other on the column holder shaft using a toothed belt.



Each column holder can be removed from the rotary frame by loosening a pair of screws on the bearing block. The multilayer coil separation column was prepared by winding a length of 2.6 mm ID PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) directly onto the holder hub making left-handed coils tightly spaced between a pair of flanges spaced 5.1 cm apart. When one layer is completed, the tubing is directly returned to the starting point to wind the second layer over the first, the process being continued to form a desired number of coiled layers on each holder. In order to minimize the dead space in the transfer tubes bridging between two layers as well as to prevent excessive deformation of the multilayer coil, each transfer tube is flattened by gently squeezing it between a pair of aluminum rods (1.25 cm OD). To avoid any dislocation of the multilayer coil on the holder, the whole column is bundled with nylon ties at four places: each nylon tie is snugly accommodated in a groove made on the holder hub and inner side of each flange so that the whole column is secured onto the holder.

**TABLE I**
**DIMENSIONS OF THE APPARATUS AND COIL HOLDER WITH DIFFERENT β VALUES**

| Apparatus | Revolution radius (cm) | Lateral shift (cm) | Coil holder | | Column capacity (ml) | β values |
|---|---|---|---|---|---|---|
| | | | Diameter (cm) | Width (cm) | | |
| X-axis CPCs | | | | | | |
| Type XLLL | | | | | | |
| | 3.8 | 13.5 | 3.8 | 5.1 | 300 | 0.50-1.30 |
| | 3.8 | 13.5 | 3.35 | 5.1 | 440 | 0.44-1.50 |
| Type XLL | 7.6 | 15.2 | 3.8 | 5.1 | 300 | 0.25-0.65 |
| | 7.6 | 15.2 | 3.35 | 5.1 | 440 | 0.22-0.75 |

β=r/R where r is the distance from the holder axis to the coil and R, the distance from the holder axis to the central axis of the centrifuge.



Two pairs of multilayer coils were prepared: the large column consists of 12 layers of the coils with a 220 ml capacity and the small column consists of 9 layers of coils with a 150 ml capacity. When in use, each pair of columns is connected on the rotary frame with a 0.85 mm ID PTFE transfer tube to make up the total column capacity of 440 ml for the large column and 300 ml for the small column. Both inflow and outflow transfer tubes exit together at the center of the top plate of the centrifuge case where they are tightly supported with a pair of silicon-rubber-padded clamps.

The revolution speed of the apparatus is regulated at 900 rpm with a speed control unit (Bodine Electric Company, Chicago, IL, U.S.A.). The dimensions of the apparatus and multilayer coils including the revolution radius (R), lateral shift (L), column capacity and its β values are listed in Table I together with those of the XLL CPC for comparison.

*Reagents*

Dextran T500 (weight-average molecular weight $Mw$=500,000), polyethylene glycol (PEG) (number-average molecular weight

*Mn*=6,000-7,500), histone, human serum albumin and α- and γ-globulines were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anhydrous monobasic and dibasic potassium phosphates were obtained from J.T. Baker Chemical Company (Phillipsburg, NJ, U.S.A.). All these chemicals were of reagent grade.

*Preparation of polymer two-phase solvent systems and sample solutions*

The aqueous-aqueous polymer phase systems were prepared by dissolving 44 g of PEG 8000 and 70 g of dextran T500 in 886 g of a 10 mM potassium phosphate buffer solution adjusted to the desired pH. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, allowing the mixture to completely separate into the two layers before use.

The sample solutions were prepared by dissolving 10-200 mg each of histones, human serum albumin, or α- and γ-globulins in 4.0 ml of the two-phase solvent system consisting of about equal volumes of each phase.

*Measurement of stationary phase retention*

Experiments were performed according to the standard procedure described elsewhere [2]. In each measurement, the coil was first filled with the upper or the lower stationary phase. Then the apparatus was rotated at the desired revolution speed while the mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. The elution was continued for 100-150 min until the total elution volume exceeded the column capacity. Then the centrifuge was stopped and the column contents emptied into a 500-ml capacity glass graduated cylinder by connecting the inlet of the column to a pressured nitrogen line. The percentage retention of the stationary phase relative to the total column capacity was calculated from the volume of the stationary phase recovered from the column.

*Measurement of partition coefficient (K) of proteins*

The partition coefficients of protein samples were determined spectrophotometrically by a simple test tube procedure. About 1.5 ml of each phase was delivered into a test tube and about 1 mg of the protein was added. The contents were thoroughly mixed and filtered with Millex-HA filter unit (Millipore Products Division, Bedford, MA, U.S.A.). The mixture was centrifuged at 800 rpm for about 5 min. After the centrifugation, an aliquot, usually 0.8 ml, of each phase was pipetted and diluted with 2.0 ml of distilled water to determine the absorbance at 280 nm using a Zeiss PM6 spectrophotometer (Zeiss Instruments, Hanover, MD, U.S.A.). The partition coefficient ($K=C_U/C_L$) was obtained by dividing the absorbance value of the upper phase by that of the lower phase.



*CCC separation of proteins*

For each separation, the coil was first completely filled with the stationary phase, which can be either the upper or the lower phase. This was followed by injection of the sample solution through the sample port. Then the mobile phase was eluted through the coil at 1.0 ml/min while the apparatus was rotated at 900 rpm. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S (LKB Instruments, Stockholm, Sweden) at 280 nm and collected into test tubes (3.0 ml/tube) with an LKB Ultrorac fraction collector (LKB Instruments).

*Analysis of fractions*

An aliquot of each fraction (1.0 ml) was diluted with 2.0 ml distilled water and the absorbance was measured at 280 nm with a Zeiss PM6 spectrophotometer.

## RESULTS AND DISCUSSION

*Retention of the stationary phase*

CCC is a genuine form of liquid-liquid partition chromatography in which the stationary phase is retained in an open column

**TABLE II**
**RETENTION OF STATIONARY PHASE ON X-AXIS COIL PLANET CENTRIFUGE**

| X-axis CPC | Elution mode | Revolution speed | Stationary phase | Retention (%) |
|---|---|---|---|---|
| Type XLLL | $P_IHO$ | 900 | UP | 24.6 |
| | $P_{II}TO$ | 900 | UP | 24.6 |
| | $P_{II}HI$ | 900 | LP | 41.1 |
| | $P_ITI$ | 900 | LP | 45.1 |
| Type XLL | $P_IHO$ | 750 | UP | 21.1 |
| | $P_{II}TO$ | 750 | UP | 19.6 |
| | $P_{II}HI$ | 750 | LP | 28.4 |
| | $P_ITI$ | 750 | LP | 28.9 |

Solvent system, 4.4%(w/w) PEG 8000, 7.0%(w/w) dextran T500, 10 mM potassium phosphate buffer (pH 6.8).
Flow rate, 1.0 ml/min. UP=upper phase, LP=lower phase.



free of solid support matrix by the aid of the gravity or a centrifugal force field. Consequently, in contrast with other chromatographic methods, the amount of the stationary phase retained in the column varies widely according to the applied experimental conditions. Since the resolution of solute peaks in CCC largely depends upon by the stationary phase volume in the column, it is extremely important to maximize the phase retention.

A series of experiments was performed to measure the retention of stationary phase for the 4.4%(w/w) PEG 8000-7.0%(w/w) dextran T500 polymer phase systems in both small and large multilayer coils mounted on the present apparatus. The experiments were performed at a 1.0 ml/min flow rate in various elution mode using both phases as the mobile phase. The results are summarized in Table II together with those obtained with the XLL CPC for comparison.

In this table, $P_I$ and $P_{II}$ indicate direction of the planetary motion; H and T, the head-tail elution mode; and O and I, the

inward-outward elution mode [2]. These three factors yield a total of 4 combinations for the left-handed coils as indicated in the table. Among these elution modes, the inward-outward elution mode plays the most important role in the stationary phase retention. In order to obtain a satisfactory retention of the stationary phase, the lower phase should be eluted outwardly along the direction of the lateral force field and the upper phase in the opposite direction. In both types of the X-axis CPC, the best results are obtained by eluting the lower phase outward ($P_IHO$ and $P_{II}TO$) or the upper phase inward ($P_{II}HI$ and $P_ITI$) while the retention of the dextran-rich lower phase substantially exceeds that of the PEG-rich upper phase in all cases. It should be mentioned here that, different from other types of the X-axis CPC such as the type XL and type X, the planetary motion and head-tail elution mode give little effect in retention of the stationary phase in the present XLLL CPC. When the percentage retention from the two types of the apparatus is compared, the XLLL CPC yield 20% (upper phase retention) to 50% (lower phase retention) greater retention over the XLL CPC in a given elution mode. The high retention value of 45.1% was obtained from the XLLL CPC by eluting the upper PEG-rich phase in the $P_ITI$ mode. The overall results indicate that the XLLL CPC is more suitable for separations of proteins with the PEG-dextran polymer phase systems than the XLL CPC.



*Partition coefficient (K) of proteins*

Table III shows the partition coefficient values ($K=C_U/C_L$) of various proteins including six types of histones, α- and γ-globulins and human serum albumin in the 4.4%(w/w) PEG 8000-7.0%(w/w) dextran T500 polymer phase system at a wide range of pH ranging from 4.7 to 9.2. The partition coefficients of histones are always less than 1.0, except for the case of histone 7S at pH 4.7 and 5.7 and of histone 8S at pH 7.7. The human serum albumin is fairly evenly distributed in the two phases at pH between 6.8 and 9.2. The coefficient values of α-globulin are close to the unity in the acidic pH from 4.7 to 6.5 but sharply increase at the basic pH above 6.8. On

**TABLE III**
**PARTITION COEFFICIENTS ($K=C_U/C_L$) OF THE SEVERAL PROTEINS IN THE PEG 8000-DEXTRAN T500 POLYMER TWO PHASE SYSTEMS AT VARIOUS pH VALUES**

| Proteins | Partition Coefficient ($C_U/C_L$) | | | | | |
|---|---|---|---|---|---|---|
| | pH 4.7 | 5.7 | 6.5 | 6.8 | 7.7 | 9.2 |
| Histone 2A | 0.79 | 0.69 | 0.22 | 0.19 | 0.13 | 0.84* |
| Histone 3S | 0.29 | 0.17* | 0.23 | 0.35 | 0.44* | 0.55 |
| Histone 5S | 0.85* | 0.78 | 0.07* | 0.14 | 0.08* | 0.05* |
| Histone 6S | 0.47* | 0.20* | 0.04* | 0.06 | 0.05* | 0.43 |
| Histone 7S | 1.02 | 1.02 | 0.13 | 0.64 | 0.15 | 0.28 |
| Histone 8S | 0.12 | 0.03 | ----- | 0.01 | 1.00* | 0.75* |
| Human serum albumin | 0.36 | 0.19 | 0.31 | 0.87 | 1.25 | 1.20 |
| α-globulin | 1.27* | 1.11* | 0.93 | 7.14 | 12.50 | 8.33 |
| γ-globulin | 0.35 | 0.14* | 0.33* | 1.09 | 1.00* | 0.80 |

*slightly soluble in the solvent systems.
-- not soluble.
Sovlent systems are same as Table II but different pH values.



the other hand, the coefficients of γ-globulin are similar to those of serum albumin, remaining low at the acidic pH and increasing to the unity at the neutral pH of 6.8.

In the table, large differences in the K values between histones 8S and 2A are observed at pH 5.7 which is considered to be the optimum pH for the separation of the basic histone (histone 8S, arginine-rich subgroup f3) from another type of histone (histone 2A, calf thymus histone). Because α-globulin shows a very high coefficient values consistently between pH 6.8-9.2, it may be well separated from human serum albumin and γ-globulin at pH 9.2 and 6.8, respectively.

*CCC separation of proteins*

In order to demonstrate the capability of the XLLL CPC in protein separation, various mixtures of selected proteins were

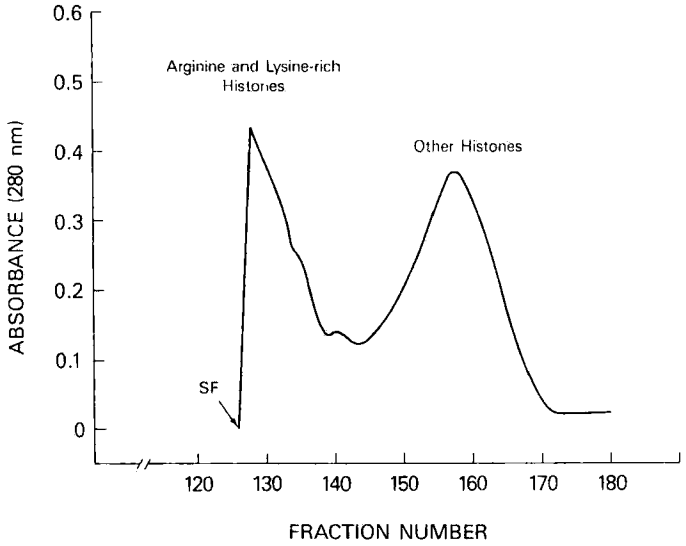


**Fig. 1. Chromatogram of histones obtained by the XLLL CPC.** Experimental conditions: Column: 2.6 mm ID PTFE multilayer coils (x 2), β=0.44-1.50, 440 ml capacity; Sample: 10.8 mg arginine-rich, 12.5 mg lysine-rich and 80 mg calf thymus histones in 4.0 ml solvent; Solvent system: 4.4%(w/w) PEG 8000-7.0%(w/w) dextran T500 in 10 mM potassium phosphate buffer at pH 5.7; Mobile phase: Lower phase; Flow rate: 1.0 ml/min; Revolution: 900 rpm, $P_IHO$; SF=solvent front.

subjected to CCC fractionation with the PEG-dextran system at the optimum pH described above.

Fig. 1 shows a chromatogram of the histone mixture obtained from the large column (440 ml capacity, β=0.44-1.50) mounted on the XLLL CPC using a polymer phase system of 4.4%(w/w) PEG 8000-7.0%(w/w) dextran T500 at pH 5.7 adjusted by a 10 mM potassium phosphate buffer. The separation was performed at 900 rpm at a flow-rate of 1.0 ml/min using the lower dextran-rich phase as the mobile phase in the head-to-tail elution mode along the action

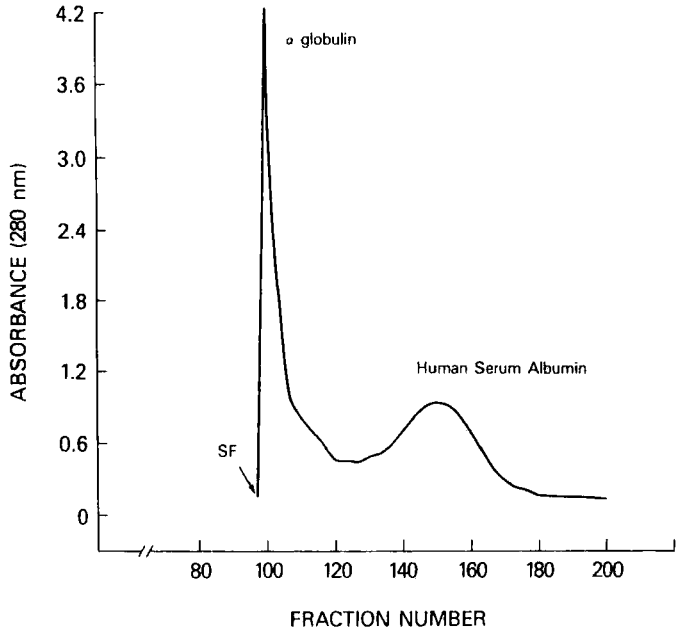


**Fig. 2. Countercurrent chromatography of human serum albumin and α-globulin.** Experimental conditions: Column: 2.6 mm ID PTFE multilayer coils (x 2), β=0.44-1.50, 440 ml capacity; Sample: 65.8 mg α-globulin and 114.1 mg HSA in 4.0 ml solvent; Solvent system: 4.4%(w/w) PEG 8000-7.0%(w/w) dextran T500 in 10 mM potassium phosphate buffer at pH 9.2; Mobile phase: Upper phase; Flow rate: 1.0 ml /min; Revolution: 900 rpm, $P_IT1$; SF=solvent front.

of the lateral force field under a counterclockwise revolution ($P_IHO$ elution mode). The histone 8S with a small K value ($C_U/C_L$=0.03) was eluted immediately after the solvent front, being separated from the histone 2A with a higher K value ($C_U/C_L$=0.69). The separation was completed within 8.5 hours.

A semipreparative-scale separation of serum proteins was performed with the large multilayer coil (440 ml capacity). A

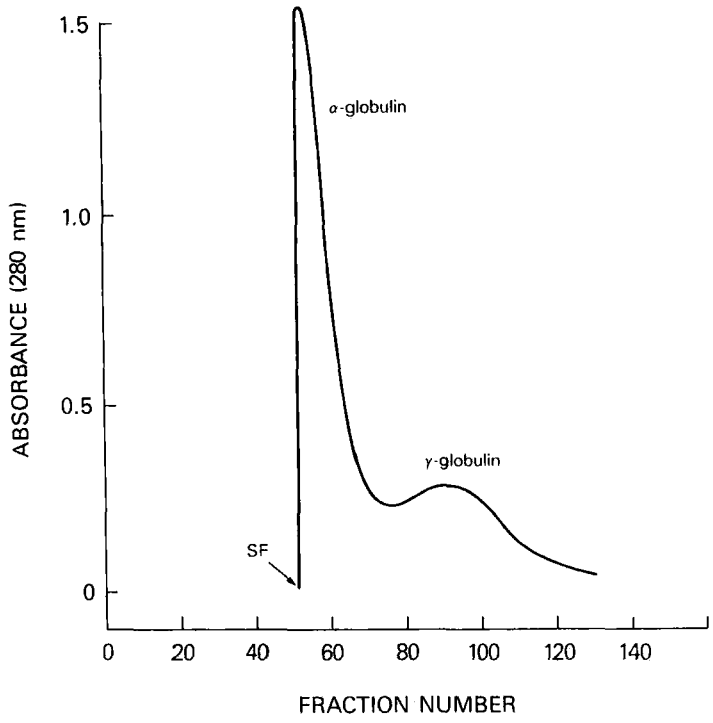


**Fig. 3. Chromatogram of human α- and γ-globulin obtained by XLLL CPC.** Experimental conditions: Column: 2.6 mm ID PTFE multilayer coils (x 2), β=0.50-1.30, 300 ml capacity; Sample: 94.5 mg α- and 107.3 mg γ-globulin in 4.0 ml solvent; Solvent system: 4.4(w/w) PEG 8000-7.0%(w/w) dextran T500 in 10 mM potassium phosphate buffer at pH 6.8; Mobile phase: Upper phase; Flow rate: 1.0 ml/min; Revolution: 900 rpm, $P_1TI$; SF=solvent front.

mixture of 65.8 mg α–globulin and 114.1 mg human serum albumin was eluted with the PEG-rich upper phase (pH 9.2) in the $P_1TI$ elution mode (Fig. 2). The revolution speed and the flow rate were same as those applied in the separation of histones. The α-globulin with a high K value (8.33) was eluted near the solvent front, whereas human serum albumin with a lower K value (1.20) emerged at a

retention volume close to the total column capacity. These proteins were separated within 9 hours.

In order to shorten the separation time of α- and γ-globulins, the small multilayer coil (300 ml capacity) was eluted with a PEG-rich upper phase at pH 6.8. A mixture of 94.5 mg α-globulin and 107.3 mg γ-globulin was separated within 6.5 hours (Fig. 3). The retention of the stationary phase (dextran-rich lower phase), measured after the separation was completed, was 40.1% of the total column capacity.

The overall results of the present studies indicate that the XLLL CPC (L/R=3.5) has substantially improved the retention of the viscous PEG-dextran polymer phase systems over the XLL CPC (L/R=2). This increased retention is attributed to the enhanced lateral shift of the column holder from L/R=2 to 3.5. The results suggest that the retention of the stationary phase may be further increased in the type L CPC with the column holder position at L/R→∞ or R=0, in which the column holder axis crosses the central axis of the centrifuge.



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