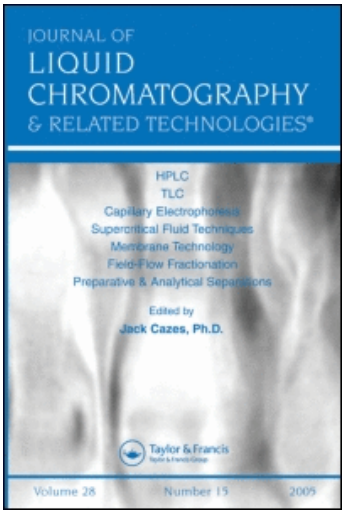

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Speed Countercurrent Chromatography of Dipeptides on a Polar Two-Phase Solvent System

Yoichi Shibusawa[ab]; Yoichiro Ito[a]
[a] Laboratory of Biophysical Chemistry National Heart, Maryland [b] Visiting Scientist from Tokyo College of Pharmacy, Tokyo, Japan

To cite this Article Shibusawa, Yoichi and Ito, Yoichiro(1991) 'High-Speed Countercurrent Chromatography of Dipeptides on a Polar Two-Phase Solvent System', Journal of Liquid Chromatography & Related Technologies, 14: 8, 1575 — 1586
To link to this Article: DOI: 10.1080/01483919108049636
URL: http://dx.doi.org/10.1080/01483919108049636

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(8), 1575-1586 (1991)

HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY OF DIPEPTIDES ON A POLAR TWO-PHASE SOLVENT SYSTEM

YOICHI SHIBUSAWA* AND YOICHIRO ITO
Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
Building 10, Room 7N322
Bethesda, Maryland 20892

ABSTRACT

The performance of the high-speed countercurrent chomatograph was evaluated by separating dipeptide samples on a polar biphasic solvent composed of n-butanol-acetic acid-water (4:1:5, v/v/v). Best results were obtained with a set of multilayer coils of small helical diameter by eluting with the upper nonaqueous phase in a head to tail direction at a flow rate of 1 or 2 ml/min. Four components were completely resolved in 5 to 11 hours. Other types of coiled columns such as a multilayer coil with large helical diameter (in a commercial model) and eccentric dual-layer coil assemblies mounted on a horizontal coil planet centrifuge also yielded satisfactory separations.

INTRODUCTION

Countercurrent chromatography (CCC) is a generic name for various liquid partition chromatographic methods which eliminate the use of solid support matrices (1,2). Among all existing CCC systems, the high-speed CCC is considered the most advanced form in terms of partition efficiency and separation times. The method has proven useful for separation of various natural products with two-phase solvent systems of moderate hydrophobicity (3).

*Visiting Scientist from Tokyo College of Pharmacy
Tokyo, Japan

One problem associated with high-speed CCC is instability in the stationary phase for hydrophilic solvent systems such as n-butanol/acetic acid/water at a volume ratio of 4:1:5 and sec.-butanol/water. These solvent systems are characterized by high viscosity and low interfacial tension between the two phases so that they have a long settling time and a high tendency to emulsify. They also display a reversed hydrodynamic trend in the coaxial multilayer coil so that the lower phase tends to move toward the head and the upper phase toward the tail. (This head-tail orientation of the rotating coil is in reference to the Archimedean screw force where all objects in different density competitively move toward the head of the coil.) Consequently, the retention of the stationary phase is effected by eluting either the lower phase from the tail toward the head or the upper phase from the head toward the tail. In both elution modes, the amount of stationary phase retained in the coil maximizes in the region with small helical diameter. This limits the column capacity in the conventional design which is equipped with a single column holder. The effective column capacity in the apparatus may be increased by increasing the length of the column holder and/or number of column holders on the rotary frame.

The high-speed CCC centrifuge used in the present study is equipped with three column holders each with 12cm in length to accommodate long multilayer coils with small helical diameters. The performance of the apparatus was evaluated in the separation of a series of dipeptides using a two-phase solvent system composed of n-butanol-acetic acid-water (4:1:5, v/v/v). The results were compared (in terms of theoretical plate and peak resolution) with those obtained from the conventional high-speed CCC centrifuge and the eccentric dual-layer coil assemblies mounted on the same apparatus.

EXPERIMENTAL

Apparatus:

The basic design of the apparatus has been described in detail elsewhere (4). The apparatus horizontally holds a set of three column holders on the rotary frame in the symmetrical positions at a distance of 10cm from the central axis of the centrifuge. (Fig. 1). Each column holder is equipped with a planetary gear (plastic) which is coupled to the identical stationary sun gear (stainless steel) mounted around the central axis of the apparatus. This gear

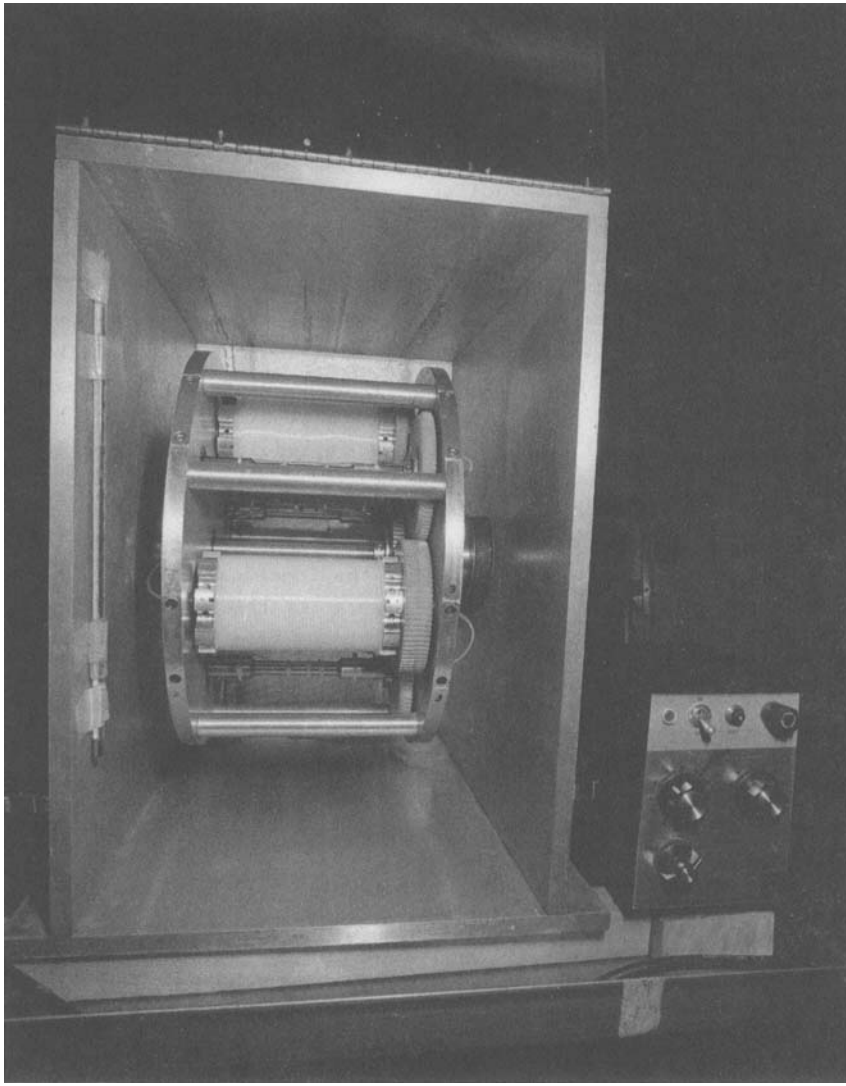

Figure 1. High-speed CCC centrifuge equipped with three multilayer coils connected in series.

arrangement produces the desired synchronous planetary motion of the holder, i.e., rotation about its own axis and revolution around the centrifuge axis in the same direction at the same rate. The rotary frame also holds a set of three rotary tube supports between the column holders. Each tube support is equipped with a planetary gear which is engaged to another planetary gear of the holder shaft so that the holder and the tube support are counterrotated to each other to unwind the flow tubes interconnecting the three columns on the holder. Each column holder assembly can be removed from the rotary frame by loosening a pair of screws on the bearing block. This facilitates both the use of interchangeable column holders and the technique for mounting the coiled column on the holder.

The columns and holders were redesigned for the present study. Each multilayer coil was prepared from a 100m-long single piece of 1.6mm ID PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Product, Raritan, NJ, U.S.A.) by winding it directly onto a holder hub (2.5cm diameter) forming multiple coiled layers between a pair of flanges spaced 11cm apart. Each multilayer coil consisted of 11 layers with an about 175ml capacity. The ß value, which is an important parameter to determine the hydrodynamic trend of the two solvent phases, ranges from 0.125 at the innermost layer to 0.30 at the outermost layer. (ß = 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.) Each end of the column is connected to a 0.85mm ID PTFE flow tube (Zeus Industrial Products). A leak-free connection was made by inserting the flow tube directly into the end of the column followed by winding a copper wire a few turns over the junction and twisting the ends. Three multilayer coils are serially connected with the flow tubes: Each connection tube runs a half way across the rotary frame along the tube support where it is connected by a commercial tube connector (Upchurch, Oak Harbor, WA, U.S.A.). As described earlier (4), the flow tubes enter the centrifuge from one side and leave it from the other side. Both inflow and outflow tubes are tightly supported on the centrifuge wall with a pair of silicone-rubber-padded clamps.

The revolution speed of the centrifuge is regulated up to 1000 rpm with a speed control unit (Bodine Electric Co., Chicago, IL, U.S.A.).

For comparative studies, we have also tested a set of three eccentric dual-layer coil assemblies mounted on the sample apparatus. The design of the column assemblies has been described previously (5). In order to study the effects of ß value on

separation, a commercial model of the high-speed CCC centrifuge (Ito Multilayer Coil Separator/Extractor, P.C. Inc., Potomac, MD, U.S.A.) has also been used. The design of the apparatus is essentially the same as the new model except that the rotary frame rotates around the vertical axis and has a single multilayer coil (300ml capacity) with greater ß values ranging from 0.5 to 0.8. The orientation of the centrifuge axis, whether it is horizontal or vertical, has no effect on the separation with the multilayer coils applied in the present study.

Reagents:

Glass-distilled n-butanol and methanol were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI, U.S.A. and a reagent grade of glacial acetic acid from Mallinckrodt, Inc., Paris, KY, U.S.A. Four dipetpide samples were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: they include L-tyrosylglycine (tyr-gly), L-valyl-L-tyrosine (val-tyr), L-leucyl-L-tyrosine (leu-tyr), and L-tryptophyl-L-tyrosine (trp-tyr).

Preparation of Two-Phase Solvent and Sample Solution:

A hydrophilic two-phase solvent system composed of n-butanol-acetic acid-water at a volume ratio of 4:1:5 was chosen for the present study. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature by repeating shaking and degassing several times and the two phases were separated shortly before use.

The sample solution was prepared by dissolving 30mg each of tyr-gly, val-tyr and leu-tyr, and 10mg of trp-tyr in 8ml of the above solvent consisting of equal volumes of each phase. The sample solution was filtered before application to the column.

Separation Procedure:

All experiments were performed according to the standard procedure previously reported (3). In each separation, the column was entirely filled with the stationary phase. This was followed by injection of the sample solution through the sample port. Then, the mobile phase was pumped into the column in the proper elution mode while the apparatus was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV monitor (Uvicord S, LKB Instruments, Inc., Bromma, Sweden) at 280 nm and collected with a fraction collector (Ultrorac, LKB Instruments). After all peaks

were eluted, the centrifuge was stopped and the column contents were emptied into a graduated cylinder by connecting the inlet of the column to a pressured N_2 line (about 80 psi) to measure the volume of the stationary phase retained in the column. Then the column was cleaned by flushing with ca 100ml of methanol and flushing with N_2 before starting the next run.

A series of experiments were performed by changing the flow rate of the mobile phase. In both instruments the multilayer coil was eluted from the internal tail end toward the external head end if the lower phase was mobile, and from the external head end toward the internal tail end if the upper phase was mobile. The eccentric dual-layer coil assembly was eluted with the lower phase from the head toward the tail.

RESULTS AND DISCUSSION

Partition Coefficients of Dipeptides

In both CCC and liquid chromatography, the partition coefficient is the major parameter governing resolution of peaks. In CCC the partition coefficient of solute can be easily determined in a simple test tube procedure (6). In the present study, four dipeptides were partitioned in a two-phase solvent system composed of n-butanol-acetic acid-water (4:1:5, v/v/v) and their partition coefficient values were determined. Each K value is expressed in two different ways, $K(C_L/C_U)$ or $K(C_U/C_L)$, where C_U indicates the solute concentration in the upper phase and C_L, that in the lower phase. These values are listed in Table I together with the chemical structures of the dipeptides. In the above solvent system, $K(C_L/C_U)$ of tyr-gly, val-tyr, leu-tyr, and trp-tyr are 3.23, 1.89, 1.00, and 0.45, respectively while $K(C_U/C_L)$ shown in Table I are their reciprocals. These K values indicate hydrophobicity of the solutes which determines the order of elution as shown in the right column of Table I.

These values are also used to compute the separation factor, α, which is an important parameter relating to resolution (7). ($\alpha = K_1/K_2$ where K_1 and K_2 are partition coefficients of two solutes and $K_1>K_2$.) The separation factors between two adjacent peaks of dipeptides used in the present study are 1.7 for tyr-gly and val-tyr, 1.89 for val-tyr and leu-tyr, and 2.2 for leu-tyr and trp-tyr. Similarity of all three α values facilitates fair comparison in partition efficiency between various CCC runs in the present study on the basis of Rs values of the adjacent peaks (Table II).

TABLE I Partition Coefficient of Four Dipeptides

Dipeptide	Paritition Coefficient $K(C_L/C_U)$	$K(C_U/C_L)$	Order of Elution Mobile Phase	
			Lower phase	Upper Phase
L-tyrosyl-L-glycine (tyr-gly)	3.23	0.31	Peak 1	Peak 4
L-valyl-L-tyrosine (val-tyr)	1.89	0.53	Peak 2	Peak 3
L-leucyl-L-tyrosine (leu-tyr)	1.00	1.00	Peak 3	Peak 2
L-tyrptophyl-L-tyrosine (trp-tyr)	0.45	2.20	Peak 4	Peak 1

TABLE II NUMBER OF THEORETICAL PLATE (TP) AND PEAK RESOLUTION (Rs) OF DIPEPTIDE OBTAINED BY COIL PLANET CENTRIFUGE

Column (β-value) Capacity	Flow Rate (ml/h)	Mobile phase	Elution mode	TP				Rs			SR** (%)
				Peak* 1	Peak 2	Peak 3	Peak 4	Peaks 1-2	Peaks 2-3	Peaks 3-4	
Coaxial multilayer											
(0.125-0.3)	60	Lower	Tail-Head	6500	8100	9600	460	2.37	2.03	1.81	19.2
525 ml	120	Lower	Tail-Head	6500	7400	13400	2700	2.45	2.02	1.98	16.5
	60	Upper	Head-Tail	830	400	960	5100	2.72	1.96	5.14	56.9
	120	Upper	Head-Tail	1400	1600	3600	3700	2.95	2.67	5.20	36.8
(0.5 0.8)	60	Lower	Tail-Head	1000	1200	1100	720	1.63	1.29	2.00	20.2
300 ml	120	Lower	Tail-Head	1200	1300	1900	1400	1.33	1.03	1.64	7.3
	60	Upper	Head-Tail	1400	1800	5000	2300	2.71	2.35	4.17	36.8
	120	Upper	Head-Tail	1200	2100	2400	1400	2.05	1.64	2.58	22.8
Eccentric double layer											
(0.3)	60	Lower	Head-Tail	3100	3400	2100	630	2.24	1.18	1.74	35.6
220 ml	120	Lower	Head-Tail	3400	2700	680	700	0.86	0.75	1.01	27.1

*Peaks 1-4 as in Table I. **SR : Stationary phase retention.
Solvent system : n-Butanol:Acetic acid:Water=4:1:5 ; Revolution speed : 800rpm.

Separation of Dipeptides

Fig. 2 shows typical chromatograms of the four dipeptide samples obtained from our high-speed CCC centrifuge equipped with three multilayer coils of small helical diameters (ß = 0.125-0.30). The separations were performed with a two-phase solvent system composed of n-butanol-acetic acid-water (4:1:5, v/v/v) eluting with the lower (A) and the upper (B) mobile phases, both at a flow rate of 1 ml/min using a revolution speed of 800 rpm. As expected from the nonlinear isotherm of the K values of the dipeptides in the n-butanol solvent system, the majority of the peaks exhibit skewed shapes where the peaks are skewed toward the left in the lower phase elution (A) and toward the right in the upper phase elution (B), indicating that the solutes are partitioned relatively more into the lower phase at higher concentrations.

Because of the reversed hydrodynamic trend (the lower phase distributes toward the head of the coil), the lower phase was eluted from the tail toward the head (A). This reversed elution mode produced rather intensive carryover of the stationary phase resulting in loss of peak resolution. In contrast, the normal mode of head to tail elution applied to the upper mobile phase (B), resulted in stable retention of the stationary phase in the coil yielding higher resolution. All peaks were completely resolved in 11 hours.

The partition efficiencies were computed and expressed in terms of theoretical plates (N) according to the following formula:

$$N = 5.54\ (R/d)^2 \qquad (1)$$

where R is the retention time or volume of the peak maximum and d is the peak width at one half the peak height measured in the same unit as R. The partition efficiency is also expressed in terms of peak resolution according to the following formula:

$$Rs = 2(R_2 - R_1)/(W_1 + W_2) \qquad (2)$$

Where Rs is the resolution of the adjacent two peaks expressed in the unit of 4σ in a Gaussian distribution; R_1 and R_2, the retention time or volume of the adjacent peaks ($R_1<R_2$); and W_1 and W_2, the widths (4σ) of the corresponding peaks. When Rs=1.5, it represents a baseline separation (over 99.7% pure). Although the aberration of the peak shape from the Gaussian distribution may somewhat disturb accurate computation of the partition efficiency, the obtained values can serve as a useful measure for comparing the efficiencies of various runs in the present study provided that the same set of samples is used.

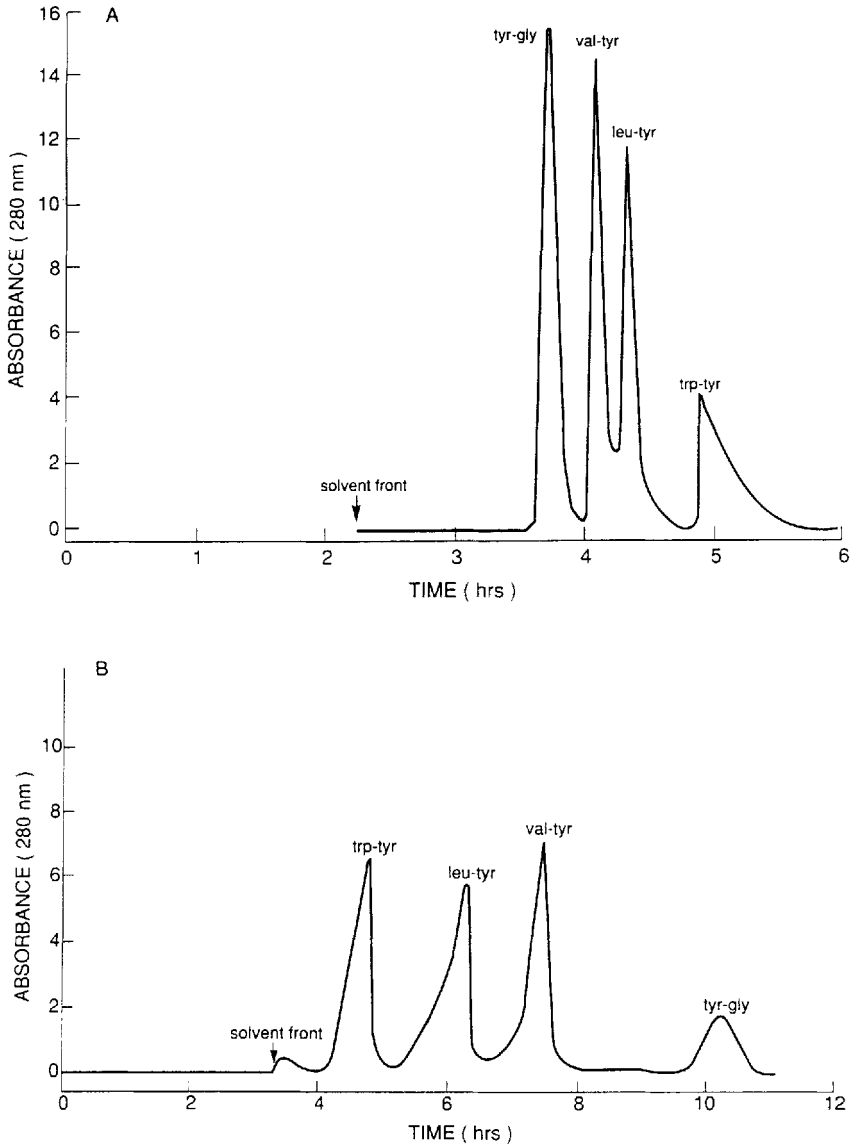

Figure 2. Separation of dipeptides by high-speed CCC. A. Lower phase mobile; B. Upper phase mobile. Experimental conditions are as follows: Apparatus: High-speed CCC centrifuge equipped with three column holders. Columns: A set of three multilayer coils connected in series, 1.6mm I.D. and 525ml capacity (β = 0.125 - 0.30); Solvent system: n-butanol-acetic acid-water (4:1:5, v/v/v): Elution mode: tail to head elution of lower mobile phase (A) and head to tail elution of upper mobile phase (B): Flow rate: 1ml/min; Revolution speed: 800 rpm; Sample: Dipeptide mixture consisting of 30mg each of tyr-gly, val-tyr, leu-tyr and 10mg of trp-tyr dissolved in 8ml of the above solvent mixture; Column pressure: Near atomospheric pressure (A) and 50 psi (B).

A series of runs were made to investigate the effects of flow rate and mobile phase on the separation using three different coiled columns. The results are summarized in Table II where efficiencies are expressed in theoretical plate number (TP) for peaks 1 to 4. Rs values between the adjacent peaks are also listed together with the percentage retention of the stationary phase obtained under various experimental conditions.

In the multilayer coils with small helical diameters (ß=0.125-0.30) TP values vary greatly according to the retention time and the applied flow rate of the mobile phase. The TP values maximize at the third peak in the lower mobile phase and at the fourth peak in the upper mobile phase. On the other hand, the Rs values, which are a more reliable parameter representing separation efficiencies, are relatively stable and quite insensitive to changes in flow rate. All peaks show complete separation (Rs>1.5). Rs values exceeding 5.0 are observed between the 3rd and 4th peaks in the upper phase mobile. Under these optimum conditions, retention of the stationary phase shows the maximum values of 56.9 - 36.8% of the total column capacity.

The TP values obtained from the multilayer coils (ß=0.5-0.8) in the commercial apparatus show much less fluctuation where increase in flow rate from 1 to 2 ml/min results in considerable loss in Rs value regardless of the choice of the mobile phase. As expected from the phase distribution diagrams reported elsewhere (3,8), the multilayer coils with large ß values in the commercial apparatus produced lower stationary phase retention compared to those with small ß values in our prototype system.

The data available from the eccentric dual-layer coil assemblies are limited to elution with the lower mobile phase which show relatively high efficiencies in both TP and Rs for its small column capacity of 220ml. However, the system produces high hydrostatic pressure of 250 psi compared with about 50 psi in the multilayer coils in the high-speed CCC centrifuge operating in the head to tail elution mode. Consequently, further increase in capacity by modifying the dual layer to the multilayer form would result in higher column pressure, eventually necessitating reduction of revolution speed, limiting the partition efficiency of the system.

The overall result of the present study indicates that multilayer coils with small helical diameters (ß=0.125-0.30) yield

the highest resolution among the three coiled columns examined, especially if the upper nonaqueous phase is used as the mobile phase. The method may be useful for separation and purification of various natural and synthetic peptides and other polar compounds.

ACKNOWLEDGEMENT

The authors of this paper wish to thank Dr. Henry M. Fales for his assistance in reviewing this paper.

REFERENCES

1. Ito, Y. Principle and Instrumentation of Countercurrent Chromatography in Countercurrent Chromatography: Theory and Practice, Chapter 3, pp. 79-442 (Edited by N.B. Mandava and Y. Ito) Marcel Dekker, New York, 1988.

2. Conway, W.D. Countercurrent Chromatography: Principle, Apparatus and Applications, VCH Publishers, 1990.

3. Ito, Y. High-Speed Countercurrent Chromatography, CRC Crit. Rev. Anal. Chem., 17, 65, 1986.

4. Ito, Y., Kitazume, E., Bhatnagar, M. and Trimble, F.D. The Cross-Axis Synchronous Flow-Through Coil Planet Centrifuge (Type XLL) I. Design of the Apparatus and Studies on Retention of Stationary Phase, J. Chromatogr., 538, 59, 1991.

5. Sandlin, J.L. and Ito, Y. Versatile Coil Planet Centrifuge for Preparative Countercurrent Chromatography. Comparative Studies on Performace of Three Types of Column, J. Liq. Chromatogr., 11, 55, 1988.

6. Conway, W.D. and Ito, Y. Resolution in Countercurrent Chromatography by Rapid Estimation of Partition Coefficients and Application to Polar Conjugates of p-Nitrophenol, J. Liq. Chromatogr., 7, 275, 1984.

7. Conway, W.D. and Ito, Y. Resolution in Countercurrent Chromatography, J. Liq. Chromatogr., 8, 2195, 1985.

8. Ito, Y. Experimental Observations of the Hydrodynamic Behavior of Solvent Systems in High-Speed Countercurrent Chromatography: Part II. Phase Distribution Diagrams for Helical and Spiral Columns, J. Chromatogr., 301, 387, 1984.