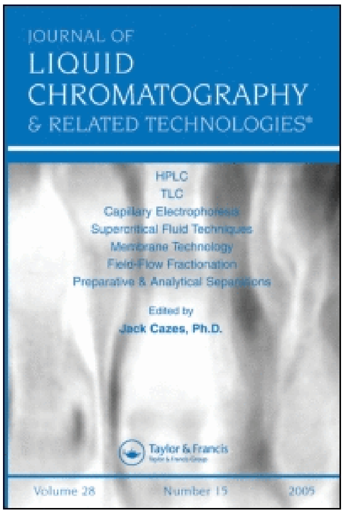

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Yang, Yi , Gu, Dongyu , Aisa, Haji Akber and Ito, Yoichiro(2010) 'NOVEL DESIGNS FOR CENTRIFUGAL COUNTERCURRENT CHROMATOGRAPHY: V. COMPARATIVE STUDIES ON PERFORMANCE OF VARIOUS COLUMN CONFIGURATIONS', Journal of Liquid Chromatography & Related Technologies, 33: 16, 1542 — 1549

To link to this Article: DOI: 10.1080/10826076.2010.503780
URL: http://dx.doi.org/10.1080/10826076.2010.503780

Journal of Liquid Chromatography & Related Technologies, 33:1542–1549, 2010

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2010.503780

NOVEL DESIGNS FOR CENTRIFUGAL COUNTERCURRENT CHROMATOGRAPHY: V. COMPARATIVE STUDIES ON PERFORMANCE OF VARIOUS COLUMN CONFIGURATIONS

Yi Yang,[1,2] Dongyu Gu,[2] Haji Akber Aisa,[2] and Yoichiro Ito[1]
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☐ *The conventional toroidal coil in centrifugal countercurrent chromatography has a low level of stationary phase retention, since a half of each helical turn is entirely occupied by the mobile phase. In order to cope with this problem, several new column designs including zigzag, saw-tooth, and figure-8 patterns have been introduced, and their performance was compared in terms of retention of the stationary phase (Sf), peak resolution (Rs), theoretical plate number (N), and column pressures. Overall results of experiments indicate that the figure-8 column yields the highest Rs when the lower phase is used as the mobile phase. Since the column pressure of all these new columns are much lower than that in the traditional toroidal coil column, the separation efficiency can be improved using a long separation column without a risk of column damage by high back pressure.*

Keywords centrifugal countercurrent chromatography, dipeptide, DNP-amino acid, figure-8 column, peak resolution, saw-tooth column, stationary phase retention, toroidal coiled column, zigzag column

INTRODUCTION

Countercurrent chromatography (CCC), being a support free liquid–liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of sample components onto the solid support as often observed in conventional liquid chromatography.[1] A variety of existing CCC schemes can be classified into two forms, i.e., hydrostatic equilibrium

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system and hydrodynamic equilibrium system.[2] High speed countercurrent chromatography that is the most advanced hydrodynamic equilibrium system has been widely used for the preparative separation and purification of natural products,[3] while hydrostatic CCC system is efficiently applied to analytical separations using a narrow bore coiled column arranged around the periphery of the centrifuge bowl in a toroidal form in a seal free flow through centrifuge.[4] In this toroidal coil CCC system, however, the retention of the stationary phase is limited to substantially less than 50% of the total column capacity, since the half of each helical turn is entirely occupied with the mobile phase. In order to cope with this problem, a triangular coiled column has been introduced that has improved the retention of the stationary phase to slightly over 40%.[5] Recently, various column designs have been introduced to further improve the retention of the stationary phase, including zigzag column,[6,7] saw tooth column,[8] and figure-8 column.[9]

In the present study, the performance of these three different columns and the traditional coiled column was compared in the separation of dipeptides and DNP-amino acid test samples, each with a suitable two-phase solvent system.

EXPERIMENTAL

Apparatus

The present study uses a rotary seal free centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, Maryland, USA. It holds an aluminum rotary platform measuring about 34 cm in diameter to hold a separation column. The column is made by hooking approximately 17 m long, a 0.46 mm ID FEP (Fluorinated ethylene propylene) (Zeus Industrial Products, Orangeburg, SC, USA) tubing onto the screws upstanding on the rotary platform to form various column configurations (Figure 1A) such as zigzag (Figure 1B), saw tooth (Figure 1C), and 8-figure (Figure 1D), each with a total capacity of about 2.8 mL. Each terminal of the column is connected to a flow tube (PTFE, Zeus Industrial Products) with a set of tubing connectors (Upchurch Scientific, Palm Spring, CA, USA). These flow tubes are put together and passed through the center of the central shaft downward and the hollow horizontal shaft of a miter gear, then led upward into the vertical hollow tube support, and finally exit the centrifuge from the center of the upper plate where they are tightly held with a pair of clamps.

Reagents

1-Butanol, hexane, ethyl acetate, and methanol of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and other solvents such

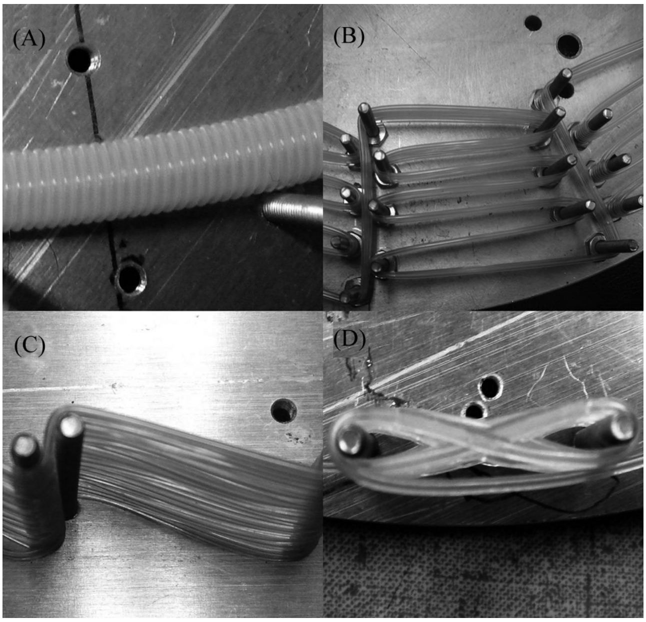


FIGURE 1 The unit of four different columns for centrifugal countercurrent chromatography. (A) Toroidal coil; (B) Zigzag; (C) Saw-tooth; and (D) Figure-8.

as acetic acid and hydrochloric acid of analytical grade from Mallinckrodt Chemicals, Phillipsburg, NJ, USA. Test samples including tryptophyl-tyrosine (Trp-Tyr), valyl-tyrosine (Val-Tyr), N-2, 4-dinitrophenyl-L-alanine (DNP-L-ala), N-2, 4-dinitrophenyl-β-alanine (DNP-β-ala), and N-2, 4-dinitrophenyl-DL-glutamic acid (DNP-DN-glu) were obtained from Sigma Chemicals (St. Louis, MO, USA).

Partition Coefficient Measurement[6]

The partition coefficient (K_U) of each sample in the two-phase solvent system was determined using the conventional test tube method with a UV spectrophotometer (Genesis 10 UV, Thermo Spectronic, Rochester, NY, USA) at 280 nm. The absorbance of the upper phase was recorded as A_U and that of the lower phase as A_L. The K_U value was calculated according to the following equation: $K_U = A_U/A_L$.

Two-Phase Solvent Systems and Sample Solutions

Two typical two-phase solvent systems including 1-butanol-acetic acid-water (4:1:5, v/v) (BAW) and hexane-ethyl acetate-methanol-0.1 M HCl

(1:1:1:1, v/v) (HEMW) were used to separate the dipeptide and DNP-amino acid test samples, respectively. Each solvent mixture was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking and degassing; and the two phases separated shortly before use. Sample solution 1 was prepared by dissolving 25 mg of Trp-Tyr and 100 mg of Val-Tyr in 20 mL of the upper phase of 1-butanol-acetic acid-water, and 40 µL of this stock solution was used for each separation. Sample solution 2 was prepared by dissolving 5.7 mg of DNP-L-ala, 5.1 mg of DNP-β-ala, and 5.3 mg of DNP-DL-glu in 10 mL of the upper phase of hexane-ethyl acetate-methanol-0.1 M HCl (1:1:1:1, v/v), and 40 µL of this solution was used for each separation.

Separation Procedure

In each separation, the separation column was entirely filled with the stationary phase, either upper or lower phase, followed by sample injection, and the column was rotated at 1000 rpm while the mobile phase was pumped into the coiled column at a given flow rate. The effluent from the outlet of the coiled column was continuously monitored with a Uvicord IIS (LKB, Stockholm, Sweden) at 280 nm and the elution curve was traced using a strip chart recorder (Pharmacia, Stockholm, Sweden). In order to improve the tracing, ethanol was added to the effluent at the inlet of the detector using a tee connector and a fine mixing tubing (PTFE 0.4 mm ID × ca 1 m) at a flow rate of 20% that of the mobile phase. After the desired peaks were eluted, the run was stopped and the column contents were forced by pressurized air into a graduated cylinder to determine the volume of the stationary phase retained in the column. The stationary phase retention (Sf) was computed by dividing the volume of the retained stationary phase by the column volume and expressed as %.

Evaluation of Partition Efficiency

The partition efficiency of the separation column was evaluated by computing theoretical plate number (N) for each peak and the peak resolution (Rs) between the peaks using the following conventional equations:

$$N = (4t_R/W)^2 \tag{1}$$

$$Rs = 2(t_2 - t_1)/(W_1 + W_2) \tag{2}$$

where t_R and W indicate the retention time and the baseline peak width in Eq. (1) and those for the specified peaks in Eq. (2), respectively.

RESULTS AND DISCUSSION

As shown in Figure 1, the performance in terms of stationary phase retention (Sf), peak resolution (Rs), theoretical plate number (N), and column pressure (P) of the conventional toroidal coil, zigzag, saw tooth, and figure-8 (fig-8) columns was investigated in the present study. The results were summarized in Table 1.

Figure 2 shows retention of the stationary phase of four different columns in two typical solvent systems, HEMW and BAW, at a flow rate of 0.05 mL/min using the revolution speed of 1000 rpm. DNP-DL-glu, DNP-β-ala, and DNP-L-ala were used as test samples for HEMW, and Val-Tyr, and Trp-Tyr, for BAW. In general, retention of the stationary phase of the HEMW system was better than that in the BAW except that the retention (Sf) in the figure-8 column using the lower mobile phase was slightly lower than that in the BAW. When lower phase was mobile phase in the HEMW, the saw tooth column yielded the best Sf at 43.7% followed by the figure-8 column with Sf at 43.1%, while Sf in the traditional coiled column was the worst at 35.7%. When the upper phase was mobile phase in the HEMW, the figure-8 column gave the best Sf at 55.6%, followed by the saw tooth, zigzag, and coiled columns n this order. The Sf in the BAW system showed a similar tendency in both

TABLE 1 Comparison of Performance of 4 Different Columns at the Flow Rate of 0.05 mL/min for Centrifugal Countercurrent Chromatography

Column	Samples	Solvent	Mobile Phase	Sf (%)	Rs	N	P (psi)
Coiled	DNP-DL-glu	HEMH	Lower phase	35.7	1.25/1.34	138/323/273	200
	DNP-β-ala		Upper phase	42.8	1.60/0.88	265/236/252	214
	DNP-L-ala						
	Val-Tyr	BAW	Lower phase	35.3	1.18	136/49	90
	Trp-Tyr		Upper phase	38.2	1.12	107/64	92
Zigzag	DNP-DL-glu	HEMH	Lower phase	39.8	1.36/1.29	171/267/266	87
	DNP-β-ala		Upper phase	46.2	1.35/0.73	100/155/209	95
	DNP-L-ala						
	Val-Tyr	BAW	Lower phase	35.7	1.22	143/52	55
	Trp-Tyr		Upper phase	39.5	0.77	32/120	63
Saw tooth	DNP-DL-glu	HEMH	Lower phase	43.7	1.54/1.55	176/284/333	109
	DNP-β-ala		Upper phase	51.9	1.27/0.85	89/140/216	121
	DNP-L-ala						
	Val-Tyr	BAW	Lower phase	43.1	1.26	102/39	63
	Trp-Tyr		Upper phase	46.8	0.92	40/46	69
Fig-8	DNP-DL-glu	HEMH	Lower phase	43.1	1.69/1.79	273/486/301	98
	DNP-β-ala		Upper phase	55.6	1.21/0.63	110/94/189	111
	DNP-L-ala						
	Val-Tyr	BAW	Lower phase	45.5	1.31	155/66	71
	Trp-Tyr		Upper phase	47.0	0.99	55/37	87

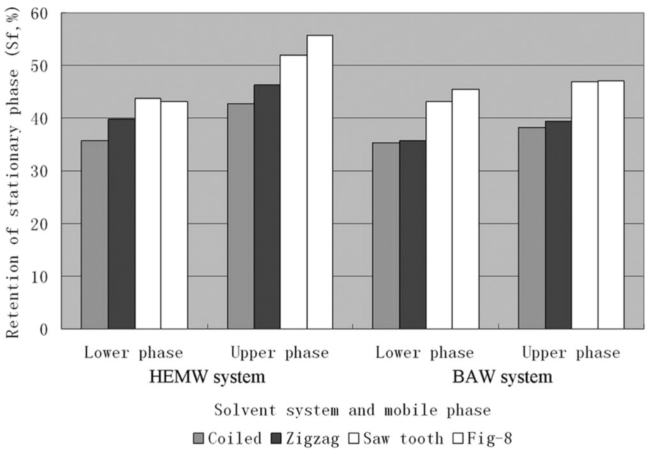
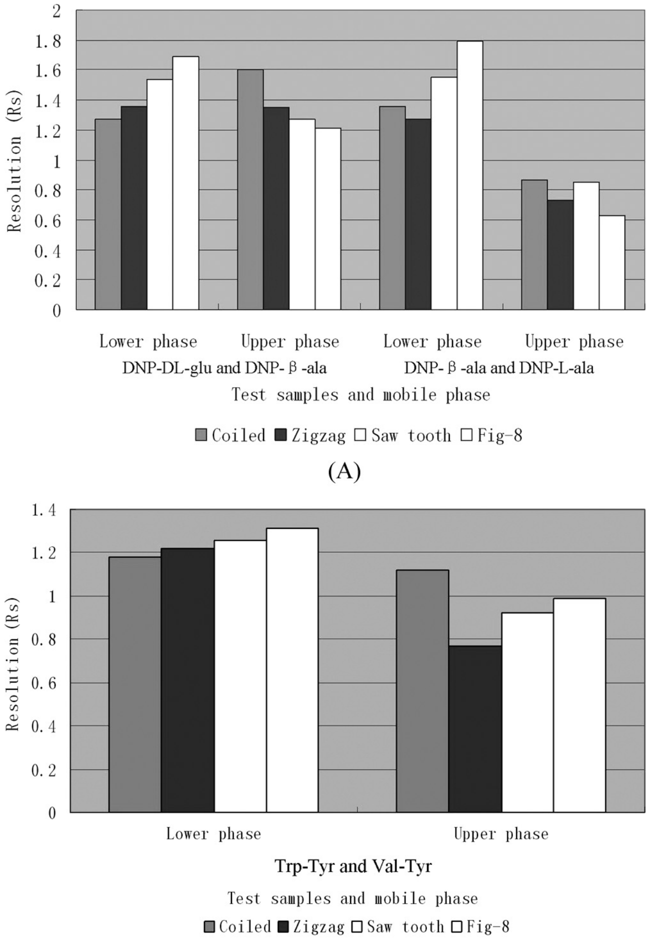


FIGURE 2 Comparison in stationary phase retention between four different columns for centrifugal countercurrent chromatography. Solvent system: HEMW and BAW; Samples: DNP-DL-glu, DNP-β-ala, & DNP-L-ala for HEMW and Val-Tyr & Trp-Tyr for BAW; Sample size: 40 µL; Flow rate: 0.05 mL/min; Revolution speed: 1000 rpm.

mobile phase groups, i.e., figure-8, saw tooth, zigzag, and coiled column in this order (Table 1).

Figure 3 shows peak resolution of test samples in the two typical two-phase solvent systems. When the lower phase was used as the mobile phase in the HEMW, figure-8 with Rs at 1.69 (1st peak/2nd peak)–1.79 (2nd peak/3rd peak) was best followed by saw tooth with Rs at 1.54–1.55, zigzag at 1.36–1.29, and coiled column at 1.25–1.34 (Figure 3A and Table 1). But when the upper phase was as the mobile phase in HEMW, Rs of the coiled column at 1.60–0.88 was better than that of zigzag at 1.35–0.73, saw tooth at 1.27–0.85, and figure-8 at 1.21–0.63 (Figure 3A and Table 1). In the BAW system, the results of Rs were also similar. When the lower phase was used as the mobile phase, Rs of figure-8 at 1.31 was best among others, whereas in the upper phase mobile the coiled column yielded the best Rs at 1.12 (Figure 3B and Table 1). Among all columns tested, the figure-8 column showed the best Rs values in the lower phase mobile.

Table 1 shows that the theoretical plate number of the figure-8 column was also best when the lower phase is mobile phase, indicating that the figure-8 column is highly efficient. The column pressure of all three new columns including zigzag, saw-tooth, and figure-8 is much lower than that of the conventional coiled column, indicating that a much longer column can be used to further improve the separation.



(A)

(B)

FIGURE 3 Comparison in the peak resolution between four different columns for centrifugal counter-current chromatography. Sample size: 40 μL; Flow rate: 0.05 mL/min; Revolution speed: 1000 rpm. (A) Solvent system: HEMW; Samples: DNP-DL-glu, DNP-*β*-ala, DNP-L-ala, (B) Solvent system: BAW; Samples: Val-Tyr, Trp-Tyr.

CONCLUSIONS

The overall results of our experiments indicated that among all columns tested, the figure-8 column yielded the best performance when the lower phase was used as the mobile phase. Since the column pressure of all new columns was much lower than that in the conventional coiled column, the peak resolution can be further improved by increasing the length of the separation column.

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