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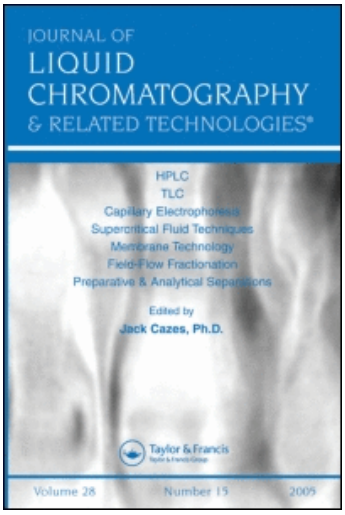

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NOVEL DESIGN FOR CENTRIFUGAL COUNTERCURRENT CHROMATOGRAPHY: IV. FIGURE-8 COLUMN

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NOVEL DESIGN FOR CENTRIFUGAL COUNTERCURRENT CHROMATOGRAPHY: IV. FIGURE-8 COLUMN

Dongyu Gu,[1,2,3] Yi Yang,[1,2,3] Jennifer D. Eng,[1] Haji Akber Aisa,[2] and Yoichiro Ito[1]

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□ *The toroidal columns using an equilateral triangular core, zigzag, and saw patterns has improved step by step both for retention of the stationary phase and peak resolution of the conventional toroidal coil in centrifugal countercurrent chromatography. To further improve the retention of stationary phase and peak resolution, a novel figure 8 toroidal coil was designed and the performance of the system was evaluated at various flow rates. In the figure-8 column with a capacity of 3 mL, the peak resolution of dipeptides (Val-Tyr and Trp-Tyr) was 1.63, and that of DNP-amino acids (DNP-DL-glu, DNP-β-ala, DNP-L-ala) were 1.92 and 1.85, respectively. The results indicated that retention of stationary phase and peak resolution were improved at lower flow rates. The Rs with lower mobile phase can be further increased by modifying the tubing to form a flat twisted configuration. With a decreased column length with a capacity of about 0.1 mL, resolution of the figure-8 column was 0.71 with about 40% retention of stationary phase at a flow rate of 0.005 mL/min.*

Keywords countercurrent chromatography, dipeptide, DNP-amino acid, figure-8 column, peak resolution, retention of the stationary phase

INTRODUCTION

High-speed countercurrent chromatography has been widely used for the separation and purification of natural products.[1,2] However, this hydrodynamic CCC system utilizing an Archimeadean screw effect

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generated by the coil planet centrifuge can not be efficiently applied to analytical separations due to a strong cohesive force between the liquid and the tube wall in small diameter tubing, which results in a loss of stationary phase from the column. This problem can be solved using a hydrostatic CCC system, which provides a stable centrifugal force field by arranging a narrow bore coiled column around the periphery of the centrifuge bowl in a toroidal form in a seal free centrifuge.[3] In the conventional toroidal coil, the retention of the stationary is limited to considerably less than 50% of the total column capacity since a half space of each helical turn is completely occupied with the mobil e phase, and in a typical separation the retention of stationary phase is no more than 30% of the total column capacity, which sharply decreases with application of a higher flow rate of the mobile phase. To cope with this problem, the triangular helical column has been designed, where the dead space in each helical turn was reduced from 1/2 to 1/3 and the stationary phase retention was improved to reach over 40%.[4] The results clearly show that the retention of the stationary phase in the toroidal coil will be further improved by increasing the height of the triangular core and/or reducing the length of the side of triangle providing the dead space.

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Recently a zigzag column was introduced in our laboratory to further improve the performance of centrifugal CCC. This column consists of the repetition of several zigzag loops which enhanced the stationary phase retention. This column was evaluated at various flow rates with typical two-phase solvent systems. The results indicated that both retention of stationary phase and peak resolution were improved as the flow rate was decreased. Modification of the tubing by pressing at given intervals with a pair of pliers improved the peak resolution without increasing the column pressure. All the separations with the zigzag column configuration were performed under low column pressure, indicating that the separation can be further improved by increasing the column length and/or rotational speed without damaging the separation column.[5] Then, the saw tooth column was designed to further decrease the dead space and improve the separation. In fact, the retention of stationary phase and peak resolution was successfully increased by these column designs.[6]

In this paper, a novel configuration of the figure-8 column is described. It consists of a repetition of short radial segments and long slanted segments, which further reduces the dead space to improve the retention of stationary phase and peak resolution (Fig. 1). The performance of this new column configuration is demonstrated by the separations of dipeptide and DNP-amino acid test samples, each with a suitable two-phase solvent system using a rotary seal free continuous flow centrifuge system.

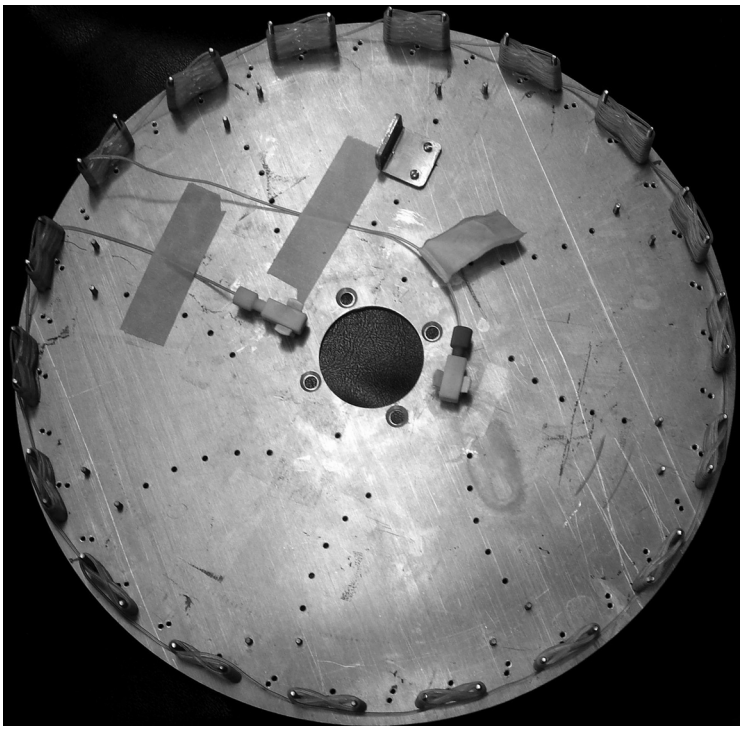

FIGURE 1 Design of the figure-8 column for centrifugal countercurrent chromatography.

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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 plate measuring about 34 cm in diameter to hold a coil separation column. Each column unit is made by hooking a 0.46 mm ID FEP (Fluorinated ethylene propylene) (Zeus Industrial Products, Orangeburg, SC, USA) tubing onto a pair of screws upstanding from the rotary plate making single or plural layers of figure-8 loops. We have prepared three columns composing 9, 18, and 36 units (all with the total capacity of 3 mL) by changing the distance between two screws (each 2.5 mm in diameter) to 5.4, 2.7, and 1.35 cm, respectively. Multiple column units are serially connected with transfer tubing to form a figure-8 separation column. Each terminal of the column is connected to PTFE flow tube (0.46 mm I.D., Zues Industrial Products) with a set of tubing connectors (Upchurch Scientific, Palm Spring, CA, USA) as shown in Fig. 1. A pair of flow tubes is 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 exits 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 were purchased from Fisher Scientific, Fair Lawn, NJ, USA and other solvents such as acetic acid and hydrochloric acid, from Mallinckrodt Chemicals, Phillipsburg, NJ, USA. Test samples including tryptophyl-tyrosine (Trp-Tyr), valyl-tyrosine (Val-Tyr), N-2,4-dinitrophenyl-L-alanine (DNP-ala), N-2,4-dinitrophenyl-*β*-alanine (DNP-*β*-ala), N-2,4-dinitrophenyl-D,L-glutamic acid (DNP-glu) were obtained from Sigma Chemicals, St. Louis, MO, USA.

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Two-Phase Solvent Systems and Sample Solutions

In the present study, 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 repeating vigorous shaking and degassing several times, and the phases separated shortly before use. The 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 50 μL was charged in each run. And the sample solution 2 was prepared by dissolving 5.7 mg of DNP-ala, 7.1 mg of DNP-*β*-ala, and 5.4 mg of DNP-glu in 10 mL of the upper phase of hexane-ethyl acetate-methanol-0.1 M HCl, and 50 μL was charged in each run.

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 mixed to the effluent at a volume ratio of 1:3 at the inlet of the detector using a tee connector and a fine mixing tube

(PTFE 0.4 mm ID × ca 1 m). After the desired peaks were eluted, the run was stopped and the column contents were collected into a graduated cylinder by pressured air to determine the volume of the stationary phase retained in the column. The retention of the stationary phase was computed by dividing the volume of the retained stationary phase with the total column volume.

Evaluation of Partition Efficiency

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

$$N = (4t_R/W)^2 \quad (1)$$

$$Rs = 2(t_2 - t_1)/(W_1 + W_2) \quad (2)$$

where t and W indicate the retention time and the peak width in Equation 1 and those for the specified peaks in Equation 2, respectively.

In order to make a fair comparison between the results of plain tubing and the modified tubing with a different capacity, the peak resolution (Rs) was adjusted using the following equation:

$$Rs\text{-}a = Rs(V_1/V_2)^{1/2} \quad (3)$$

where Rs-a is the adjusted peak resolution and V indicates the column volume being specified by the subscript 1 for the standard column and 2 for a modified column to be compared.

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RESULTS AND DISCUSSION

As shown in Fig. 2, there are two solvent entrances, A, and B in the figure-8 column. In order to obtain better retention of the stationary phase, the lower mobile phase should be eluted from entrance A and the upper mobile phase from entrance B. Hence, these elution modes are called the proper elution mode. And the reversed elution modes are called the improper elution mode, in which the ratio between the effective column space and the dead space was reversed. A series of experiments was performed using the BAW solvent system at a flow rate of 0.05 mL/min to confirm the above assumption, the results of which are shown in Fig. 2. In the proper elution mode, the retention of stationary phase was at 43.0%

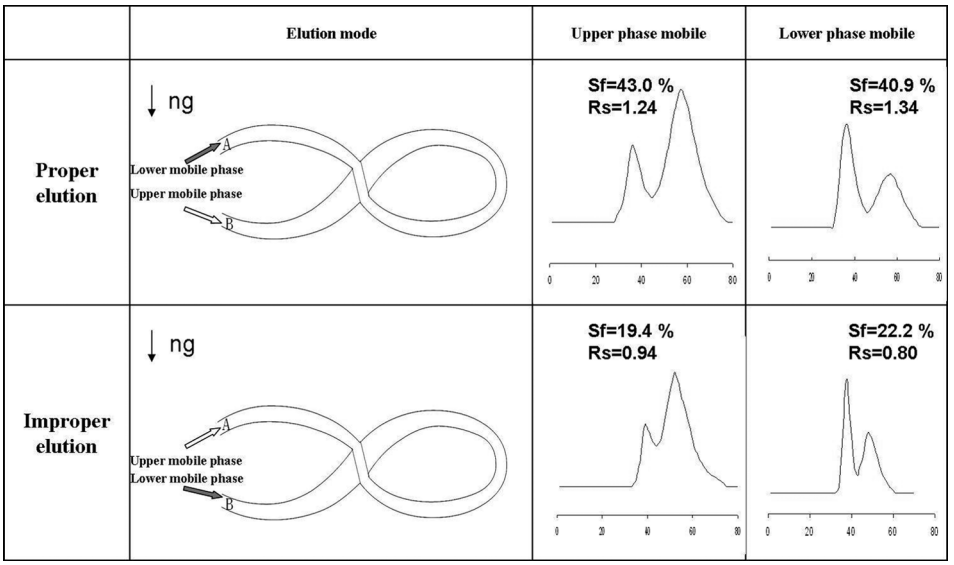

FIGURE 2 Comparison of performance of the figure-8 column between four elution modes. Sample: Val-Tyr and Trp-Tyr; Sample size: 50 μL; Detection: 280 nm; Capacity: 3 mL; Solvent system: BAW; Flow rate: 0.05 mL/min; Revolution: 1000 rpm.

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and 40.9% with similar peak resolution at 1.24 and 1.34, respectively, whereas in the improper elution mode, retention of stationary phase was reduced to 19.4% and 22.3% with peak resolution of 0.94 and 0.80, respectively.

Then, DNP-amino acids, DNP-L-ala, DNP-*β*-ala, and DNP-DL-glu, were used for further testing the column performance with the proper elution mode. Table 1 shows the results obtained from the moderately hydrophobic solvent system of HEMW at a flow rate of 0.05 mL/min. The retention of stationary phase with the upper mobile phase was much higher than that with the lower mobile phase, whereas peak resolution obtained was just opposite. The peak resolution is 2.01 between DNP-DL-glu and DNP-*β*-ala, and 1.44 between DNP-*β*-ala and DNP-L-ala with the retention of stationary phase at 41.7% using the lower mobile phase (Table 1), whereas the peak

TABLE 1 Performance of BAW and HEMW with the Proper Elution Modes

Solvent System	Test Samples	Mobile Phase	Sf (%)	Rs
BAW	Val-Tyr	Lower phase	40.9	1.34
	Trp-Tyr	Upper phase	43.0	1.24
HEMW	DNP-DL-glu	Lower phase	41.7	2.01/1.44
	DNP-*β*-ala	Upper phase	55.6	1.11/0.64
	DNP-L-ala			

Note: Flow rate: 0.05 mL/min; rotational speed: 1000 rpm.

resolution is 1.11 between DNP-β-ala and DNP-DL-glu and 0.64 between DNP-β-ala and DNP-L-ala with the retention of stationary phase at 55.6% using the upper mobile phase (Table 1). Table 1 also indicates that retention of the stationary phase in the HEMW system was better than that in the BAW system.

After finding the best eluting mode, the performance of the figure-8 column was tested at the different given flow rates. Figure 3 shows that peak resolution and retention of stationary phase increased with a decreased flow rate. The experiments were performed on the separation of dipeptides, Val-Tyr and Trp-Tyr, with the polar BAW solvent system composed at a volume ratio of 4:1:5. All of the stationary phase retention was over the 40%. It should be noted that a lower flow rate can yield higher retention of the stationary phase (Sf) and higher peak resolution (Rs). The results clearly showed that the lower mobile phase yielded lower retention of stationary phase (Fig. 3a), but better peak resolution (Fig. 3b) at a given flow rate than the upper mobile phase.

Figure 4 shows the results of retention of stationary phase and peak resolution using the different figure size (distance between the two screws) of the figure-8 column. The experiments were performed on the separation of dipeptides with the BAW solvent system at the different given flow. The results show that the larger figure size gives higher retention of stationary phase (Fig. 4a), but it isn't beneficial to the peak resolution (Fig. 4b). In the maximum figure size at 5.4 cm, Sf is remarkably better than that in the two other figure size columns, whereas Rs is the worst. In the smallest figure size of 1.35 cm, the retention of stationary phase is the worst, but Rs (1.49) is the best at the higher flow rate, indicating that it provides much better mixing of the two phases in the column.

The different tubing geometries were tested in the BAW solvent system for the figure-8 column (Table 2). The experiments were performed at a flow rate of 0.05 mL/min with the rotational speed of 1000 rpm. The retention of stationary phase in the plain tubing is the best. As the tubing was clamped, then flattened and finally twisted, the retention of stationary phase decreased gradually. The retention of stationary phase with lower phase mobile is always lower than that with upper phase mobile. In order to compare with the Rs of the plain tubing at the same capacity, the Rs of tubing with different geometries was adjusted according to Equation 3. The results showed that flat twisted tubing yielded the best resolution of Rs at 1.66 when the lower phase was mobile, whereas it showed the lowest Rs of 0.76, when the upper phase was mobile. Overall results of the experiments at a flow rate of 0.05 mL/min revealed that the flat twisted tubing displayed much better performance than the plain tubing with the lower mobile phase.

Performance of the flat twisted tubing was further evaluated in separation of dipeptides (Val-Tyr and Trp-Tyr) in BAW solvent system at different

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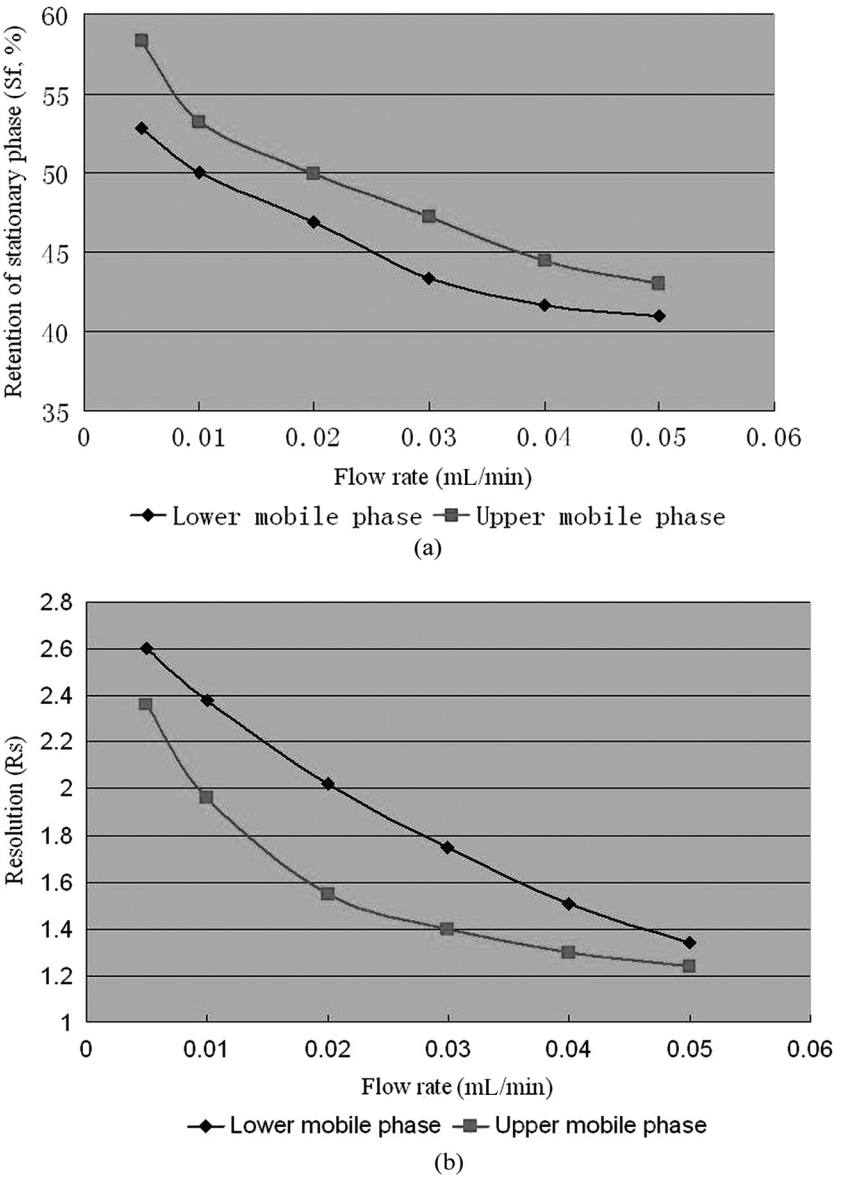

FIGURE 3 Comparison of retention of the stationary phase (a) and Rs (b) of various flow rates in the dipeptide separation using the figure-8 column. Sample: Val-Tyr and Trp-Tyr; Sample size: 50 μL; Detection: 280 nm; Capacity: 3 mL; Solvent system: BAW; Flow rate: 0.05 mL/min; Revolution: 1000 rpm.

flow rates. Figure 5 shows the peak resolution and retention of the stationary phase in the flat twisted tubing using the figure-8 column with different flow rates. Compared with the results obtained from the plain tubing, the retention of stationary phase of flat twisted tubing was remarkably lower

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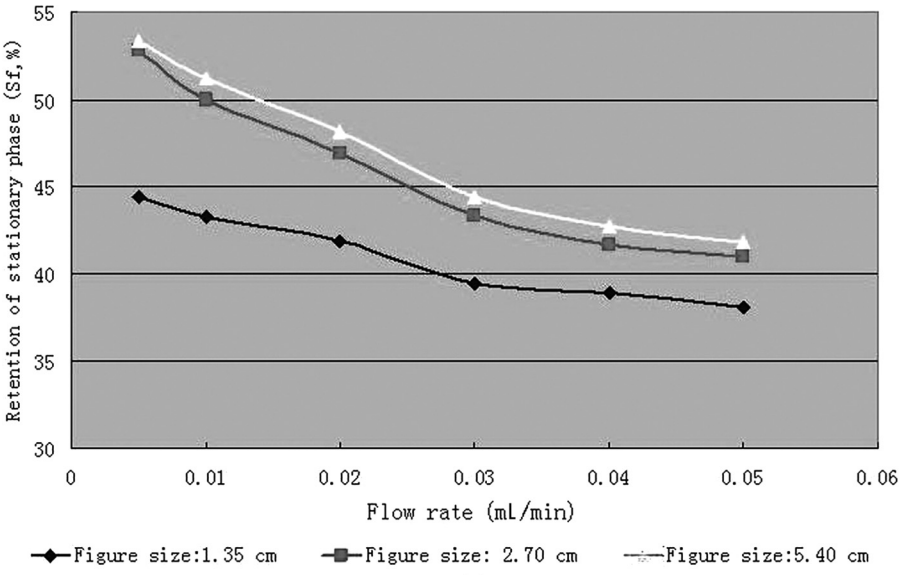
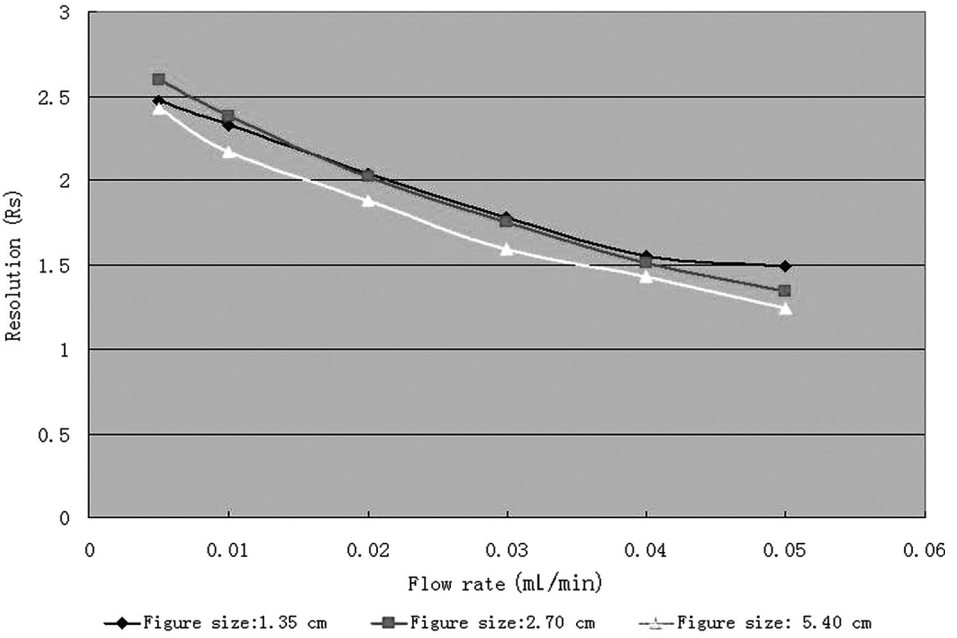


(a)



(b)

FIGURE 4 Comparison of the different figure size of 8 figure column in the BAW solvent system. Sample: Val-Tyr and Trp-Tyr; Sample size: 50 µL; Detection: 280 nm; Capacity: 3 mL; Solvent system: BAW; Flow rate: 0.05 mL/min; Revolution: 1000 rpm.

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TABLE 2 Evaluation on Resolution of Val-Tyr (1) and Trp-Tyr (2) in the BAW at the Same Capacity Using Different Geometries of figure-8 Tubing by Centrifugal Countercurrent Chromatography

Tubing	Mobile Phase	Flow Rate (mL/min)	Capacity (mL)	Length (m)	Sf (%)	Rs-a[a]
Plain	Lower phase	0.05	3.0	18	40.9	1.34
	Upper phase				43	1.24
Para-mid-clamping	Lower phase		2.8	18	35.3	1.26
	Upper phase				38.2	0.92
Vert-flattened	Lower phase		1.9	18	32.3	1.46
	Upper phase				36.1	0.82
Flat-twisted	Lower phase		1.8	18	27	1.66
	Upper phase				29.2	0.76

[a]Rs-a = $Rs(V_1/V_2)^{1/2}$, where Rs-a is the adjusted peak resolution and V indicates the column volume being specified by the subscript 1 for the standard plain column and 2 for the modified column.

(Fig. 5a). Nevertheless, it yielded much higher peak resolution than that obtained from plain tubing as shown in Figure 5b. This result indicates that the flat twisted tubing gives more efficient mixing of the two phases in the column, which leads to a smaller amount of the stationary phase retained in the column.

Figure 6 schematically illustrates the peak resolution of flat twisted tubing with the figure-8 column at different column lengths. The general formula used for computing the peak resolution in countercurrent chromatography is

$$Rs = 0.25(\alpha - 1)N^{1/2}\ K_1/[K_1 + (1 - S_F)/S_F] \quad (4)$$

where Rs is the peak resolution, α is the separation factor or $K_1/K_2 (K_1 > K_2)$, N is the theoretical plate number, K is the partition coefficient and S_F is the % retention of stationary phase. According to Equation 4, Rs is proportional to $N^{1/2}$, while N is proportional to the coil length. Then, the square of Rs should be proportional to the tubing length.[7] Figure 6 shows the linear relationship between the square of Rs and the tubing length using the flat twisted tubing of figure-8 column. It produced the peak resolution of Rs = 0.71 for the decreased column length with a capacity of about 0.1 mL.

Finally, the column performance was compared between the figure-8 and saw tooth column, the latter of which has shown the best performance among all other columns used in centrifugal countercurrent chromatography. When the lower phase is the mobile phase, the retention of stationary phase is higher in the saw tooth column (Table 3). However, the resolution of figure-8 is very difficult to compare with that of the saw tooth column due to the difference in column capacity. In order to make a fair comparison in the peak resolution between these two columns, Rs was

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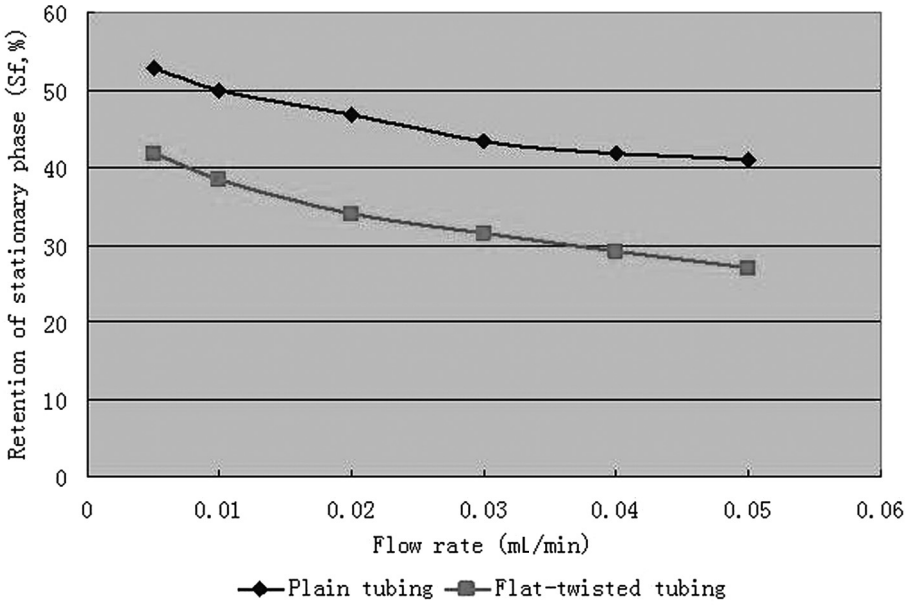
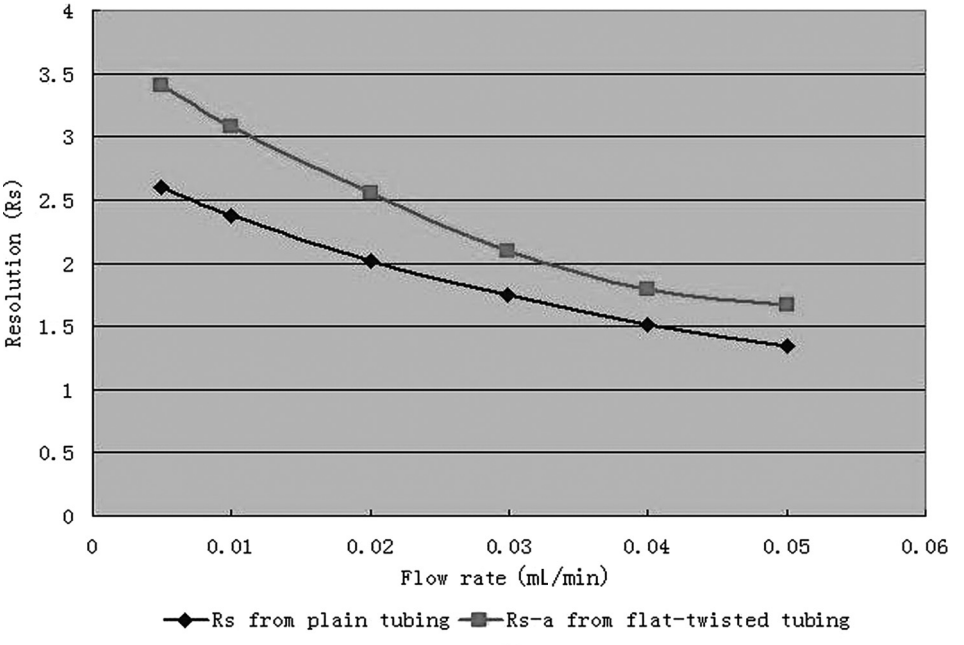


(a)



(b)

FIGURE 5 Comparison of performance between plain tubing and flat twisted tubing using the adjusted Rs values. Sample: Val-Tyr and Trp-Tyr; Sample size: 20 μL; Detection: 280 nm; Capacity: 2.3 mL; Solvent system: BAW; Flow rate: 0.005–0.05 mL/min; Revolution: 1000 rpm. Rs of plain tubing was adjusted to the capacity of 2.3 mL by Equation (3).

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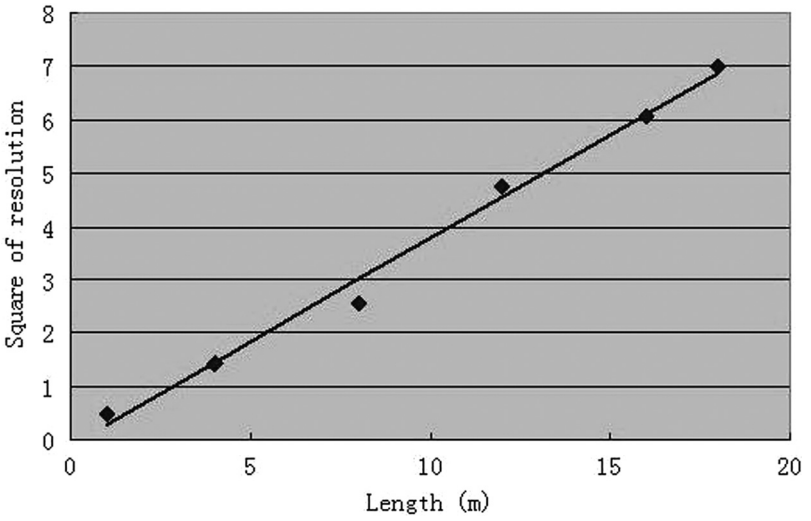

FIGURE 6 Comparison of performance of flat-twisted tubing of figure-8 column at the different column length. Sample: Val-Tyr and Trp-Tyr; Detection: 280 nm; Solvent system: BAW; Revolution: 1000 rpm.

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TABLE 3 Comparison of Performance of figure-8 and Saw Tooth Column by Centrifugal Counter-Current Chromatography[6]

			figure-8		Saw Tooth	
Solvent System	Test Samples	Mobile Phase	Sf (%)	Rs-a[a]	Sf (%)	Rs
BAW	Val-Tyr	Lower phase	40.9	1.62	43.2	1.63
	Trp-Tyr	Upper phase	43.0	1.50	47.8	1.56
HEMW	DNP-DL-glu	Lower phase	41.7	2.43/1.74	43.2	1.92/1.85
	DNP-β-ala	Upper phase	55.6	1.34/0.77	51.7	2.02/1.22
	DNP-L-ala					

Note: flow rate: 0.05 ml/min; rotational speed: 1000 rpm.
[a]Rs-a = $Rs(V_1/V_2)^{1/2} = Rs(4.4/3)^{1/2} = 1.21Rs$.

adjusted according to the Equation 3. The Rs in the dipeptide separation using the saw tooth column is slightly better which, perhaps, is because of slightly better retention of stationary phase. However, when the lower phase was used as the mobile phase in the HEMW solvent system, the figure-8 column yielded higher Rs as shown in Table 3.

CONCLUSIONS

Satisfactory peak resolution and stationary phase retention were obtained from the figure-8 column using the plain tubing. The results

indicate that the small figure size yields better peak resolution at a higher flow rate. It was found that the flat twisted tubing further improved the partition efficiency. The overall results indicate that the figure-8 column is useful for analytical separation.

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