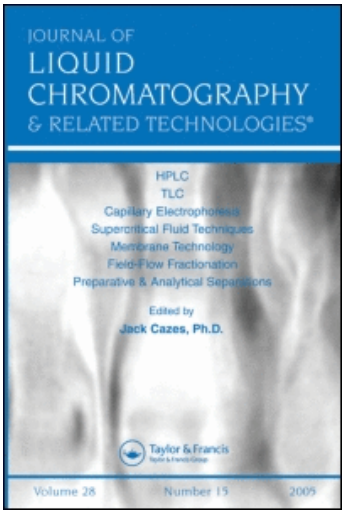

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NOVEL DESIGN FOR CENTRIFUGAL COUNTER-CURRENT CHROMATOGRAPHY: III. SAW TOOTH COLUMN

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□ *The toroidal coil using an equilateral triangular core and zigzag pattern column have improved both retention of the stationary phase and peak resolution of the conventional toroidal coil in centrifugal counter-current chromatography. To further improve the retention of stationary phase and peak resolution, a novel saw tooth column was designed and the performance of the system was evaluated at various flow rates. The results indicated that both retention of the stationary phase and peak resolution were improved as the flow rate was decreased and at a flow rate of 0.005 mL/ min the resolution is remarkably increased. Modification of the tubing called flat-twisted tubing further improved the peak resolution without increasing the column pressure. With decreased column length at a capacity of about 0.2 mL, resolution of the saw tooth column was 1.02.*

Keywords counter-current chromatography, dipeptide, DNP-amino acid, resolution, retention of the stationary phase, saw tooth column

INTRODUCTION

High-speed counter-current chromatography (HSCCC) has been widely used for the separation and purification of natural products.[1–4] However, this hydrodynamic CCC system cannot be efficiently applied to analytical separations, because the Archimedean screw effect is diminished in small diameter tubing by a strong cohesive force between liquid and the inner wall of tubing resulting in loss of stationary phase from the column. This problem can be solved by subjecting the separation column to a stable

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centrifugal force field in a hydrostatic CCC system that uses a narrow-bore toroidally-coiled column mounted around the periphery of the centrifuge bowl in a seal-free flow-through centrifuge.[5]

In this toroidal coil, the retention of the stationary phase is limited to considerably less than 50% of the total column capacity since half of the volume of each helical turn is entirely occupied by the mobile phase. In a typical separation, retention of the stationary phase is no more than 30% of the total column capacity and it sharply decreases with higher flow rates of the mobile phase. In order to cope with this problem, a triangular helical column has been designed, where the dead space in each helical turn was reduced to 1/3 and the stationary phase retention was increased to over 40%.[6] Recently, a zigzag toroidal column was introduced to further improve the performance of centrifugal CCC as reported.[6] 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.[7]

Although the retention of stationary phase and resolution of the zigzag column were improved, we found there is still a large dead space in this column design, leading to the loss of stationary phase. In this paper, a novel configuration of the saw tooth column is described. It consists of a repetition of short radial segments and long slanted segments, which further reduce 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 counter-current chromatographic separation of dipeptide and DNP-amino acid test samples using a rotary-seal-free continuous-flow centrifuge system.

EXPERIMENTAL

Apparatus

The present study uses a rotary-seal-free flow-through centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, Maryland, USA. It is equipped with an aluminum rotary plate measuring about 34 cm in diameter to hold a saw tooth separation column on its periphery. The columns are made by hooking a set of plain or modified 0.46 mm FEP

tubing (fluorinated ethylene propylene tubing from Zeus Industrial Products, Orangeburg, SC, USA) onto the screws upstanding over the rotary plate forming the saw tooth pattern as shown in Fig. 1 and Table 1. The tubing modifications include tubing clamped at the middle portion of the loop parallel to the centrifugal force (para-mid-clamping tube), tubing flattened perpendicular to the centrifugal force (vert-flattened tubing) and twisted flat tubing (flat-twisted tubing) (Fig. 2). The saw tooth column consists of a repetition of short radial segments (0.4 cm) and long slanted segments (approximately 3 cm). Each terminus of the toroidal column is connected to flow tubes with a set of tubing connectors (Upchurch Scientific, Palm Spring, CA, USA) as shown in Fig. 1. These flow tubes are put together and passed through the center of the hollow central shaft downward and the hollow horizontal shaft of a miter gear, then led upward into the vertical tube support pipe, and finally exited the centrifuge from the center of the upper plate where they are tightly held with a pair of clamps.[8] The total volume of the feed and return tubing (dead volume) is approximately 0.5 mL.

The pump (Shimadzu LC-10ADVP, Columbia, MD, USA) was used for pumping the solvents, and the effluent was continuously monitored with a UV detector (LKB Instruments, Stockholm, Sweden).

Reagents

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

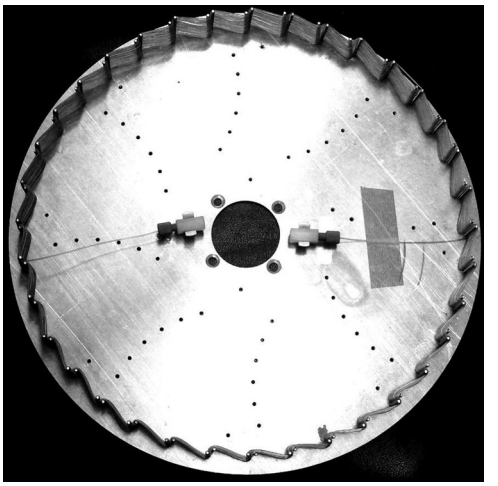


FIGURE 1 Design of the saw tooth column for centrifugal counter-current chromatography.

TABLE 1 Performance of BAW and HEMW with Proper Elution Modes

Solvent system	Test samples	Mobile phase	Sf (%)	Rs
BAW	Val-tyr	Lower phase	43.2	1.63
	Trp-tyr	Upper phase	47.8	1.56
HEMW	DNP-DL-glu	Lower phase	43.2	1.92/1.85
	DNP-β-ala	Upper phase	51.7	2.02/1.22
	DNP-L-ala			

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

such as acetic acid and hydrochloric acid from Mallinckrodt Chemicals, Phillipsburg, NJ, USA. Dipeptide samples including tryptophyl-tyrosine (Trp-Tyr), valyl-tyrosine (Val-Tyr) and N-2, 4-dinitrophenyl-L-alanine (DNP-L-Ala), N-2, 4-dinitrophenyl-β-alanine (DNP-β-Ala), N-2, 4-dinitrophenyl-DL-glutamic acid (DNP-DL-Glu) were obtained from Sigma Chemicals, St. Louis, MO, USA.

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 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. 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

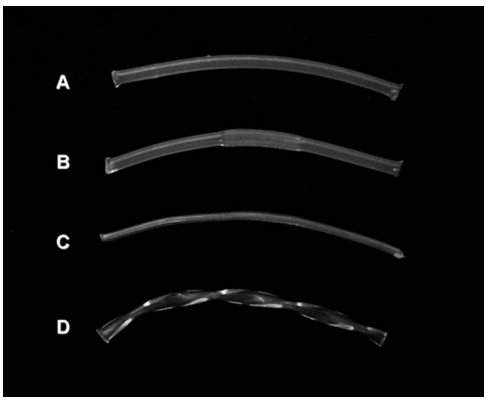


FIGURE 2 Different tubing geometries used for the zigzag toroidal column. (A) plain tubing; (B) mid-clamping tubing; (C) flattened tubing; (D) flat-twisted tubing.

10 mL of the upper phase of hexane-ethyl acetate-methanol-0.1 M HCl (1:1;1:1, v/v).

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 (5 μL/min, 10 μL/min, 20 μL/min, 30 μL/min, 40 μL/min or 50 μL /min). 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 30% 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 retained stationary phase by the column volume.

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.

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:

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

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.

RESULTS AND DISCUSSION

As shown in Fig. 1, the saw tooth pattern consists of repetition of the short radial segment and the long slanted segment. In order to obtain better retention of the stationary phase, the lower mobile phase should be eluted in a descending mode and the upper mobile phase in the ascending mode as indicated in Fig. 3. Hence, these elution modes are called the proper elution mode. And the reversed elution modes are called the improper elution mode. A series of experiments was performed in the saw tooth column using the BAW solvent system at a flow rate of 0.05 mL/min, the results of which are shown in Fig. 3. The chromatograms of dipeptides (Val-Tyr and Trp-Tyr) obtained from the proper elution mode were compared with those obtained from the improper elution modes in which the ratio between the effective column space and the dead space was reversed. When experiments were performed by eluting the upper or the lower phase in the proper elution mode, the retention of stationary phase was at 47.8% and 43.2% with similar peak resolution at 1.56, 1.63, respectively, whereas in the improper elution mode, retention of stationary phase was reduced to 24.4% and 27.3% with peak resolution of 1.40 and 1.26, respectively. The best peak resolution was obtained with lower mobile phase eluted in the proper elution mode, although it produced slightly lower retention of stationary phase than upper phase mobile. The fact that the peak resolution in the proper elution mode is only slightly higher than the resolution obtained from the improper elution mode in spite of sharply increased

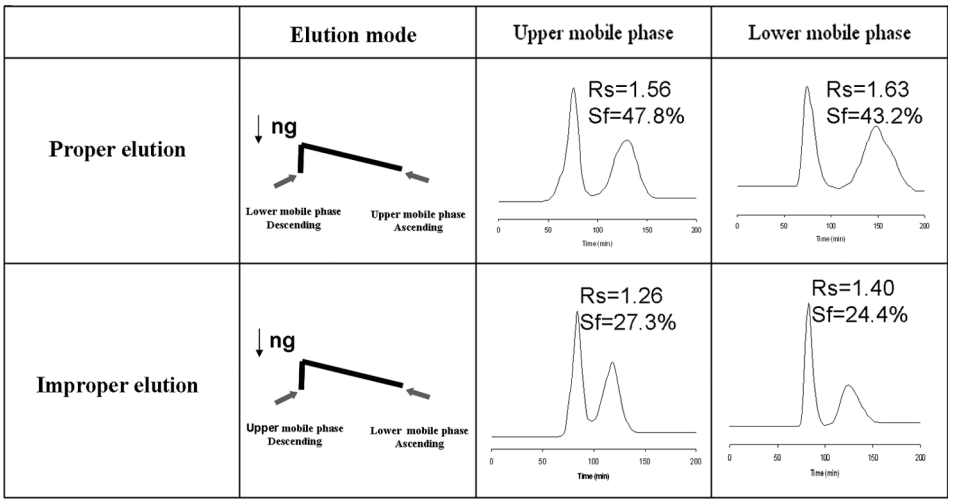


FIGURE 3 Comparison in the stationary phase retention and peak resolution in the saw tooth column between four elution modes. Sample: Val-Tyr and Trp-Tyr; Sample size: 40 μL; Detection: 280 nm; Capacity: 4.4 mL; Solvent system: BAW; Flow rate: 0.05 mL/min; Revolution: 1000 rpm.

stationary phase retention may indicate that the long flow path between the stationary segments in the efficient column space may produce laminar flow which tends to broaden the solute band.[7]

After finding the best eluting mode, all the studies were tested in the proper elution mode. Figure 4 shows the results obtained from the moderately hydrophobic solvent system of HEMW at a flow rate of 0.05 mL/min. In this study on separation of three test solutes of DNP-L-ala, DNP-β-ala and DNP-DL-glu, 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. When the lower phase is mobile phase, the peak resolution is 1.95 between DNP-DL-glu and DNP-β-ala, and 1.87 between DNP-β-ala and DNP-L-ala with the retention of stationary phase at 43.2% (Fig. 4a, Table 1). When the upper phase is mobile phase, the peak resolution is 2.02 between DNP-β-ala and DNP-DL-glu and 1.22 between DNP-β-ala and DNP-L-ala with the retention of stationary phase at 51.7% (Fig. 4b, Table 1). The resolution between DNP-β-ala and DNP-DL-glu with the lower phase mobile is similar to that with the upper phase mobile. But, the resolution between DNP-L-ala and DNP-β-ala with the lower phase mobile is much better than that with the upper phase mobile. Table 1 indicates that both retention of stationary phase and Rs in the HEMW system were better than those in the BAW system.

Column design is at the heart of the chromatographic system that leads to the successful separation. In our previous studies, the modified tubing showed a good performance,[9,10] especially in our studies on the zigzag column.[7,11] After evaluation of the peak resolution of different geometries of zigzag tubing in BAW solvent system, the vert-flattened tubing revealed much better performance than the plain tubing. So, in the present study, the different tubing geometries were tested using the saw tooth pattern column with the BAW solvent system. At first, plain, para-mid-clamping, vert-flattened and flat-twisted tubing were tested at a flow-rate of 0.05 mL/min. Table 2 summarizes the retention of stationary phase, peak resolution and N in four sets of the above saw tooth columns. Para-mid-clamping tubing yielded the similar peak resolution with plain tubing in spite of the higher retention of stationary phase with the lower mobile phase. The vert-flattened tubing which produced a good performance in zigzag column showed lower efficiency. When the lower phase was the mobile phase, flat-twisted tubing yielded the best results in retention of the stationary phase, peak resolution and N.

Next, the second series of experiments were designed using the saw tooth column according to the above results. Different tubing geometries, including plain tubing, vert-flattened tubing, and flat-twisted tubing were evaluated with dipeptides (Val-Tyr and Trp-Tyr) in BAW polar solvent system at different flow rates. Figure 5 shows the resolution and retention

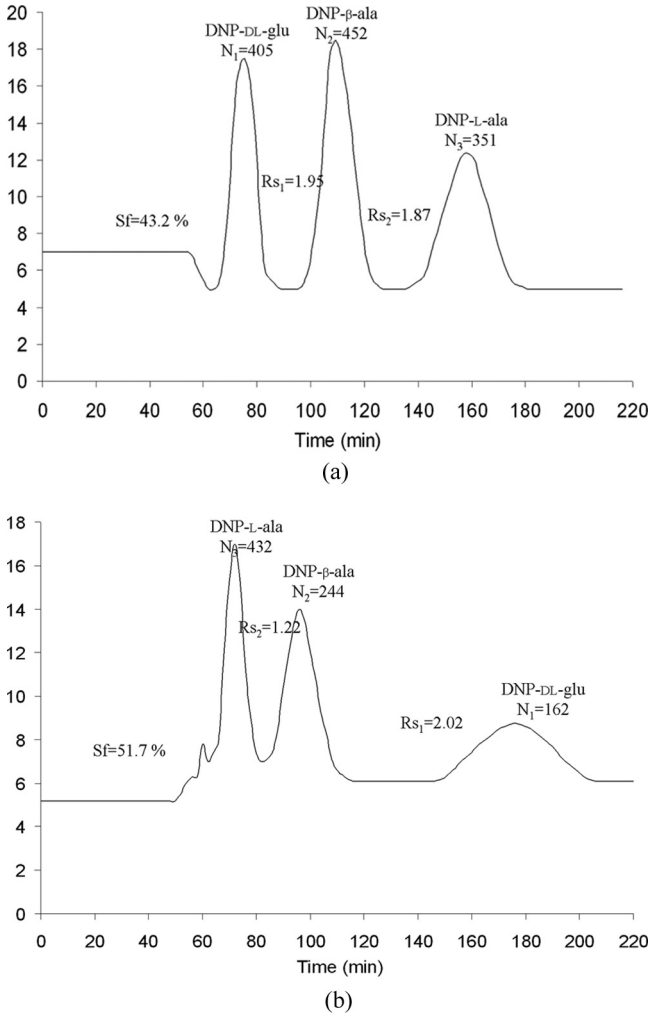


FIGURE 4 CCC chromatograms of DNP-amino acids separation using saw tooth column. Sample: DNP-L-Ala, DNP-*β*-Ala and DNP-DL-Glu; Sample size: 50 µL; Detection: 280 nm; Capacity: 4.4 mL; Solvent system: HEMW; Flow rate: 0.05 mL/min; Revolution: 1000 rpm; (a) Lower mobile phase; (b) Upper mobile phase.

of the stationary phase of plain tubing using the saw tooth column with 27.5 layers at different flow rates. The peak resolution (Fig. 5a) and retention of the stationary phase (Fig. 5b) were increased with the decreased flow rate. The resolution obtained from lower mobile phase is better than that of upper mobile phase while retention of the stationary phase is opposite. This may be explained as follows: Because of a lack of the tubing wall affinity the lower phase tends to form a droplet flow through the upper stationary phase providing a large interface area for mass transfer. In

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

Tubing	Mobile phase	Flow rate (mL/min)	Capacity (mL)	Layers[a]	Length (m)	Sf (%)	Rs-a[b]	N_1-a[c]	N_2-a[c]
Plain	Lower phase	0.05	4.4	17.5	25	43.18	1.63	120	84
	Upper phase					47.82	1.56	126	191
Para-mid-clamping	Lower phase		4	17.5	25	51.39	1.63	257	102
	Upper phase					37.50	1.46	84	133
Vert-flattened	Lower phase		2.3	13.5	19	32.13	1.69	224	165
	Upper phase					34.45	0.86	104	176
Flat-twisted	Lower phase		2.3	13.5	19	53.85	1.80	426	195
	Upper phase					34.62	1.56	59	90

[a]The layer is the vertical layers at the same radius.

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

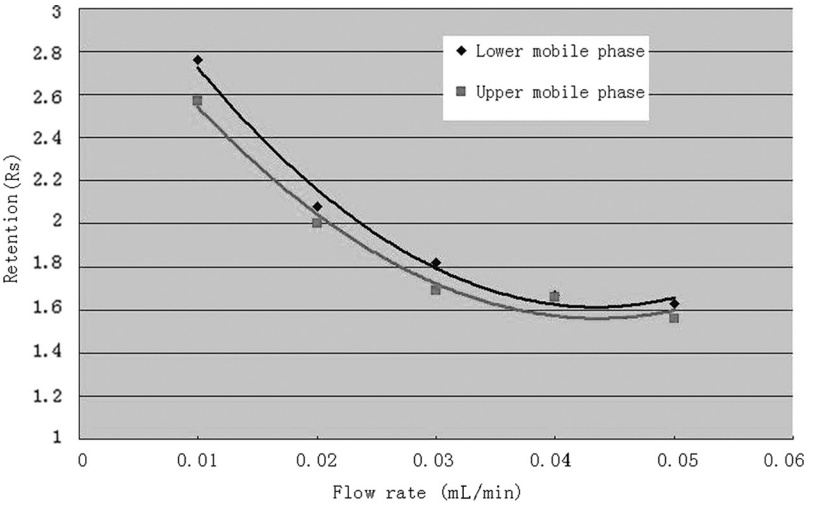
[c]$N\text{-}a = N\ L_1/L_2 = N\ (17.5/13.5) = 1.3\,N$, where N-a is the adjusted theory plate number and L indicates the column length or layers being specified by the subscript 1 for the plain column or layers and 2 for a column of compared length or layers.

contrast, due to the strong affinity to the plastic tubing, the upper phase smoothly flows along the wall surface of the tubing, providing high retention of the stationary phase while giving a limited interface area for mass transfer.[9] The best peak resolution of plain tubing obtained from lower mobile phase was 2.76 at a flow rate of 0.01 mL/min. Vert-flattened tubing and flat-twisted tubing were further tested and compared with plain tubing with the corrected capacity using 13.5 layers (approximately 2.3 mL capacity) of saw tooth tubing with lower mobile phase at different flow rates (Fig. 6). The peak resolution and retention of the stationary phase were improved with the decreased flow rate. The retention of stationary phase was higher than that of the plain tubing. Rs was also higher than the adjusted Rs of plain tubing. The highest peak resolution of flat-twisted tubing was 2.78 with the stationary phase retention of 72.2% at a flow rate of 5 μL/min. In the flat-twisted tubing configuration, the mobile phase repeat percolation in the stationary phase in every turn to improve solute partitioning. These results are much better than those obtained from other column designs reported earlier.[6,7]

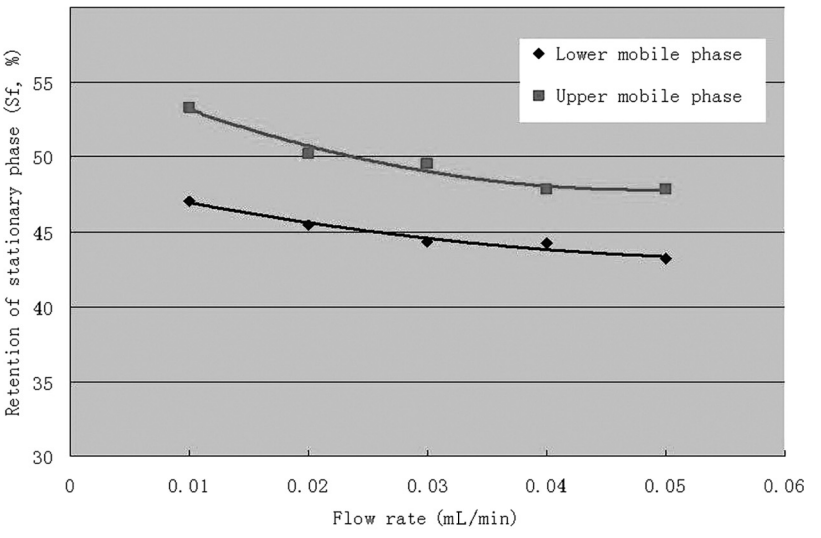
Figure 7 schematically illustrates the peak resolution of plain, vert-flattened and flat-twisted tubing with the saw tooth column at different column lengths. The general formula used for computing the peak resolution in counter-current chromatography is:

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

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



(a)



(b)

FIGURE 5 Comparison of performance of plain saw tooth column in separation of dipeptides. Sample: Val-Tyr and Trp-Tyr; Sample size: 40 μL; Detection: 280 nm; Capacity: 4.4 mL; Solvent system: BAW; Flow rate: 0.01–0.05 mL/min; Revolution: 1000 rpm.

retention of stationary phase. According to Eq (4) Rs is proportional to $\sqrt{N}$. while N is proportional to the coil length times the number of layers. Then, the square of Rs should be proportional to the number of layers.[12] Figure 7 shows the linear relationship between the square of actual Rs and the number of layers using Eq (3) to compare Rs of two columns with different lengths of tubing.

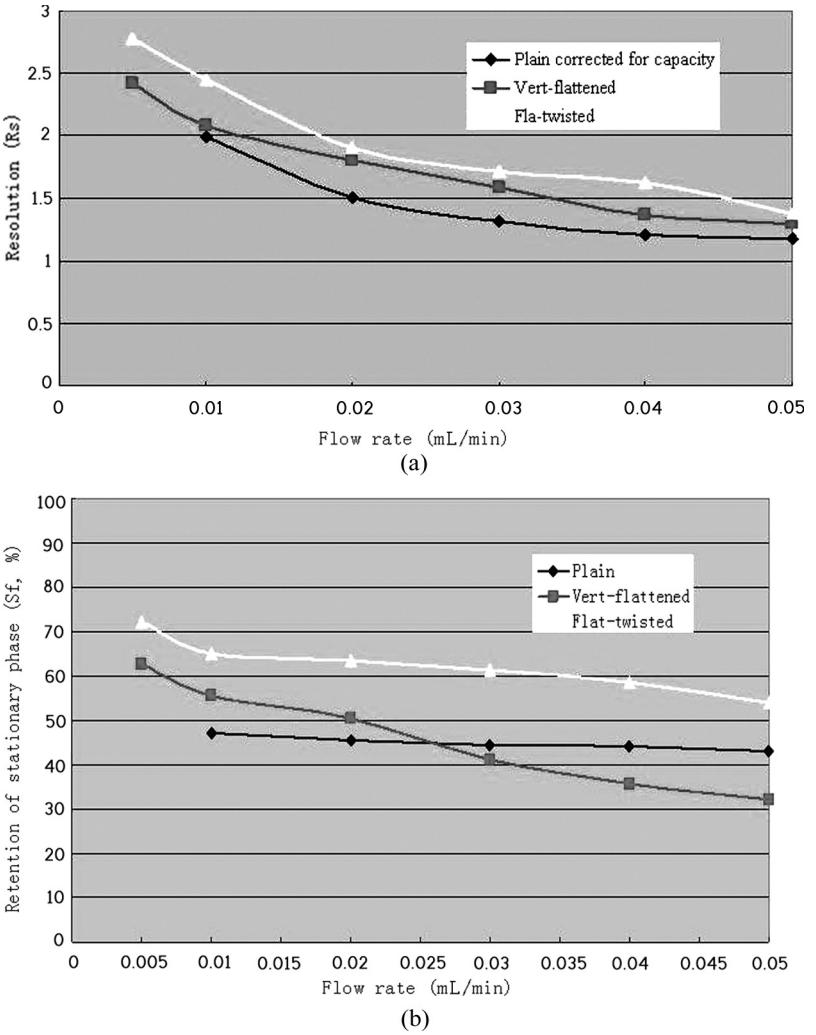


FIGURE 6 Comparison of performance between plain tubing, vert-flattened tubing and flat-twisted tubing using the adjusted Rs values. Sample: Val-Tyr and Trp-Tyr; Sample size: 20 μL; Detection: 280 nm; Capacity: 2.3 mL; Solvent system: BAW; Flow rate: 0.005–0.05 mL/min; Revolution: 1000 rpm. Rs of plain tubing was adjusted to the capacity of 2.3 mL by Eq (3) $Rs\text{-}a = Rs(V1/V2)^{1/2}$, where Rs-a is the adjusted peak resolution and V indicates the column volume being specified by the subscript 1 for the plain column (4.4 mL) and 2 for a column of 2.3 mL volume.

The dipeptides were separated in the BAW solvent system at a flow rate of 5 μL/min. The trend of resolution with flat-twisted tubing is the best, plain tubing is next and the vert-flattened tubing is the worst. In this series of experiments, when the number of tubing layers was decreased to 1, the column capacity was only about 0.2 mL. But, flat-twisted tubing still yielded a peak resolution of 1.02 at a flow rate of 5 μL/min.

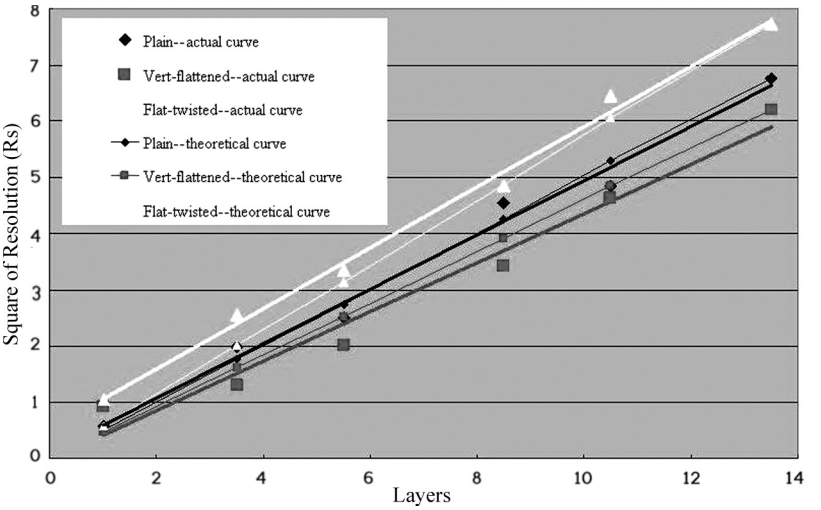


FIGURE 7 Comparison of performance of the plain, vert-flattened and flat-twisted tubing of saw tooth column at the same column length. Sample: Val-Tyr and Trp-Tyr; Detection: 280 nm; Solvent system: BAW; Flow rate: 0.005 mL/min; Revolution: 1000 rpm.

We also used the Rs of 13.5 layers of three types of tubing to calculate the theoretical Rs according to Eq (3) to obtain the theoretical Rs curves, which were compared with the actual Rs curves (Fig. 7). There are very similar trends between the theoretical and actual curves, although slightly higher deviation was at the very small column capacity of flat-twisted tubing between the theoretical and actual results.

CONCLUSIONS

Satisfactory peak resolution and stationary phase retention were obtained by the saw tooth column using the plain tubing. The flat-twisted tubing further improved the partition efficiency. Even with a small capacity at about 0.2 mL, it yields a peak resolution at 1.02 for the dipeptide separation with the lower aqueous phase mobile which can be efficiently applied for analytical separation of minute amounts of test samples for CCC/MS.

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