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Studies on the effect of column angle in figure-8 centrifugal counter-current chromatography

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ABSTRACT

The performance of the figure-8 column configuration in centrifugal counter-current chromatography was investigated by changing the angle between the column axis (a line through the central post and the peripheral post on which the figure-8 coil is wound) and the centrifugal force. The first series of experiments was performed using a polar two-phase solvent system composed of 1-butanol–acetic acid–water (4:1:5, v/v) to separate two dipeptide samples, Trp-Tyr and Val-Tyr, at a flow rate of 0.05 ml/min at 1000 rpm. When the column angle was changed from 0° (column axis parallel to the centrifugal force) to 45° and 45° to 90° (column axis perpendicular to the centrifugal force), peak resolution (Rs) changed from 1.93 (Sf = 37.8%) to 1.54 (Sf = 30.6%), then to 1.31 (Sf = 40.5%) with the lower mobile phase and from 1.21 (Sf = 38.8%) to 1.10 (Sf = 34.4%), then to 0.99 (Sf = 42.2%) with the upper mobile phase, respectively, where the stationary phase retention, Sf, is given in parentheses. The second series of experiments was similarly performed with a more hydrophobic two-phase solvent system composed of hexane–ethyl acetate–methanol–0.1 M hydrochloric acid (1:1:1:1, v/v) to separate three DNP-amino acids, DNP-glu, DNP-β-ala and DNP-ala, at a flow rate of 0.05 ml/min at 1000 rpm. When the column angle was altered from 0° to 45° and 45° to 90°, Rs changed from 1.77 (1st peak/2nd peak) and 1.52 (2nd peak/3rd peak) (Sf = 27.3%) to 1.24 and 1.02 (Sf = 35.4%), then to 1.69 and 1.49 (Sf = 42.1%) with the lower mobile phase, and from 1.73 and 0.84 (SF = 41.2%) to 1.44 and 0.73 (Sf = 45.6%), then to 1.21 and 0.63 (Sf = 55.6%) with the upper mobile phase, respectively. The performance of figure-8 column at 0° and 90° was also compared at different flow rates. The results show that Rs was increased with decreased flow rate yielding the highest value at the 0° column angle with lower mobile phase. The overall results of our studies indicated that a 0° column angle for the figure-8 column enhances the mixing of two phases in the column to improve peak resolution while decreasing the stationary phase retention by interrupting the laminar flow of the mobile phase.

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1. Introduction

High-speed counter-current chromatography (HSCCC) has been widely applied for the separation and purification of biological samples with conventional two-phase solvent systems [1–4]. However, this hydrodynamic CCC system fails to perform an analytical separation because of a diminished Archimedean screw effect 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 hydrostatic CCC using a coiled column mounted around the periphery of the centrifuge bowl. This analytical CCC system can produce a highly efficient analytical separation as reported earlier [5–8].

In this conventional toroidal coil CCC system, however, the retention of the stationary phase is limited to substantially less than 50% or typically about 30% of the total column capacity, since the half of each helical turn is entirely occupied by the mobile phase [8]. Consequently, the low retention of stationary phase limits the partition efficiency of this method. In order to cope with this problem, a triangular coiled column has been introduced which has improved the retention of the stationary phase to slightly over 40% [9]. Recently, a series of novel column designs has been introduced to further improve the retention of the stationary phase, including zigzag column [10,11], saw tooth column [12] and figure-8 column [13]. The retention of stationary phase was increased step by step [14].

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In the traditional toroidal coil CCC system, the coiled column is mounted with its axis perpendicular to the acting centrifugal force. However, in our recent study, we found that mounting the column at different angles, greatly alters its performance. In general, as the column angle against the centrifugal force was decreased, the retention of the stationary phase increased while peak resolution decreased and when the column was mounted parallel to the centrifugal force, the retention of the stationary phase became maximum [15].

In the present study, the performance of the figure-8 column in centrifugal counter-current chromatography was investigated by changing the angle between the column axis and centrifugal force in the separation of dipeptides and DNP-amino acids each with a suitable two-phase solvent system.

2. Experimental

2.1. Apparatus

The present study uses a rotary-seal-free centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, Maryland, USA. It has an aluminum rotary plate measuring about 34 cm in diameter which holds a 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 perpendicular to the rotary plate making single or plural layers of figure-8 loops. A total of 18 column units are serially connected with transfer tubing to form a figure-8 separation column. The single layer column has a total capacity of 3.4 ml. Each figure-8 column is mounted around the periphery of the rotary platform at various angles against the acting centrifugal force field as shown in Fig. 1. 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). 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 it is tightly held with a pair of clamps [16]. The total space in the feed and return tubing (dead volume) is approximately 0.5 cm^3.

A metering 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).

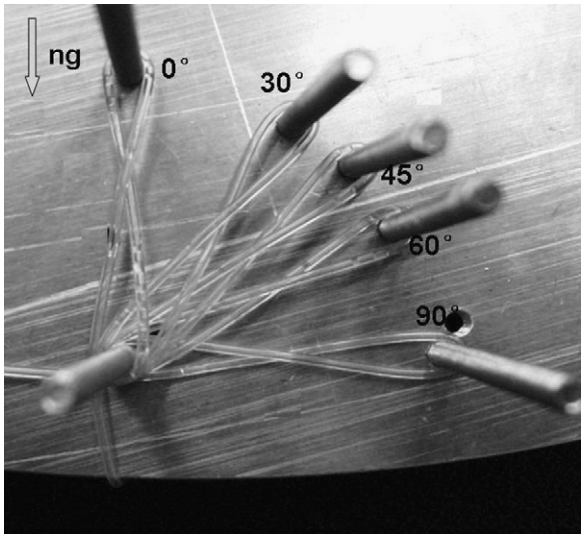

Fig. 1. The figure-8 column mounted on the plate at different column angles against the centrifugal force.

Table 1
Two-phase solvent systems and K values of the test samples.

Two-phase solvent system	Abbreviations	Test samples	*K* value
n-Hexane–ethyl acetate–methanol–0.1 M HCl (1:1:1:1, v/v)	HEMH	DNP-L-ala	2.36
		DNP-β-ala	1.18
		DNP-L-glu	0.44
1-Butanol–acetic acid–water (4:1:5, v/v)	BAW	Trp-Tyr	1.69
		Val-Tyr	0.53

2.2. Reagents

1-Butanol, hexane, ethyl acetate and methanol were all of HPLC grade and purchased from Fisher Scientific, Fair Lawn, NJ, USA and other solvents such as acetic acid and hydrochloric acid of an 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-DL-glu) were all obtained from Sigma Chemicals, St. Louis, MO, USA.

2.3. Partition coefficient measurement [17]

The partition coefficients (K_U) of each sample in the two-phase solvent system were 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. Then the K_U value was calculated according to the following equation: $K_U = A_U/A_L$ (Table 1).

2.4. Two-phase solvent systems and sample solutions

Two typical two-phase solvent systems composed of 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 several times, 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 BAW. 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 HEMW.

2.5. 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 continuously 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 terminated 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.

2.6. Evaluation of partition efficiency

The partition efficiency of 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:

$$N = \left(\frac{4t_R}{W}\right)^2 \quad (1)$$

$$\mathrm{Rs} = 2\left(\frac{t_2 - t_1}{W_1 + W_2}\right) \quad (2)$$

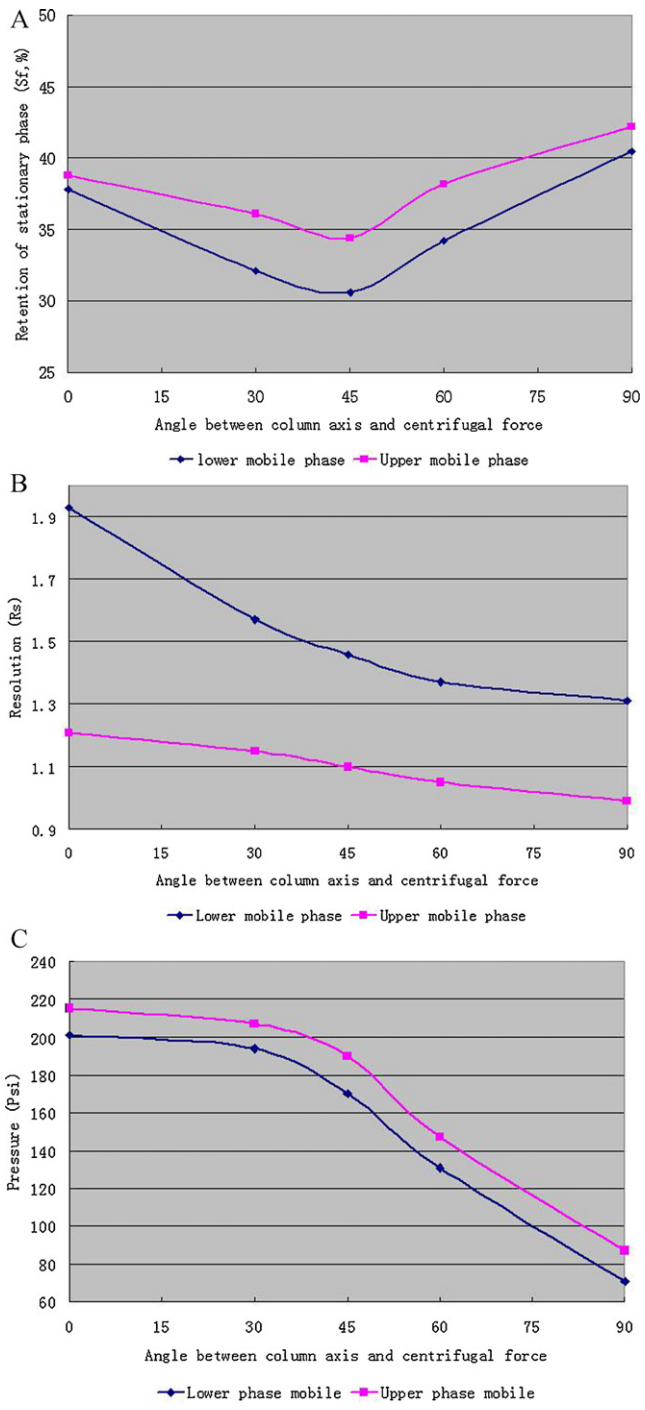

Fig. 2. Comparison of performance of dipeptide separation using figure-8 column with different column angles against the centrifugal force by centrifugal counter-current chromatography. Sample: Trp-Tyr, Val-Tyr; sample size: 40 μl; solvent system: BAW; revolution speed: 1000 rpm; flow rate: 0.05 ml/min; column capacity: 3.4 ml; (A) relationship between retention of stationary phase and column angle; (B) relationship between Rs and column angle; (C) relationship between column pressure and column angle.

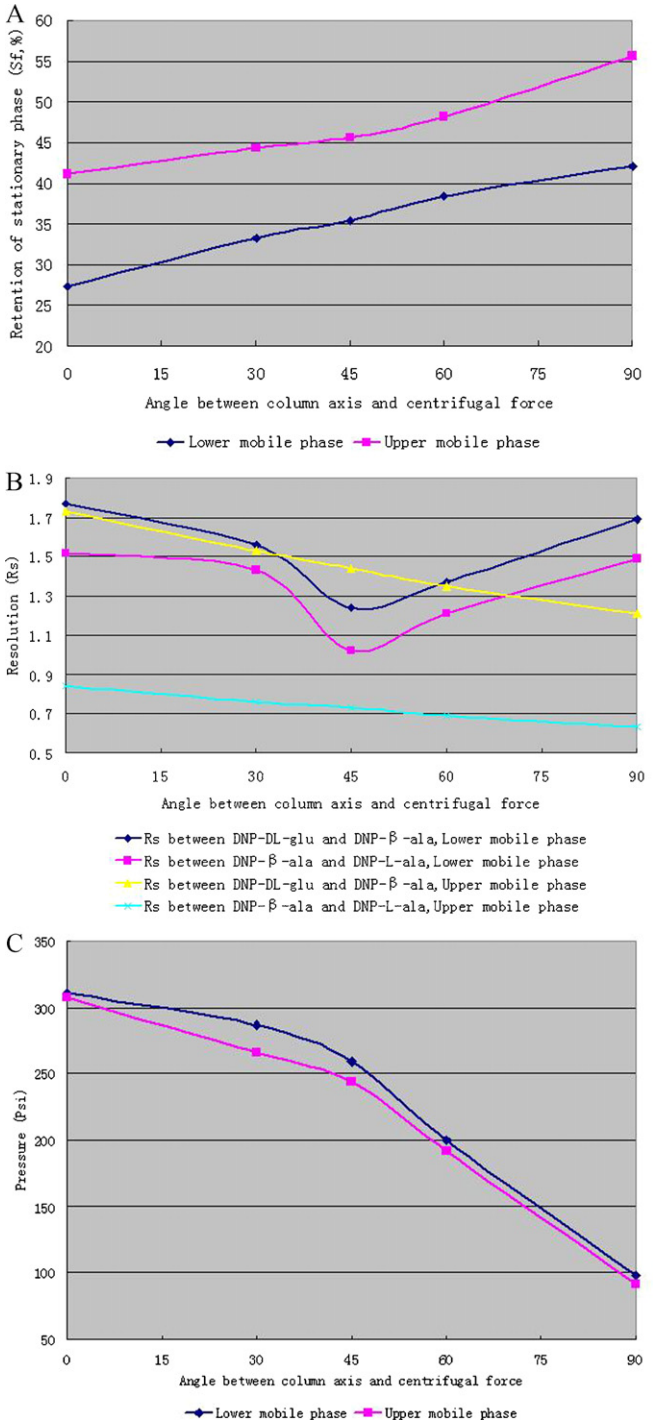

Fig. 3. Comparison of performance of DNP-amino acid separation using figure-8 column with different column angles against the centrifugal force by centrifugal counter-current chromatography. Sample: DNP-DL-glu, DNP-β-ala and DNP-L-ala; sample size: 40 μl; solvent system: HEMW; revolution speed: 1000 rpm; flow rate: 0.05 ml/min; column capacity: 3.4 ml; (A) relationship between retention of stationary phase and column angle; (B) relationship between Rs and column angle; (C) relationship between pressure and column angle.

Table 2
The effect of different column angles on the separation of dipeptides and DNP-amino acids by centrifugal counter-current chromatography using a figure-8 column.

Angle	Mobile phase	BAW system				HEMW system			
		Sf (%)	Rs	*N*	Pressure (psi)	Sf (%)	Rs	*N*	Pressure (psi)
90°	Lower phase	40.5	1.31	135/66	71	42.1	1.69/1.49	273/486/301	98
	Upper phase	42.2	0.99	55/37	87	55.6	1.21/0.63	110/94/189	91
60°	Lower phase	34.2	1.32	245/85	131	38.4	1.37/1.21	688/829/391	200
	Upper phase	38.2	1.05	56/58	147	48.2	1.35/0.69	200/119/270	192
45°	Lower phase	30.6	1.54	442/143	170	35.4	1.24/1.02	426/390/280	259
	Upper phase	34.4	1.10	56/136	190	45.6	1.44/0.73	203/280/400	244
30°	Lower phase	32.1	1.57	236/94	194	33.3	1.56/1.43	841/829/418	287
	Upper phase	36.1	1.15	68/144	207	44.4	1.53/0.76	146/220/313	266
0°	Lower phase	37.8	1.93	316/166	201	27.3	1.77/1.52	1422/576/434	311
	Upper phase	38.8	1.21	72/153	215	41.2	1.73/0.84	170/358/467	307

Note: Sample in BAW system: Val-Tyr, Trp-Tyr; sample in HEMW system: DNP-DL-glu, DNP-β-ala, DNP-L-ala; sample size: 40 μl; rotational speed: 1000 rpm; flow rate: 0.05 ml/min.

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.

3. Results and discussion

The performance of the figure-8 column with the different column angles against the centrifugal force was evaluated by the separation of two standard pair of dipeptides, Trp-Tyr and Val-Try with the BAW solvent system and three standard DNP-amino acids, DNP-L-ala, DNP-β-ala and DNP-DL-glu using the HEMW solvent system at various flow rates.

3.1. Separation of dipeptides with BAW

Fig. 2 shows the results of the studies on the separation of the dipeptides at 1000 rpm with the flow rate of 0.05 ml/min under various column angles ranging from 0° to 90°. Fig. 2A shows that retention of stationary phase decreased from 0° to 45°, and then increased from 45° to 90°. And the retention of stationary phase with lower and upper mobile phases at 90° was 40.5% and 42.2% which was slightly higher than 37.8% and 38.8% at 0°, respectively (Table 2). It is interesting to note that the retention of stationary phase becomes the worst at 45° being 30.6% for the upper mobile phase and 34.4% for the lower mobile phase (Fig. 2A and Table 2). However, peak resolution (Rs) continuously decreased from 0° to 90°. When the lower phase was mobile phase, Rs = 1.93 at 0° is much higher than Rs = 1.31 at 90° (Fig. 2B and Table 2). However, Rs with the upper mobile phase is much less than that with the lower mobile phase, while retention of stationary phase is better approaching our previously results [10,12,13] that are also consistent with the explanation given in our report [17]. Rs = 1.21 at 0° is slightly better than Rs = 0.99 at 90° (Fig. 2B and Table 2). The separation pressure was also remarkably altered by changing column angle from 0° to 90° (Fig. 2C). The column pressure slightly decreased from 0° to 45° and then sharply decreased from 45° to 90°. Higher pressure is associated with enhanced mixing of the two phases, which improves the separation efficiency, but leads to lower retention of the stationary phase. In the counter-current chromatography, Rs is determined by both the retention of stationary phase and theoretical planter number (N) as shown in the following equation [18]:

$$\mathrm{Rs} = \frac{0.25K_1(\alpha - 1)\sqrt{N}}{0.5K_1(\alpha + 1) + (1 - \mathrm{Sf})/\mathrm{Sf}} \quad (3)$$

where α is the separation factor between two peaks, $\alpha = K_2/K_1$ where K_2 and K_1 are the partition coefficients for the second and first peaks, respectively, N is the number of theoretical plates and Sf is the stationary phase fraction.

Table 3
The effect of flow rate on the performance of the figure-8 column mounted at 90° and 0° on separation of dipeptides by centrifugal counter-current chromatography.

Angle	Mobile phase	Flow rate (ml/min)	Sf (%)	Rs	N	Pressure (psi)
90°	Lower phase	0.07	37.2	1.21	163/67	83
		0.06	38.7	1.28	115/66	75
		0.05	40.5	1.31	135/66	71
		0.04	42.7	1.45	124/67	68
		0.03	45.3	1.57	143/74	61
	Upper phase	0.07	38.1	0.82	38/135	92
		0.06	39.7	0.86	46/68	90
		0.05	42.2	0.99	55/37	87
		0.04	44.4	1.13	94/167	76
		0.03	47.2	1.17	109/116	69
0°	Lower phase	0.07	35.3	1.48	309/115	205
		0.06	36.4	1.67	322/154	202
		0.05	37.8	1.93	316/166	201
		0.04	39.1	2.34	380/231	198
		0.03	39.8	3.28	326/317	193
	Upper phase	0.07	36.3	0.93	68/111	222
		0.06	37.2	1.07	61/108	220
		0.05	38.8	1.21	72/153	215
		0.04	40.1	1.37	94/120	211
		0.03	41.2	1.53	105/102	207

Note: Solvent system: BAW system; sample: Val-Tyr, Trp-Tyr; sample size: 40 μl; rotational speed: 1000 rpm.

According to Eq. (3), the retention of the stationary phase (Sf) is a significant contributor to Rs, but its contribution is independent of theoretical plate number (*N*). Therefore, *N* was also tabulated in Table 2. When the lower phase was mobile phase, *N* at 0° and 45° is remarkably higher than that at 90°. When the upper phase was mobile phase, *N* was always very small leading to low Rs values.

3.2. Separation of DNP-amino acids with HEMW

Fig. 3 shows the results of the studies on the separation of the DNP-amino acids at 1000 rpm with a flow rate of 0.05 ml/min under various column angles ranging from 0° to 90°. The retention of stationary phase increased from 0° to 90° in both mobile phases (Fig. 3A and Table 2). When the lower phase was mobile, Rs decreased from 0° to 45°, and then increased up to 90° whereas in the upper mobile phase Rs decreased continuously (Fig. 3B and Table 2). The column pressure was also greatly altered by changing column angle from 0° to 90°. From 0° to 45°, the pressure was only slightly decreased and then sharply decreased from 45° to 90° (Fig. 3C). When the lower phase was mobile phase, *N* becomes maximum at 0° angle, and it becomes minimum at 45° (Table 2).

3.3. Effects of column angle on hydrodynamic motion of the two phases

As described above, Rs becomes minimum at 45° column angle in the lower mobile phase DNP-amino acid separation while Sf becomes minimum at the same angle in the dipeptide separation.

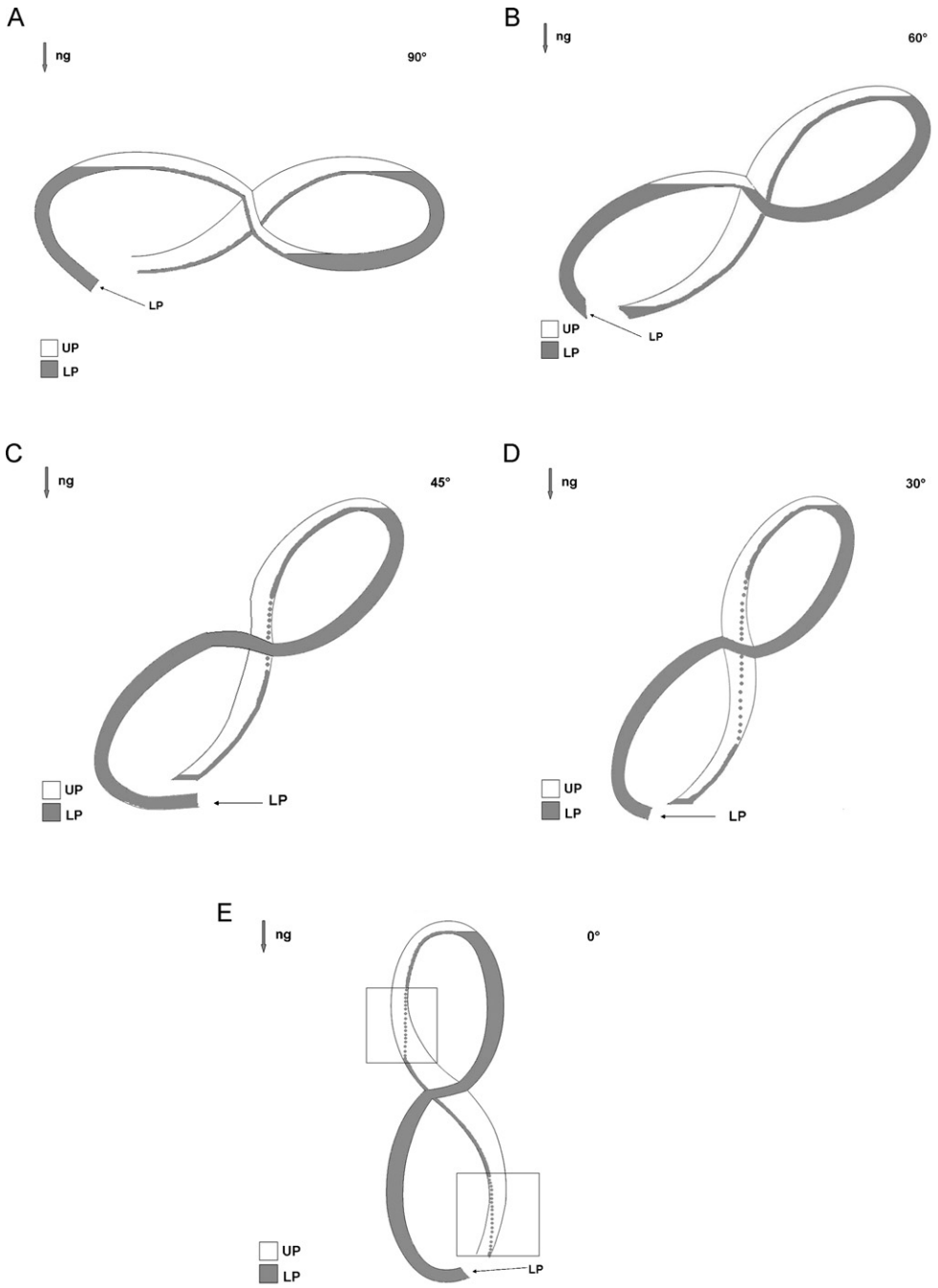

Fig. 4. Hydrodynamic motion and interaction of the two phases in the column at various angles. (A) 90° angle; (B) 60°; (C) 45°; (D) 30°; (E) 0°.

These curious phenomena may be partially explained by speculating the two phase motion in the figure-8 column mounted at various angles against the acting centrifugal force. Fig. 4 diagrammatically illustrates hydrodynamic motion and interaction of the two phases in the column at various angles. In Fig. 4A, the column mounted at 90° provides a long effective column space with relatively short dead space entirely occupied by the lower mobile phase. In both loops, the lower phase forms a laminar flow against the upper phase which gives minimum interface area against a large volume of the retained upper phase. This explains high stationary phase retention, low partition efficiency and low column pressure in this column orientation. When the column angle is reduced to 60° (Fig. 4B), the effective column space in the first loop is diminished while the space in the second loop maintains the laminar flow, resulting in lower retention of the stationary phase. When the column angle becomes 45°, as shown in Fig. 4C, the first loop is completely occupied by the mobile phase and the partition process is solely carried out in the second loop. This causes further reduction of the stationary phase retention in both solvent systems. The flow pattern at the middle portion of the second loop is now modified from laminar to droplet flow which affects Rs values according to the physical properties of the two phases. In the BAW system, the low interfacial tension, high viscosity and small difference between the two phases may form small droplets with a large interface area which greatly favors mass transfer in the droplet flow, hence cancelling out the loss of the partition space in the first loop to improve Rs for both mobile phases. In the HEMW system which has a low viscosity in the upper phase and larger density difference between the two phases, lower mobile phase forms relatively large droplets which move quickly in the less viscous upper phase and fails to improve the separation giving the minimum Rs values at this angle of 45°. With further decrease of the column angle at 30°, the mobile phase forms a droplet flow through a long segment at the middle portion of the second loop resulting in improvement of Rs in both solvent systems (Fig. 4D). Finally, when the column angle reaches 0°, the second loop provides two separate segments for droplet flow as shown in Fig. 4E, improving the Rs especially with the lower mobile phase in the BAW system.

3.4. Effects of flow rates in dipeptide separation by BAW at 0° and 90° column angles

A series of experiments was performed to evaluate the effects of flow rates on the retention of stationary phase, peak resolution, theoretical plate number and column pressure at 0° and 90° column angles in the dipeptide separation with the BAW solvent system. Table 3 summarizes the results of dipeptide (Trp-Tyr and Val-Tyr) separation with the BAW solvent system at various flow rates under a given rotational speed of 1000 rpm. The results show that retention of the stationary phase (Sf) with both lower and upper mobile phases decreases with increased flow rate (Fig. 5A). The retention of stationary phase at 0° angle is less than 5%. It is much lower than the retention of stationary phase at 90° angle [13]. The retention of stationary phase produced by the upper mobile phase is better than that of the lower mobile phase under otherwise identical experimental conditions. As expected from the results of our previous studies, the retention of stationary phase remarkably increased with decreased flow rate [12,13]. Fig. 5B shows the effect of the figure-8 column angle on the peak resolution (Rs) at various flow rates. When the lower phase was mobile, Rs at 0° was slightly better than that at 90° at the highest flow rate of 0.07 ml/min. However, as the flow rate is reduced to 0.03 ml/min, Rs at 0° sharply increased from 1.48 to 3.28 in spite of only slight increase in the stationary phase retention (Fig. 5A) whereas Rs at 90° showed only small changes. This indicates that the column yields higher partition efficiency at 0° angle at an extremely low flow rate, suggesting that the

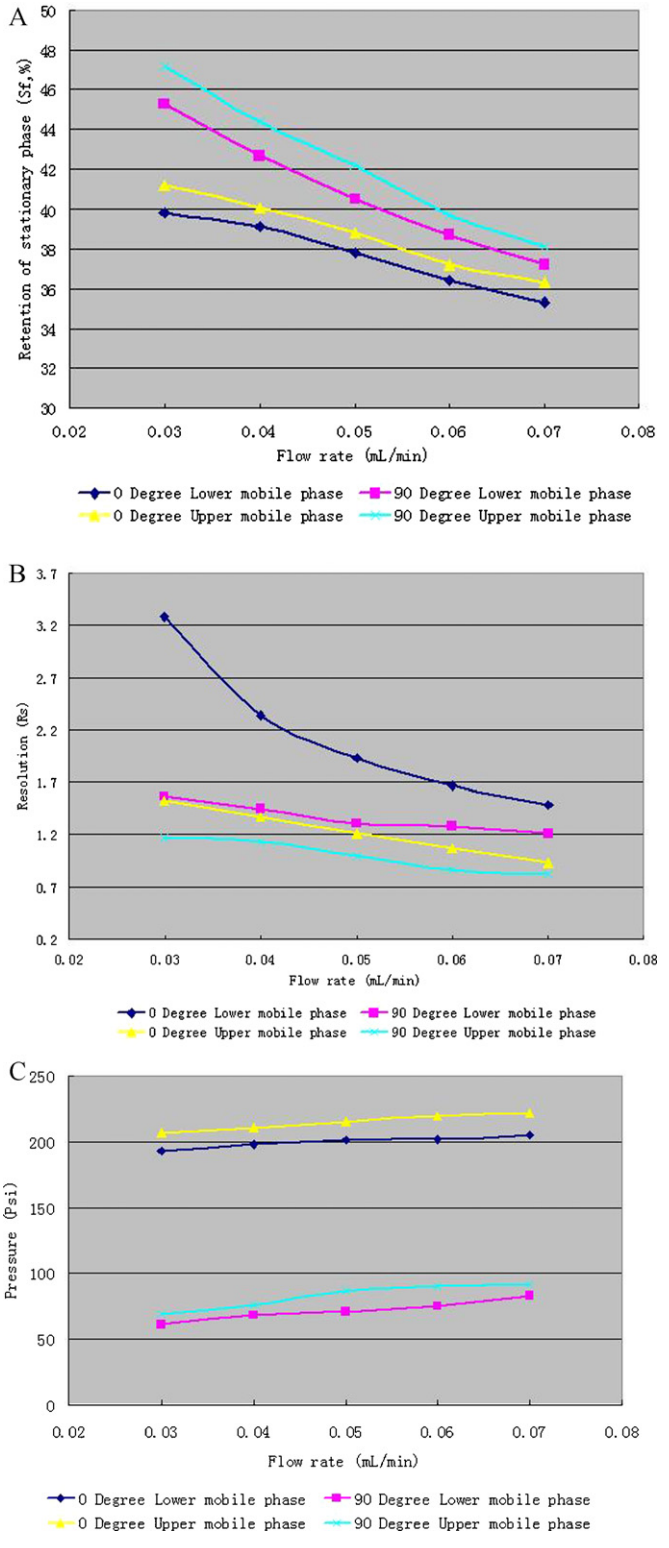

Fig. 5. Comparison of performance of dipeptide separation with 0° and 90° angles at various flow rates in centrifugal counter-current chromatography with figure-8 column. Sample: Trp-Tyr, Val-Tyr; sample size: 40 μl; solvent system: BAW; revolution speed: 1000 rpm; column capacity: 3.4 ml. (A) Relationship between retention of stationary phase and flow rate; (B) relationship between resolution and flow rate; (C) relationship between column pressure and flow rate.

partition efficiency would be improved by further decreasing the flow rate of the mobile phase. When the upper phase was mobile phase, Rs also increased with decreased flow rate of mobile phase, and Rs at 0° was better than that at 90°. *N* at 0° with lower mobile phase was remarkably higher than that at 90° (Table 3), which indicated that the separation efficiency is enhanced. As shown in Fig. 5C, the separation pressure was steadily increased with the flow rate in all groups while the pressure in the 0° angle group was much higher than that in the 90° angle group due to the increased hydrostatic pressure in each loop.It is interesting to note that the figure-8 column yields much higher Rs but lower Sf at 0° than at 90°, whereas this relationship is reversed in the coiled separation column.

4. Conclusions

Overall results indicates that the larger column angle gives higher stationary phase retention by undergoing laminar flow of the two phases through the column which in turn limits the interface area of the two phases for mass transfer resulting in lower Rs. On the other hand, a smaller column angle reduces the stationary phase retention and increases the column pressure, but yields higher Rs by producing droplet flow of the mobile phase which provides a large interface area to enhance the mass transfer process. The best Rs value was attained from the 0° angle column orientation at a low flow rate of the lower mobile phase.

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