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

Yi Yang[a,b,c], Dongyu Gu[a,b,c], Haji Akber Aisa[b], Yoichiro Ito[a,*]

[a] Bioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Drive, Building 10, Room 8N230, Bethesda, MD 20892, USA
[b] Xinjiang Key Laboratory of Plant Resources and Natural Products Chemistry, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Urumqi 830011, China
[c] Graduate University of the Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

The performance of the coiled column of centrifugal counter-current chromatography was investigated by changing the angle between column axis and centrifugal force in the separation of dipeptides or DNP-amino acids each with suitable two-phase solvent systems. In general, retention of the stationary phase (Sf) decreased, and peak resolution (Rs) increased as the column angle was increased. 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/v) to separate two dipeptide samples, Trp-Tyr and Val-Tyr, at a flow rate of 1 ml/min at 1000 rpm. When the column angle was changed from 0° to 90°, Rs increased from 1.05 (Sf = 60.1%) to 1.17 (Sf = 38.7%) with the lower phase mobile and from 1.02 (Sf = 67.8%) to 1.14 (Sf = 47.4%) with the upper phase mobile, respectively. 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/v/v) to separate three DNP-amino acids, DNP-glu, DNP-β-ala and DNP-ala, at a flow rate of 1 ml/min at 1000 rpm. When the column angle was changed from 0° to 90°, Rs increased from 1.38 (1st peak/2nd peak) and 1.20 (2nd peak/3rd peak) (Sf = 61.1%) to 1.66 and 1.45 (Sf = 34.4%) with the lower phase mobile and from 1.14 and 0.63 (Sf = 72.2%) to 1.53 and 0.87 (Sf = 51.1%) with the upper phase mobile, respectively. The overall results of our studies indicate that increasing the column angle against the radially acting centrifugal force enhances the mixing of two phases in the column to improve the peak 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 used for the separation and purification of biological samples with the conventional two-phase solvent systems [1–4]. However, this hydrodynamic CCC system fails to perform an analytical separation because of 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 subjecting the separation column to a stable 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 the conventional toroidal coil counter-current chromatography, the coiled column is mounted with its axis perpendicular to the acting centrifugal force, but it is not known whether it is the best column position for performing separation. In the present system, using a set of short coiled columns the effect of column angle relative to the centrifugal force field was investigated. Using two typical solvent systems with suitable sets of test samples, the performance of the coiled columns was investigated at various angles in terms of peak resolution and retention of the stationary phase.

2. Experimental

2.1. Apparatus

The present study uses a rotary-seal-free flow-through centrifuge fabricated by Pharma-Tech Research Corporation, Baltimore, MD, USA. It is equipped with an aluminum rotary platform measuring about 34 cm in diameter to hold a set of multiple short coiled separation columns on its periphery (Fig. 1A). The separation column was made by winding a single piece of 0.85 mm I.D.

* Corresponding author at: National Institutes of Health, National Heart, Lung, and Blood Institute, Building 10, Room 8N230, 10 Center Drive, MSC 1762, Bethesda, MD 20892-1762, USA. Fax: +1 301 402 0013.
E-mail address: itoy2@mail.nih.gov (Y. Ito).

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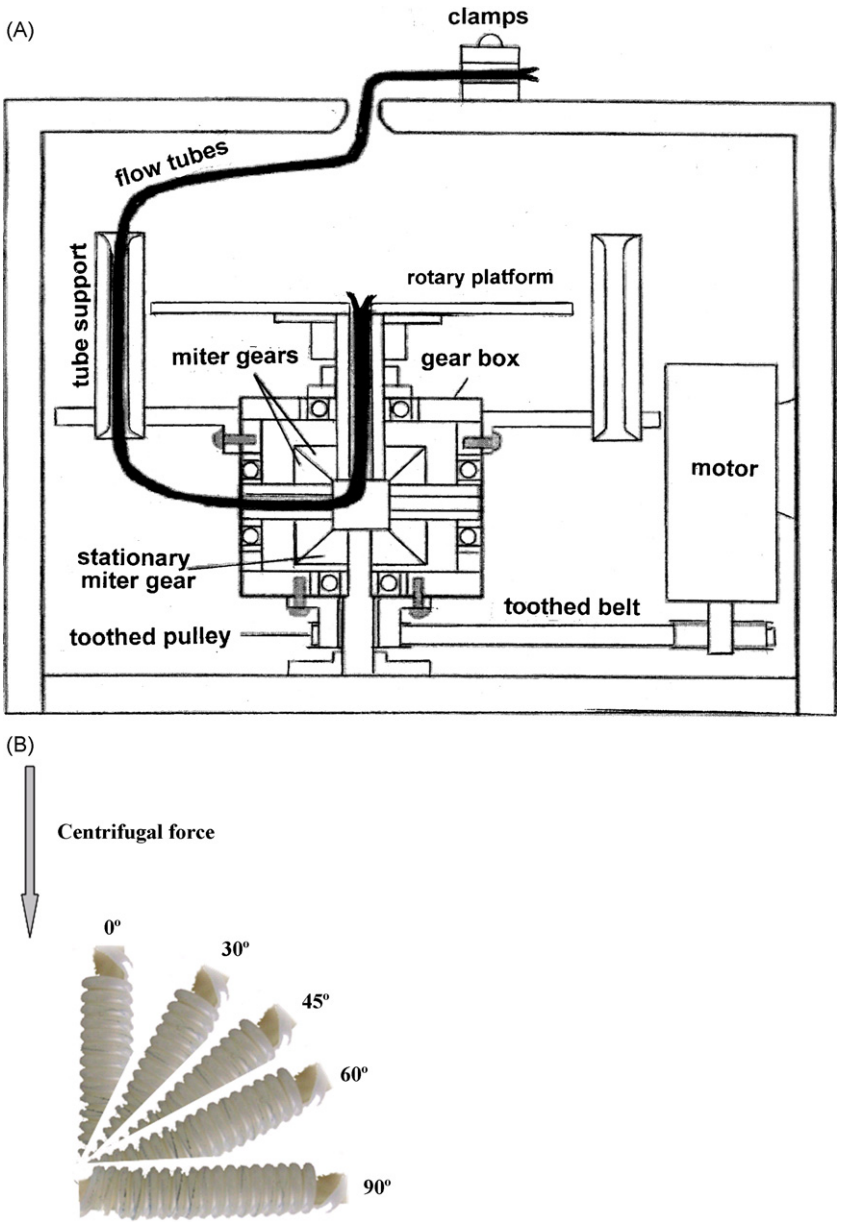

Fig. 1. Centrifugal counter-current chromatograph. (A) Cross-sectional view of the apparatus. (B) The coiled column mounted on the rotary platform at different column angles.

tubing (PTFE SW No. 20, Zeus Industrial Products, Orangeburg, SC, USA) onto 34 pieces of 5 mm O.D. and 5 cm long nylon pipes with about a 5 cm length of transfer tubing between the neighboring units. The length of the tubing is approximately 14 m with a total capacity of 8 ml. The segments of the coiled column are mounted horizontally around the rotary platform at various angles as shown in Fig. 1. Each terminus of the column is connected to flow tubes (0.46 mm I.D.) 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 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 (Fig. 1A). The total volume of the feed and return tubing (dead volume) is approximately 0.5 ml. The apparatus was operated at 1000 rpm (mean centrifugal force: $110 \times g$).

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).

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), N-2,4-dinitrophenyl-DL-glutamic acid (DNP-DL-glu) were all obtained from Sigma Chemicals, St. Louis, MO, USA.

2.3. Distribution coefficient measurement [6]

The distribution coefficients (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 was

recorded as A_L. The K_U value was calculated according to the following equation: $K_U = A_U/A_L$.

2.4. Two-phase solvent systems and sample solutions

Two typical two-phase solvent systems including 1-butanol–acetic acid–water (4:1:5, v/v/v) (BAW) and hexane–ethyl acetate–methanol–0.1 M HCl (1:1:1:1, v/v/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 10 ml of the upper phase of hexane–ethyl acetate–methanol–0.1 M HCl (1:1:1:1, v/v/v/v).

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 added to the effluent at the inlet of the detector using a tee connector and a fine mixing tubing (PTFE 0.4 mm I.D. × 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 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:

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

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

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.

3. Results and discussion

A series of experiments was performed to evaluate the effects of column angle between 0° and 90° against the centrifugal force on the retention of stationary phase and peak resolution. The dipeptides (Trp-Tyr and Val-Tyr) separation using BAW system was first investigated at the rotational speed of 1000 rpm at various flow rates. The results (Fig. 2A) show that retention of stationary phase (Sf) decreases with increased flow rate. The retention of stationary phase at 0° angle is approximately 20% higher than that the retention at 90° angle that is identical to the column orientation in the traditional centrifugal counter-current chromatography. The retention of stationary phase produced by the upper phase mobile is better than that of the lower phase mobile under the same separation conditions. As expected from our previous studies, the retention of stationary phase increased with decreased flow rate [6,7]. It is interesting to note that the retention of stationary phase at a flow rate of 0.25 ml/min at 0° column angle is much better than that at the flow rate of 0.05 ml/min at 90° angle. Fig. 2B shows the effect of the column angle on the peak resolution (Rs) at various

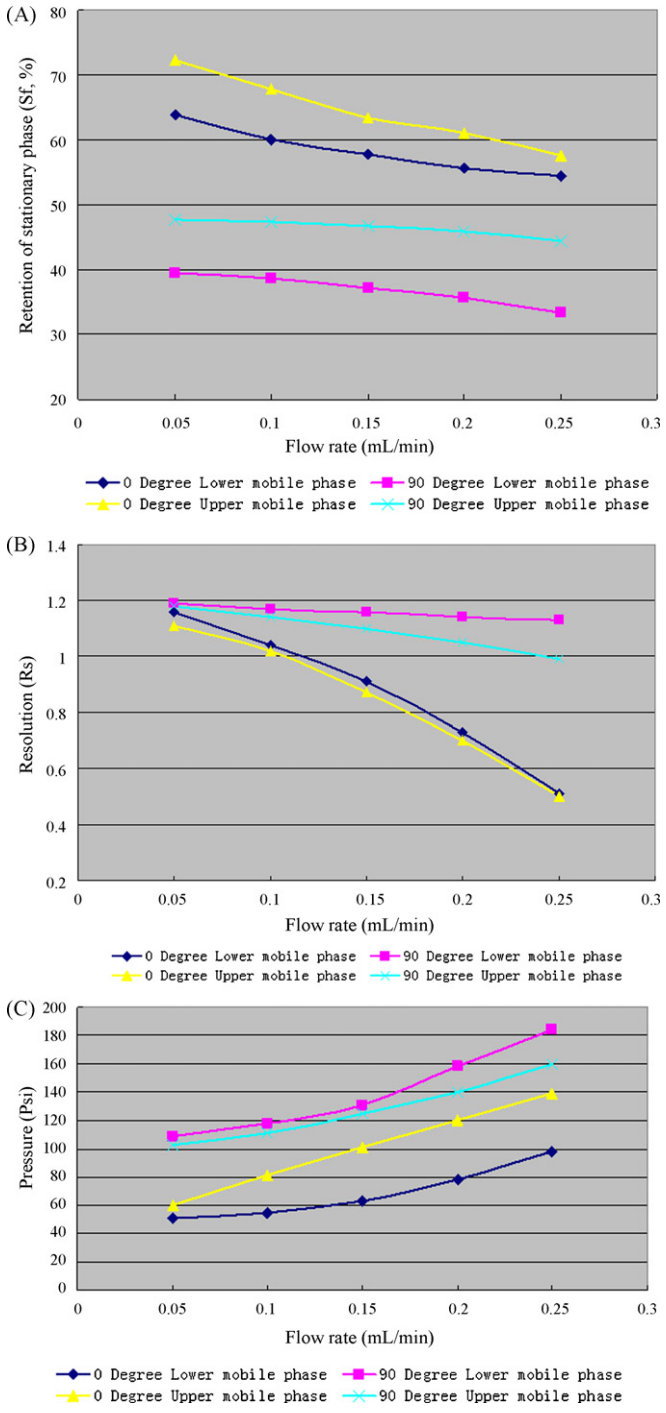

Fig. 2. Comparison of performance of dipeptide separation with 0° and 90° angles at different flow rates in centrifugal helix counter-current chromatography. Sample: Trp-Tyr and Val-Tyr; sample size: 0.2 ml; solvent system: BAW; revolution speed: 1000 rpm; column capacity: 8 ml. (A) Relationship between retention of stationary phase and flow rate; (B) relationship between resolution and flow rate; (C) relationship between column pressure and flow rate.

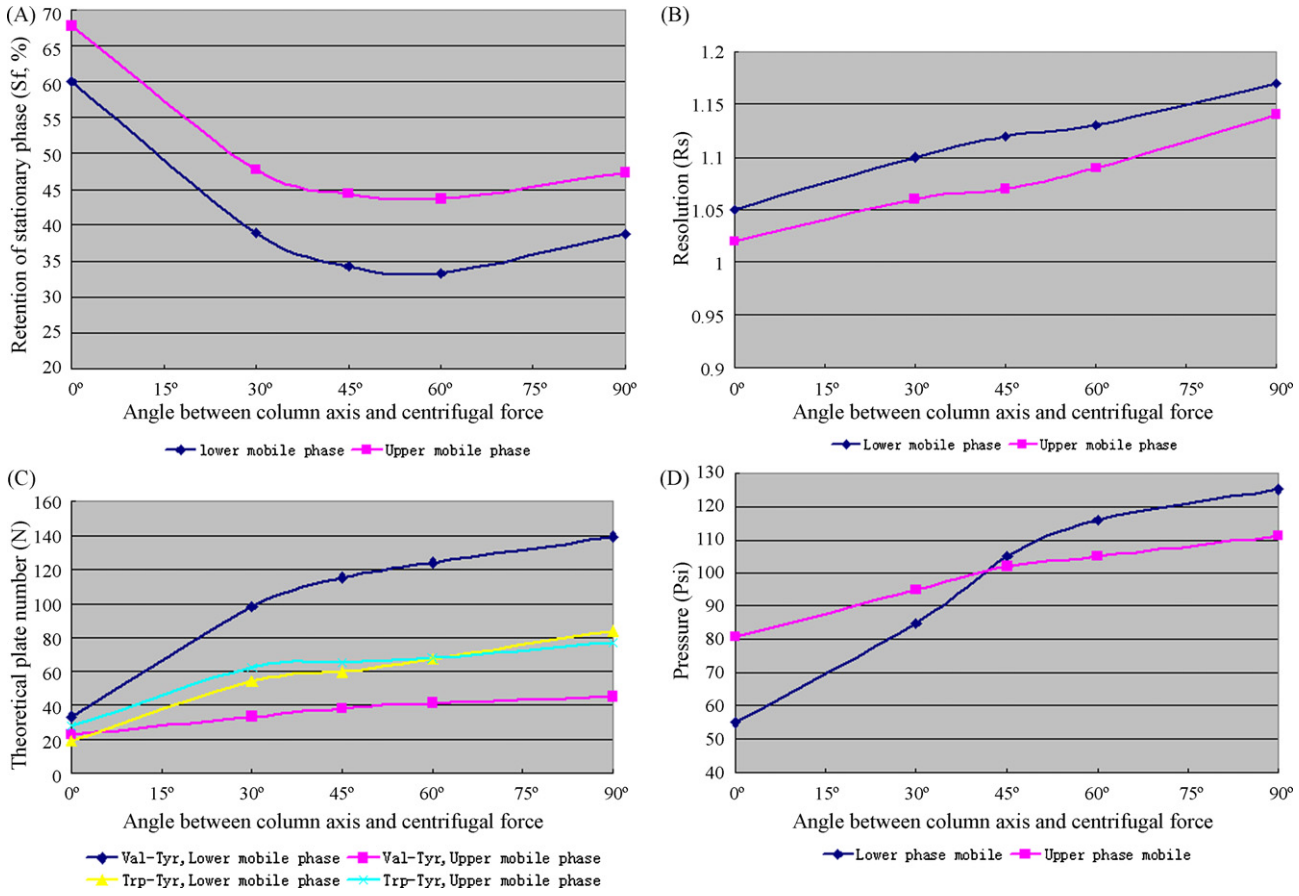

Fig. 3. Comparison of performance of dipeptide separation at different column angles in centrifugal helix counter-current chromatography. Sample: Trp-Tyr and Val-Tyr; sample size: 0.2 ml; solvent system: BAW; revolution speed: 1000 rpm; flow rate: 0.1 ml/min; column capacity: 8 ml. (A) Relationship between retention of stationary phase and column angle; (B) relationship between Rs and column angle; (C) relationship between theoretical plate number (*N*) and column angle; (D) relationship between column pressure and column angle.

flow rates. At a low flow rate of 0.05 ml/min, Rs at 0° angle is very similar to that at 90° angle. However, as the flow rate is increased, Rs at 0° angle sharply decreased down over 50% from 1.2 to 0.5 in both mobile phase curves in spite of maintaining high retention of the stationary phase (Fig. 2A) whereas Rs at 90° column angle shows only a slight decrease. This indicates that the column yields low partition efficiency at 0° angle at a high flow rate of the mobile phase. As shown in Fig. 2C, the column pressure was increased with the flow rate in all groups while the pressure in the 90° angle is much higher than that in the 0° group. This indicates that the laminar flow at the 0° angle is changing to a droplet flow of the mobile phase through the stationary phase in each helical turn at 90° column angle providing much broader interface for mass transfer by the expense of higher column pressure.

Next, the dipeptides (Trp-Tyr and Val-Tyr) separation with BAW system was performed at the flow rate of 0.1 ml/min with various column angles between 0° and 90° at the rotational speed of 1000 rpm. Fig. 3 shows the retention of stationary phase (A), Rs (B), *N* (C) and column pressure (D) at the different column angles. This flow pattern is easily visualized by a simple model of coiled tubing in the gravitational field. When tubing is vertical, either mobile phase can flow smoothly forming one continuous interface (laminar flow). When the orientation of the tubing is gradually changed to the horizontal position, at some critical column angle a pair of interfaces is formed in each helical turn and the mobile phase must flow passing through the stationary phase in each helical turn. As shown in Fig. 3A, the retention of stationary phase was sharply decreased as the column angle was increased until around 60° where it became lowest, then increasing gradually up to 90°. The retention of stationary phase with upper phase mobile is always better than that with lower phase mobile. Fig. 3B illustrates the effect of column angle on Rs. As the column angle is decreased, Rs is steadily improved in both elution modes, where the lower phase mobile gives substantially higher Rs values than those in the upper mobile phase. The Rs of dipeptide samples obtained from 0° to 90° column angles were improved from 1.05 to 1.17 with reduced stationary phase retention from 60.1% to 38.7% respectively in the lower phase mobile, and from 1.02 to 1.14 with the reduced stationary phase retention from 67.8% to 47.3% respectively in the upper phase mobile. As shown in Fig. 3C, the theoretical plate number (*N*) sharply rose from 0° to 30° column angles which is coincided with the sharp drop of the stationary phase retention as shown in Fig. 3A.

Here it is interesting to note that there is a large difference in *N* for Val-Tyr depending upon whether the lower or upper phase is mobile, whereas hardly any difference between *N* for Trp-Tyr. This may be explained on the basis of two factors inherent in CCC as follows: First, *N* measured from the same chromatogram is not uniform where the peak eluted near the solvent front shows the highest and it decreases as the retention time of the peak is increased. Second, in hydrostatic CCC, where the mixing is only produced by the mobile phase flow, the upper mobile phase forms laminar flow providing the low interfacial area for mass transfer whereas the mobile lower phase flows through the inner wall of narrow tubing coated with the upper organic stationary phase to enhance mass transfer. These two phenomena worked together to produce a extremely high *N* value of Val-Tyr peak in the lower phase

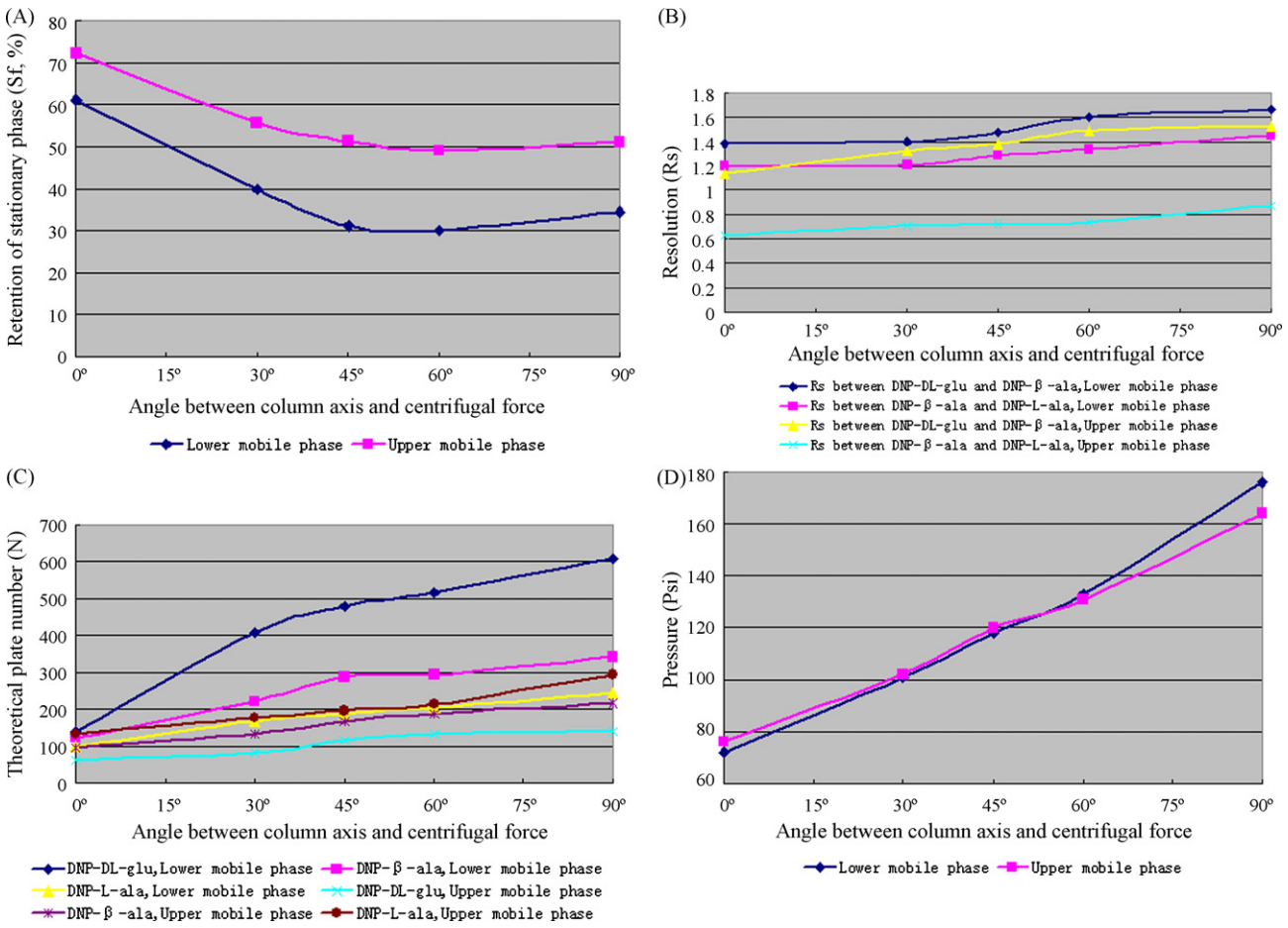

Fig. 4. Comparison of performance at different column angles in centrifugal helix counter-current chromatography. Sample: DNP-DL-glu, DNP-β-ala and DNP-L-ala; sample size: 0.2 ml; solvent system: BAW; revolution speed: 1000 rpm; flow rate: 0.1 ml/min; column capacity: 8 ml. (A) Relationship between retention of stationary phase and column angle; (B) relationship between Rs and column angle; (C) relationship between theoretical plate number and column angle; (D) relationship between pressure and column angle.

mobile ($K_{\text{stationary/mobile}} = 0.53$) and a very low N value in the upper phase mobile ($K = 1.89$), while in Trp-Tyr ($K = 1.69$ in the lower phase mobile and $K = 0.59$ in the upper phase mobile) these two factors were cancelled out to give nearly the same N values between both mobile phases. Similar results are also seen in DNP-amino acid separations illustrated in Fig. 4D (DNP-DL-glu: $K = 0.44$ in the upper phase mobile; $K = 2.27$ in the lower phase mobile).

Thereafter N increases gently up to the 90° column angle (Fig. 3C). The column pressure also showed remarkable variation (Fig. 3D). In both upper and lower phase mobile, the column pressure increased with increased column angle from 0° to 90°. At the beginning the column pressure in the lower phase mobile is lower than that in upper phase mobile, then it sharply increases until 45° where it becomes higher than that in the upper phase mobile.

The column performance at different angles was also investigated using DNP-amino acid separation in the moderately polar HEMW system with the flow rate of 0.1 ml/min at the revolution speed of 1000 rpm. Fig. 4A–D similarly shows the results of the retention of stationary phase (A), Rs (B), N (C) and pressure (D) at the different angles between column axis and centrifugal force. The trends of results are very similar with the dipeptide separation using BAW system described above. The retention of stationary phase sharply decreased and then slightly increased making a lowest point around 60° (Fig. 4A). The retention of stationary phase in the upper mobile phase is almost always much higher than that in the lower phase mobile. In both mobile phase groups the retention of stationary phase becomes more constant and slightly rises above 45° column angle with less than 5% variation. The peak resolution (Rs) was gradually increased with increased column angle where Rs in the lower phase mobile is always better than that in the upper mobile phase (Fig. 4B). From 0° to 90° column angle DNP-amino acids were separated with the resolution from 1.38 and 1.20 (glu/β-ala and β-ala/ala) to 1.66 and 1.45 with the reduced stationary phase retention from 61.1% to 34.4% in the lower mobile phase, and from 1.14 and 0.63 (ala/β-ala and β-ala/glu) to 1.53 and 0.87 at the reduced stationary phase retention of 72.2% to 51.1% in the upper phase mobile. The theoretical plate number (N) also showed the similar trend with that in the dipeptides separation (Fig. 4C): N increased with increased column angle and is always higher in the lower phase mobile than in the upper phase mobile. From 0° to 90° column angle, the separation pressure steadily increased about 100 psi in both mobile phase groups (Fig. 4D).

4. Conclusions

The overall results of our experiments indicate that the mixing is enhanced and separation efficiency is improved as the column angle is increased. Although the stationary phase retention was increased at 0° column angle, the partition efficiency was decreased by the formation of laminar flow of the mobile phase. The best performance of the coiled column was attained by placing the column axis perpendicular to the direction of the centrifugal force.

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