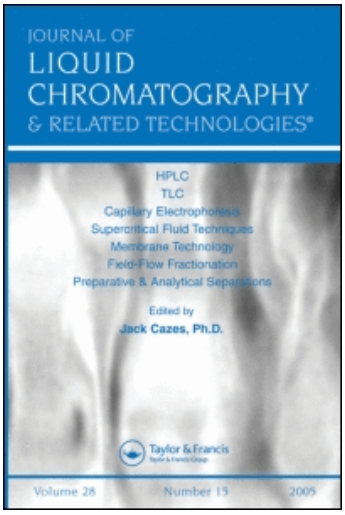


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# Spiral Tube Support for High-Speed Countercurrent Chromatography

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**Abstract:** A novel spiral tube support is introduced which allows accommodating a multlilayer spiral column made of a long piece of fluorinated plastic tubing. Two spiral columns tested in this study consist of 1.6 mm ID and 0.85 mm ID FEP (fluorinated ethylenepropylene) and PTFE (polytetrafluoroethylene) tubing with the total capacity of 100 mL and 40 mL, respectively. Performance of these spiral columns was examined with a two phase solvent system composed of 1-butanol/acetic acid/water at a volume ratio of 4:1:5 using tryptophyl-tyrosine (try-tyr) and valyl-tyrosine (val-tyr) as test samples under various revolution speeds and flow rates. Among 4 different elution modes tested, L-I-T (lower phase pumped from the internal head end of the spiral column) and U-O-H (upper phase pumped from the external tail of the spiral column) at a low flow rate produced the best results especially for both columns. The highest peak resolution ($R_s$) of over 3.5 was obtained from U-O-H at a flow rate of 1 mL/min with 74% stationary phase retention. At a flow rate of 5 mL/min, the highest revolution speed at 1,200 rpm improved the peak resolution in both elution modes. The present system may be useful for purification of various polar compounds in biomedical researches.

**Keywords:** Spiral tube support, High speed countercurrent chromatography, Peptide separation

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## INTRODUCTION

For a number of years, high-speed countercurrent chromatography (HSCCC) has been successfully performed for separation and purification of natural products using a multilayer coil column.[1–7] The application of the method to polar.

Compounds, such as peptides and proteins, however, encountered a problem of insufficient retention of the stationary phase. In order to cope with this problem, we have developed a spiral disk assembly, which can provide a centrifugal force gradient along the radius of the spiral channel to enhance the retention of the stationary phase. As expected, this new column design was able to retain a satisfactory amount of the stationary phase, and produced efficient separation of peptides and proteins.[8–11] The fabrication of the spiral disk, however, is rather expensive due to the following reasons: The disk should be made from inert plastic such as Kel-F (monochlorotrifluoroethylene) since it is directly exposed to the sample and solvent, and the column assembly should be leak free, which requires careful machining of the surface of the disk and the PTFE (polytetrafluoroethylene) septa. The cost of the fabrication of the disk assembly is greatly reduced by making a coiled column from a piece of inert plastic tubing such as PTFE (polytetrafluoroethylene) and FEP (fluorinated ethylene propylene) using a rigid spiral tube support as described in this article. Since the material of the support is not directly exposed to the solvent it can be made of any kind of rigid plastic or metallic material. Furthermore, the inert tubing such as PTFE and EFP is inexpensive, and the system is leak free so that careful sealing of the assembly is not required.

The present paper describes the design of the spiral tube support and its performance in the separation of peptides using a two phase solvent system composed of 1-butanol, acetic acid, and water (4:1:5, v/v/v), which is not satisfactorily retained in the conventional multilayer coil separation column used in HSCCC.

## EXPERIMENTAL

### Apparatus

The present studies were performed with a type-J high-speed CCC centrifuge (Ito Multilayer Separator and Extractor) manufactured by PC Inc., Potomac, MD, USA. It holds a separation column and a counterweight at a distance of 10 cm from the central axis of the centrifuge. The column is made by winding a single piece of FEP or PTFE tubing (Zeus Industrial Products, Raritan, NJ, USA) around the spiral tube support, which is fabricated as follows: A block of aluminum disk (17.5 cm diameter and 5 cm high) was machined with a milling board to form 4 spiral grooves (2.6 mm wide and

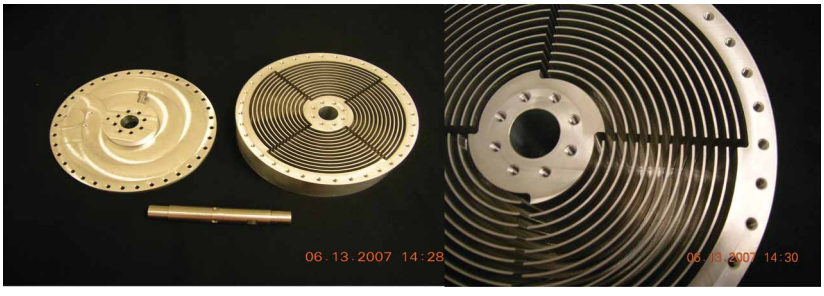


***Figure 1.*** Photograph of the spiral tube support. Multiple spiral layers can be made from one piece of plastic tubing. Material: aluminum; dimensions, 18 cm in diameter and 5 cm in height; grooves, 4 spiral grooves and 4 radial paths all 4.8 cm deep each 2.8 mm wide, each spiral groove can accommodate about 1 m of tubing.



4.7 cm deep with spiral pitch of 1.6 cm) as shown in Figure 1. Each spiral groove is interconnected with a radial groove so that multiple spiral layers can be made from a single piece of plastic tubing without junction. In the present study, two types of tubing was used: 1.6 mm ID in 9 spiral layers (36 spirals) with a total capacity of 103 mL, and 0.85 mm ID in 14–15 spiral layers (56–60 spirals) with a total capacity of around 40 mL. Beta values for each column range from 0.20 at the internal terminal and 0.75 at the external terminal of the spiral layer. In each column, the inlet and outlet tubing was connected to PTFE tubing (0.85 mm ID), which was led through the central axis of the centrifuge and rigidly supported on the cover plate of the centrifuge by clamps without twisting. The FEP tubing is considered to withstand against higher pressure than PTFE tubing[12] while it is less resistant for repetitive reflexing motion. The apparatus can be rotated up to 1200 rpm with a speed controller (Bodine Electric Co., Chicago, IL, USA).

## Reagents

1-Butanol was obtained from Fisher Scientific Co., Fair Lawn, NJ, USA and glacial acetic acid from Mallinckrodt Baker, Phillipsburg, NJ, USA. The standard dipeptide samples of tryptophyl-tyrosine (trp-tyr) and valyl-tyrosine (val-tyr) were purchased from Sigma Chemical Co., St. Louis, MO, USA.

## Preparation of the Two-Phase Solvent System and Sample Solution

A two phase solvent system was prepared by mixing 1-butanol, glacial acetic acid and water at a volume ratio of 4:1:5 in a separatory funnel, and the two phases separated shortly before use. The stock sample solution is prepared by

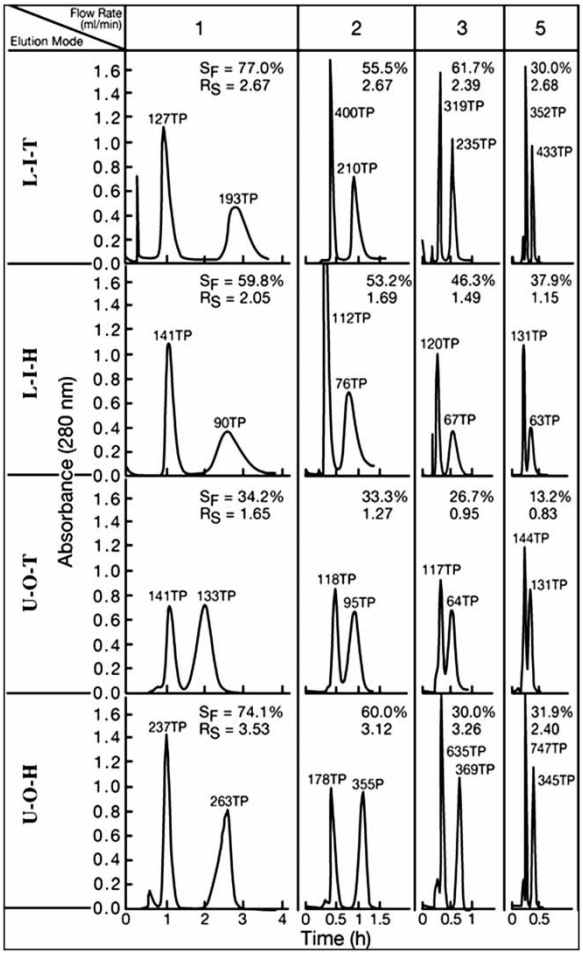


***Figure 2.*** Performance of 1.6 mm ID spiral tube at various flow rates and elution modes at 800 rpm. Experimental conditions are as follows: spiral tube: 1.6 mm ID, and ca 36 m long forming 9 spiral layers with a total capacity of ca 100 mL; solvent system: 1-butanol-acetic acid-water (4:1:5, v/v/v). Sample: tryptophyl-tyrosine (1.25 mg) and val-tyr (5 mg) in 0.5 mL of upper phase. Monitoring system: Uvicord IIS at 280 nm; elution mode: L-I-T indicates lower phase pumped from the inner tail terminal of the spiral tube; L-I-H, lower phase pumped from the internal head terminal of the spiral tube; U-O-T: upper phase pumped from the outer tail terminal of the spiral tube; U-O-H: upper phase pumped from the outer head terminal of the spiral tube.

dissolving 25 mg of trp-tyr and 100 mg of val-tyr in 20 mL of the upper phase used in separation. A few drops of the lower phase were added until the solution became cloudy, which ensured that the sample solution forms two phases against the mobile lower phase in the column. In each separation 0.5

***Table 1.*** Experimental conditions and separation efficiencies of dipeptides with 1.6 mm ID FEP coil at 800 rpm

| Elution mode | Flow rate | TP (1st/2nd peaks) | $R_s$ | $S_F$ (%) |
|---|---|---|---|---|
| L-I-T (CCW) | 1 mL/min | 127/93 | 2.67 | 77.0 |
| | 2 mL/min | 400/210 | 2.67 | 55.5 |
| | 3 mL/min | 319/235 | 2.39 | 61.7 |
| | 5 mL/min | 352/433 | 2.68 | 43.3 |
| L-I-H (CW) | 1 mL/min | 141/90 | 2.05 | 59.8 |
| | 2 mL/min | 112/76 | 1.69 | 53.2 |
| | 3 mL/min | 120/67 | 1.49 | 46.3 |
| | 5 mL/min | 131/63 | 1.15 | 37.9 |
| U-O-T (CW) | 1 mL/min | 141/133 | 1.65 | 34.2 |
| | 2 mL/min | 118/95 | 1.27 | 33.3 |
| | 3 mL/min | 117/64 | 0.95 | 26.7 |
| | 5 mL/min | 144/131 | 0.83 | 13.2 |
| U-O-H (CCW) | 1 mL/min | 237/263 | 3.53 | 74.1 |
| | 2 mL/min | 178/355 | 3.12 | 60.0 |
| | 3 mL/min | 635/369 | 3.26 | 30.0 |
| | 5 mL/min | 747/345 | 2.40 | 31.9 |

L-I-T: lower mobile phase pumped into the inner tail terminal; L-I-H: lower mobile phase pumped into the inner head terminal; U-O-T: upper mobile phase pumped into the outer tail terminal; U-O-H: upper mobile phase pumped into the outer head terminal; direction of rotation viewing the spiral disk: CCW, counterclockwise rotation; CW, clockwise rotation; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%): % stationary phase retention; 1st peak: val-tyr in L-I-T or L-I-H and trp-tyr in U-O-T or U-O-H; 2nd peak: trp-tyr in L-I-T or L-I-H and val-tyr in U-O-T or U-O-H.



to 1 mL (for 1.6 mm ID coil) and 0.2 mL (for the 0.85 mm ID coil) of the sample solution was loaded into the column.

## Partition Coefficient Measurement

The above two phase solvent system was selected on the basis of the partition coefficient (K) of a pair of dipeptide samples. The measurement of the partition coefficient for the above dipeptides was performed by dissolving a few mg of each sample equilibrated in the two phase solvent system, ca 2 mL of each phase, and measuring the absorbance of each phase at 280 nm using a spectrophotometer (Genesis 10 uv, Thermo Spectronic, Rochester, NY, USA). K values are expressed as the concentration of the solute in the upper phase divided by that of the lower phase. The K values of these two dipeptides are trp-tyr for 1.7 and val-tyr for 0.53 making the separation factor $\alpha = 3.2$.

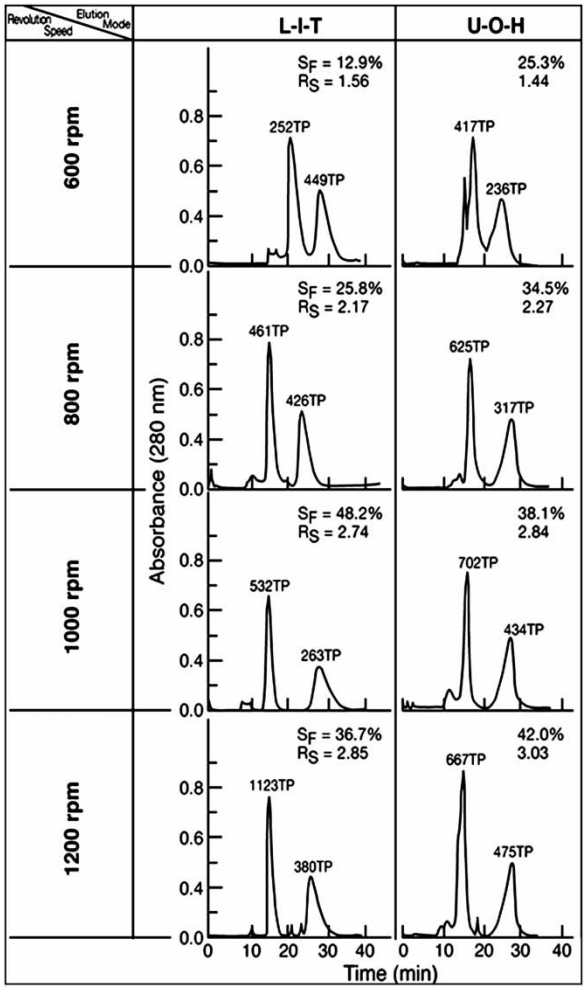


***Figure 3.*** Performance of 1.6 mm ID spiral tube at various revolution speeds at a flow rate of 5 mL/min in two elution modes. Experimental conditions: see the Figure 2 caption.

## HSCCC Separation Procedure

In each separation, the entire spiral column was filled with the stationary phase, either the upper or the lower phase, followed by sample loading through a sample loop.

Then the centrifuge was run at a given revolution speed while the column was eluted with the mobile phase at a desired rate. The effluent from the outlet of the column was monitored with a UV detector (Univord SII, LKB Instrument, Stockholm, Sweden) at 280 nm to record the elution curve (Pharmacia LKB REC102, Pharmacia, Stockholm, Sweden). In order to

***Table 2.*** (A) Experimental data for separation of val-tyr (1st peak) and trp-tyr (2nd peak) in L-I-T elution mode at a flow-rate of 5 mL/min[a]; (B) Experimental data for separation of try-tyr (1st peak) and val-tyr (2nd peak) in U-O-H elution mode at a flow-rate of 5 mL/min[b]

| Revolution speed | TP (1st/2nd peaks) | $R_s$ | $S_F$ (%) |
|---|---|---|---|
| A | | | |
| 600 rpm | 259/449 | 1.56 | 12.9 |
| 800 rpm | 462/426 | 2.17 | 25.8 |
| 1000 rpm | 532/263 | 2.74 | 48.2 |
| 1200 rpm | 1129/380 | 2.85 | 36.7 |
| B | | | |
| 600 rpm | 417/236 | 1.44 | 25.3 |
| 800 rpm | 625/317 | 2.27 | 4.5 |
| 1000 rpm | 702/434 | 2.84 | 38.1 |
| 1200 rpm | 667/475 | 3.03 | 42.0 |

[a]L-I-T: lower mobile phase pumped into the inner tail terminal; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%): % stationary phase retention.

[b]U-O-H: upper mobile phase pumped into the outer head terminal; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%): % stationary phase retention.



improve the tracing of the chromatogram, a 22 cm long hollow fiber dialysis filter (MicroKros, pore size 10 kD, Spectrum, New Brunswick, NJ, USA) was inserted on line at the inlet to the monitor. Ethanol was pumped through the line at a rate of 1/5 of the mobile phase flow rate to dilute the effluent. After the two peaks were eluted, the run was stopped and the column contents (Vc) were collected into a graduated cylinder using pressured $N_2$ to measure the volume of the stationary phase retained in the column (Vs). The % retention of the stationary phase was expressed as:

$$S_F = (Vs/Vc) \times 100 \quad (1)$$

## Measurement of Partition Efficiency

The efficiency of separation was expressed by two parameters, i.e., theoretical plate number (N or TP) and peak resolution ($R_s$) as follows:

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

$$R_s = 2(R_2 - R_1)/(W_1 + W_2) \quad (3)$$

where R denotes the retention time and W, the peak width for the specified peak.

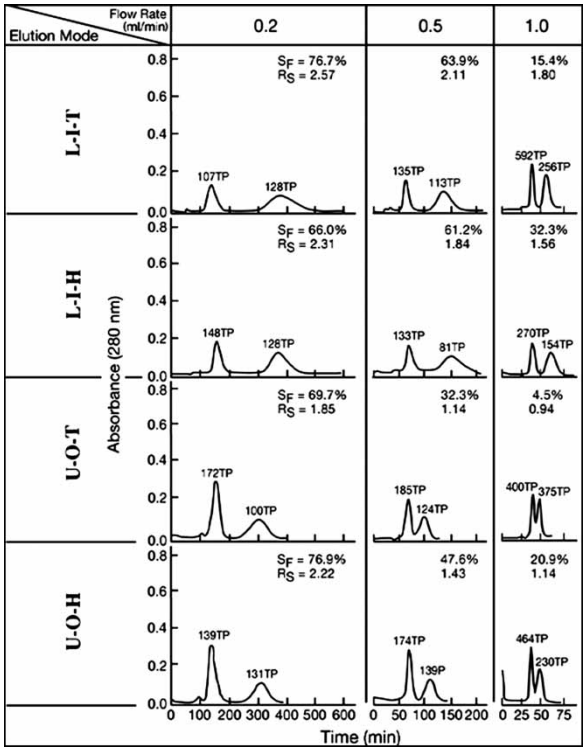


***Figure 4.*** Performance of 0.85 mm ID spiral tube at various flow rates and elution modes at 800 rpm. Experimental conditions are as follows: spiral tube: 0.85 mm ID, and ca 60 m long forming 15 spiral layers with a total capacity of ca 40 mL; solvent system: 1-butanol-acetic acid-water (4:1:5, v/v/v). Sample: tryptophyl-tyrosine (0.25 mg) and val-tyr (1 mg) in 0. 2 mL of upper phase. Monitoring system: Uvicord IIS at 280 nm. Elution mode: see the Figure 2 caption.

## RESULTS AND DISCUSSION

The performance of the two spiral columns (1.6 mm and 0.85 mm ID) was evaluated by the separation of two standard pair of dipeptides, trp-tyr and val-tyr, with suitable K values, using a two phase solvent system composed of 1-butanol/acetic acid/water at a volume ratio of 4:1:5 at various flow rates and revolution speeds.

### Performance of 1.6 mm ID Spiral Column

Figure 2 and Table 1 show the results of the studies on the separation of the dipeptides at 800 rpm under various flow rates ranging from 1 to

***Table 3.*** Experimental conditions and separation efficiencies of dipeptides with 0.85 mm ID FEP and PTFE coils at 800 rpm

| Elution mode | Flow rate | TP (1st/2nd peaks) | $R_s$ | $S_F$ (%) |
|---|---|---|---|---|
| L-I-T (CCW) | 0.2 mL/min | 107/128 | 2.57 | 76.7 |
| | 0.5 mL/min | 135/113 | 2.11 | 63.9 |
| | 1.0 mL/min | 362/243 | 1.78 | 25.0 |
| L-I-H (CW) | 0.2 mL/min | 148/128 | 2.31 | 66.0 |
| | 0.5 mL/min | 133/81 | 1.84 | 61.2 |
| | 1.0 mL/min | 270/154 | 1.56 | 32.3 |
| U-O-T (CW) | 0.2 mL/min | 172/100 | 1.85 | 69.7 |
| | 0.5 mL/min | 185/124 | 1.14 | 32.3 |
| | 1.0 mL/min | 400/375 | 0.94 | 4.5 |
| U-O-H (CCW) | 0.2 mL/min | 139/131 | 2.22 | 76.9 |
| | 0.5 mL/min | 142/139 | 1.43 | 47.0 |
| | 1.0 mL/min | 464/230 | 1.14 | 20.9 |

L-I-T: lower mobile phase pumped into the inner tail terminal; L-I-H: lower mobile phase pumped into the inner head terminal; U-O-T: upper mobile phase pumped into the outer tail terminal; U-O-H: upper mobile phase pumped into the outer head terminal; direction of rotation viewed from top of the spiral disk: CCW, counterclockwise rotation; CW, clockwise rotation; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%): % stationary phase retention; 1st peak: val-tyr in L-I-T or L-I-H and trp-tyr in U-O-T or U-O-H; 2nd peak: trp-tyr in L-I-T or L-I-H and val-tyr in U-O-T or U-O-H.



5 mL/min. On the left of the composite diagram, four different elution modes are shown where L-I-T indicates lower mobile phase pumped into the inner tail terminal; L-I-H, lower mobile phase pumped into the inner head terminal; U-O-T, upper mobile phase pumped into the outer tail terminal; and U-O-H, upper mobile phase pumped into the outer head terminal. CCW and CW in Table 1 indicate the direction of rotation of the spiral disk viewed from the top of the centrifuge: CCW denotes counterclockwise rotation and CW, clockwise rotation. The partition efficiency expressed in terms of theoretical plate number (TP) and peak resolution ($R_s$), as well as the % retention of the stationary phase ($S_F$) are given in each chromatogram. The first peak eluted in L-I-T or L-I-H is val-tyrosine, and that in U-O-T or U-O-H is trp-tyr.

As clearly shown from the diagrams, L-I-T and U-O-H yield higher peak resolution than their counterparts where the efficiencies on U-O-H substantially exceed those from L-I-T. It is interesting to note that in L-I-T the Rs values stay unaltered while the flow-rate is increased in five fold from 1 mL/min to 5 mL/min, indicating that the high efficiency separation can be attained in a short period of time by applying a high flow rate.

Figure 3 and Table 2 summarize the effect of the revolution speed ranging from 600 rpm to 1200 rpm on the separation under a flow rate of 5 mL/min in

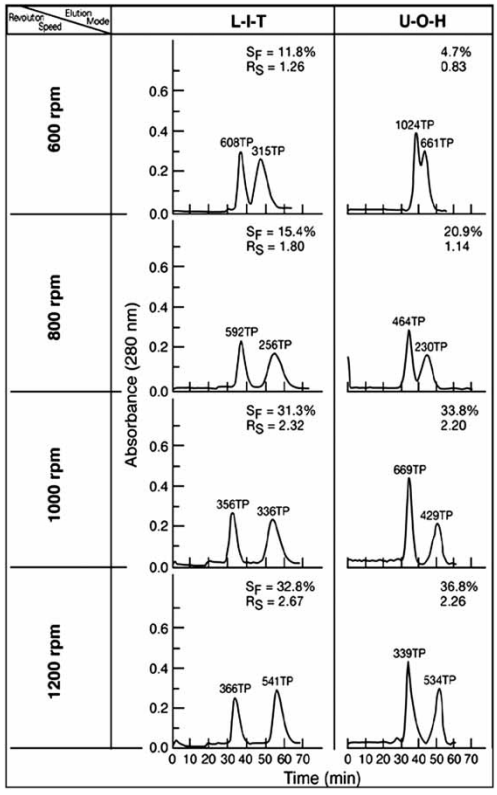


***Figure 5.*** Performance of 0.85 mm ID spiral tube at various revolution speeds at a flow rate of 1 mL/min in two elution modes. Other experimental conditions: see the Figure 4 caption.

the L-I-T and U-O-H elution modes. The results show that both stationary phase retention and separation efficiency improve as the revolution speed is increased. The best peak resolution is obtained at the maximum revolutions speed in both elution modes. At a flow rate of 5 mL/min several hundred TP with peak resolution (Rs $\approx$ 3) is obtained within 30 min of elution in both elution modes.

## Performance of 0.85 mm ID Spiral Column

Figure 4 and Table 3 similarly show the effect of elution modes and flow rate on partition efficiency at 800 rpm in a 0.85 mm ID column. All elution modes were tested at flow rates from 0.2 to 1.0 mL/min. As expected from the results obtained from the 1.6 mm ID column, the present data show that L-I-T and U-O-H are the best choice among the 4 elution modes.

***Table 4a.*** Experimental data for separation of val-tyr (1st peak) and trp-tyr (2nd peak) with 0.85 mm ID coil in L-I-T elution mode at a flow-rate of 1 mL/min

| Revolution speed | TP (1st/2nd peaks) | $R_s$ | $S_F$ (%) |
|---|---|---|---|
| 600 rpm | 608/315 | 1.26 | 11.8 |
| 800 rpm | 362/243 | 1.78 | 25.0 |
| 1000 rpm | 355/336 | 2.32 | 31.3 |
| 1200 rpm | 366/546 | 2.67 | 32.8 |

L-I-T: lower mobile phase pumped into the inner tail terminal; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%): % stationary phase retention.

***Table 4b.*** Experimental data for separation of trp-tyr (1st peak) and val-tyr (2nd peak) with 0.85 mm ID coil in U-O-H elution mode at a flow-rate of 1 mL/min

| Revolution speed | TP (1st/2nd peaks) | $R_s$ | $S_F$ (%) |
|---|---|---|---|
| 600 rpm | 1024/661 | 0.83 | 4.7 |
| 800 rpm | 464/230 | 1.14 | 20.0 |
| 1000 rpm | 667/429 | 2.20 | 33.8 |
| 1200 rpm | 339/534 | 2.26 | 36.8 |

U-O-H: upper mobile phase pumped into the outer head terminal; TP: theoretical plate number; $R_s$: peak resolution; $S_F$(%); % stationary phase retention.



The effects of revolution speed on the partition efficiency and retention of the stationary phase in L-I-T and U-O-H elution modes at a flow rate of I mL/min are shown in Figure 5 and Table 4. In both elution modes, the peak resolution and stationary phase retention improve as the revolution rates are increased. Peak resolution ($R_s$) at 1200 rpm at a high flow rate of 5 mL/min exceeds those obtained by a low flow rate of 0.2 mL/min at 800 rpm in both elution modes.

## CONCLUSION

The new column support can securely hold the multiple spiral layers made of a single piece of plastic tubing without junction. The high spiral pitch can ensure satisfactory retention of the stationary phase of polar solvent systems for separation of biologically active compounds such as peptides. Performance of two types of spiral columns with 1.6 mm ID and 0.85 mm ID was tested on the separation of dipeptides (tryptophyl-tyrosine and valyl-tyrosine) with a

polar two phase solvent system composed of 1-butanol, acetic acid, and water at a volume ratio of 4:1:5. A series of experiments conducted under various conditions including elution mode, flow rate, and revolution speed showed satisfactory stationary retention and peak resolution ($R_s$) results.

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