Published in final edited form as:

J Sep Sci. 2011 October ; 34(19): 2611–2617. doi:10.1002/jssc.201100205.

Evaluation and application of a spiral tube column for high-speed counter-current chromatography

Xueli Cao^{a,*}, Hairun Pei^a, Liangsheng Huo^a, Guanghui Hu^a, and Yoichiro Ito^b

^aBeijing Technology and Business University, Beijing Key Lab of Plant Resource Research and Development, Beijing 100037, China

^bBioseparation Technology Laboratory, Biochemistry and Biophysics Center, NHLBI, NIH, Bethesda, MD 20892-1762, USA

Abstract

A new type-J CCC planet centrifuge with two spiral tube columns (2×15 mL, β value 0.3–0.7) was developed and evaluated for its retention ability of different solvent systems including less polar, moderately polar and polar organic-aqueous solvent systems, and aqueous two-phase systems in different operation conditions. The results suggested that the spiral tube column can produce good retention of stationary phase for less polar to moderately polar solvent systems, such as heptane-methanol (1:1, v/v) and hexane-ethyl acetate-methanol-water (1:1:1:1, v/v) (1 mL/min, S_F 70%) by eluting lower mobile phase from head (H) to tail (T), or upper mobile phase from tail (T) to head (H), regardless of whether the inlet is located at the inner (I) or outer (O) terminals. It can also provide satisfactory retention for polar to aqueous two-phase systems such as *n*-butanol-acetic acid-water (4:1:5, v/v/v) (0.5 mL/min, S_F 60%) and PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w) (0.5 mL/min, S_F 50%) by eluting lower mobile phase from inner terminal (I) to outer terminal (O), or upper mobile phase from outer terminal (O) to inner terminal (I), except for U-O-T mode. The results also clearly indicated that the spiral tube column could significantly increase the retention of four typical solvent systems compared with a multilayer coil column with similar parameters, the S_F for the former two solvents can be increased by about 10%, and for the latter two by 30–40%. The preliminary applications of this spiral tube column on the separation of small molecular compounds such as moderately polar theaflavins, polar anthocyanins and dipeptides were successful. Acceptable resolution can be obtained between cytochrome c and myoglobin, lysozyme and myoglobin when it was applied on protein separation, however it still needs to be improved regarding to its low column efficiency.

Keywords

High-speed counter-current chromatography; Spiral tube column; Theaflavins; Anthocyanins; Peptides; Proteins

1. Introduction

High-speed countercurrent chromatography (HSCCC) is based on liquid-liquid extraction and centrifugal distribution^[1]. One phase (stationary phase) is retained in the column by the high-speed planetary movement of the column, while the other phase (mobile phase) is pumped in to the column. The sample was separated according to the different distribution coefficient. The instrument (type-J HSCCC) usually uses a multilayer coil as a separation

* caoxl@th.btbu.edu.cn, Fax: 00-86-10-69984898.

column, which is made simply by winding a single piece of polytetrafluoroethylene (PTFE) tubing in several layers on a spool^[1]. It's widely used for the separation of small molecular compounds in natural products with a variety of two-phase solvent systems, especially for those organic-aqueous systems^[2–6]. However it often fails to retain the stationary phase for the polar two-phase systems and highly viscous, low interfacial tension aqueous two-phase systems (ATPSs), which greatly limits the application of HSCCC in the area of biological macromolecules separation.

The retention of stationary phase can be improved by forming a spiral tube configuration, and during recent years a spiral disk column design was devised to increase the pitch of the spiral^[7–10]. Using a multiple spiral disk assembly designed and manufactured in our laboratory, we have found the retention of a series of solvent systems including both typical organic-aqueous systems and ATPSs can be increased to different extent, and specially it can be used for fast separation of small molecular compounds at high flow rate^[10]. However, it was also found the fabrication of spiral disk column was of some problems: The disk should be made from expensive inert plastic such as Kel-F (monochlorotrifluoroethylene) to withstand the corrosion of different solvents, and the column assembly should be leak free, which requires high precision machining of the surface of the disk and the PTFE (polytetrafluoroethylene) septa.

In order to retain the advantages of this spiral tube configuration design and reduce the cost and difficulty of fabrication, a spiral tube support design was promoted recently by Dr. Ito^[11,12]. In this paper, a spiral tube column designed and manufactured in our laboratory was investigated for the retention of a series of solvent systems including both typical organic-aqueous systems and ATPSs. In addition, its preliminary application in the separation of small molecular theaflavins (TFs), anthocyanins, standard peptides and proteins was demonstrated.

2. Experimental

2.1. Development of the apparatus

A type-J CCC planet centrifuge with a 7.0 cm revolution radius was designed. Two spiral tube columns were symmetrically mounted on the centrifuge as the photo showed in Fig. 1. The β values range from 0.3 to 0.7. The capacity of each column is 15 mL. The spiral tube support (12.0cm diameter and 5.0cm high) was made of Acrylonitrile Butadiene Styrene (ABS) with four spiral grooves (1.7 mm wide and 45 mm deep with a spiral pitch 11.6mm) as shown in Fig. 2. A single piece of PTFE tubing (0.8 mm i.d. and 1.6 mm o.d.) was embedded into the grooves by 14 layers, starting from I₁ to O₁, then I₂ to O₂, I₃ to O₃, I₄ to O₄, each spiral and each layer of tubing was interconnected through the four radial grooves. Two columns were connected in series through tubing without twist.

A J-type CCC with a traditional multilayer coil column was employed for the comparison with the new spiral tube column machine, and their geometrical parameters were listed in table 1.

2.2 Reagents

The solvents used in HSCCC such as heptane, hexane, ethyl acetate, methanol, *n*-butanol, acetonitrile, potassium dihydrogen phosphate, potassium hydrogen phosphate, glacial acetic acid were purchased from Beijing Chemical Reagent Company in analytical grade.

Theaflavins (TFs) were standard samples prepared in our laboratory (certified by Standardization Administration of China/Technical Committee); *tert*-butyl methyl ether used

in HPLC, PEG1000, and cytochrome C, myoglobin, lysozyme, leu-tyr, val-tyr and other standard samples were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Preparation of solvent systems and sample solution

The following two-phase solvent systems were used in the experiments: heptane-methanol (1:1, v/v), hexane-ethyl acetate-methanol-water (1:1:1:1, v/v), *n*-butanol-acetic acid-water (4:1:5, v/v), PEG1000- K₂HPO₄-water (12.5:12.5:75, w/w) (pH=9.0), and *tert*-butyl methyl ether-*n*-butanol-acetonitrile-water, 0.1% TFA (1:2.5:1.5:5, v/v). Each solvent system was prepared by thoroughly equilibrating it in a separatory funnel at room temperature and two-phases separated shortly before use.

The sample solutions were prepared by dissolving the sample mixture in the lower or upper mobile phase used for the separation.

2.4. Retention ability evaluation

A series of experiments was performed to evaluate the retention of different solvent systems in different rotation directions and speed (g-levels), different mobile phases, different flow rates, different flow ways. In each experiment, the spiral tube column was firstly filled with the stationary phase, and then the apparatus was rotated at a desired speed in a certain direction while the mobile phase was pumped into the column at a given flow rate and flow direction. A graduated cylinder was used to collect the stationary phase eluted out from the column. After the dynamic equilibrium was established without carryover of the stationary phase, the volume of stationary phase pushed out from the column was measured to determine the percentage retention of stationary phase (S_p).

The definition of head (H) and tail (T) is dependent on the rotation direction. When the column is rotated clockwise, viewing from cover side of the column, the inner terminal (I) of the tube was the head (H), and outer terminal (O) was the tail (T), and vice versa. All of eight possible operation modes were examined as follows: counter clockwise rotation was used for modes 1–4 and clockwise rotation for modes 5–8.

- (1) Lower mobile phase pumped from inner tail to out head (L-I-T);
- (2) Upper mobile phase pumped from inner tail to out head (U-I-T);
- (3) Lower mobile phase pumped from outer head to inner tail (L-O-H);
- (4) Upper mobile phase pumped from outer head to inner tail (U-O-H);
- (5) Lower mobile phase pumped from inner head to out tail (L-I-H);
- (6) Upper mobile phase pumped from inner head to out tail (U-I-H);
- (7) Lower mobile phase pumped from outer tail to inner head (L-O-T);
- (8) Upper mobile phase pumped from outer tail to inner head (U-O-T).

2.5 Comparison of the two HSCCC columns

A HSCCC unit equipped with a traditional multilayer coil column (same tubing bore and similar volume) was employed for the comparison of the retention ability of stationary phase with that of spiral tube column under the same condition. Four typical solvent systems were examined in the optimized operation modes under different rotation speed.

2.6 Separation procedures of samples

Based on the above retention experiments, preliminary application of the spiral tube column was performed on the separation of both small molecular compounds and macromolecules.

In the each separation, the column was firstly filled with the stationary phase, and then the mobile phase was pumped through the column while the machine was rotated at 1200 rpm (112 *g*-level). Once the equilibrium was established, the sample solution was loaded through an injector valve. The effluent was continuously monitored at 280 nm using a UV detector and the fractions collected.

3. Results and discussion

3.1 Study on the retention of stationary phase

The retention of stationary phase of different solvent systems under different operation conditions was summarized in Fig. 3–6.

For less polar and moderately polar solvent systems such as heptane-methanol (1:1, v/v) (Fig. 3) and hexane-ethyl acetate-methanol-water (1:1:1:1, v/v) (Fig. 4), better retention was obtained in the following four elution modes: U-I-T; L-O-H; L-I-H; U-O-T. In another word, for these solvent systems, better retention of stationary phase can be achieved only by pumping lower mobile phase in the head (H) to tail (T) direction, or upper mobile phase in the tail (T) to head (H) direction, regardless of whether the inlet is located at the inner (I) or outer (O) terminals. For example, for heptane-methanol (1:1, v/v), the highest S_f is about 60–65% at 1.0 mL/min and 55–60% at 2.0 mL/min in L-I-H and U-O-T modes. For hexane-ethyl acetate-methanol-water (1:1:1:1, v/v), the highest S_f is about 70% at 1.0 mL/min and 60% at 2.0 mL/min in L-I-H and U-O-T modes.

For polar solvent systems such as *n*-butanol–acetic acid–water (4:1:5, v/v) (Fig. 5), better retention was obtained in the following four elution modes: L-I-T; U-O-H; L-I-H; U-O-T. It means for these polar solvent systems, higher retention of stationary phase can be achieved by pumping lower mobile phase from inner terminal (I) to outer terminal (O), and upper mobile phase from outer terminal (O) to inner terminal (I), no matter that when the terminal is the head or the tail. For example, the best S_f is about 55–60% at 0.5 mL/min and 40% at 1.0 mL/min in L-I-T and U-O-H modes. A similar phenomenon was also observed for the PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w) system except for the U-O-T mode (Fig. 6). For example, the highest S_f is about 50% at 0.5 mL/min and 30% at 1.0 mL/min in L-I-T mode. However, the retention of stationary phase decreased due to the higher viscosity and lower interfacial intension.

The above results agree with that from spiral disk assembly reported by Ito and Cao^[7,10]. The lower S_f compared with that of spiral disk assembly may due to the smaller cross-section area of the tubing. The S_f could be increased further when using wider diameter tubing to make bigger volume of column according to Wood's prediction model^[13].

3.2 Comparison of two HSCCC columns

As seen in Fig. 7, a comparison has been conducted between a traditional multilayer coil column and the spiral tube column for the retention of different solvent systems. The results clearly indicated that the spiral tube column could significantly increase the retention of four typical solvent systems compared to multilayer coil column. For heptane-methanol (1:1, v/v) and hexane-ethyl acetate-methanol-water (1:1:1:1, v/v), the S_f can increase about 10%, while for *n*-butanol-acetic acid-water (4:1:5, v/v/v) and PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w), the S_f can increase about 30–40% under different *g*-levels.

3.3 Preliminary separation applications

3.3.1 Separation of theaflavins (TFs) of black tea—Fig. 8 shows the separation of four theaflavins (TF, TF-3-MG, TF-3'-MG and TFDG) from black tea using the hexane-

ethyl acetate-methanol-water (1:5:1:5, v/v) system by pumping the lower mobile phase at two different flow rates in the L-I-H mode. As the flow-rate was increased from 1.0 to 2.0 mL/min, over 50% of stationary phase was retained, and almost the same resolution was achieved while the separation time was reduced over two times from 160min to about 70 min. The resolution is the same as we obtained before on multilayer column^[14].

3.3.2 Separation of anthocyanidin from *Lycium ruthenicum* Murr.—*Lycium ruthenicum* Murr. is a salt tolerant and drought resistant plant with black fruit growing in north-west of China. The pigment extract of its fruit was subjected to the separation for anthocyanidins. As seen in Fig. 9A, there is a main peak at about 63min in its HPLC chromatogram, which is our target anthocyanidin according to HPLC-MS analysis (Fig. 9C). The solvent system composed of *tert*-butyl methyl ether-*n*-butanol-acetonitrile-water, 0.1% TFA (1:2.5:1.5:5, v/v) was employed under the optimized operation condition (L-I-H) in the separation as illustrated in Fig. 10. The results from HPLC analysis indicated the target anthocyanidin could be isolated in about 90% purity through one step HSCCC separation, see Fig. 9B.

3.3.3. Separation of peptides—Fig.11 shows the separation of two peptides including leucyl-tyrosine (Leu-Tyr), valyl-tyrosine (Val-Tyr) using *n*-butanol-acetic acid-water (4:1:5, v/v) system by pumping the upper mobile phase in U-O-H mode.

3.2.4. Separation of proteins—The spiral tube column was applied to the separation of several protein mixtures using the PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w) (see Fig. 12). Fig. 12A shows the separation of cytochrome c and myoglobin in the L-I-T mode. Fig. 12B displays the separation of lysozyme and myoglobin in the U-O-H mode. The resolution between each pair of two proteins were acceptable, however, the peak efficiency was lower compared to that of small molecular. This may due to the lower mass transfer rate of macromolecules.

4 Conclusions

The above studies indicated that spiral tube column can produce higher retention of stationary phase for less polar, moderately polar, polar organic-aqueous solvent systems, to ATPS compared to traditional multilayer coil column under the same conditions. Remarkable improvement can be observed especially for polar to ATPS systems. The retention rule is generally consistent with that of that of spiral disk column, while its S_f are not comparable with that of spiral disk column due to the lower cross section area of tubing. The spiral tube column can give efficient separation for small molecular compounds from less polar theaflavins to polar anthocyanidin, and this is of great significance for analysis and preparation of small bioactive metabolites from plant and natural resources. Acceptable resolution can be achieved when it was used for the separation of dipeptides and proteins, which is almost impossible for the traditional multilayer coil column mentioned above. However, it still needs to be improved in term of separation efficiency especially for proteins.

Acknowledgments

The authors acknowledge financial support of this work by the National Natural Science Foundation of China (No. 20877005) the National High Technology Research and Development Program ("863" Program No. 2007AA061601), and Beijing Natural Science Foundation (No. 2102016).

References

- [1]. Ito Y. CRC Crit. Rev. Anal. Chem. 1986; 17:65.
- [2]. Ito Y. J. Chromatogr. A. 2005; 1065:145. [PubMed: 15782961]
- [3]. Winterhalter P. Trends Food Sci. Tech. 2007; 18:507.
- [4]. Pauli GF, Pro SM, Friesen JB. J. Nat. Prod. 2008; 71:1489. [PubMed: 18666799]
- [5]. Sutherland IA, Fisher D. J. Chromatogr. A. 2009; 1216:740. [PubMed: 19108842]
- [6]. Berthod A, Ruiz-Ángel MJ, Carda-Broch S. J. Chromatogr. A. 2009; 1216:4206. [PubMed: 18986655]
- [7]. Ito Y, Yang FQ, Fitze PE, Sullivan JV. J. Liq. Chromat. and Rel. Technol. 2003; 26:1355.
- [8]. Ito Y, Qi L, Powell J, Sharpnack F, Metger H, Yost J, Cao X-L, Dong Y-M, Huo L-S, Zhu X-P, Li T. J. Chromatogr. A. 2007; 1151:108. [PubMed: 17316660]
- [9]. Ito Y, Yang FQ, Fitze PE, Powell J, Ide D. J. Chromatogr. A. 2003; 1017:71. [PubMed: 14584692]
- [10]. Cao X-L, Hu G-H, Huo L-S, Zhu X-P, Li T, Powell J, Ito Y. J. Chromatogr. A. 2008; 1188:164. [PubMed: 18339394]
- [11]. Ito Y, Clary R, Powell J, Knight M, Finn TM. J. Liq. Chromat. and Rel. Technol. 2008; 31:1346.
- [12]. Ito Y, Clary R, Powell J, Knight M, Finn TM. J. Chromatogr. A. 2009; 1216:4193. [PubMed: 19062024]
- [13]. Wood PL, David H, Lee J, Sutherland IA. J. Liq. Chromat. and Rel. Technol. 2003; 26:1373.
- [14]. Cao X-L, Lewis JR, Ito Y. J. Liq. Chromat. and Rel. Technol. 2003; 27:1893.

Fig. 1.
Photo of the type J planet centrifuge equipped with two spiral tube columns

Fig. 2.
Design of spiral tube columns (A) spiral tube support; (B) cover

Fig. 3.
Stationary phase retention of heptane-methanol (1:1, v/v) on spiral tube column

Fig. 4. Stationary phase retention of hexane-ethyl acetate-methanol-water (1:1:1:1, v/v) on spiral tube column

Fig. 5. Stationary phase retention of n-butanol-acetic acid-water (4:1:5, v/v) on spiral tube column

Fig. 6. Stationary phase retention of PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w) on spiral tube column

Fig. 7.

Comparison of two different HSCCC columns for the retention of different solvent systems

A. heptane-methanol (1:1, v/v), L-I-H, 1.0 mL/min

B. hexane-ethyl acetate-methanol-water (1:1:1:1, v/v), L-I-H, 1.0 mL/min

C. n-butanol-acetic acid-water (4:1:5, v/v), U-O-H, 0.5 mL/min

D: PEG 1000-12.5% K_2HPO_4 -75% water (w/w), L-I-T, 0.5 mL/min

Fig. 8.

Separation of TFs using hexane-ethyl acetate-methanol-water (1:1:1:1, v/v) Conditions: sample: TFs 2 mg/4mL; rotation speed: 1200 rpm (112 g-level); detection: 280 nm; L-I-H mode. 1. TF; 2. TF-3-MG and TF-3'-MG; 3. TFDG

Fig. 9.
HPLC-DAD-MS analysis of anthocyanidin in *Lycium ruthenicum* Murr.
(A) crude pigment extract; (B) target anthocyanidin fraction from HSCCC; (C) MS spectrum of target anthocyanidin HPLC Conditions: column: TSK Gel ODS-80 Ts QA (250×4.6 mm i.d.), temperature: 35°C; mobile phase: 10% formic acid+0.1% TFA (A), 85% acetonitrile +15% methanol (B); gradient elution: 0–30 min, 97% ~ 88.5% A; 30–40 min, 88.5% A; 40–60 min, 88.5%~84.5% A; 60–70 min, 84.5%~84% A; 70–80 min, 84%~77% A; 80–100 min, 77%~97% A; flow-rate: 0.8 mL/min; detection: 280 nm. MS conditions: nebulizer pressure 35 psi, drying gas flow 8 L/min, drying gas temperature 350°C, scan range from m/z 100 to 2200.

Fig. 10.

Separation of anthocyanidin from pigment extract of *Lycium ruthenicum* Murr. Conditions: solvent system: *tert*-butyl methyl ether-*n*-butanol-acetonitrile-water, 0.1% TFA (1:2.5:1.5:5, v/v); L-I-H; sample: 20 mg of pigment extract; flow-rate: 1.0 mL/min; rotation speed: 1200 rpm (112 g-level); detection: 280 nm

Fig. 11.

Separation of dipeptides mixture using n-butanol-acetic acid-water (4:1:5, v/v) in U-O-H mode

Conditions: sample: Leu-Tyr (1.7 mg) and Val-Tyr (1.2 mg) in 1 mL; flow-rate: 0.5mL/min; rotation speed: 1200 rpm (112 g-level); detection: 280 nm

Fig. 12.

Separation of protein mixtures using PEG1000-K₂HPO₄-water (12.5:12.5:75, w/w)
Conditions: flow-rate: 0.5 mL/min; rotation speed: 1200rpm (112 g-level); detection: 280 nm; A. sample: 0.6 mg cytochrome c and myoglobin in 2 mL lower phase, L-I-T mode; B. sample: 0.8 mg lysozyme and 0.8 mg myoglobin in 2 mL lower phase, U-O-H mode.

Table 1

the geometrical parameters of two HSCCC machine

	Traditional multilayer coil column HSCCC	Spiral tube column HSCCC
Revolution radius R	50 mm	70 mm
β value	0.3–0.7	0.3–0.7
Pitch of screws	1.6mm	11.6mm
Winding way	multilayer	Embedded in spiral groove
Material of tube	PTFE	PTFE
I.D.×O.D.	0.8×1.6mm	0.8×1.6mm
Volume	35 mL	15mL×2=30mL