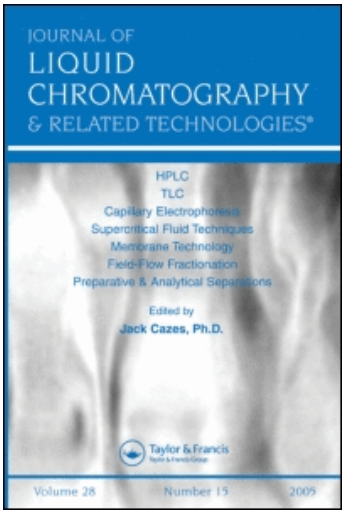

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Knight, Martha , Ito, Yoichiro and Finn, Thomas M.(2008) 'Separation of Peptides by Spiral Countercurrent Chromatography', Journal of Liquid Chromatography & Related Technologies, 31: 4, 471 — 481
To link to this Article: DOI: 10.1080/10826070701812699
URL: http://dx.doi.org/10.1080/10826070701812699

Journal of Liquid Chromatography & Related Technologies®, 31: 471–481, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701812699

Separation of Peptides by Spiral Countercurrent Chromatography

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Abstract: Spiral countercurrent chromatography (CCC) utilizes a new separation rotor composed of 8 high density polyethylene plates with spiral flow channels, mounted in the type-J coil planet centrifuge (CPC). Synthetic peptides were evaluated for partition coefficients in various solvent systems. Approximately 30 mg amounts were chromatographed and the fractions analyzed for peptide content and purity. The solvent systems which contained sec-butanol or n-butanol and the mobile phase selected produced good purification. The stationary phase was highly retained over 60%, thus the spiral separation coil is able to retain these more viscous, low interfacial tension solvents that have not been able to be used well in the multi-layer coil for high speed CCC.

Keywords: Spiral disk separation coil, Countercurrent chromatography, Preparative purification, Peptide separation, Coil planet centrifuge, Partition coefficient

INTRODUCTION

Peptides as a group are readily able to be fractionated or purified by partition methods. Included in this are the CCC methods and most of the past instruments have been used for peptide purification. The Ito coil planetary centrifuges have proven useful for peptides, due to the instruments' high loading capacity and versatility with solvent systems.[1] Because of their different side chains and wide range of MW, peptides are diverse in their structures which affect their solubility and stability. Many of the smaller peptides, less than 20 amino acid residues, are water soluble and partition easily in the

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butanol solvent systems, considered very polar in the current classifications.[2] This means that the solubility is high and partitioning occurs between the phases. Early instruments, such as the horizontal flow through CPC,[1,9] and a modified 6-layered (multi-coil) CPC[3] have been used for preparative peptide purification.

However, the later developed popular multi-layer CPC used for high speed CCC (used mostly for small molecules from plant extracts) has difficulty in retaining the butanol solvent systems, thus peptide chromatography was not easily achieved. Although solvent systems based on n-butanol and acetonitrile seem to work,[4] the solubility of peptides is limited in these solvent systems. CCC has been useful for the purification of less water soluble or hydrophobic peptides, as these are subjected to high losses in preparative HPLC because of the solid stationary phase. Since CCC has a liquid stationary phase that can be pumped out, total recovery is possible. Previous studies of solvent systems for these types of peptides have been reported.[5,6] The chromatography of peptides is studied here with the new spiral separation coil[7] in the hopes that the butanol-based solvent systems would be retained and, thereby, this application would be possible.

The solvent flow path through the spiral disk coil is similar to the Teflon tubing coils in that the solvent flow entry is at the center of the disk and the channel spirals clockwise to the outer end. Whereas the designation of the head and tail ends can be controlled by the direction of rotation, in the present instrument the paramount consideration for the operating condition is the elution of the mobile phase through the inside entry or the outside entry. It is important to know the partition coefficient (K or PC concentration in upper phase/concentration in lower phase) of the sample to select the right solvent system and to choose the mobile phase with higher stationary phase retention for maximum resolution and elution within a sufficient time or column volume. The direction of the mobile phase flow, either the lower phase from the inner entry or the upper phase from the outer end or periphery, is important for high stationary phase retention.

In the development of methods for the separation of peptides, the partition coefficient was determined by mixing the solvent system, adding equal volumes of each phase to the dry peptide, dissolving, mixing, and after separation of the phases, measuring the concentration in each phase. This was done by analytical reverse-phase HPLC of equal volumes of each phase and measurement of the ratio of the area or peak heights of the chromatograms. This is the most accurate method for determining the K of the major component of a crude peptide. The strategy for selecting the optimal solvent system is to find a K of one, thus, the peptide will elute in a column volume in not too long a timeframe and impurities can be separated. Some examples of peptides are shown here. One peptide was found to have a K value near one for n-butanol/acetic acid/water, 4:1:5 (BAW), a commonly used solvent system. The other peptide required more searching for a

suitable solvent system. This latter peptide is a highly water soluble sequence so the capability of this method for a polar molecule is also demonstrated.

EXPERIMENTAL

Instrument

The instrument used for countercurrent chromatography is a coil planetary centrifuge CPC with a 10 cm revolution radius manufactured by P.C. Inc. (Potomac, MD, USA). In this type-J coil planet centrifuge was mounted the spiral disk separation coil in place of the multi-layer coil. The spiral disk column coil assembly (Machine Instrumentation Design and Fabrication, National Institutes of Health, Bethesda, MD, USA) shown in Figure 1 is a stack of eight disks of 17.5 cm OD.[7,8] Each plate is made of 4 mm thick high density polyethylene with a single spiral groove 2.0 mm deep, 2.6 mm wide, and 4 mm space (pitch) between the channels (Figure 2).[7] The disks are sandwiched between Teflon sheeting that have holes for solvent entry and exit into the channels. The solvent enters the center, flows through the spiral to the periphery where there is a straight line (1 mm × 1 mm × 50 mm) channel underneath the disk to the center, where the flow enters the next spiral disk, and so on. In the spiral disk

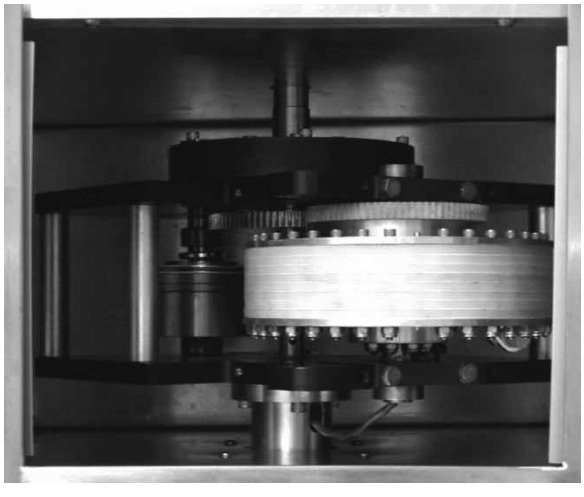

Figure 1. Close-up of spiral disk assembly mounted in a coil planet centrifuge. Side view of the spiral separation rotor with the nylon planetary gear at the top and counterbalance on other side of rotor shaft. The assembly has eight plates. Flow tubing comes out at bottom and goes up central shaft to top where the tubing is clamped (not shown). The total volume of the spiral module is 153 mL.

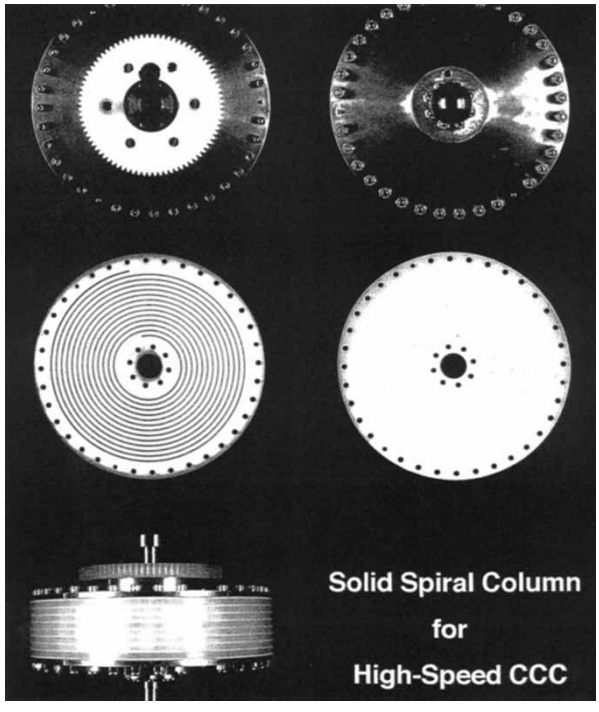

Figure 2. The components of the spiral disk column.[7] Top left is end flange (aluminum alloy) with the nylon gear (circular opening for flow tube) and right is other flange with tubing exit compression screw hole (top center). The spiral groove (single simple design) in the high density polyethylene plate (2nd row left) and Teflon sheet separating each plate (2nd row right). Finally, in the 3rd row is the side view of assembled rotary column module (called the spiral disk column coil or rotor) with eight plates.

where from the center, the spiral is CW (right handed) and from the outer end the spiral is CCW (left handed), the light phase goes to the head (top, inner entry) and the heavy phase goes to the tail (bottom, outer entry) in CW rotation (Figure 3).[9] Generally, the highest stationary phase retention occurs in the CCW rotation, where the inner entry is the tail (Figure 3, left) into which a mobile lower phase is pumped against its direction in the centrifugal force field and holds the stationary phase in equilibrium, while the excess exits the other end. Conversely, a mobile upper phase pumped into the outer head entry, can hold the lower stationary phase. The mechanism retains one phase stationary while the other phase passes through during the planetary centrifugation mixing, separating substances according to their partitioning between the phases. The rotation of the coil planet centrifuge was set at 800 rpm by a controller (Bodine Electric Co., Chicago, IL, USA). The mobile phase delivery of 2 mL/min was by a Waters 510 solvent pump (Milford, MA, USA).

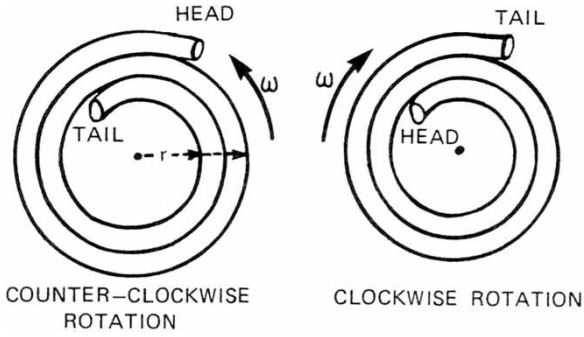

Figure 3. Orientation of mobile phase flow in coiled tubing applied to spiral separation coil. The inner entry or outer exit terminal of flow tubing is defined as head or tail depending on centrifugal direction.[9]

Materials

Solvents and water were HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ, USA). Peptides were out of the inventory retained from Peptide Technologies Corp. (Gaithersburg, MD, USA). The peptides had been synthesized by solid phase methods,[3] HPLC purified and stored for a few years. For these experiments the correct MW was identified by mass spectrometry (Applied Biosystems, Foster City, CA, USA) and HPLC analysis was performed to assess the impurities present.

Methods

The countercurrent purification procedures were as described previously.[3,5] Briefly, the peptide sample was dissolved in 3 to 5 mL of the solvent system and loaded in-line into the inner or outer end of the coil, already filled with the stationary phase. The centrifugation was set at 800 rpm, then, the mobile phase was pumped at 2 mL/min. After the elution of up to 2 or 3 column volumes, the centrifugation was stopped and the contents were pushed out with nitrogen gas. The effluent passed through a LKB UVicord S monitor set at 280 nm into a LKB 7000 Ultorac fraction collector (LKB Instruments, Bromma, Sweden), which collected 2 min fractions, 4-mL/tube. A Pharmacia LKB Rec 102 recorder was used. In addition, manual determinations of the absorbance of the fractions were made in a spectrophotometer (GeneSys 10 UV, Thermo Spectronic, Rochester, NY, USA). The absorbance measurements were plotted using Excel©. The peaks were identified by HPLC analysis in a Shimadzu dual pump system (SCL-10AV VP controller, two LC-10AD pumps, a SPC-6AV variable UV detector, and a CR501 recording integrator, Shimadzu Instruments Co., Columbia, MD, USA). A reverse-phase

Table 1. Determination of solvent systems for peptides

Solvent system	Partition coefficient
Peptide: KLESMETYLKAVLLF	
BAW (4:1:5)	0.93
Peptide: KKANELIAYLKQATK	
n-Butanol/0.5 M NH_4OAc	0.02
n-Butanol/Pyr/A/W (8:2:1:9)	0.09
BAW (4:1:5)	0.25
n-Butanol/1% aq. TFA	0.28
sec-Butanol/1% aq. TFA	0.45
CHL/A/W (2:2:1)	45.5

Abbreviations used: BAW = n-butanol/acetic acid/water, CHL = chloroform, NH_4OAc = ammonium acetate, Pyr = pyridine, TFA = trifluoroacetic acid.

column was used (dC_{18} 5 μm Atlantis 0.39 × 15 cm, Waters, Milford, MA, USA) with 0.1% aq. TFA (solvent A) and gradients of 0.1% TFA/acetonitrile (solvent B). Some HPLC analyses were conducted in a D-Star binary gradient system (Manassas, VA, USA) with a variable UV detector and StarChrom data analysis software using the column, YMC AP-302 s-5, 5 μm, C_{18}, 300Å, 0.46 × 15 cm (Waters, Milford, MA, USA). The fractions containing pure peptide were dried in a Savant centrifugal evaporator connected to a Virtis lyophilizer and Heto −90°C condenser and Precision vacuum pump (ATR, Laurel, MD, USA).

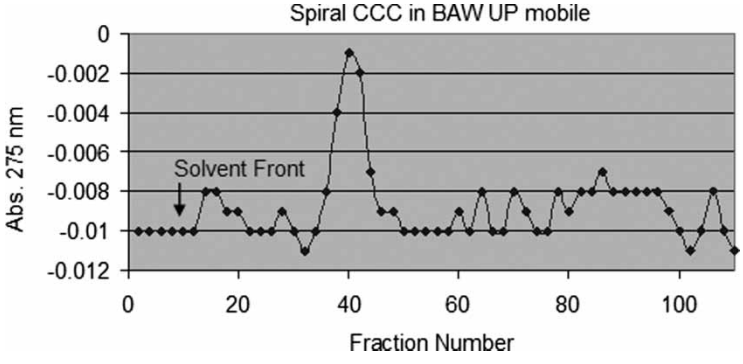

Figure 4. Chromatography of 19 mg KLESMETYLKAVLLF in the spiral CCC in the BAW solvent system with the upper phase mobile into the outer end in the head to tail direction (CCW) or U-o-H. The manual absorbance measurements of 100 μL aliquots in 2 mL methanol are plotted. The CCC flow rate was 2 mL/min and 2-min fractions were taken. Analyses of the fractions across the peak were made by HPLC (not shown). Other details of the experiment in the text.

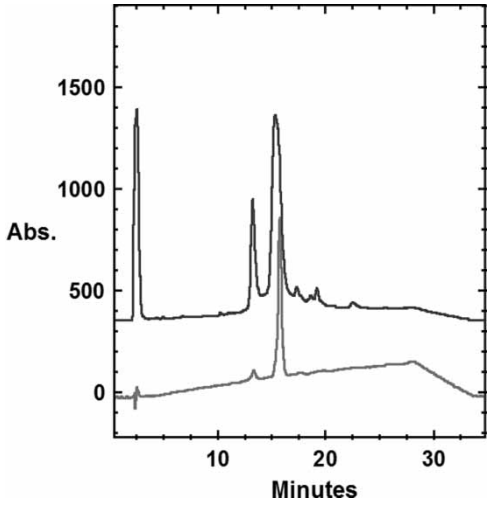

Figure 5. HPLC analysis of the pooled and lyophilized fractions (35–45) compared to the unpurified sample (upper trace). Sample approximately 50 μg dissolved in 50% aq. acetonitrile and unpurified sample dissolved in glacial acetic acid. Analysis performed in D-Star instrumentation as described in the text with detection at 215 nm at a flow of 1 mL/min in a gradient of 5% B to 20% B 0–5 min; 20% B to 60% B 5–25 min.

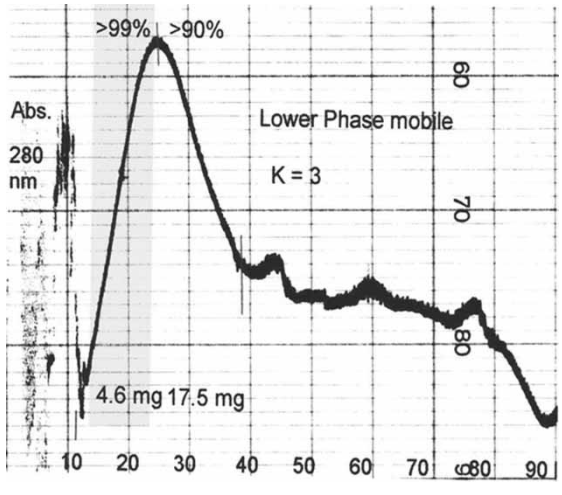

Figure 6. Separation of 27 mg KKANELIAYLKQATK with the lower phase mobile entering through the inner end; the direction of flow, tail to head or L-i-T (CCW rotation). Solvent system used was 0.5% aq. TFA/sec-butanol at 1 mL/min, 800 rpm. The solvent front came out at fraction 11, thus, the stationary phase retention was 61%. In the shaded part of the peak the fractions were >99% pure by HPLC and the amount recovered is shown. In the rest of the peak the amount recovered is shown as well. Tracing is recording of 280 nm absorbance per fractions collected.

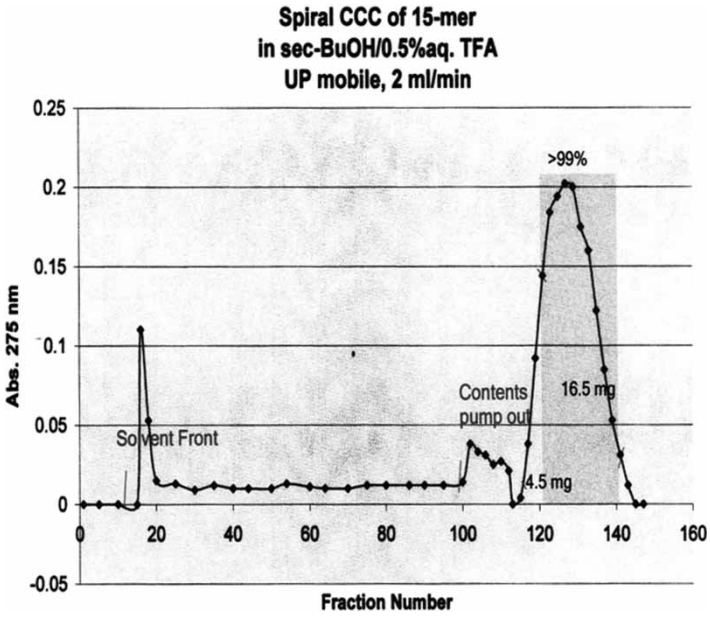

Figure 7. Chromatography of a similar amount, 30 mg of peptide, with the upper phase mobile at a flow of 2 mL/min. The mobile phase was introduced into the outer terminal with CCW rotation from the head to tail direction or U-o-H. At fraction 100, the centrifugation was ceased and the contents extruded with fractions collected. The absorbance was detected manually as described in the text. The recovery and purity level of the first part and shaded part of the major peak are noted.

RESULTS AND DISCUSSION

The peptide KLESMETYLKAVLLF had a K of 0.93 in the BAW system (Table 1). The peptide was run in the conditions of upper phase mobile whereby the sample was loaded in the outer entry and eluted in the head to tail mode, which was the CCW rotation. The stationary phase retention was 74%. The peptide was eluted in one peak (32–45) correlating to one elution volume predicted by a K close to one (Figure 4). After HPLC analysis of fractions within the peak (not shown), the most pure fractions (35–45) were combined and lyophilized and were analyzed to be 96.2% pure as shown in Figure 5. The HPLC analysis was done some time after the CCC and the small impurity is likely sulfoxide of the methionine amino acid residue. The peptide is not completely soluble in water. The absence of a solid support ensures higher recovery of peptides of limited water solubility.

A more polar peptide KKANELIAYLKQATK of high water solubility was found to have a closely eluting impurity by HPLC. Determination of K's in various solvent systems was made to find a K value close to one for best

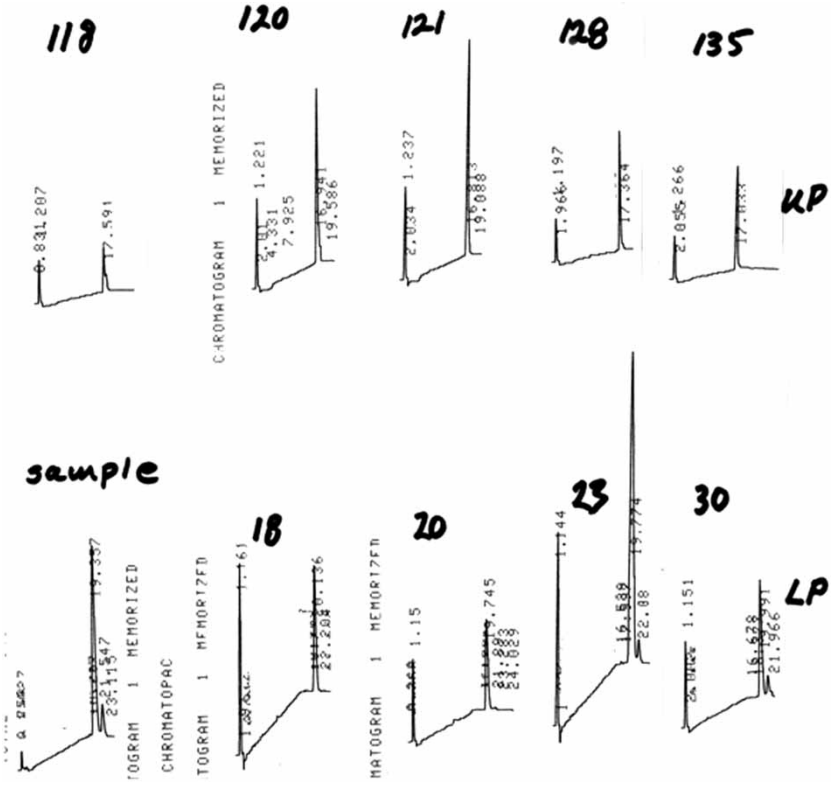

Figure 8. HPLC analysis of 15-mer peptide in Shimadzu instrumentation. On the lower left is a chromatogram of sample loaded showing a small impurity in HPLC conditions of 0.5 mL/min flow with a gradient of 0% to 25% B in 15 min. On the same row are the analyses of the CCC run with lower phase mobile (Figure 6). In the row above, the analyses of CCC run with upper phase mobile (Figure 7) is shown. Gradient was from 0% to 27% B in 15 min; impurity was still observed in fractions 118 and 120.

fractionation. As listed in the table, the peptide had a K of 0.45 in the solvent system consisting of sec-butanol/1% TFA. Experiments were done with this solvent system in the conditions of both phases mobile to determine recovery of the purified compound. Although this is an experiment to obtain purification it was also intended to observe the power of resolution relative to HPLC. An amount of 30 mg was loaded into the coil after filling with the upper phase used as the stationary phase. The run conditions are as described in Figure 6. This was in the 1/K mode, which calculates to approximately 2, however, the peptide was eluted at a smaller elution volume than expected calculated in the run to about 3. Nevertheless, the early fractions were the purest (shaded part of peak, Figure 6) and the later fractions contained the more hydrophobic impurity consistent with the elution of the aqueous phase as the mobile

phase. In Figure 8 is the HPLC analysis with the results of fractions 22 and 23 in lower part of the figure showing the closely eluting impurity. In the next experiment an amount of 30 mg was chromatographed in the condition of upper phase mobile (Figure 7). The peptide was eluted later than expected; the centrifugation was stopped and the contents were extruded and fractions collected. Due to the detection noise the fractions were read manually. Abs. ethanol was added to the bi-phasic fractions (column contents) which coalesced to one phase because of the solubility of sec-butanol, and these could be read directly. Consistent with the organic mobile phase, the more hydrophobic impurity was eluted first and higher recovery of the purified peptide resulted because of the longer elution time. The upper part of Figure 8 contains the HPLC results of the pure fractions 121 to 135. These results show the development of methods for better resolution.

CONCLUSIONS

Peptide purification is accomplished in the new spiral disk rotor in the coil planet centrifuge. The new separation coil design is comprised of a flow channel in each disk in a single spiral that connects to the adjoining plate. More complicated spirals have been incorporated into the plates and will be studied in the future.[10] However, very significantly, at high flow and centrifugal rates the butanol-containing solvent systems are able to be retained at well over 60%, enabling chromatography of larger molecular weight compounds. The spiral rotor extends the use of high speed CCC in the type J-coil planet centrifuge to all molecules.

ACKNOWLEDGMENTS

The research was conducted under a CRADA between CC Biotech LLC and the National Heart, Lung, and Blood Institute, of the National Institutes of Health. The research was supported in part by a National Science Foundation SBIR Phase I grant 0638082 to CC Biotech LLC. This company had a commercialization evaluation license L-083-2006/0 and presently has a commercialization license L-144-2007/0 from the National Institutes of Health for the spiral countercurrent chromatography.

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Received September 2, 2007
Accepted September 6, 2007
Manuscript 6176