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Spiral counter-current chromatography of small molecules, peptides and proteins using the spiral tubing support rotor

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ABSTRACT

An important advance in countercurrent chromatography (CCC) carried out in open flow-tubing coils, rotated in planetary centrifuges, is the new design to spread out the tubing in spirals. More spacing between the tubing was found to significantly increase the stationary phase retention, such that now all types of two-phase solvent systems can be used for liquid–liquid partition chromatography in the J-type planetary centrifuges. A spiral tubing support (STS) frame with circular channels was constructed by laser sintering technology into which FEP tubing was placed in 4 spiral loops per layer from the bottom to the top and a cover affixed allowing the tubing to connect to flow-tubing of the planetary centrifuge. The rotor was mounted and run in a P.C. Inc. type instrument. Examples of compounds of molecular weights ranging from <300 to approximately 15,000 were chromatographed in appropriate two-phase solvent systems to assess the capability for separation and purification. A mixture of small molecules including aspirin was completely separated in hexane–ethyl acetate–methanol–water. Synthetic peptides including a very hydrophobic peptide were each purified to a very high purity level in a *sec*-butanol solvent system. In the STS rotor high stationary phase retention was possible with the aqueous *sec*-butanol solvent system at a normal flow rate. Finally, the two-phase aqueous polyethylene glycol–potassium phosphate solvent system was applied to separate a protein from a lysate of an *Escherichia coli* expression system. These experiments demonstrate the versatility of spiral CCC using the STS rotor.

1. Introduction

Research in rotor or column-coil design for CCC separation in the J-type planetary centrifuges produced the spiral disk assembly in 2003. The spiral disk was made of high density polyethylene plates with spiral flow channels with small ID return channels under each plate to connect the flow to the next plate. The spiral groove in each plate spreads out from the center at a radial distance every 4 mm or a 4-mm pitch [1]. The distance between each spiral flow channel is greater than the distance between the liquid flows in adjacent tubing in a multi-layer coil. Standard wall of extruded tubing #14 FEP (Zeus Inc., Orangeburg, SC, USA) is 0.41 mm, therefore the pitch is <1 mm, the sum of the 2 tubing walls of adjacent tubing. The initial application studies showed very high retentions of stationary phases of organic-aqueous solvent systems and the polar butyl alcohol–aqueous solvent systems, 91% and 79% respectively, at 2 ml/min flow rate [2]. A means to increase the pitch even higher was to make 4 interweaved spirals on a plate with return channels to the center and subsequent exit to the next disk. This was the 4-spiral disk assembly. In this way the pitch was increased to 16 mm resulting in high retention of the polar two-phase solvent systems at 1 ml/min and importantly, the aqueous two-phase solvent systems (ATPS) such as polyethylene glycol (MW = 1000) 12.5% and K_2HPO_4 12.5% by weight [3].

The 4-spiral interweave design was created with continuous tubing by placing the tubing in channels of a circular frame with openings at the quadrants where the tubing returns to the center crossing over tubing in the channels. In 2008, the first spiral tubing support (STS) made in a 17-cm OD aluminum cylinder with the tubing held in with a top flange cover was applied to CCC separations [4]. The results were successful in that all commonly used solvent systems including the ATPS were able to be used [5]. Advantages include no leaking through sandwiched plates and higher resolution due to smaller tubing OD than 2-mm wide flow channels in the disks. Tubing of 1.6 mm ID was used in this frame. The first STS had radial channels that were straight making right angles to the circumference channels. Sharp tubing bends could impede flow and the metal edges could cut the tubing [4]. Next an STS rotor was made by laser sintering which incorporated many features to improve the tubing arrangement and eliminate much weight [6]. Importantly, the radial channels were curved eliminating sharp bends. The new plastic spiral tubing support CCC rotor was applied to the

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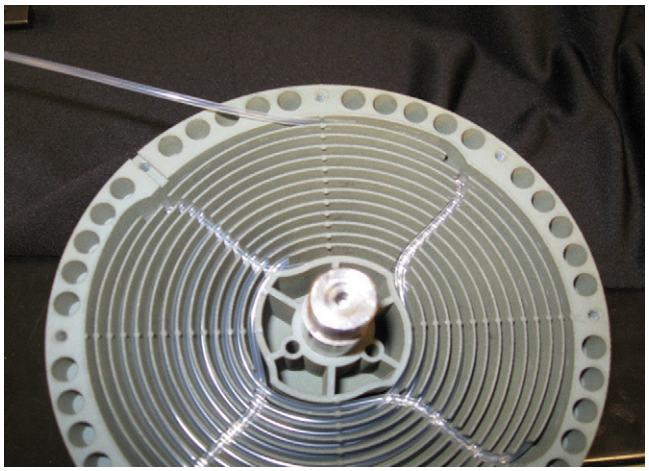

Fig. 1. View of a spiral tubing support rotor with the cover removed and the rotor shaft in center. Tubing is placed in circular channels, wound to center and is guided to periphery through curved radial channels. Halfway between the radial channels in the wall rims are protrusions that keep the tubing from popping out while being inserted. Further details are in Section 2.

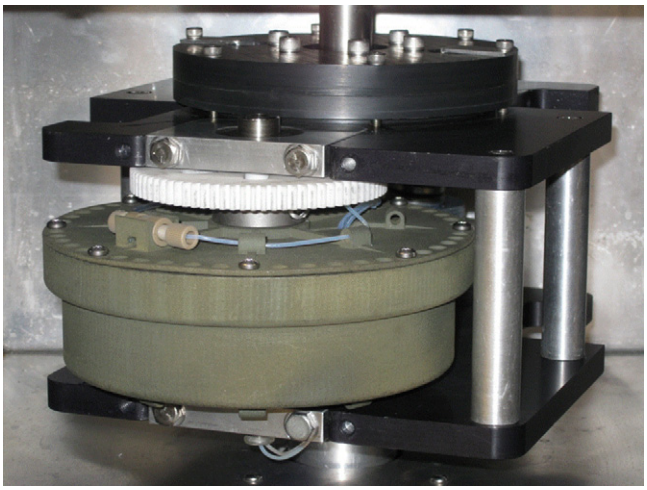

Fig. 2. The STS rotor fully assembled and mounted in the planetary centrifuge. The cover has a press hold for a tubing compression screw union. The tubing threaded through holds goes into the shaft and out the bottom into the center centrifuge shaft. The gear is also laser sintered and the bearing blocks are fitted to the rotating frame.

separation of different compounds including higher MW ones, using the low-polarity, polar and ATPS solvent systems to evaluate its capability.

The aqueous butanol solvent systems are found to be versatile for separating many types of molecules in spiral CCC. The butyl alcohols are biofuels, produced from renewable biological sources [7]. This characteristic, along with no solid support requiring less resources, make spiral CCC a "green" technology.

2. Experimental

2.1. Instrumentation

The rotary frame of the STS was made by the laser sintering technique using a rapid prototyping machine (Sinterstation 2300 plus) and incorporated shape changes that improved the purpose of the rotor and its ease of manufacture [6]. This 3-dimensional printing formed a complex shape by laser hardening of a monomeric powder EOS Precision polyamide PA220, a nylon co-polymer. The round frame has concentric circular walls and radial channels that come to the center, curved at ends to prevent tubing from crimping and impedance to solvent flow as shown in Fig. 1. In this cross-sectional view, tubing is shown being inserted into a channel and at the top rims of the channels are small protrusions that hold the tubing in during winding. The top rim of the frame is wider in diameter than the rest of the body of the frame (Figs. 2 and 3). There are on the rim screw holes to secure the cap and 5 holes in between to fill with weights, if necessary for balancing (Fig. 1). In the radial path to the center, the tubing while being fit into the frame can be pressed hard to flatten and include more layers. This is aided by a tool built by the laser sintering method that fits the radial channels and presses down the tubing (Fig. 4). Through flattened tubing in the return channels, the solvent still flows with the advantage of laminar flow disrupted.

By the same laser lithography process, a cover was made for the rotor. In Fig. 2 is shown the final assembled rotor containing the cover with tubing guides and a tubing union holder. The overall dimensions of the rotor excluding the gear are 17.5 cm wide and 7.0 cm high. The β values range from 0.25 to 0.73. The cap cover is secured by screws onto the rotor around the central shaft as well as at the periphery. In Fig. 1 are seen the screw holes around the shaft. The cap cover has a notch to fit it in one position on the rotor body for all holes for tubing and screws to be properly aligned. In Fig. 4 on the underside of the cover turned upside down is a notch. A slot for the cover notch is present on the left rim in the cross section view (Fig. 1). A soft coating of Teflon is applied under the cap cover to prevent rubbing of tubing (not shown). A CAD drawing of the underside of the rotor (Fig. 3) shows a hold for the compression screw union as well as raised supports for the tubing guide rings. This design maintains support without added weight. The tubing is inserted into the hole at the bottom and is fitted in a channel that curves from the outside to the center where the tubing is next guided out through the radial channel to the periphery and is guided around through another spiral channel, and so forth until at the top and center the tubing goes up through a hole in the cover to connect to the flow tubing at the union. From the top the spiral direction is clockwise from the center out.

The in-flow and out-flow tubing (0.85 mm ID PTFE) are passed through the rotor shaft to the central shaft of the centrifuge and out the top and clamped. The rotor filled with 1.6 mm ID tubing has a total volume of 135 ml. The planetary centrifuge used is made by Conway CentriChrom Inc. (Williamsville, NY, USA) as reported previously [2]. There is a 10-ml sample loading valve at the inlet and

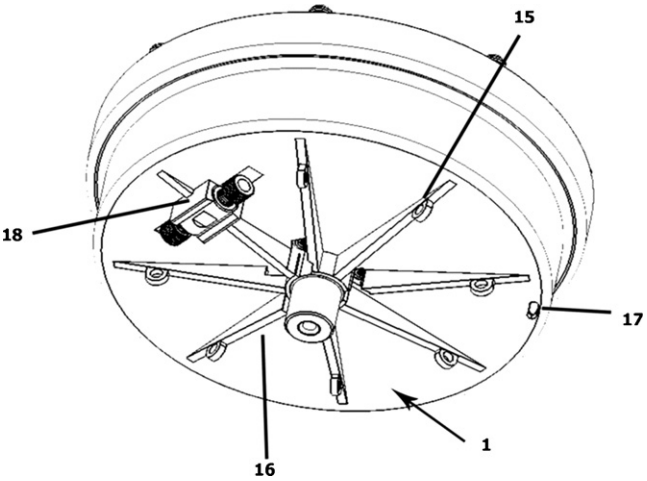

Fig. 3. Underside view of the STS rotor (1) with features indicated such as the hold for compression screw union (18) that joins the rotor tubing coming out of the hole at (17) to the flow-tubing that goes through the rotor shaft. Guide loops (15) for the tubing are mounted on supports (16).

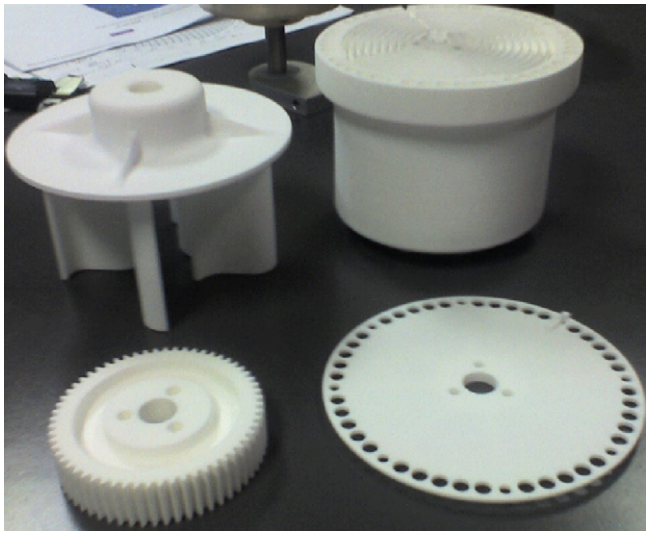

Fig. 4. Photo of laser sintered parts of a STS rotor and a pressing tool. Upper left is the tool with prongs that fit into radial channels. A gear is below. The STS rotor that fits a Pharma-Tech Research instrument is in the upper right. On the lower right is the cover upside down with a notch at about 1 o'clock. Holes for attaching around the center shaft and perimeter are described in the text.

the outflow is to a LKB 2070 Ultrorac II fraction collector (Piscataway, NJ, USA). The solvent flow is provided by a D-1463 Knauer pump (Berlin, Germany). A flow rate of 1 ml/min was used for most experiments. The planetary centrifuge is set for either CW or CCW revolution. The revolution of the planet centrifuge was set at 800–840 rpm for all runs. For expelling contents and drying the system, helium gas is used from a cylinder connected into the sample valve manifold.

2.2. Materials

Solvents were HPLC grade from Mallinckrodt/Baker (Phillipsburg, NJ, USA). The solvent *sec*-butanol was certified grade from Fisher Scientific (Fair Lawn, NJ, USA). Chemicals including acetyl salicylic acid, salicylic acid, naringenin, salicin were obtained from Fisher Scientific or Sigma–Aldrich (St. Louis, MO, USA). Synthetic peptides were samples from aapptec Inc. (Louisville, KY, USA) or Peptide Technologies Corp. (Gaithersburg, MD, no longer active). Water was purified through a reverse-osmosis and ion-exchange filtration system with irradiation (Neu-Ion, Baltimore, MD, USA). From an ubiquitin-related protein expression system

Table 1
Composition of small molecule sample.

Compound	MW	Amount (mg)	*K* (lower phase/upper phase)
Naringenin	272.3	18	0.12
Ac-salicylic acid (aspirin)	180.16	44	0.75
Salicin	286.27	33	4.34

in *Escherichia coli* [8], 75 ml of the cell lysate was freeze dried to 0.98 g. The psalmotoxin tosyn2 fusion protein of MW = 15 kDa was the target protein to be purified.

2.3. Methods

In selecting solvent systems and elution modes, the partition coefficients of the analytes were determined by dissolving the sample in the solvent systems and analyzing by HPLC, the concentration in the upper phase and lower phase according to previously published procedures [2,9]. The equipment used for the small molecules experiments was D-Star Gradient Solvent Delivery System with variable UV detection and Starchrom software for instrument control and data analysis (D-Star, Manassas, VA, USA). Besides partition coefficient measurements, HPLC was used for the analysis of fractions to determine identity and purity according to previously published procedures [2,9]. For the peptide and protein partition coefficient and fraction analysis experiments, Shimadzu instrumentation (Columbia, MD, USA) consisting of SCL-10Avp controller, LC-10ATvp dual head pump, FCV-10ALvp low pressure gradient mixer for 4 solvents, DGU-14A solvent degasser, SPD-10Avp variable wavelength detector and a SIL-10AC autoinjector with EZSTARTv1.2.1SP1 chromatography software was used. The solvent system used for HPLC was $A = 0.1\%$ aq. trifluoroacetic acid and gradients of $B = 0.1\%$ TFA in acetonitrile at a flow of 1 ml/min and detection at 215 nm. A YMC-Pack ODS-A column (S-5 μm, 150 mm × 4.6 mm ID) (Allentown, PA, USA) was used for small molecule and peptide analytical separation, and for proteins the YMC-Pack Protein-RP column (C_4, S-5 μm, 250 mm × 4.6 mm ID) was used. The fractions of the lysate separation were submitted to polyacrylamide gel electrophoresis (PAGE) analysis [10]. The details of the sample prep are included in the legend of Fig. 12.

The spiral countercurrent chromatography procedures are as previously published [2,9]. The solvent system is mixed, equilibrated, separated and the stationary phase is loaded into the rotor. The sample is dissolved in equal volumes of each phase and loaded

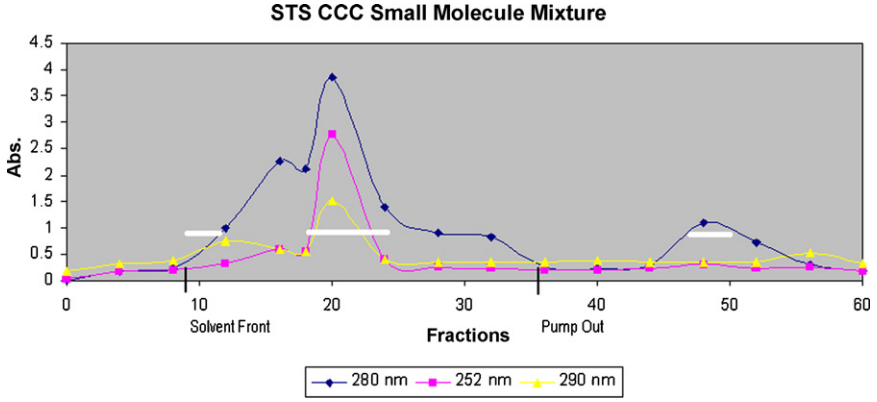

Fig. 5. Separation of compounds in the HEMW (3:5:3:5) solvent system in the U-o-T elution mode at 1 ml/min with 4-min fractions collected. The upper phase emerged at 40 ml or fraction 9, thus the stationary phase was retained at 70.4% of the coil volume. The centrifugation was ceased and contents pushed out with water at fraction 35. The localization of each compound was by HPLC analysis and is identified by bars on the chromatogram. Naringenin was eluted in fractions (9–10) followed by aspirin in fractions (18–27) and salicin in fractions (48–50).

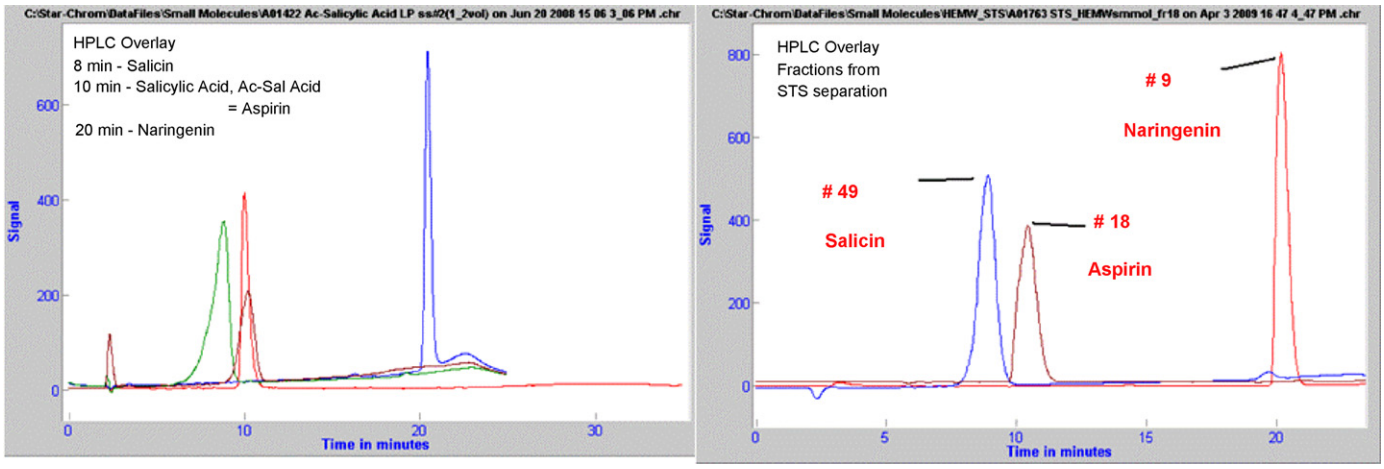

Fig. 6. Analytical HPLC analysis of compounds and countercurrent chromatography fractions using the YMC-Pack ODS column and conditions as described in Section 2. At a flow of 1 ml/min the gradient 0–15% B in 15 min; 15–30% B at 20 min was applied. (Left) Analysis of standards with detection at 252 nm except for salicin at 215 nm. Salicylic acid overlaps with aspirin in HPLC, so it was not included in the experiment. An overlay of chromatograms is shown. (Right) Analysis of peak fractions identified the contents and showed complete separation. Overlay of individual HPLC analysis of CCC fractions 49, 18 and 9.

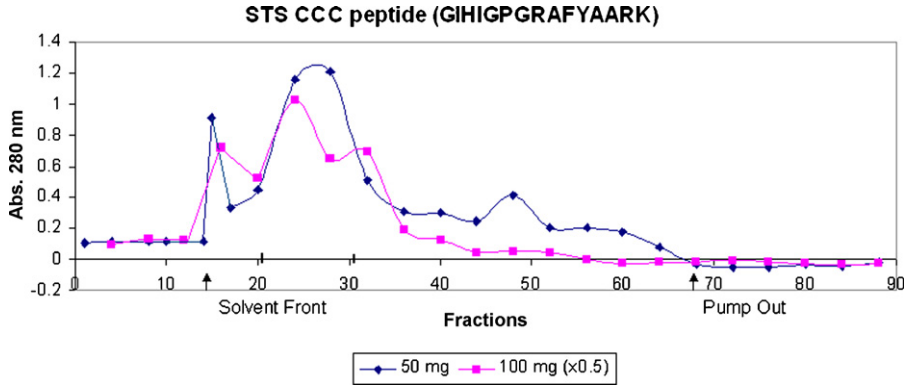

Fig. 7. The results of 2 CCC separations of synthetic 15-mer peptide in *sec*-butanol-0.1% aq.TFA (1:1) run with the lower phase mobile (L-i-T) at 1 ml/min and 830 rpm. Recoveries of pure peptide are described in Section 3.2.

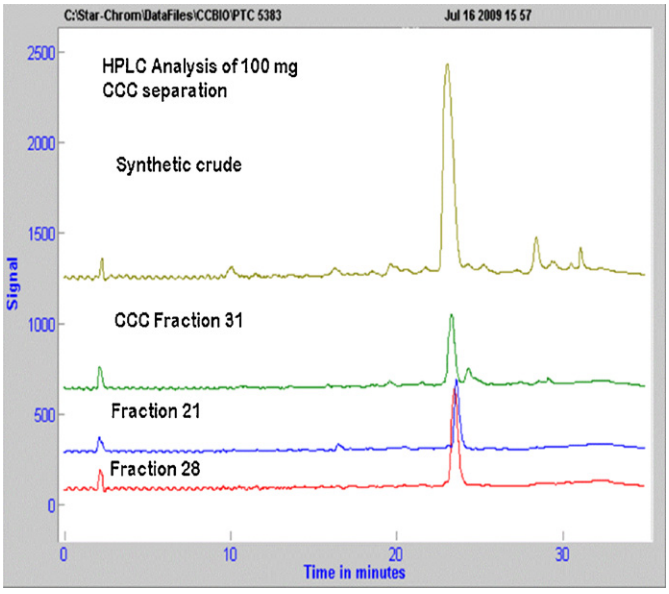

Fig. 8. Analytical HPLC of unpurified (approximately 50 μg) and fractions (approximately 15 μl) of 100-mg CCC run of 15-mer peptide. Analysis performed in D-Star instrument with gradient conditions: 0–5 min, 5% B; 5–20 min, 5–20% B; 25–30 min, 20–50% B, 1 ml/min and 215 nm detection.

into the sample valve connected to either the inner or outer terminal flow tubing of the STS rotor. The rotation at 800 rpm is started, as well as the flow at 1 ml/min then the sample is injected. When the stationary phase is the upper phase, the mobile lower phase is pumped into the inner or top end; conversely, for a stationary lower phase, the upper mobile phase is introduced into the outer or bottom terminal flow-tubing, as previously described for the spiral disk rotors [2]. The elution is head to tail or vice versa by changing the rotation between clockwise or counterclockwise. The elution is carried out for one or two column volumes then the rotation is stopped and the contents are pushed out by helium gas and fractions of the column contents collected, if desired. Fractions of 4 min are collected and UV absorbance readings are made manually (Spectro UV-240PC, Shimadzu) and plotted using Excel©. For compounds to be recovered, the fractions determined to have pure compound are freeze dried to a powder using a RVT4104 vapor condenser, −110 °C (Thermo Savant, Holbrook, NY, USA) and Sargent-Welch Direct Torr vacuum pump (Model 8814A, Skokie, IL, USA).

3. Results and discussion

3.1. Separation of small molecules

Small molecules of MW < 500 were investigated by measuring the *K* in various solvent systems by HPLC experi-

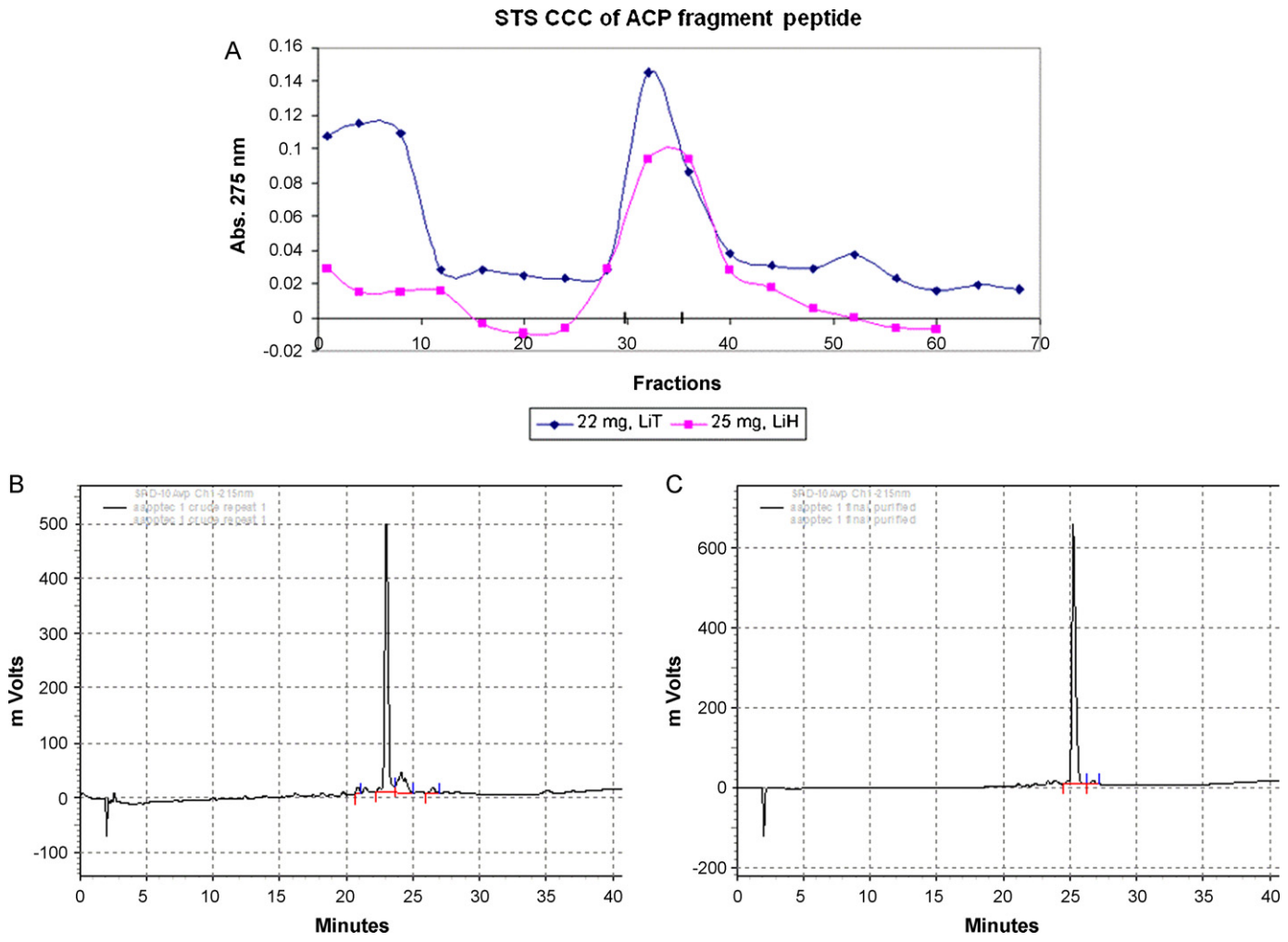

Fig. 9. A. Separation of the ACP fragment peptide in 2 elution modes. Absorbance readings were taken of 0.2 ml samples diluted in 0.6 ml 50% ethanol. The high values in early fractions of the L-i-T chromatogram are due to the reference cell not balanced with stationary phase. The fractions in each peak were analyzed by HPLC and high purity fractions were combined. The solvent front of the L-i-T run was at fraction 9 and the solvent front of the L-i-H run came out at fraction 16. B. HPLC of crude peptide showing 82% purity. HPLC performed in Shimadzu instrument with gradient conditions; 1% B to 15% B in 20 min and 15% B to 60% B from 30 to 60 min. C. Analysis of the final purified peptide recovered from both runs. Purity level is 98.6%.

ments. The compounds were selected from the solvent system study for small molecules previously reported [11]. Naringenin (4′,5,7,trihydroxyflavanone) is the most hydrophobic compound followed by aspirin and salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside). The two-phase organic-aqueous solvent system HEMW (3:5:3:5), n-hexane, ethyl acetate, methanol, water by volume had a spread of values of partition coefficients for these compounds. In Table 1 the *K* values are listed as the ratio of concentration in lower phase (stationary phase) to upper phase (mobile phase). In this way the expected elution volume can be determined. The amount of each compound loaded in the experiment is listed in the table, as well.

The mixture of the compounds in 3 ml total of both phases was loaded. The chromatography was conducted at a flow rate of 1.3 ml/min with 4-min fractions collected (Fig. 5). The rotation was 840 rpm with the elution mode of U-o-T [2]. This means the upper phase was pumped through the outer terminal in the tail to head direction (CW). The absorbance readings of the fractions are plotted in the chromatogram. Naringenin was present in fractions (9–10) predicted by the *K* value. Aspirin was identified in fractions (18–27) with high content in (18–20) (Fig. 6). Its peak calculated by the *K* is 21. The elution was carried out for about 200 ml which is before the elution volume for the last compound salicin which was localized in the last peak (column contents). A mass load of 95 mg was successfully separated into the 3 components in the expected elution volumes by spiral CCC in the new STS rotor.

3.2. Separation of synthetic peptides

The new STS rotor was evaluated for the capability to purify synthetic peptides. Previously, using the spiral disk rotor, various peptides, including very hydrophobic peptides, in the amounts of 30 to 65 mg were recovered in good mass yields at very pure levels [8,11]. A 15-mer peptide (GIHIGPGRAFYAARK) was determined to have a $K = 0.29$ in the *sec*-butanol-0.1% aq.TFA solvent system. The UV chromatograms of 50 and 100 mg samples in the L-i-T elution mode are presented in Fig. 7. Recovery of pure peptide was 25 mg from fractions (23–31) of the 50-mg run and 63 mg from fractions (21–30) from the 100-mg separation. The loading capacity of the 135-ml volume rotor is high in comparison with an equal volume filled LC column. Furthermore, the high sample load did not have a surfactant effect to change the stationary phase retention which was over 66% in both runs. The HPLC analysis of fractions of the 100-mg run shows the high level of purity obtained (Fig. 8).

The ACP (acyl carrier protein) fragment, VQAAIDYING is synthesized as a test product for peptide synthesizers. A sample obtained was 82% pure as shown in the HPLC (Fig. 9B). This was submitted to spiral CCC in the *sec*-butanol-1% aq.TFA solvent system (Fig. 9A).

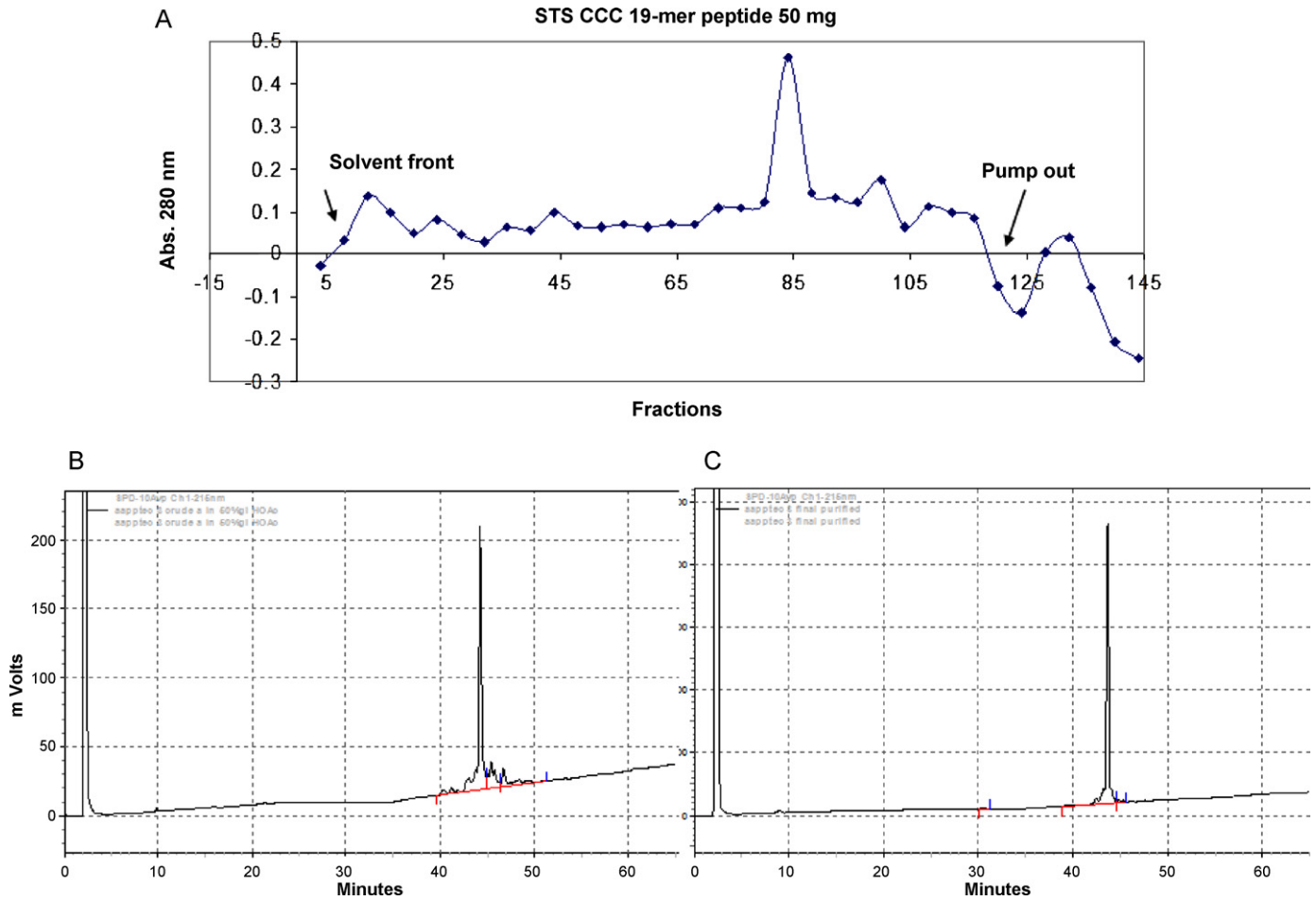

Fig. 10. A. Separation of the hydrophobic peptide in *sec*-butanol-0.1% aq.TFA in the L-i-T conditions. The solvent front came out at fraction 10 and after fraction 120, the contents were pushed out. Besides the peptide eluting in fractions (72–102) there was a residue in the last peak (125–139). B. HPLC analysis of crude peptide indicating a 74% purity level. C. Analysis of final purified peptide, 96.8% pure from pooled fractions (80–97). HPLC gradient conditions are the same as in Fig. 9.

This is one of the most polar solvent systems used in CCC and is less unilaterally distributed to one end than the non-polar organic-aqueous two-phase solvent systems. Therefore the lower phase mobile in both the head to tail and tail to head directions was tried. In Fig. 9A the plots of both runs 23 mg L-i-T (CCW) and 25 mg in the L-i-H (CW) elution modes are shown. The *K* values calculated from both runs are the same, 1.01. The most pure fractions by HPLC were combined from both runs and 23 mg 98.6% pure ACP fragment peptide was obtained (Fig. 9C). The stationary phase retention of the L-i-H run was 55% which is lower than that of the L-i-T elution mode which was 74%. The stationary phase retention of the single-spiral disk rotor was measured to be 70.3% in the L-i-T and 54.1% in the L-i-H elution mode at 2 ml/min [2]. The STS, a 4-spiral per layer design, is known to have higher stationary phase retention than the single-spiral disk rotors and these results are consistent with this.

The very hydrophobic 19-mer peptide (fPRGGGGNGD-FEEIPEEYL, f = D-Phe) is not water soluble due to the presence of 2 phenylalanine residues, but could be dissolved in 50% acetic acid for HPLC analysis (Fig. 10B). An amount of 50 mg was loaded in the *sec*-butanol-1% aq.TFA solvent system and run in the L-i-T elution mode for at least 3 column volumes (Fig. 10A). The peptide was localized between fractions 72 and 102. The purest fractions (80–97) determined by analytical HPLC, were combined and lyophilized to yield 16.4 mg (Fig. 10C). The stationary phase retention was 71% in this experiment. It was advantageous that this polar solvent system could purify this peptide of low-water solubility. If the upper phase were used as the mobile phase, the peptide would have come out close to the solvent front and probably not well separated from other residue present in a later eluting peak (Fig. 10A).

These results show that the solvent system of *sec*-butanol-1% aq.TFA (1:1), similar to previous work with the single-spiral disk separation rotor, serves well in purifying many different peptides with varying charge and water solubility. Previous publications have included purification of other hydrophobic peptides [12]. The studies so far indicate that using the lower phase mobile in the L-i-T elution mode is the most useful for separating and purifying peptides which can be recovered easily from this aqueous phase by lyophilization.

3.3. Separation of proteins

The STS rotor was applied to the separation of proteins with the aqueous two-phase solvent systems. First a stationary phase retention study of the 12.5% PEG (MW = 1000)–12.5% dibasic potassium phosphate in water (by weight) was performed and the results are in Table 2. As expected there was no stationary phase retention with the lower phase introduced into the outer terminal and conversely the upper phase pumped into the inner end. The S_F of other ATPS and *n*-butanol-phosphate buffer (a relatively non-polar solvent system) are also included in the table. The percent stationary phase retentions are high at least 70% with the lower phase mobile and above 49.9% with the upper phase used as a mobile phase. Myoglobin and lysozyme were completely

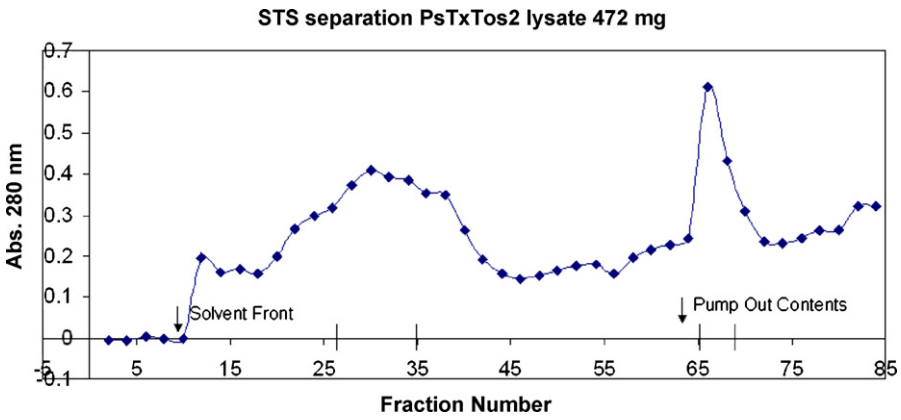

Fig. 11. Spiral CCC separation of psalmotoxin–tosyn2 fusion expression lysate in 12.5% PEG (1000)/12.5% K_2HPO_4 with the lower phase mobile at 1 ml/min. Absorbance readings at 280 nm were taken after diluting 200 μl aliquots of each fraction with 600 μl water.

Table 2
Solvent systems and conditions used for proteins.

Solvent system composition 800–840 rpm	Flow rate (ml/min)	Elution mode	Stationary phase retention (%)
ATPS—12.5% PEG (MW = 3350):12.5% K_2HPO_4 all by weight in water	1	L-i-T	70.5
		U-o-H	59.3
		L-o-T, L-o-H	0
		U-i-H, U-i-T	0
ATPS—12.5% PEG (MW = 3350):12.5% K_2HPO_4	1	L-i-T	72
		U-o-H	49.9
ATPS—16% PEG (MW = 8000):6.25% K_2HPO_4:6.25% KH_2PO_4	0.5	L-i-T	80
n-Butanol/0.1 M K_2HPO_4, KH_2PO_4 (1:1)	1	L-i-H	70

separated in the aforementioned ATPS at 1 ml/min in the L-i-T elution mode [13].

In an *E. coli* protein expression system, psalmotoxin–tosyn2 an ubiquitin-like fusion protein with the target 40-mer peptide psalmotoxin linked to the carboxyl end, is usually isolated by metal chelate affinity chromatography (IMAC) from the cell lysate. Then a protease cleaves the peptide from the fusion protein and the resulting peptide is purified by an appropriate chromatography method. Previously, antiflammin nonapeptide was purified by spiral CCC from the cleaved fusion protein using *n*-butanol-1% aq.TFA [13]. This study assessed if the fusion protein can be purified directly from the lysate by spiral CCC. A lysate prepared from a 4-L fermentation was provided and 75 ml was freeze-dried to 0.98 g. About half of this was dissolved in 5 ml of each phase of 12.5% PEG (MW = 1000)–12.5% K_2HPO_4–water and submitted to spiral CCC in the L-i-T elution mode at 1 ml/min (Fig. 11). After the solvent front at fraction 10, there was a broad peak from fractions 20 to 40 and another peak starting at fraction 66 that was sharper came out. In this run the contents were pumped out starting at fraction 64 after which two-phases were present, but most of the protein was in the upper PEG phase. In another experiment when the mobile phase was eluted for a longer time the second peak came out with a similar height and peak width. The contents of fractions across the run were analyzed by PAGE and the results are shown in Fig. 12. The 15 kDa fusion protein is seen in fractions of both peaks, the first peak has higher MW impurities and the later peak has a lower MW protein that is the tosyn2 without the psalmotoxin peptide. In fraction 12 there is little protein content and the coil contents (fraction 84) contained heterogeneous proteins.

Peak fractions 30 and 66 of peaks (27–35) and (66–68) respectively, have been analyzed by Western blot and both react with anti-ubiquitin-like protein antibodies meaning that the fusion target protein is enriched in both peaks (not shown). Since sample prep for the PAGE analysis involves reduction, some analyses were done without reduction and the results are the same (not shown) eliminating the possibility that one peak is a dimer. The first peak and second peak fractions of other runs were each dialyzed in a membrane of MW cutoff = 3500 and then freeze dried. The weights of the peaks were still very high (541 mg first peak and 2.49 g second peak), thus the PEG was not removed, probably due to a large water of hydration radius. More extensive dialysis in a membrane with larger pore size may be necessary to remove the PEG, if desired. The ubiquitin-like fusion protein may have two different conformations that result in different partition coefficients in this ATPS.

In Fig. 13 the SDS PAGE analysis of pooled fractions compared to the lysate indicates a significant purification over the lysate stage,

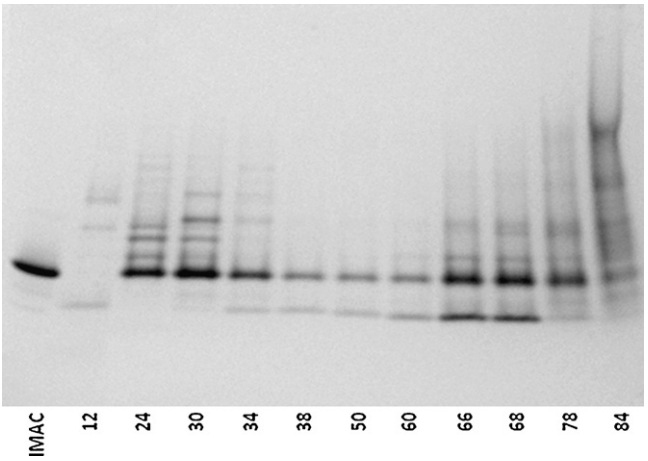

Fig. 12. PAGE analysis of the fractions of the spiral CCC chromatogram in Fig. 11. Aliquots of 60 μl were TCA precipitated and washed with acetone. The resulting pellets were suspended in SDS, reduced with DTT, boiled for 5 min. Subsequently, they were run in a 10–20% tricine gel and stained with Coomassie Blue. First lane is psalmotoxin–tosyn2 fusion protein purified by metal chelate chromatography.

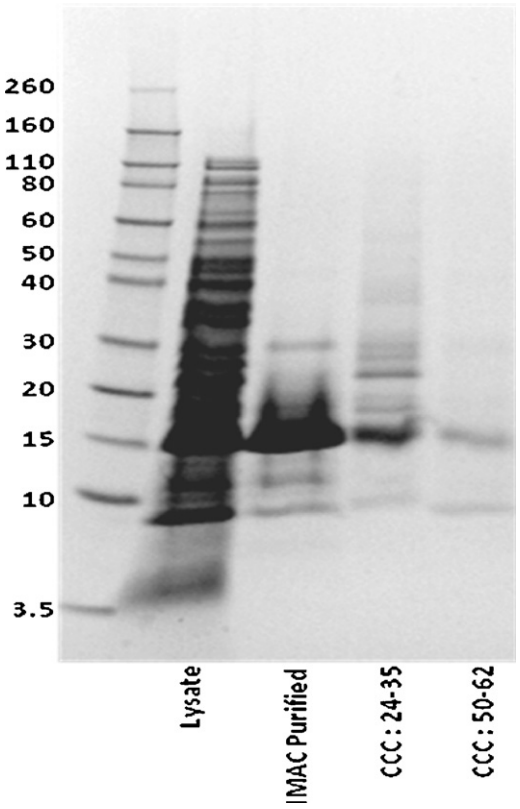

Fig. 13. PAGE analysis of pooled peaks of another spiral CCC separation of lysate compared to the lysate and IMAC purified fusion protein. First lane is MW standards; fourth lane is pooled first peak fractions (24–35) and last lane is pooled second peak fractions (50–62).

although the lysate and IMAC lanes are heavily loaded. These results indicate that the target protein can be purified out of the lysate in one step, although not purified to homogeneity, the protein will be digested with the cleavage enzyme and the final peptide will be isolated in another chromatography step. The appearance of two peaks was also observed in the separation of this fusion protein in the *n*-butanol-0.1 M potassium phosphate, pH 7.0 (1:1) solvent system.

4. Conclusion

A new useful device is introduced for laboratory preparative separations. Using laser sintering technology the spiral tubing support CCC separation rotor was manufactured with appropriate structural features. With computer aided design, various sized rotors with improved function can be innovated in a short timeframe. The STS rotor is found to retain well all of the types of two-phase solvent systems, making possible the chromatographic separation of small molecules in the organic-aqueous solvent systems and peptides in the more polar butanol solvent systems. To date small proteins of MW up to 15 kDa have been separated in the ATPS in spiral CCC. There remains more research to perform with larger MW proteins and recoveries of high biological activity [14]. Applying spiral CCC with the ATPS for one step isolation of a protein product out of a cell lysate while not achieving the purity of the affinity step did achieve significant removal of impurities and host cell proteins [15]. The spiral CCC isolated the fusion protein sufficiently pure for the next enzymatic release reaction from which the final product can be purified in another spiral CCC step. This approach is in development.

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