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ISOLATION OF SUTHERLANDINS A, B, C AND D FROM *SUTHERLANDIA FRUTESCENS* (L.) R.Br. BY COUNTER-CURRENT CHROMATOGRAPHY USING SPIRAL TUBING SUPPORT ROTORS

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Abstract

Spiral countercurrent-chromatography has great potential for improving the capacity and efficiency of purification of secondary metabolites, and here we describe applications useful for the isolation of flavonoids from the widely used South African medicinal plant, *Sutherlandia frutescens* (L.) R. Br. In the spiral tubing support rotor, STS-4 for high-speed counter-current chromatography, several polar butanol aqueous solvent systems were selected using a Log K plot, and the novel flavonol glycosides (sutherlandins A-D) were well separated by the optimized solvent system (ethyl acetate; *n*-butanol; acetic acid; water; 5:1:0.3:6 by vol.). The yield of purified flavonoids from 0.9 g extract varied from 8.6 mg to 54 mg of the sutherlandins for a total of 85.3 mg. The same extract was fractionated in the new STS-12 rotor of the same outside dimensions but with more radial channels forming 12 loops of the tubing instead of 4. The rotor holds more layers and increased length of tubing. From 0.9 g extract the STS-12 rotor yielded more recovery of 110.4 mg total with amounts varying from 11.2 mg to 64 mg of the sutherlandins. and apparent increased separation efficiency as noted by less volume of each fraction peak. Thus from 1-g amounts of extract, good recovery of the flavonoids was achieved in the butanol aqueous solvent system

Keywords

Spiral counter-current chromatography; STS-4 and STS-12 rotors; log K plots; solvent selection; flavonoids; Sutherlandins A-D

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1. Introduction

Recent developments of the spiral design rotors for counter-current chromatography (CCC) have made possible the use of the heavy alcohol solvents to fractionate and isolate in high yield the water-soluble polar small molecule metabolites in plant extracts [1–3]. The major metabolites from the widely used South African medicinal plant *S. frutescens*, are being investigated by many for biological activities [4,5]. Various components such as L-canavanine, pinitol, γ -amino butyric acid and the characteristic iridoid glycosides and cycloartane glycosides have been suggested to be responsible for some of the claimed benefits [4,5]. Previously we utilized the spiral tubing support column or rotor with 4 radial channels (STS-4) to fractionate an *n*-butanol extract, and isolated in high yield the cycloartane glycoside Sutherlandioside B [6,]. In the HPLC analysis of the *S. frutescens* extract, four other distinct metabolites were eluted before the sutherlandiosides, that were determined to be flavonoids, the sutherlandins A – D (Fig. 1) [7–10]. As the STS-4 rotor retains well the butanol aqueous 2-phase solvent systems (BTPS) in High Speed CCC, we considered that BTPS could be applied to fractionation of these even more water-soluble metabolites. The BTPS have been used in other natural product purifications [11–13]. Consequently, a new design of the STS rotor for CCC was developed and fabricated which we filled with 1.6 mm ID FEP tubing and applied to the separation of *S. frutescens* metabolites and compared with yield and separation efficiency of the original STS-4 rotor.

2. Materials and methods

2.1 Materials

Solvents used for spiral CCC and HPLC were analytical or chromatographic grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified in a Neu-Ion system (Baltimore, MD, USA). Milled, dried vegetative plant leaves and stems of *S. frutescens* was obtained from Big Tree Nutraceutical (Fish Hoek, So. Africa).

2.2 CCC instrument and STS rotors

A planetary centrifuge (CentriChrom, Inc. Buffalo, NY) was mounted with a spiral tubing support rotor (17 cm OD, STS-4, cat. no. 200-10001, CC Biotech, Rockville MD)[26] filled with 1.6 mm ID FEP (fluorinated ethylene-propylene) tubing (Zeus Industrial Products, Orangeburg, SC) as described previously [14]. An improved spiral rotor with 12 return channels, the STS-12 (Cat. No. 203-10001, CC Biotech, Rockville MD) was developed for the pilot analysis [15,16] and is shown in Fig. 2. The STS rotors are manufactured under a license from the National Institutes of Health [17–18]. The STS rotor has circular channels holding tubing that is wound in a spiral path to the center, skipping 3 concentric channels. The tubing is then guided from the center through a curved radial channel to the periphery then it continues in another circular channel to the center then goes through another radial channel to the periphery. After 4 spirals, the same path is followed on top of this layer and in the radial channels the tubing crosses the first layer of tubing in 4 spirals. In the STS-12 as seen in the cross-sectional drawing, each quad frame is divided into 2 more channels. Thus tubing is wrapped making 12 loops per layer and since less tubing is overlapping in each radial channel where it is pressed down, more layers are fit in the body of the rotor. In Fig. 3

there is also shown a pressing tool with prongs, which when inserted into the radial channels, flattens the tubing. This is used when manually filling the rotor with tubing. Flow still passes through the flattened FEP tubing. In filling the rotor the tubing is wound counterclockwise from a hole in the bottom at the outer rim and follows around the circular channel to the center where it follows a radial channel to the outer rim and follows another circular channel to the center having been guided through the first spiral. In one layer the tubing has made 4 interweaved spirals and in the STS-12, 12 interweaved spirals. The volume of the tubing in the STS-4 rotor was measured to contain 110 ml and the volume of the STS-12 rotor was determined to contain 138 ml. On the outside, the 1.6 mm ID (or 1/8 in ID) tubing from the rotor is connected to the inlet and outlet PTFE flow tubing, 1/32 in ID (Zeus Industrial Products, Orangeburg, SC) which pass into the rotor shaft and out the bottom into the central axis shaft of the planetary centrifuge and out the top of the chamber where they are clamped to prevent twisting. The other side of the planetary centrifuge arm has counterbalancing metal rings equal in weight to the rotor, placed the same distance from the center axis as described previously [19].

2.3 Counter-current separation procedure

An isocratic pump (D-Star Instruments, Manassas, VA) is connected to a 2-valve assembly; a 10-ml sample loop valve and another valve with helium gas for changing the flow to gas pressure to clear out the rotor contents. The sample is dissolved in 1 ml upper phase and 1 ml lower phase and loaded into the sample loop. The flow at 1 ml/min is started with the mobile phase, the centrifugation at 800 rpm is started and the sample injected into the flow tubing to the CCC instrument. The out-flow from the instrument central axis goes to a fraction collector (LKB, GE Healthcare, Piscataway, NJ) as described previously [19]. Fractions of 4 ml are collected until about 2 column volumes have eluted, then rotation is stopped and the column contents are expelled by He gas (with fractions collected). Aliquots are taken of fractions and diluted in 50% ethanol for manual absorbance determination (Cary 3E VC-Vis Spectrophotometer, Varian, division of Agilent, Santa Clara, CA). Fractions were dried by evaporation in a Thermo Savant SPD2010 SpeedVac System (Waltham, MA).

2.4 Extraction of milled *S. frutescens* powder

Amounts of the milled *S. frutescens* (30 g) were extracted for 24 hr with 300 ml methanol (MeOH), filtered and extracted twice again with 300 ml MeOH stirring for 4 hr. The volumes were combined and evaporated in a rotary evaporator to a syrup which was dissolved in 180 ml water. This was extracted with 180 ml hexane, then 180 ml chloroform, next with water saturated *n*-butanol, 135 ml, then 2 times with 90 ml of *n*-butanol. The water layer was acidified by adding 9 ml gl. acetic acid and then extracted 2 times with 90 ml *n*-butanol. The acidified *n*-butanol step served to partition well the flavonoids into the butanol phase. All the *n*-butanol extracts were combined and evaporated to a syrup which was dried under vacuum and weighed. The average yield of extract per 10 g powder was 1.2 g and extractions were performed multiple times to provide samples for chromatography.

2.5 HPLC analysis

The extract and CCC fractions were analyzed by reverse phase HPLC with a D-Star binary gradient pump with a manual 100 µl injector, Waters 486 variable UV detector, D-Star

Instruments StarChrom software for pump control and data analysis. An HPLC gradient protocol was used for analyzing the extract to determine the sutherlandins and sutherlandiosides (Fig. 4) and a shorter gradient for the sutherlandins, only (Fig. 5). The solvents used were A=0.1% aq. acetic acid; B=0.1 % acetic acid/acetonitrile and flow was at 1 ml/min on a YMC 5 μ , C-18 15 $\times$ 0.46 cm column (YMC America, Allentown, PA). For analysis of the combined isolated flavonoids shown in Fig. 13, the same column and elution conditions were used with Shimadzu instrumentation (LC-10AT VP pump, low pressure gradient and a DGU-14A degasser, SPD-10A VP UV-Vis detector, SCL-10AD VP system controller and EZ-Chrom software and computer for operation and data analysis). To detect all the compounds, detection at 220 nm was used; when only the flavonoids were being analyzed in shorter gradient times, detection was at 260 nm.

2.6 Nuclear magnetic resonance analysis

NMR analysis of the separated peaks was analyzed on a Bruker AVIII 600 MHz (sample 1, 2 and 3) and AVIII 800 MHz (sample 4), both operating with a cryogenically cooled HCN probe. The samples were dissolved in methanol-d₄ and analyzed at 25 °C. Chemical shift scale was calibrated by setting the solvent peak at 3.3 PPM.

3 Results and Discussion

3.1 Solvent system selection

In order to choose a CCC solvent system to separate the flavonoids, we prepared different solvent systems and dissolved the dry extract sample in equal volumes of the two phases of a solvent system. The concentrations in the upper phase and in the lower phase were measured by HPLC (Fig. 5), and the ratio of the peaks [peak heights] give the partitioning of each compound in the solvent system. Solvent systems with the most differences in the values of the partition coefficients [K, conc.UP/conc.LP] of the four peaks are likely to best separate the compounds [20].

Solvent systems were mixed by combining the solvents in the volume ratios shown in Table 1, and the designations in column 1 are plotted as s1, s2, etc. in Fig. 6. Analysis of the log K plots give solvent systems s5 and s7 as showing the maximal differences among the K values.

3.2 CCC separations using the STS-4

Initial CCC experiments were performed with the solvent system made of *n*-butanol, gl. acetic acid, and water (BAW). After trying s5 (bold in Table 1) we selected the solvent system BAW (4.75:0.25:5) with the conditions of the upper phase mobile, such that the compounds would come out around a column volume and after the solvent front. In the results shown in Fig. 7 A, however, lots of UV absorbing material came out in the early fractions after the solvent front at tube 20 and the flavonoids came out mixed in the fractions after 30. When run in the opposite elution mode, lower phase mobile, the flavonoids were too highly retained (Fig. 7 B) and eluted between fractions 161 to 196, with poorly resolved peaks 1 with 2 and peaks 3 mixed with 4. Consequently, the solvent system was changed so that the flavonoids were eluted with the lower phase mobile. An experiment with EBAW

[E=ethyl acetate] (Figure 8) shows that the CCC peaks correlated with flavonoid peaks, except for peak 4. The inclusion of the ethyl acetate served to drive the compounds to the lower phase enough so that in the opposite elution mode, lower phase mobile, the compounds came out after the solvent front and separated.

3.3 Separation comparing STS-4 and STS-12 rotors

It was decided to alter the EBAW solvent system (s4 in Table 1) by increasing ethyl acetate relative to *n*-butanol. The concentration of acetic acid was the same as s7. In running with the lower phase mobile, 0.87 g extract was chromatographed as shown in Fig. 9 (A). With these conditions all 4 flavonoid peaks were separated after the solvent front and pure amounts 8.6 to 54 mg were recovered of each as shown in Table 2. In Fig 10 are shown some of the HPLC analyses of fractions identifying the separated peaks.

A similar fractionation experiment was run in the newly available STS-12 rotor (Fig. 9 (B)). Analyses of fractions of each flavonoid peak (Fig 10) indicated this achieved successful fractionation of each compound, and highly purified and semi-purified fractions were dried down (Table 2). The structures of these compounds were verified by UV-Vis spectra (Fig. 11) and 1-H NMR spectra (Fig. 12) using literature values [8].

The solvent fronts of both runs indicated a stationary phase retention of 63%, which is very high for this type of solvent system. Even with the increased volume of the STS-12 rotor due to the larger number of tubing layers, the elutions are very similar. Although the flavonoids were well separated in both experiments, the highly purified fractions of the STS-12 run were identified in fewer fractions (28% lower volume) than the STS-4 run; results in Table 2. The high stationary phase retention of the spiral rotors and large difference in K of the solvent system contribute to higher resolution or α obtained with these rotors. The recovery of the highly purified fractions in STS-12 was more than that of the STS-4 rotor, which means the efficiency of the STS-12 rotor is higher. The total amount of flavonoids recovered by the STS-4 was 85 mg whereas from the STS-12 it was 110 mg, (Table 2).

A disadvantage of the STS-12 rotor is that it is more difficult to fill with the tubing; directing tubing to the correct radial channel is difficult. But with practice the process can be readily achieved. Recently, another rotor has been made for smaller OD tubing that has 12 separate channels (not the channels coming from 4 spirals as done here) and good results were obtained with this more analytical-scale rotor [21].

Samples of the dried pooled fractions of the run of STS-4 were combined in 200 μ l water, diluted 1:3 and samples analyzed by HPLC, indicating four peaks separated, confirming isolation of each flavonoid (Fig. 13). The partition coefficients of the purified flavonoids were determined in HPLC experiments in the solvent system EBAW (5:1:0.3:6) used in the CCC and designated as s10 in Table 1. Fig. 14 is the plot of the log K values of all the solvent systems and placing s10 between s3 and s4 shows the K values indicate a suitable solvent system. For the STS rotors in general it is best to have the K values span 0.4 to 1.0 and not necessarily go higher.

2 Conclusion

A goal of the project is to develop methods for chromatography of small molecules that are more water soluble and less likely to be successfully separated by more established techniques, such as prep HPLC or currently marketed CCC rotors. These results provide suitable conditions to use spiral CCC to separate the flavonoids/sutherlandins of *S. frutescens*, and this is likely to be true for other similar metabolites of plants. For better recoveries of highly pure compounds, the side fractions of many runs could be pooled and re-chromatographed. The CCC method using the spiral tubing support rotors to chromatograph extracts allows isolation of large quantities of the four flavonoids present. The results with the STS-12 warrant further experimentation. With the next version of the spiral CCC instrument that is scaled up to >0.9 liter volume, it should be possible to chromatograph in single runs, 10 g perhaps, to isolate the bio-active metabolites that are present in low concentrations.

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Large scale chromatography of natural products extracts

- 2** Isolation of polar small molecule metabolites by polar heavy alcohol solvents in CCC
- 3** Log K plot for solvent selection
- 4** Improved STS CCC rotor for increased loading capacity and efficiency

Fig. 1. Structures of the Sutherlandins; Sutherlandin A (peak 1), Sutherlandin B (peak 2), Sutherlandin C (peak 3), Sutherlandin D (peak 4).

Fig. 2.

A Drawing of a fully assembled STS rotor with bearing blocks on top and bottom, gear below the top bearing block, the cover affixed with screws on to the body. On the cover are loops for tubing and a hold for tubing fittings union. B STS-4 rotor body with circular channels that hold the tubing. The curved radial channels guide the tubing between the center and outer channel and make 4 loops. C Rotor body for the STS-12, with 12 curved radial channels. The center has a more open space for the tubing.

Fig. 3.
Photo of the STS-12 rotor 6 7/8"OD rotor assembled [right] and tubing pressor [left] used to compress tubing in the radial channels when filling rotor body with the flow tubing (1.6mm ID FEP). Both items are made by laser lithography. The pressing tool has prongs to fit the radial channels of the rotor body. The tool for the STS-4 has 4 protrusions (not shown).

Fig. 4.
HPLC analysis of the *n*-butanol extract. The conditions of HPLC are described in the text.
Detection is at 220 nm. Gradient conditions: 0–10min, 5–10%B; 10–25min, 10–15%B; 25–55min, 15–35%B; 55–85min, 35–80%B; 85–90min, 80–100%B; 95–100min, 100–5%B.
Absorbance to 80 min shown.

Fig. 5. Measurement of K of individual flavonoids in a solvent system by HPLC. The ratio of the peak heights = K. The gradient used is 0–10min, 5%B; 10–40min, 5–15%B; 40–50min, 15–35%B; 50–65min, 35–80%B; 65–70min, 80–100%B; 75–80min, 100–5%B.

Fig. 6. Selection of solvent systems for the sutherlandins. The log K of each peak of a solvent system from Table 1 is plotted.

Sp CCC of *Suth. f.* extract **Fig. 7.**

Counter-current separation of the extract in the *n*-butanol, gl. acetic acid, water (4.75:0.25:5) in both elution modes. In A the upper phase was pumped into the rotor bottom in the tail to head direction, CW rotation. The solvent front at fraction 20 is indicated and the rotation is stopped at fraction 91 and contents are extruded. The flavonoids came out in fractions 30 to 40 not well separated. In B the lower phase was used as the mobile phase pumped into the top in the head to tail direction, CW. The flavonoids came out late from fraction 161 to 196 and were not completely separated. Further details in the text section 3.2.

Fig. 8.

Separation results with another solvent system and elution mode. EBAW (4:1:0.25:5) with the lower phase mobile into the top, head to tail direction at a flow of 1 ml/min and 840 RPM (CW) The pure flavonoids were found as peak 1 [82–92](fractions); peak 2[116–118]; peak 3[150–174].

Fig. 9.

CCC chromatograms of extract in the STS-4 rotor (A) and STS-12 rotor (B). The pure fractions of the peaks 1 to 4 are shaded and identified below the graphs. In STS-4, fractions identified to have pure flavonoid; peak 1[55–63]; peak 2[72–83]; peak 3[102–113]; peak 4[165–181] and in the STS-12 pure fractions are peak 1[58–65]; peak 2[71–79]; peak 3[98–104]; peak 4[166–177]. The solvent system EBAW (5:1:0.3:6) was used with the LP mobile into the top in the head to tail elution mode at a flow of 1 ml/min and at 800 RPM (CW).

STS-4**Pk 1****Pk 2****Pk 3****Pk 4** **STS-12** **Fig. 10.**

HPLC analysis of fractions of the CCC runs of Fig. 11. The HPLC was conducted as described in the legend of Fig. 5 with detection at 260 nm. For the STS-4 CCC run peak 1 is fraction 55, peak 2 is fractions [81–83], peak 3 is [102–110] and peak 4 is [165–170]. For the STS-12 CCC run, peak 1 is fraction 64, peak 2 is fraction 76, peak 3 is fraction 100 and peak 4 is [166–177].

Fig. 11.

UV scan of separated peaks from the STS-12 CCC run. Samples of the dried pooled peaks were dissolved in water and spectra from 220 nm to 400 nm were recorded in instrument described in section 2.3.

Fig. 12.

NMR spectra plots in scales A region to the left of water, B region to the right of water. The stacking order is peak 1, 2, 3, and 4 starting from the bottom. The NMR was acquired on a Bruker AVIII 600 MHz (sample 1, 2 and 3) and AVIII 800 MHz (sample 4), further details in section 2.6.

Fig. 13. Analysis of the reconstituted separated peaks in the gradient conditions of Fig. 5 with the same solvents and column. The instrument and software used was Shimadzu equipment as described in section 2.5.

Fig. 14.

The log plot of the K values of the solvent systems, including the solvent system s10 developed in this study.

Table 1

Solvent system	Volume ratio	Peak 1	Peak 2	Peak 3	Peak 4
EBMW (S1)	15:1:3:15	0.039	0.078	0.087	0.086
BW (S2)	1:1	0.072	0.101	0.174	0.242
SecBW (S3)	1:1	0.135	0.228	0.334	0.355
<i>EBAW (S10)</i>	<i>5:1:0.3:6</i>	<i>0.405</i>	<i>0.715</i>	<i>0.810</i>	<i>1.100</i>
EBAW (S4)	1:9:0.1:10	0.761	0.793	0.949	1.746
BAW (S5)	5:0.05:5	0.602	0.614	0.949	1.800
SecBAW (S6)	5:0.05:6	0.594	0.683	0.915	1.159
BAW (S7)	4.75:0.25:5	0.649	1.342	1.869	1.608
BAW (S8)	4:1:5	0.744	1.187	1.497	1.750
B 0.1% aq.TFA (S9)	1:1	0.953	1.007	1.310	2.029

Table 2
Mass Recovery of Purified Sutherlandins from 0.9 g Extract and Comparison of Peak Volumes

Flavonoid	STS-4 Rotor			STS-12 Rotor		
	Yield (mg)	Fractions pooled	ml	Yield (mg)	Fractions pooled	ml
Sutherlandin A	8.6	55–63	36	64	58–65	32
Sutherlandin B	54	72–83	48	22	71–79	36
Sutherlandin C	13.6	102–113	48	13.2	98–104	28
Sutherlandin D	9.1	165–181	68	11.2	166–177	48
Total	85.3		200	110.4		144