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Evaluation of Analytical Countercurrent Chromatographs: High-Speed Countercurrent Chromatograph-4000 Vs. Analytical Toroidal Coil Centrifuge

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EVALUATION OF ANALYTICAL COUNTERCURRENT CHROMATOGRAPHS: HIGH-SPEED COUNTERCURRENT CHROMATOGRAPH-4000 VS. ANALYTICAL TOROIDAL COIL CENTRIFUGE

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ABSTRACT

Performance of two countercurrent chromatographic models, high speed countercurrent chromatograph (HSCCC-4000) and analytical toroidal coil centrifuge (TCC), is evaluated in terms of theoretical plate number, resolution factor and separation time to assess their analytical capability. A series of experiments was conducted to investigate the effects of internal diameter and length of the coiled column, and flow rate of the mobile phase on the separation of indole auxins in two-phase solvent systems composed of n-hexane-ethyl

acetate-methanol-water at different volume ratios. The three components of indole auxins were completely resolved in 16 min with a HSCCC system equipped with a multilayer coil of a 0.55 mm I.D. PTFE tube with theoretical plates ranging from 1290 to 829. Similar separation was achieved in 24 min with a TCC systems equipped with a 0.3 mm I.D. PTFE tube with theoretical plates ranging from 1811 to 969. It is concluded that both systems have comparable analytical capability at the present stage of development.

INTRODUCTION

Countercurrent chromatography (CCC) has many desirable features for performing analytical separations, because it eliminates various complications such as sample loss, tailing of solute peaks, contamination, etc., all arising from the use of solid supports. However, due to the relatively long separation times required, this method has been used primarily for preparative separations of natural products [1,2].

Existing CCC instruments may be classified into two categories, i.e., hydrodynamic and hydrostatic systems [3]. High-speed countercurrent chromatograph (HSCCC), the most advanced form of hydrodynamic CCC system, can yield high resolution in short retention times. On the other hand, the toroidal coil centrifuge (TCC), a typical hydrostatic CCC system, has two major advantages over HSCCC in that (1) the system permits universal application of two-phase solvent systems and (2) gives stable elution curves under UV monitoring.

In this paper, performance of two CCC models, HSCCC-4000 and analytical TCC, is evaluated in terms of theoretical plate

number, resolution factor and separation times to assess their analytical capabilities. A series of experiments was conducted to investigate the effects of internal diameter and length of the coiled column, flow rate of the mobile phase on the separation of indole auxins with two-phase solvent systems composed of n-hexane-ethyl acetate-methanol-water at different volume ratios.

EXPERIMENTAL

Apparatus

The analytical HSCCC used for the present study is a multi-layer coil planet centrifuge which produces a synchronous planetary motion at 4000 rpm. The design of the apparatus has been described previously [4] and is briefly given below. Fig. 1 shows a photograph of our apparatus. The HSCCC centrifuge holds a column holder and counterweight holder symmetrically on the rotary frame at a distance of 2.5 cm from the central axis of the centrifuge. The column holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. Three different types of multilayer coiled separation columns were prepared from 0.85 mm I.D., 0.55 mm I.D., and 0.4 mm I.D. thick-wall (0.45 mm) PTFE (polytetrafluoroethylene) tubes (Zeus Industrial Products, Raritan, NJ, USA) by winding each tube directly onto the column holder with a 2.5 cm hub diameter making 4 coil layers with the total capacity of 8, 5, and 1.5 ml, respectively.

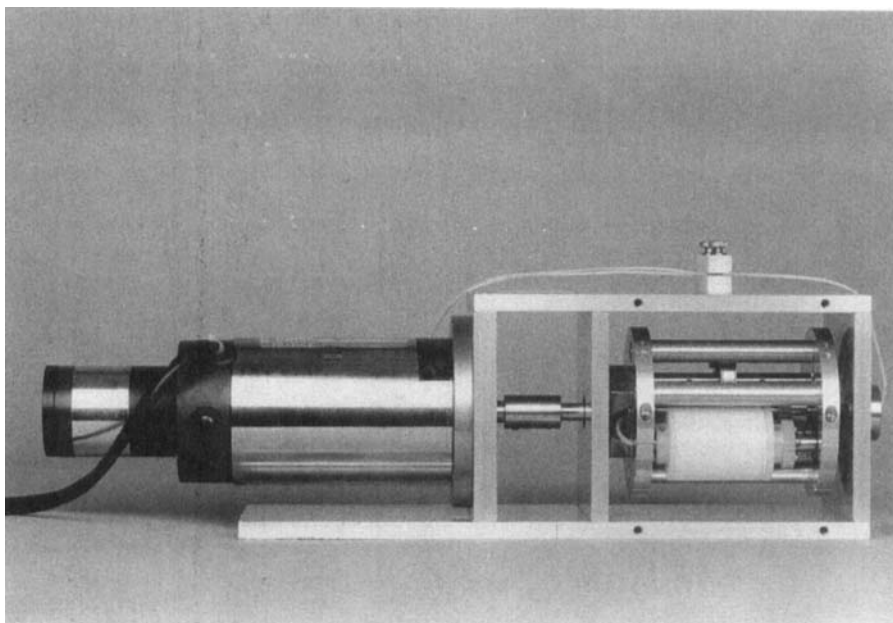


Fig. 1 Photograph of HSCCC-4000.

Fig. 2 shows a photograph of the analytical TCC used for the present study. The design of this apparatus has been already reported previously [5,6] and is briefly described here. The rotor frame consists of three parallel horizontal plates rigidly linked together and holds a column container, a counterweight, and a tube holder. When the frame is driven by the motor shaft at an angular velocity ω , a set of gears and toothed pulleys coupled with toothed belts causes rotation of the column container at 2ω and counterrotation of the tube holder around its own axis at ω . The toroidal

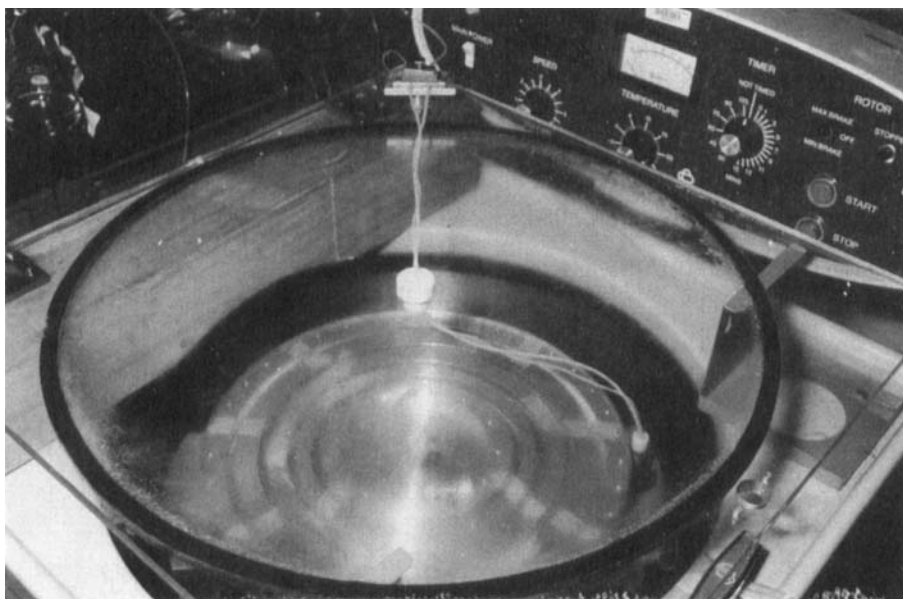


Fig. 2 Photograph of analytical TCC.

column was accommodated around the periphery of the column container at the distance of 17 cm from central axis of the centrifuge. Four columns were prepared by winding 0.55 mm I.D., 0.4 mm I.D., 0.3 mm I.D., and 0.1 mm I.D. PTFE tubes onto 1.5 mm I.D. nylon tubes to make desired helical turns. Except the 0.3 mm I.D. column, all toroidal columns were made from thick wall (0.45 mm) PTFE tubes.

Reagents

All organic solvents, including n-hexane, ethyl acetate, and methanol, were glass-distilled chromatographic grade and purchased from Burdick and Jackson Laboratories Inc., Muskegon, MI, USA.

Indole-3-acetamide (IA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Preparation of two-phase solvent system and sample solution

Two phase solvent systems composed of n-hexane-ethyl acetate-methanol-water at the various ratios (3:5:3:5, 4:5:4:5, and 1:1:1:1) were used in the present study. Each solvent system was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking and degassing at room temperature and two phases were separated before use.

The sample solution for analytical separations was prepared by dissolving 5 mg of IA, 15 mg of IAA, and 15 mg of IBA in 3 ml of the nonaqueous upper phase of the above solvent system.

Procedure for analytical separation of indole auxins

The columns were first entirely filled with the upper non-aqueous stationary phase. Then, the centrifuge was rotated at the optimum speed of 3500 rpm for HSCCC, and 1000 rpm for TCC, while the mobile phase was pumped into the head of the column for HSCCC, or one end of the column for TCC (the hydrostatic equilibrium system has no head-tail orientation of the column) at the

desired flow rate by a Shimadzu Model 6A HPLC pump (Shimadzu Corp., Kyoto, Japan). After a steady state hydrodynamic or hydrostatic equilibrium was reached, 5-20 μl of the sample solution were injected with a microsyringe via a Rheodyne Model 7125 syringe loading sample injector. Effluent from the outlet of the column was continuously monitored by the absorbance at 260 nm using a Shimadzu Model SPD-6A detector and a Pharmacia 482 recorder (Pharmacia, Uppsala, Sweden).

Measurement of partition coefficients

In the present paper, the partition coefficient is expressed in two different ways, i.e., $K(U/L)$ (solute concentration in the upper phase divided by that in the lower phase) or $K(S/M)$ (solute concentration in the stationary phase divided by that in the mobile phase). The partition coefficient for each component was determined as follows: Approximately 0.1 ml of methanol containing about 1 mg of the test sample was evaporated with an air stream in a 13 mm diameter culture tube. A 2 ml volume of each phase of the preequilibrated two-phase solvent system was pipetted into the test tube. The test tube was stoppered with a teflon-lined cap and shaken vigorously for 1 min to thoroughly equilibrate the sample with the two phases. A 0.5 ml volume of each layer was transferred to the second test tube containing 2 ml of methanol, followed by thorough mixing of the contents. The absorbance of each solution was determined at the suitable wavelength of 280 nm with a Zeiss PM6 spectrophotometer. The partition coefficient is given by the ratio of the two absorbances measured.

RESULTS AND DISCUSSION

Analytical HSCCC-4000

The HSCCC technique utilizes a particular combination of coil orientation and planetary motion to produce a unique hydrodynamic effect to form a unilateral phase distribution of two immiscible solvents in a coiled column [7]. HSCCC with a 2.5 cm revolutional radius and a 0.85 mm I.D. column is capable of operating at a maximum speed of 4000 rpm. This represents the most advanced model for analytical work [4]. When indole auxin mixture (IA, IAA, and IBA) was initially separated by HSCCC, the theoretical plate numbers for the three peaks showed low values of 373 (IA), 309 (IAA), and 247 (IBA), respectively (Table I). We considered that the use of a smaller I.D. PTFE tube is essential to improve peak resolution. However, when the column and its flow tubes were made of a single continuous piece of the standard-wall (0.3 mm wall thickness) PTFE tubing narrower than 0.85 mm I.D., we observed that the flow tube repeatedly broke at the point leading to the column shown by an arrow in Fig. 3. This is apparently due to an increased back pressure acting on the sharp flexing portion of the flow tube undergoing a steady rolling motion. This problem was solved by employing a 0.45 mm wall thickness PTFE tubing which provided a satisfactory operation under a high pressure up to 50 kg/cm². The chromatograms of indole auxins obtained from the multilayer coil with various I.D.s are shown in Fig. 4. The theoretical plate numbers, resolution factor between IA and IAA, analysis time and

Table I
Chromatographic evaluation of HSCCC-4000 under various analytical conditions

Conditions	Theoretical plate number		Resolution factor between peaks IA and IAA	Analysis time (min)	Retention of stationary phase (%)	Chromatogram
	IA	IAA				
Tube I.D.: 0.85 mm Column capacity: 8.0 ml Flow rate: 0.5 ml/min	373	309	247	1.71	16	54.0
Tube I.D.: 0.55 mm Column capacity: 5.0 ml						
Flow rate: 0.3 ml/min	1290	1068	829	2.06	16	55.3
Flow rate: 0.6 ml/min	1129	773	683	1.63	8	48.3
Tube I.D.: 0.40 mm Column capacity: 1.5 ml						
Flow rate: 0.1 ml/min	943	719	603	1.86	18	48.8
Flow rate: 0.2 ml/min	784	692	576	1.00	9	37.6

Solvent systems were composed of n-hexane, ethyl acetate, methanol, and water at a volume ratio of 1:1:1:1.
Mobile phase: lower phase.

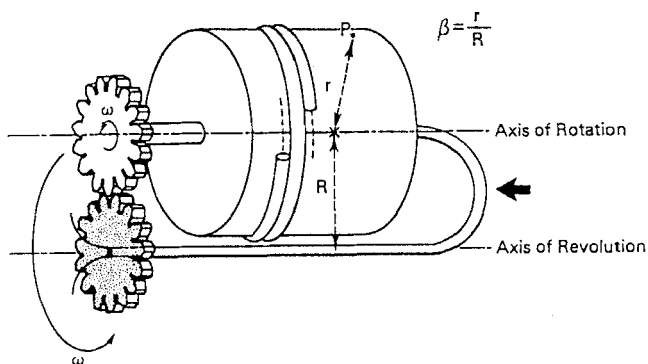


Fig. 3 Design of the HSCCC-4000.

A)
I.D.: 0.55 mm
Flow rate: 0.3 ml/min

B)
I.D.: 0.55 mm
Flow rate: 0.6 ml/min

C)
I.D.: 0.40 mm
Flow rate: 0.1 ml/min

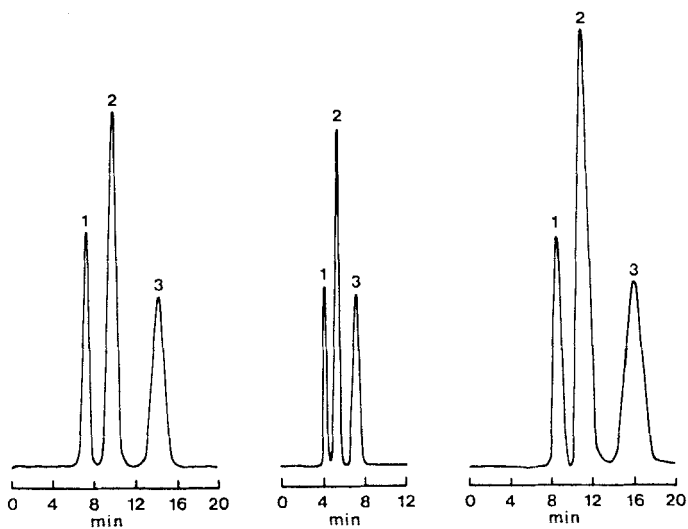


Fig. 4 Chromatograms of indole auxins by HSCCC-4000.
Key: 1, IA; 2, IAA; 3, IBA
Analytical conditions A, B, and C: See Table 1.

experimental conditions are summarized in Table I. The theoretical plate number, N , of each peak was calculated according to the following equation:

$$N=(4Tr/W)^2$$

where Tr is the retention time, and W is the peak width in the same unit as Tr . Resolution factor, R_s , was computed by the following equation:

$$R_s=2(Tr_1-Tr_2)/(W_1+W_2)$$

where Tr_1 and Tr_2 are the retention times, and W_1 and W_2 are the corresponding peak widths expressed in the same unit as Tr_1 and Tr_2 .

With regard to the internal diameter of the column, the best result was obtained from the 0.55 mm I.D. column eluted at a flow rate of 0.3 ml/min under the present experimental conditions. The 0.4 mm I.D. column produced less stationary phase retention (<50 %) and considerably lower values for both theoretical plate number and resolution factor even at a reduced flow rate of 0.1 ml/min.

The flow rate of the mobile phase greatly affects the stationary phase retention in the column. Better retention is obtained with a lower flow rate but at the expense of longer analysis time. The results of our studies indicate the optimum flow rate is 0.3-0.6 ml/min for the 0.55 mm I.D. column and 0.1 ml/min for the 0.4 mm I.D. column.

Partition coefficient is one of the most important factors in determining retention time and resolution of the solute peaks. In general, when retention of the stationary phase substantially exceeds

Table II

Partition coefficients of indole auxins

Solvent system ^{a)}	Partition coefficients ^{b)}		
	IA	IAA	IBA
1:1:1:1	0.07	0.37	0.87
4:5:4:5	0.15	0.88	2.37
3:5:3:5	0.33	2.20	6.40

a) Solvent systems were composed of *n*-hexane, ethyl acetate, methanol, and water at the indicated volume ratios.

b) K(U/L).

50% of the total column capacity, the best separation can be attained with the partition coefficient, $K(S/M)$, between 0.5 and 1. When the stationary phase retention is lower than 50 %, the peak resolution is effectively improved by higher partition coefficient values ranging between 1 and 2. Partition coefficients of indole auxins were determined in the *n*-hexane-ethyl acetate-methanol-water solvent systems by varying the volume ratios to 1:1:1:1, 4:5:4:5, and 3:5:3:5 (Table II). In the *n*-hexane-ethyl acetate-methanol-water (1:1:1:1) solvent system, $K(U/L)$ values of IA, IAA, and IBA were 0.07, 0.37, and 0.87, respectively. When the upper nonaqueous phase was used as a stationary phase, the retention of the stationary phase was over 50 %. Therefore, we have chosen the above solvent systems for HSCCC in the present study. However, if retention of the stationary phase becomes lower than 50 % in a smaller I.D. column, the solvent system should be modified so that the $K(S/M)$ value of the sample become 1.0 or greater.

The 0.55 mm I.D. column eluted at a flow rate of 0.3 ml/min produced the best results among the present experimental conditions as shown in Fig. 4A, the indole auxins were separated in 16 min with high theoretical plates ranging from 1290 to 829 and a resolution factor of $R_s=2.06$ between peaks IA and IAA. Increasing the flow rate to 0.6 ml/min shortened the analysis time to 8 min without an appreciable loss in peak resolution (Fig. 4B). This represents an analytical condition suitable for routine analysis. The combination of the 0.4 mm I.D. column and a 0.1 ml/min flow rate yielded an excellent peak resolution in 18 min (Fig. 4C). This analytical condition is most suitable for interfacing with mass spectrometer, which requires an extremely low flow rate of the mobile phase.

Analytical TCC

As described earlier, TCC is a typical hydrostatic equilibrium system and has a potential capability of yielding a high partition efficiency. The use of a toroidal coil minimizes longitudinal sample band broadening by the presence of a pair of interfaces in each helical turn together with the mild mixing effect of the two solvent phases under a stable centrifugal force field. In addition, the system permits universal application of two-phase solvent systems and provides stable UV-tracing of the elution curves. However, the system has a limited capacity of the stationary phase retained in the column (less than 50 % of the total column capacity) and usually produces a high back pressure much greater than that in HSCCC for a unit length of the column. Consequently, the limiting factor of

the TCC system is a mechanical strength of the separation coil and flow tubes to withstand a high hydrostatic pressure which linearly increases with the column length and the applied centrifugal force field [5].

The evaluation of the TCC system was initiated with a relatively large bore column (0.55 mm I.D. and 4440 helical turns) to limit the column pressure while applying a high flow rate of 1.5 ml/min. Under this experimental condition, the toroidal coil maintained its integrity and the indole auxins were separated in 9 min with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (4:5:4:5, v/v/v/v). However, partition efficiencies obtained from the above separation were relatively low in terms of both theoretical plates and peak resolution as shown in Table III. In order to improve the above results, we prepared a new toroidal coil from a thick-wall (0.45 mm), smaller I.D. (0.4 mm) PTFE tube as used for the multilayer coil in HSCCC described earlier. Although the 0.4 mm I.D. column successfully held a high back pressure, the precolumn flow tube (which was made from the same tube as in the column) frequently broke at the flexing points as indicated by arrows A and B in Fig. 5. A similar problem was observed with the use of a PEEK (polyetheretherketone) tube (Alltech Associates, Inc., Deerfield, IL, USA) which can hold a high back pressure up to 350 kg/cm² in ordinary use. This clearly indicated that the precolumn flow tube also requires a good flexibility to withstand the constant flexing and rolling motion which is inherent to the rotary-seal-free flow-through mechanism.

Table III Chromatographic evaluation of analytical TCC under various analytical conditions

Conditions	Theoretical plate number		Resolution factor between peaks IA and IAA	Analysis time (min)	Retention of stationary phase (%)	Chromatogram
	IA	IAA IBA				
Tube I.D.: 0.55 mm Column capacity: 9.5 ml Flow rate: 1.5 ml/min Helical turns: 4440 Solvent system: B	1972	900 219	1.11	9	22.1	Fig. 6A
Tube I.D.: 0.40 mm Flow rate: 1.0 ml/min Solvent system: B	1849	911 350	1.00	7	12.5	Fig. 6B
Helical turns: 4140 Column capacity: 8.0 ml	2960	1600 682	1.21	11	12.5	Fig. 6C
Helical turns: 6540 Column capacity: 12.0 ml						
Tube I.D.: 0.30 mm Helical turns: 10000 Column capacity: 7.0 ml Flow rate: 1.0 ml/min	799	412 183	2.48	36	-	
Solvent system: A	1811	1849 969	1.64	24	-	Fig. 6D
Solvent system: B	1872	1296 652	0.86	12	-	
Solvent system: C						

Solvent systems were composed of n-hexane, ethyl acetate, methanol, and water at a volume ratio as follows:
 Solvent system A: 3:5:3:5, B: 4:5:4:5, and C: 1:1:1:1.
 Mobile phase: lower phase

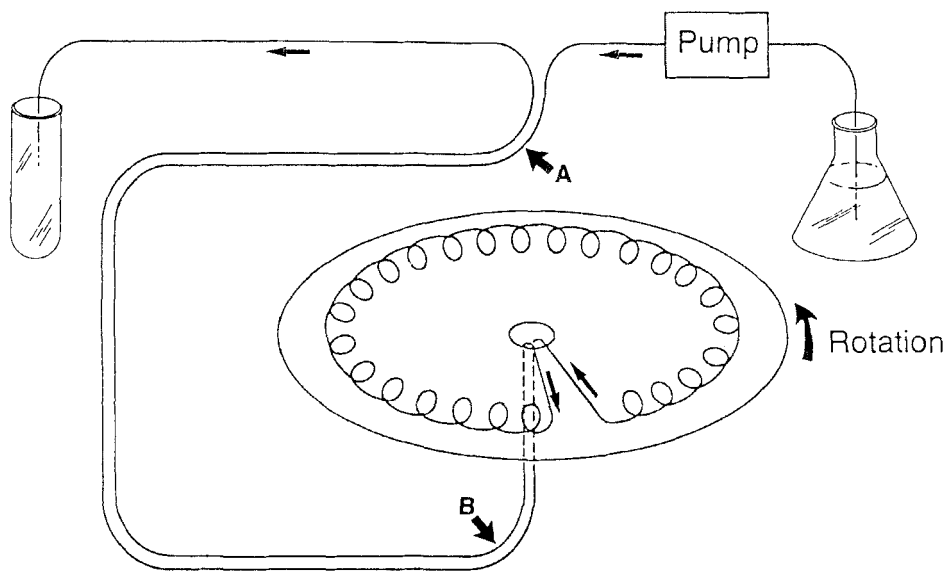


Fig. 5 Schematic design of analytical TCC.

Finally, we have tested Tefzel tubes (Alltech Associates, Inc.) which provide an excellent pressure resistance ($175\text{--}280\text{ kg/cm}^2$) and much higher flexibility than the PEEK tube. Among four commercially available Tefzel tubes with 0.175, 0.3, 0.5, and 0.75 mm I.D.s and all with 0.56 mm O.D., only the smallest I.D. tube (0.175 mm) with the greatest wall thickness could successfully maintain its integrity for several consecutive runs. In order to further improve the durability of the Tefzel tube, the centrifuge cover was raised from a distance of 10 cm and 20 cm above the centrifuge bowl to moderate the flexing angle of the flow tube at point A shown in Fig. 5, while the entire length of the Tefzel tube (precolum flow

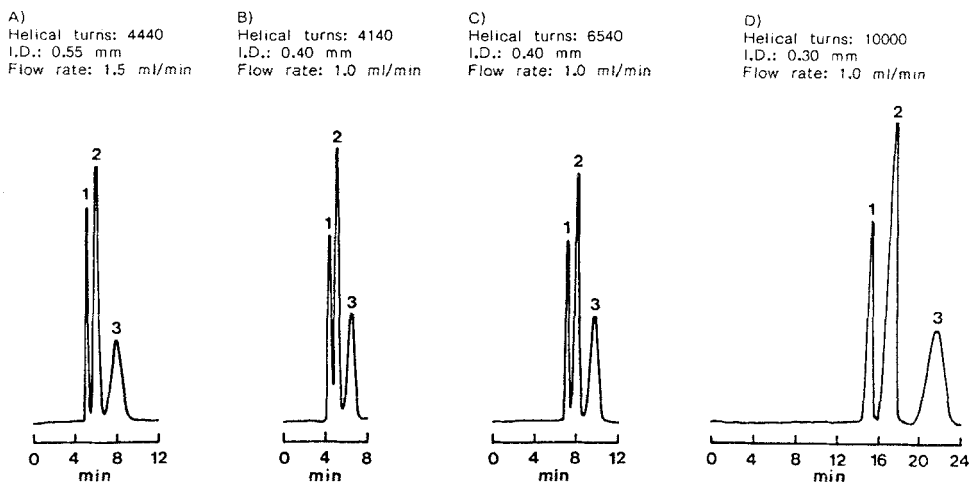


Fig. 6 Chromatograms of indole auxins by analytical TCC.
Key: See Fig. 4.
Analytical conditions A, B, C, and D: See Table III.

tube) was reinforced with a single piece of olefin heat shrinkable tubing (Alpha Wire Corporation, Elizabeth, NJ, USA). With the above modification, we have successfully carry out a series of experiments under a high column pressure of up to 100 kg/cm^2 .

Figs. 6A-D show a set of TCC chromatograms of indole auxins obtained under various analytical conditions. The experimental conditions including column I.D., number of helical turns, solvent system and flow rate, and the data on theoretical plates, resolution factors, and analysis times for each experiment are summarized in Table III.

When the results are compared from two different I.D. columns with a similar number of helical turns, the smaller I.D.

column (0.4 mm) produced lower stationary phase retention and slightly less efficient peak resolution between peaks IA and IAA than the 0.55 mm I.D. column, while both columns yielded equivalent theoretical plates. However, increasing in the number of helical turns of the 0.4 mm I.D. column from 4140 to 6540 substantial improved both the theoretical plates and peak resolution but with about 50 % increase in the separation time (Fig. 6C). We have further examined a 0.3 mm I.D. and a 0.1 mm I.D. columns each with 10000 helical turns. The 0.3 mm I.D. column produced excellent peak resolution in 24 min (Fig. 6D) whereas the 0.1 mm I.D. column failed to retain a satisfactory volume of the stationary phase under the applied experimental condition.

The above experiments were performed with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (4:5:4:5, v/v/v/v) which gave partition coefficient values, $K(S/M)$, for the indole auxins ranging from 0.15 to 2.37 (Table II). As mentioned earlier, TCC produces lower retention of the stationary phase and necessitates the choice of higher $K(S/M)$ values in contrast to HSCCC which can produce much higher retention of the stationary phase.

The above experimental results with TCC indicate that efficient separations of indole auxins can be attained with a coiled column of either 0.55 or 0.40 mm I.D. in 10 min whereas the highest peak resolution ($R_s=1.64$ between IA and IAA) is obtained from a small-bore column of 0.3 mm I.D. in a longer elution time in 24 min.

CONCLUSION

Although TCC needs higher pressure to pump the mobile phase and longer analysis times than HSCCC, it has advantages over HSCCC in that the system permits universal application of two-phase solvent systems and gives stable elution curves under UV monitoring. In the present study, HSCCC and TCC showed almost equivalent theoretical plates and resolution factors in the separation of indole auxins under their optimal conditions. Therefore, we have demonstrated that at the present stage of development both systems are capable of performing comparable analytical tasks. However, it is more likely that TCC will become a powerful analytical tool in the future when the problems associated with high back pressure are solved.

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