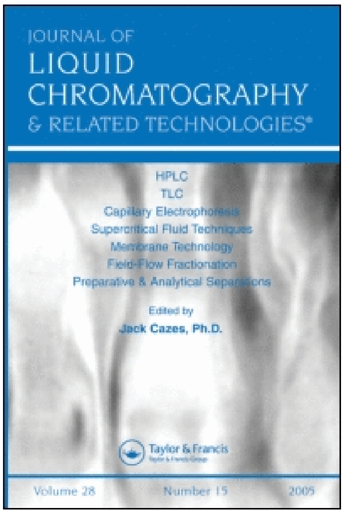

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COMPARISON OF COLUMNS FOR THE ANALYTICAL HIGH SPEED COUNTER-CURRENT CHROMATOGRAPH

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ABSTRACT

Use of a high speed countercurrent chromatograph (CCC) for analytical-scale separations is described. Separations are reported for columns of 0.85, 1.2, and 1.6 mm internal diameter. The effect of varying the experimental conditions was also investigated. It was found necessary to modify the CCC for the 0.85 and 1.2 mm i.d. columns, where no modification was necessary for the 1.6 mm i.d. columns. Carryover of the stationary phase frequently occurred with the 0.85 mm i.d. column, but carryover was not a problem with the 1.2 mm i.d. columns. The solute-to-solvent ratios of the eluates obtained with the 1.2 mm i.d. columns were higher than those obtained with the larger-bore columns. The 1.6 mm i.d. columns, however, proved to be better suited for preparative separations as faster flow rates were possible, which led to faster separations.

INTRODUCTION

High speed countercurrent chromatography (CCC) is effected by chromatographic column diameter as are other column chromatographies. The relationship between column diameter and performance is controlled by different parameters in CCC than other column chromatographies. The results of a study using different column diameters in high speed countercurrent chromatography are presented in this paper. Previous high speed CCC studies employed columns of 1.6 mm and 2.6 mm i.d. only, which are generally used for preparative-scale chromatography (1,2). In order to use high speed CCC for analytical purposes more efficient columns are necessary, which implies columns of smaller diameter. In this study columns of 0.85 and 1.2 mm i.d. PTFE (polytetrafluoroethylene) tubing were used for the first time in high speed CCC. The results obtained with these smaller diameter columns are compared to results obtained with 1.6 mm i.d. columns.

The term high speed CCC refers to the type of countercurrent chromatography that employs a multilayer PTFE column. The multilayer column rotates around its own axis while it revolves around the main axis of the apparatus. The two rotations are synchronized and they rotate at the same rate. Ito refers to this motion as the scheme IV motion (3). The use of multilayer columns under scheme IV motion (high speed CCC) leads to faster separations than those previously obtained with the Horizontal Flow-Through Coil Planet Centrifuge (HFTCPC).

Two columns were used in the HFTCPC for countercurrent chromatography (4-6). Ito referred to these columns as the pulley side and gear side columns. The gear side column was under a scheme IV motion. The pulley side column revolved around the main axis of the instrument but a second pulley prevented it from rotating around its own axis. Ito referred to this motion as the scheme I motion. He recommended the use of the pulley side column for analytical-scale separations and the gear side for preparative-scale separations.

The flow-through coil planet centrifuge (FTCPC) preceded HFTCPC. The difference between the two is that the horizontal instrument carried two columns horizontally opposed to the earlier vertical single column design. Flow-through CPC was used in the only detailed study of the role of column diameter in countercurrent chromatography (7). In this study Ito and Bowman compared results obtained with 0.38, 0.55, 0.85, and 1.2 mm i.d. columns.

Ito and Bowman pointed out that as the column diameter is reduced, three variables play an increasingly significant role (7). These variables are: the centrifugal field, interfacial surface tension, and the nature of the mobile phase. The centrifugal field has to be sufficiently strong to promote phase mixing of the two phases and leads to an equilibrium where the mobile phase displaces only itself. In smaller inner diameter columns, stronger centrifugal fields are required because the reduced diameter of the column makes phase mixing more difficult. The strength of the centrifugal field can be increased by using higher revolutional speeds and providing the column with a longer radius of revolution.

Interfacial surface tension also becomes a very important parameter as the column diameter is reduced. The interface between the two mutually immiscible liquids contracts because of interfacial surface tension. The interface contracts because the molecules at the surface of a liquid are attracted into the body of the liquid. The attraction of the underlying molecules is greater than the attraction by molecules on the other side of the surface which are immiscible. The greater the interfacial tension the more the interface contracts. A high interfacial tension system requires a sufficiently strong centrifugal field (high revolutional speeds at a large radius of revolution) to obtain enough phase mixing to achieve the equilibrium. Once equilibrium is established the mobile phase displaces only itself. If a high interfacial tension system is used in a narrow-bore column an even greater centrifugal field is necessary because of the small column diameter.

The interfacial surface tension of a solvent system may be reduced by adding a third solvent that is soluble in both phases. For example, the interfacial surface tension of a chloroform, water solvent system can be reduced by adding methanol, ethanol, or acetic acid to the system. Ito and Bowman were able to use a 2:2:1 chloroform, acetic acid, water system with columns of 0.38 and 0.55 mm because the acetic acid lowered the interfacial surface tension between water and chloroform (7).

The final parameter of importance as the diameter of the column is reduced is the nature of the mobile phase. As the column diameter is reduced it is easier to obtain a high retention of the stationary phase if the aqueous phase is used as the mobile phase. Unfortunately when the aqueous phase is used as the stationary phase the percentage of retained stationary phase is very low. At the lower column diameters such as 0.38, 0.55, and 0.85 mm i.d., the organic phase strongly adheres to the PTFE walls and displaces the aqueous phase. This effect is strong enough that even after the interfacial surface tension is reduced and the centrifugal field increased, the retention of the aqueous stationary phase is low.

The use of high speed CCC for analytical purposes is described in this paper. Lower diameter, more efficient, columns of 0.85 and 1.2 mm i.d. have been used for the first time in high speed CCC, for analytical-scale separations. Significant differences were observed between these columns and the wider 1.6 mm i.d. columns in terms of the interfacial surface tension of the solvent system, the centrifugal field, and the mobile phase required for a successful separation. These results are discussed below.

MATERIALS AND METHODS

The PTFE tubing was obtained from Zeus Industrial Products (Raritan, N.J.). Narrow-bore (0.85 mm i.d.) columns were initially studied using a 140 meter long column with a volume of 90 mL. The 0.85 mm i.d. column was prepared with a column spool where β=0.5 (β=rotational radius / revolutional radius). Columns of 1.6 and 2.6 mm i.d. were also built at β=0.5 (1,2).

The centrifugal field applied to the 0.85 mm i.d. column was increased by modifying the column spool. A wider spool drum was built to bring the column out about 2.5 cm. This changed the rotational radius and increased β to 0.74. The high speed countercurrent chromatograph has been described by Ito (1,2); only the spool was modified.

Columns of 1.2 mm i.d. were studied using a 130 meter long column with a volume of 160 mL. The β=0.74 spool that was built to increase the centrifugal field of the 0.85 mm i.d. column, was also used for the 1.2 mm i.d. columns.

The 1.6 mm i.d. column was 170 meters long and had a volume of 385 mL. The smaller spool (β=0.5) was used for the studies with this column.

Two sets of separations were studied with the 0.85, 1.2, and 1.6 mm i.d. columns. The separation of naphthalene, *p*-nitrotoluene, and *o*-nitrophenol was investigated using a hexane, methanol, and water system. The ratio in which these solvents were mixed depended on the column diameter, because lower interfacial surface tension systems are required for the smaller diameter columns. The lower aqueous phase was introduced as the stationary phase, and hence the organic upper phase was introduced through the tail end of the column. The second set of separations; acetophenone, benzoic acid, *p*-nitrophenol, and phenol, was performed by using a chloroform, water and methanol solvent system. The upper aqueous phase was used as the stationary phase. In this solvent system the aqueous phase is the "upper" phase. The lower mobile phase was introduced through the head end of the column. Once again, the ratios in which these solvents were mixed depended on the column diameter.

Reagents. Naphthalene, acetophenone and benzophenone were obtained from Fisher Scientific Co. (Fairlawn, N.J.), phenol from Baker and Adamson (Morristown, N.J.), benzoic acid from Matheson, Coleman & Bell (Norwood, Ohio), *o*-nitrophenol, and *p*-nitrophenol from Eastman Kodak Co. (Rochester, New York). Doubly distilled water was used. Chloroform, hexane and methanol, HPLC grade, were obtained from the J. T. Baker Chemical Company.

RESULTS AND DISCUSSION

Use of Narrowbore Columns

Several hexane, methanol, and water solvent systems were employed for the separation of naphthalene, benzophenone, and *o*-nitrophenol. Table I shows the solvent systems employed for this separation. Some of these solvent systems led to the retention of a large percentage of the stationary phase. Unfortunately, the percentage of retained stationary phase was not constant as some of the stationary phase eluted along with the mobile phase (carryover of the stationary phase).

To eliminate the carryover of the stationary phase higher centrifugal speeds were used. As noted above a decrease in column diameter makes phase

TABLE I.

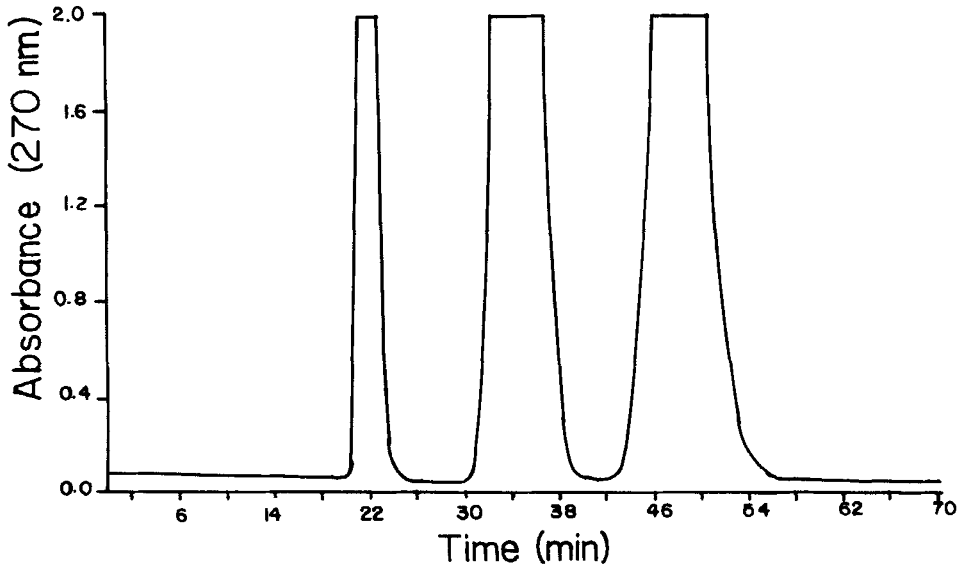
Solvent Systems Used with Narrowbore Column

Solvent System Composition				Revolutions per Minute (RPM)	Flow Rate (mL/min)	Retained Stationary Phase (Percent)
Hexane	Methanol	Water	Ethanol			
2	1	1		600	1.0	35
5	2	2	1	1000	0.5	92
5	2	2	2	800	1.0	75
5	2	2	2	1000	0.5	93
12	5	5		600	1.0	38
35	15	4		800	1.0	35

mixing more difficult and shifts the equilibrium towards higher centrifugal fields. Revolutional speeds ranging from 600 to 1,000 rpm were employed for the solvent systems shown in Table I. Even at 1,000 rpm, carryover of the stationary phase occurred.

An attempt was made to eliminate the loss of stationary phase by reducing the surface tension between the two phases and using lower flow rates. The surface tension of the hexane, methanol and water system was reduced by the addition of ethanol, which is soluble in both phases. These lower surface tension systems still led to carryover of the stationary phase. At the lower flow rates, the amount of stationary phase initially displaced was lower but the loss of stationary phase still continued throughout the separation.

In order to increase the centrifugal field acting on the 0.85 mm i.d. column, a β=0.74 column spool was built to replace the smaller spool (β=0.5) as stated above. With this new spool, separations without carryover of the



1. Chromatogram for the separation of naphthalene, benzophenone and *o*-nitrophenol using a 0.85 mm i.d. column and a 15:10:8 hexane:methanol:water solvent system. The β=0.74 spool was used.

stationary phase were achieved at 1,000 rpm. Figure 1 shows the separation of naphthalene, benzophenone, and *o*-nitrophenol using a 15:10:8 system of hexane, methanol, and water.

Separations in which a large portion of the solvent system was water remained a problem, even with an increased centrifugal field. Due to the reduced diameter of the column the organic mobile phase strongly adheres to the PTFE walls and displaces the aqueous phase; therefore, the percentage of retained stationary phase is very low. For example, when the 3:3:1 chloroform, water, methanol system was employed at 1,000 rpm with a flow rate of 1.0 mL/min, only 47% of the stationary phase was retained. This same separation at β=0.5 gave only 19% retention of the stationary phase at a flow rate of 0.3

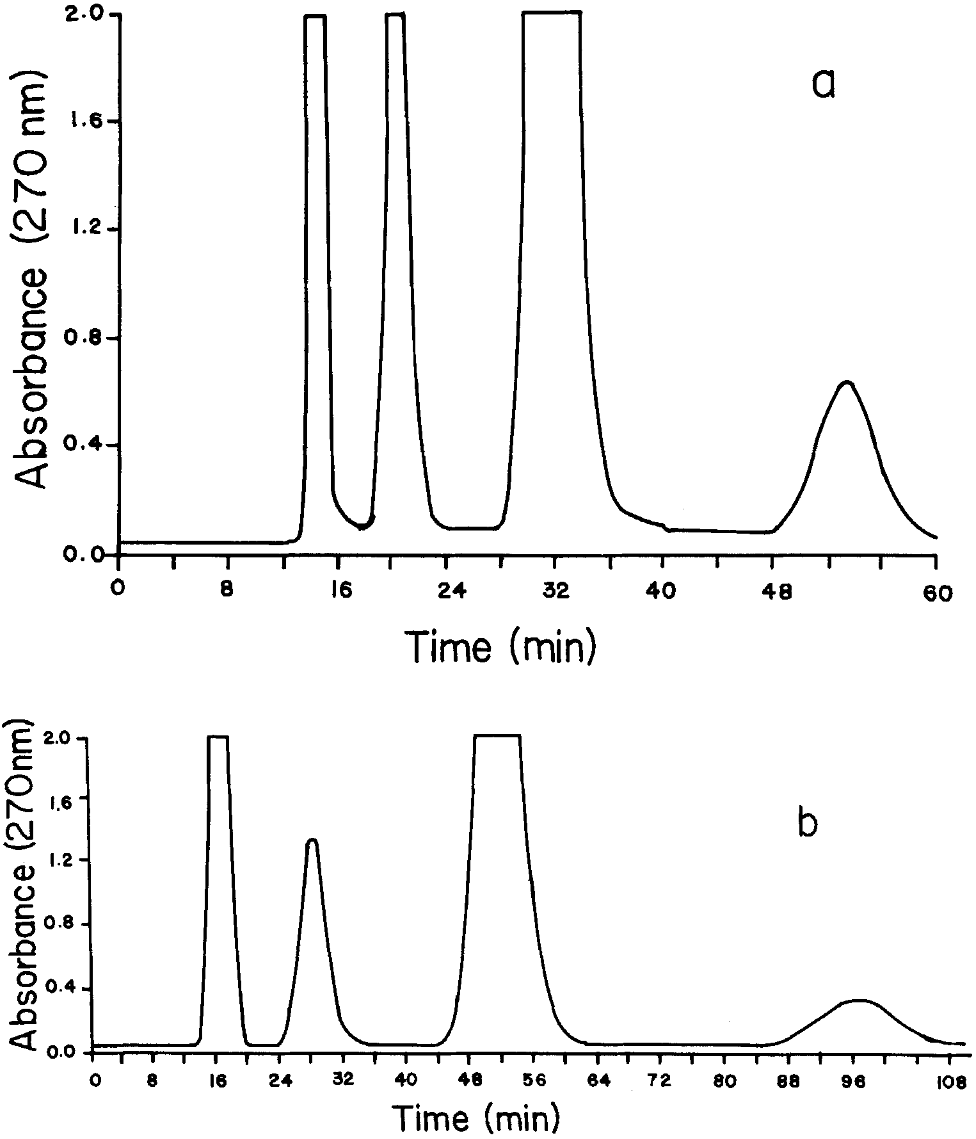
mL/min. This shows that the higher centrifugal field is increasing phase mixing. Nevertheless, the centrifugal field is not strong enough to overcome the attraction of organic solvents to the PTFE tubing.

Since the retention of aqueous stationary phases is very low in 0.85 mm i.d. columns, the choice of solvent systems with the narrowbore columns is limited. This led to the use of 1.2 mm i.d. columns that were expected to be more flexible than narrowbore columns, and more efficient than 1.6 mm i.d. columns.

1.2 mm and 1.6 mm i.d. columns

The retention of the stationary phase was not a problem with 1.2 mm i.d. columns as was found with 0.85 mm i.d. columns. Approximately 80% of the aqueous stationary phase was retained when naphthalene, benzophenone, *o*-nitrophenol, and acetophenone were separated with the 3:3:2 hexane, methanol, and water solvent system (Figure 2a). The separation was run at a flow rate of 3.0 mL/min without carryover of the stationary phase. The same solvent system was also used to carry out the separation in the 1.6 mm i.d. column (Figure 2b). The wider column also retained 80% of the stationary phase.

The main advantage of the 1.2 mm i.d. column to the 1.6 mm i.d. column is the reduced diffusion of the sample due to the smaller column diameter. Fractions collected from the smaller diameter column have higher concentrations of the components of interest than those obtained from a wider column. Table II shows the average peak concentrations for the two columns when 4.0 mg per component were injected into each of the columns. The concentrations



2. Chromatograms for the separation of naphthalene, benzophenone, *o*-nitrophenol, and acetophenone with a 3:3:2 hexane:methanol:water solvent system. a) 1.2 mm i.d. column at a flow rate of 3.0 mL/min, b) 1.6 mm i.d. column at a flow rate of 4.0 mL/min.

TABLE II.

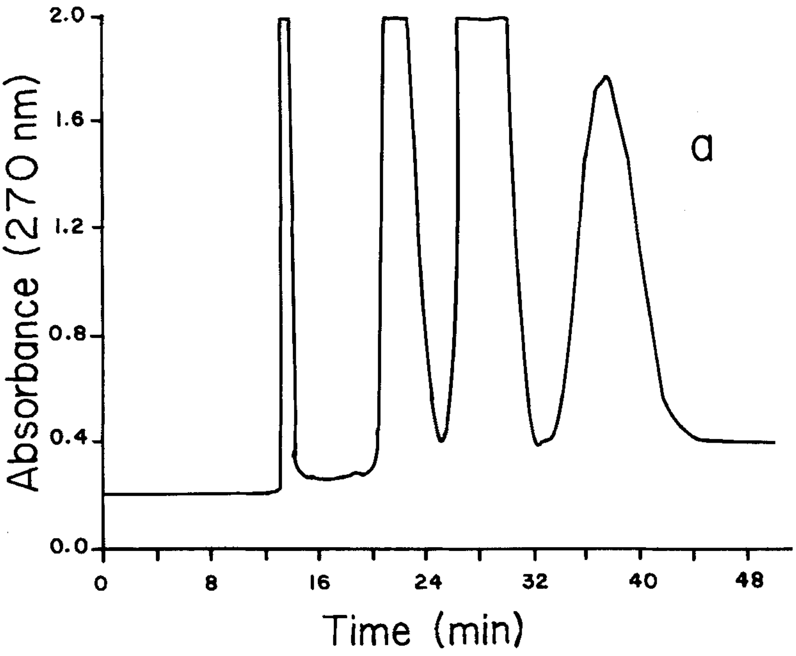
Average Peak Concentrations

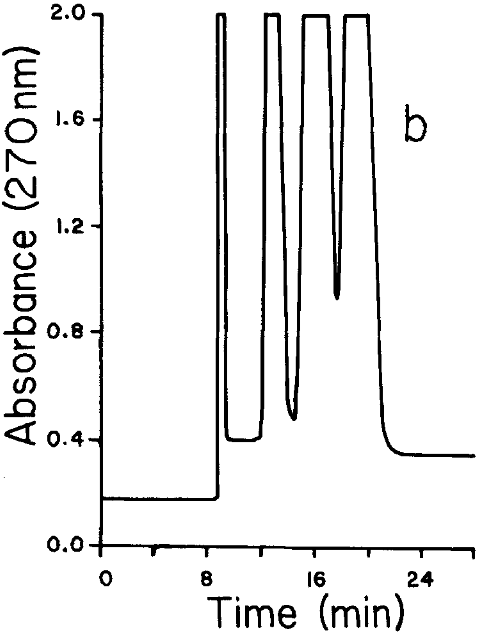
Compound	Average Peak Concentrations (mg/mL)	
	1.6 mm i.d.	1.2 mm i.d.
Naphthalene	0.21	0.67
Benzophenone	0.14	0.41
o-Nitrophenol	0.089	0.24
Acetophenone	0.054	0.16

obtained with the 1.2 mm i.d. column are about 3 times higher than those from the 1.6 mm i.d. column. The higher concentrations obtained with the narrower column are useful in spectrometric studies, for example, infrared spectrometry of the eluates.

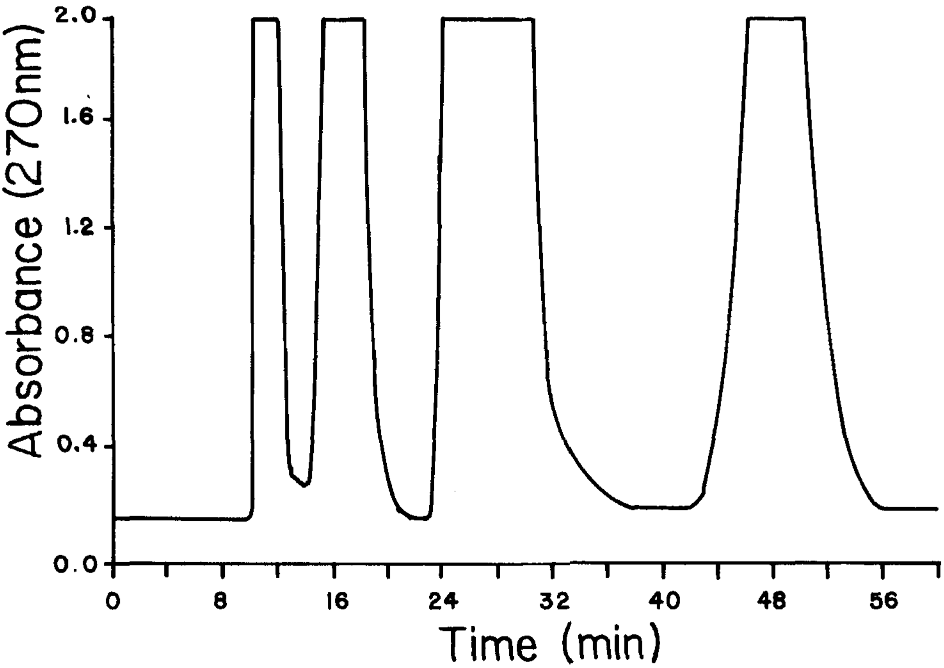
Separations of acetophenone, benzoic acid, *p*-nitrophenol, and phenol were accomplished in the 1.2 mm i.d. column with a 3:3:1 chloroform, water (acidified to pH 2 with hydrochloric acid), methanol solvent system. The chromatograms for this separation at a flow rate of 2.0 mL/min and 4.0 mL/min are shown in Figure 3. For the lower flow rate 86% of the stationary phase was retained, while 79% was retained at 4.0 mL/min. Even at 4.0 mL/min no loss of stationary phase occurred.

The percentage retained stationary phase for the 3:3:1 separation in the 1.6 mm i.d. columns greatly varies according to the centrifugal field applied to the column. When this separation is done at 600 rpm, the percentage of retained stationary phase is close to 80%. If the separation is performed at 800 rpm, then less than 50% of the stationary phase is retained. The inter-





3. Chromatograms for the separation of acetophenone, benzoic acid, *p*-nitrophenol, and phenol in a 1.2 mm i.d. column with a 3:3:1 chloroform:water (acidified to pH 2):methanol solvent system. a) 2.0 mL/min flow rate, b) 4.0 mL/min flow rate.



4. Chromatogram for the separation of 16 mg/component in a 1.2 mm i.d. column using a 3:3:2 hexane:methanol:water solvent system and a flow rate of 3.0 mL/min.

facial surface tension of the 3:3:1 system is probably low enough that at 800 rpm the strong centrifugal field forms emulsions.

The use of 1.2 mm i.d. columns for preparative purposes has also been investigated. Figure 4 shows the chromatogram obtained for 16 milligrams per component for the 3:3:2 hexane, methanol, and water system. The fastest useful flow rate available with the 3:3:2 separation was 3.0 mL/min. If this separation had been carried out in a wide bore column much faster flow rates could have been used. Although 1.2 mm i.d. columns can be used for preparative purposes, wider columns are better suited for this task as they allow much higher flow rates.

CONCLUSION

The results obtained in this study show that the current design of the high speed CCC is not suitable for the use of 0.85 mm i.d columns. The reduced diameter of the column impairs phase mixing and makes it necessary to use a stronger centrifugal field to achieve proper phase mixing. The centrifugal field on the column was increased by spinning it at 1,000 rpm, and by increasing β from 0.5 to 0.74. Even with this increased centrifugal field it was very difficult to retain aqueous stationary phases in the 0.85 mm i.d. column and to avoid carryover of the stationary phase.

The HFTCPC is better suited for separations in 0.85 mm i.d. columns than the high speed CCC. The revolutional radius of the column is adjustable when the HFTCPC is used, and can be extended up to 30 centimeters to increase the centrifugal field. This study also demonstrates that columns of 1.2 mm i.d. can be used for high speed CCC. More concentrated fractions can be collected from these narrower columns due to the reduced sample diffusion. The use of the narrower column also results in a reduction in the quantities of solvent used. The 1.6 and 2.6 mm i.d. columns are better suited for preparative purposes than the 1.2 mm i.d. column. Wider columns have higher sample capacities, and can be run at faster flow rates.

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