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NEW ANGLE ROTOR COIL PLANET CENTRIFUGE FOR COUNTER-CURRENT CHROMATOGRAPHY

II. DESIGN OF THE APPARATUS AND STUDIES ON PHASE RETENTION AND PARTITION CAPABILITY

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SUMMARY

A new angle rotor coil planet centrifuge ($\Psi = 25°$), which produces a Type J-L synchronous planetary motion, has been constructed to examine its capability in terms of stationary phase retention and solute partitioning. Studies on phase distribution diagrams obtained from various two-phase solvent systems indicated that the present system can be adapted to a wide variety of solvent systems by adjusting the centrifugal conditions. Excellent partition capability of the apparatus was successfully demonstrated in separations of dinitrophenyl amino acid samples with chloroform–acetic acid–0.1 N hydrochloric acid (2:2:1).

INTRODUCTION

Counter-current chromatography (CCC) utilizes complex hydrodynamic interaction of two immiscible solvent phases in a tubular column free of solid support matrix[1]. Consequently, partition efficiency of CCC relies highly on the column configuration and applied force field which, together, provides optimum degree of phase mixing and retention of the stationary phase. Recently, application of a particular type (Type J) of synchronous planetary motion to the coiled column has produced highly efficient, rapid chromatographic separations which are comparative to those attained by preparative-scale high-performance liquid chromatography (HPLC)[2,3].

As described in Part I, the new type (Type J-L) of synchronous planetary motion was found to form a similar pattern of the centrifugal force field, which may be quite useful for performing high-speed CCC. In order to study hydrodynamic effects produced by this centrifugal force field, a new Type J-L coil planet centrifuge has been constructed. The present paper describes the mechanical design of the new angle rotor coil planet centrifuge and its capability of phase retention for a set of commonly used two-phase solvent systems. The partition capability of the apparatus has been demonstrated by separation of a standard set of dinitrophenyl (DNP) amino

acid samples on a solvent system composed of chloroform–acetic acid–0.1 N hydrochloric acid (2:2:1) with multilayer coil separation columns.

APPARATUS

Fig. 1 shows a cross-sectional view through the central axis of the apparatus. The motor (Bodine Electric Company) drives the rotary frame around the central stationary pipe (shaded) by means of a pair of toothed pulleys coupled with a toothed belt. The rotary frame consists of a pair of aluminum plates rigidly bridged with links (not shown in the diagram) and holds a column holder (right) and the counter-weight holder (left) in symmetrical positions at a 25° inclination from the vertical. Distance from the middle portion of the column to the central axis of the centrifuge measures about 10 cm. The column holder is equipped with a planetary miter gear (12.5°), which is interlocked with a stationary sun gear of an identical shape (shaded) tightly mounted around the central stationary pipe. This gear coupling produces a desired synchronous planetary motion of the column holder: the holder revolves around the central axis of the apparatus and simultaneously rotates around its own axis at the same angular velocity in the same direction. While doing so, the holder always maintains its inclined orientation of 25° from the central axis of the apparatus. Both the column holder and the counter-weight holder are removable from the rotary frame by loosening a set of screws on the upper plate of the rotary frame. This enables the use of an interchangeable column holder of different hub diameters and also facilitates mounting the coiled column on the holder.
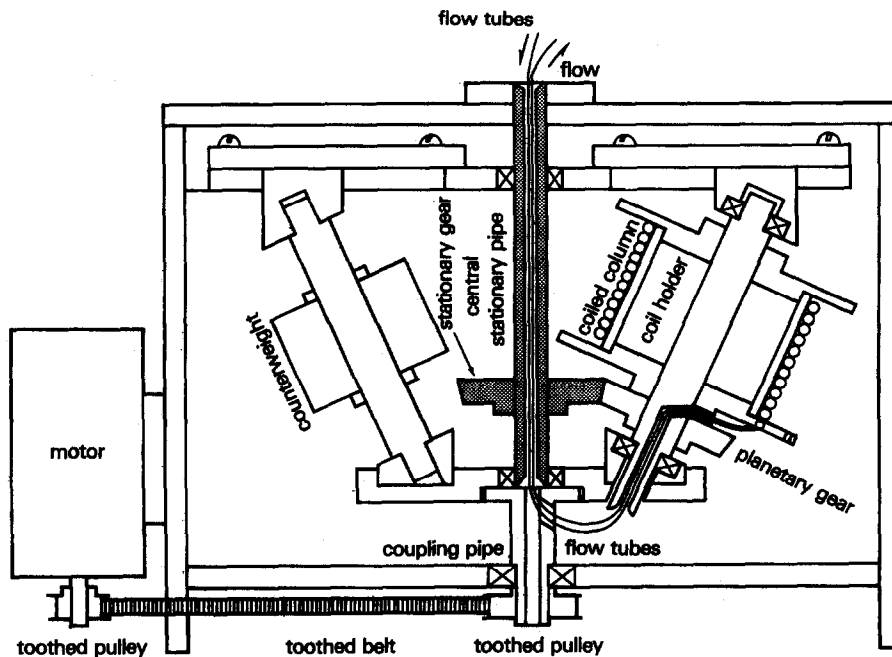

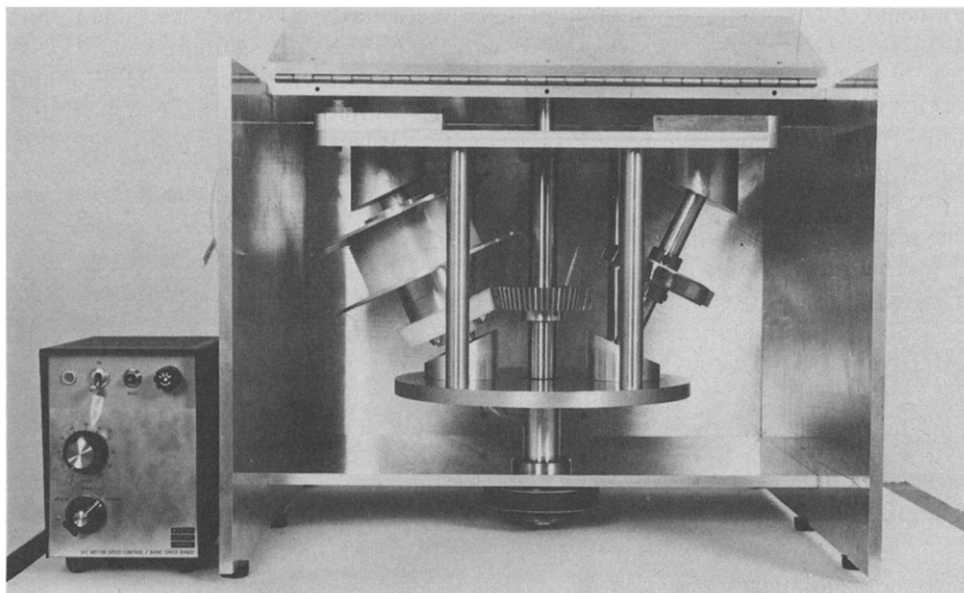Fig. 1. Cross-sectional view through the central axis of the apparatus.

Fig. 2. Photograph of the prototype angle rotor coil planet centrifuge.

For the present studies two types of PTFE tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) were selected, one with 1.6 mm I.D. and the other with 0.85 mm I.D. Each column was prepared by winding a long piece of the above tubing directly around the holder hub, forming either a single or multiple layers of the continuous coil. A pair of flow tubes (0.85 mm I.D.) from the coiled column passes through the holes on the column holder and then, forming a loop, enters the side hole of the coupling pipe to reach the opening of the central stationary pipe as illustrated in Fig. 1. As mentioned earlier in Part I, the present flow-through centrifuge system requires no rotating seals, because the synchronous rotation of the column holder steadily unwinds the twist of the flow tubes formed by revolution. These flow tubes are thoroughly lubricated with grease and protected with flexible plastic tubing (Tygon) to prevent direct contact with metal parts.

Fig. 2 shows a photograph of our prototype angle rotor coil planet centrifuge used for the present study. The revolution speed of the apparatus is continuously adjustable from 0 to 1000 rpm, with a speed control unit (Bodine Electric Company) shown on the left.

EXPERIMENTAL

Reagents

Organic solvents for the preparation of the two-phase solvent systems, including n-hexane, ethyl acetate, chloroform, n-butanol, sec.-butanol and methanol, are all glass-distilled chromatographic grades (Burdick and Jackson Labs., Muskegon, NJ, U.S.A.) while glacial acetic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.) and hydrochloric acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.) are reagent grades. Dini-

trophenyl (DNP) amino acids samples including delta-N-2,4-DNP-ornithine (DNP-orn), N-2,4-DNP-L-aspartic acid (DNP-asp), N-2,4-DNP-D,L-glutamic acid (DNP-glu), N,N-di(2,4-DNP)-cystine (DiDNP-cys$_2$), N-2,4-DNP-β-alanine (DNP-β-ala), N-2,4-DNP-L-alanine (DNP-ala), N-2,4-DNP-L-proline (DNP-pro), N-2,4-DNP-L-valine (DNP-val), and N-2,4-DNP-L-leucine (DNP-leu), are all reagent grades (Sigma, St. Louis, MO, U.S.A.).

Two-phase solvent systems

Using the above solvents, nine kinds of volatile two-phase solvent systems were prepared for phase retention studies. They are, in order of hydrophobicity of the non-aqueous phase: *n*-hexane–water, *n*-hexane–methanol, ethyl acetate–water, ethyl acetate–acetic acid–water (4:1:4), chloroform–water, chloroform–acetic acid–water (2:2:1), *n*-butanol–water, *n*-butanol–acetic acid–water (4:1:5) and *sec.*-butanol–water. In addition, a two-phase solvent system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) was used for separation of DNP-amino acid samples. Each solvent mixture was gently degassed and thoroughly equilibrated in a separatory funnel at room temperature and separated before use.

Measurement of phase retention

PTFE tubing with two different diameters, 1.6 mm I.D. and 0.85 mm I.D., was selected for coil preparation. Each tubing was tightly wound around the holder with a hub diameter of 10 or 15 cm, making a single-layer coil with the respective helical diameter. Due to inclination of the holder axis (25°), the distance (R) between the coil axis and the central axis of the centrifuge changes with the location of the coil on the holder, while the helical diameter ($2r$) of the coil remains constant. Consequently, the β value (r/R) increases from the internal lower terminal to the external upper terminal of the coil. Therefore, the averaged β value ($\bar{\beta}$) is used as the parameter for the coil location on the holder. Thus $\bar{\beta}$ values for the 10- and 15-cm diameter coils described above are 0.5 and 0.75, respectively.

Phase retention measurement was performed according to the procedure previously described[4]. For each measurement, the coiled column was first entirely filled with the stationary phase. Then, the apparatus was run at the desired revolutional speed while the mobile phase was pumped through the column at a given flow-rate. The effluent from the outlet of the column was collected in a graduated cylinder to measure the volume of the stationary phase eluted from the column, as well as the total volume of the mobile phase eluted. The run was continued until an effluent volume of *ca.* 1.5 times the total column capacity was collected. Then, the apparatus was stopped and the column was emptied by connecting the inlet to the nitrogen gas line at 80 p.s.i. pressure.

For each solvent system, both upper and lower phases were used as the mobile phase, and in the 1.6 mm I.D. columns the runs were carried out for each mobile phase in four different elution modes, *i.e.*, external head to internal tail, internal head to external tail, external tail to internal head, and internal tail to external head, as listed in Table IA. Each run was repeated under four different revolutional speeds: 400, 600, 800 and 1000 rpm. A flow-rate of 120 ml/h was applied for the 1.6 mm I.D. columns, except for the viscous butanol solvent systems in which a reduced flow-rate of 60 ml/h was applied. For the 0.85 mm I.D. columns, the runs were performed in

TABLE I

SYMBOLIC DESIGN OF THE RETENTION CURVES IN THE PHASE DISTRIBUTION DIA-
GRAM

H = Head; T = tail; I = internal; E = external.

	Elution mode		
	Upper phase mobile	Lower phase mobile	
(A) 1.6 mm I.D. coil	EH → IT	IH → ET	Thick solid line
	IH → ET	EH → IT	Thin solid line
	IH ← ET	EH ← IT	Thick broken line
	EH ← IT	IH ← ET	Thin broken line
	Elution mode	Flow-rate	
(B) 0.85 mm I.D. coil	H → T	low	Thick solid line
	H → T	high	Thin solid line
	H ← T	low	Thick broken line
	H ← T	high	Thin broken line

one optimum elution mode, whereas two different flow-rates were tested for each
solvent system at 60 and 24 ml/h, which were reduced to 24 and 12 ml/h for the
butanol solvent systems.

In each experiment, the volume of the stationary phase retained in the column
was calculated from the volume of the stationary phase eluted from the column and
expressed as a percentage of the total column capacity according to the expression
$100 (V_c + V_f - V_s)/V_c$, where V_c denotes the total column capacity; V_f, the volume
of the flow tubes; V_s, the volume of the stationary phase eluted from the column.
The hydrodynamic distribution of the two solvent phases in the column was summa-
rized by a phase distribution diagram constructed by plotting the percentage reten-
tion of stationary phase as a function of revolutional speed for a particular mobile
phase flow-rate. A group of retention curves produced by different elution modes but
otherwise identical experimental conditions can be illustrated in a single diagram
using the respective symbolic lines indicated in Table I.

DNP-Amino acid separation

Experiments were performed with two different types of multilayer coils. The
first column for semipreparative-scale separations consists of 1.6 mm I.D. PTFE
tubing mounted on a 12.7-cm diameter holder-hub with a total capacity of *ca.* 190
ml. The second column for analytical-scale separations consists of 0.85 mm I.D.
PTFE tubing wound around a 14-cm diameter holder-hub with a total capacity of
ca. 55 ml.

Two sets of DNP-amino acid samples were selected for separation, one for the
run with the upper phase mobile and the other for the lower phase mobile. Sample
solutions were prepared by dissolving each set of DNP amino acid mixtures in the
stationary phase to make a concentration of each component ranging from 100 to
1000 mg%, with a total concentration of 2–3 g%. The sample volume charged in
each run was 1 ml for the semipreparative column and 0.1 ml for the analytical
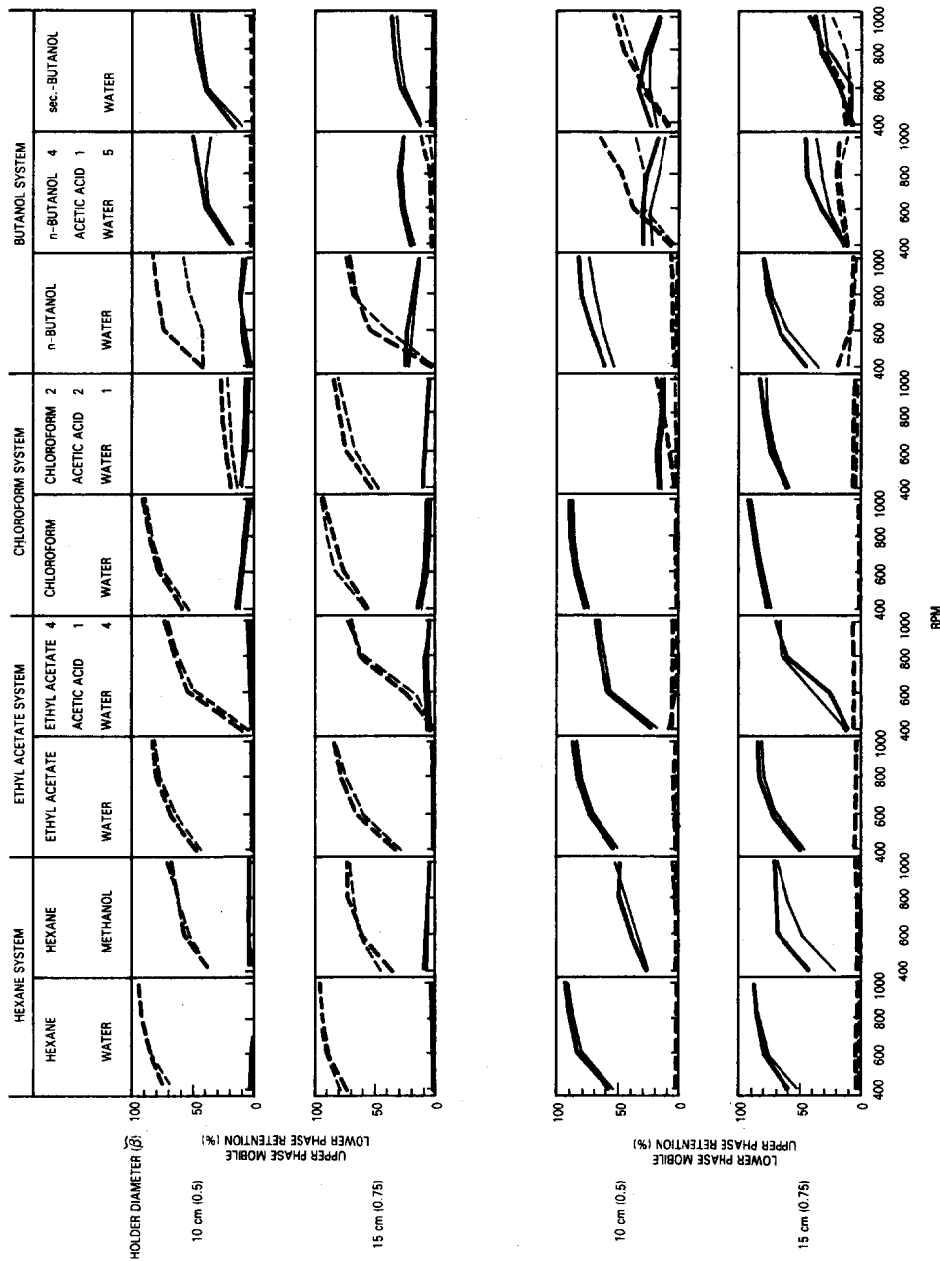column.

Fig. 3. Phase distribution diagrams of nine volatile solvent systems obtained from the 1.6 mm I.D. coil. Experimental conditions are as follows: planetary motion: Type J-L ($\Psi = 25°$) (see Fig. 6, Part I), revolution: 400–1000 rpm, holder hub diameter (β): 10 cm (0.5) for the 1st and 3rd rows and 15 cm (0.75) for the 2nd and 4th rows, column: 1.6 mm I.D. single-layer coil, flow-rate: 120 ml/h for hexane, ethyl acetate and chloroform systems and 60 ml/h for butanol systems, mobile

CCC separation was performed for each column under the predetermined optimum operational conditions of revolutional speed, flow-rate and elution mode, using both the upper aqueous and the lower non-aqueous phases as the mobile phase. For each separation, the column was first entirely filled with the stationary phase followed by sample injection through the sample port. Then, the apparatus was run at a given revolutional speed while the mobile phase was pumped into the column at the optimum flow-rates of 240 ml/h for the 1.6 mm I.D. column and 24 ml/h for the 0.85 mm I.D. column. Effluent from the outlet of the column was monitored continuously with an LKB Uvicord S at 278 nm and fractionated with an LKB fraction collector. An aliquot of each fraction was diluted with methanol and the absorbance was determined at 430 nm with a Zeiss spectrophotometer.

RESULTS AND DISCUSSION

Studies on phase retention

The effects of this new synchronous planetary motion on the retention of the stationary phase in the coiled column have been investigated for nine volatile two-phase solvent systems with a wide range of hydrophobicity. The results of the phase retention studies performed on the 1.6 mm I.D. coil are summarized in Fig. 3, where the phase distribution diagrams are arranged from left to right in order of their hydrophobicity of the non-aqueous phase used in the solvent system. Four sets of horizontal rows of the phase distribution diagrams were obtained from the run, each with different experimental conditions. The top two rows were produced by the upper phase mobile and the bottom two rows by the lower phase mobile, whereas the first and the third rows were obtained from a 10-cm diameter holder or under $\beta = 0.5$ and the second and fourth from a 15-cm diameter holder or under $\beta = 0.75$, as indicated on the left edge of Fig. 3.

Four retention curves drawn in each diagram indicate the different modes of elution. As listed in Table IA, a pair of solid lines represents the head to tail elution and a pair of broken lines represents the tail to head elution. Within each group, the thick line indicates an elution mode favored by the centrifugal force gradient formed through the coil from the internal terminal toward the external terminal, *i.e.*, the upper phase eluting from the external terminal toward the internal terminal or the lower phase eluting from the internal terminal toward the external terminal. Similarly, the thin line indicates an elution mode against the force gradient, *i.e.*, the upper phase eluting from the internal terminal toward the external terminal or the lower phase eluting from the external terminal toward the internal terminal. Consequently, the thick line is always situated above the thin line as shown in the diagrams, while the distance between these two lines semiquantitatively indicates the effect of the centrifugal force gradient on the retention of the stationary phase.

In the top two diagrams for hexane–water obtained by upper phase mobile, the two broken lines rise with increased revolutional speed to reach almost 100% at 1000 rpm while the two solid lines always stay close to the 0% line. This retention profile is characteristic of the typical unilateral hydrodynamic distribution of the two solvent phases in the coil, where the upper phase is distributed toward the head and the lower phase toward the tail. Under this hydrodynamic trend, the upper phase introduced at the tail can move rapidly toward the head leaving a large volume of
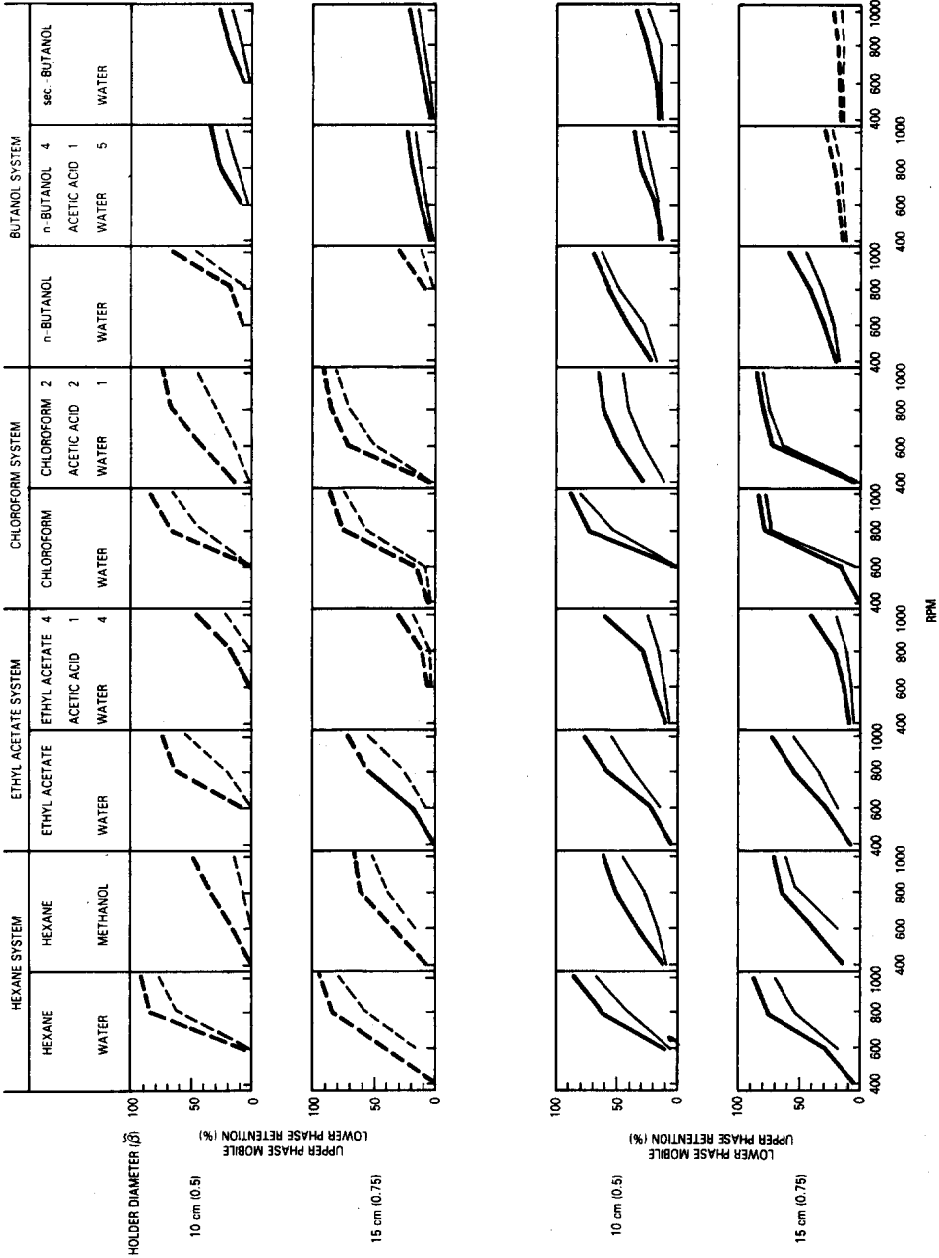
Fig. 4. Phase distribution diagrams for nine volatile solvent systems obtained from the 0.85 mm I.D. coil. Experimental conditions are as follows: planetary motion: Type J-L ($\Psi = 25°$) (see Fig. 6, Part I), revolution: 400–1000 rpm, holder hub diameter (β): 10 cm (0.5) for the 1st and 3rd rows and 15 cm (0.75) for the 2nd and 4th rows, column: 0.85 mm I.D. single layer coil, flow-rate: 60 and 24 ml/h for hexane, ethyl acetate and chloroform systems and 24 and 12 ml/h for the butanol systems, mobile phase: upper phase for the top two rows and lower phase for the bottom two rows, elution mode: see Table I.B.

the lower phase stationary in the coil (see tail to head elution shown by the broken line). Contrary to the above, the upper phase introduced at the head pushes the entire lower phase through the coil toward the tail, resulting in total loss of the stationary phase from the coil (see head to tail elution shown by the solid line).

The above findings are consistent with the phase retention profile obtained from the runs with the lower phase mobile shown at the bottom two diagrams for hexane–water, where the relationship between the two retention curves is completely reversed, i.e., the solid lines show high retention and the broken lines show ca. 0% retention. Under the same unilateral hydrodynamic condition, the lower phase introduced at the head can move quickly toward the tail, permitting a large volume of the stationary phase to stay behind (see head to tail elution shown by the solid lines) while the same lower phase introduced at the tail pushes the entire upper phase out of the coil, resulting in no retention of the stationary phase (see tail to head elution shown by the broken lines).

This typical unilateral hydrodynamic distribution ideal for performing CCC is also found in other solvent systems such as ethyl acetate–water and chloroform–water. These three binary solvent systems share a common nature characterized by high hydrophobicity of the non-aqueous phase and high interfacial tension between the two phases.

A similar hydrodynamic distribution, but with a reversed retention profile, is found in two butanol solvent systems, n-butanol–acetic acid–water (4:1:5) and sec.-butanol–water. The hydrodynamic trends of these two solvent systems are reversed, i.e., the lower phase is distributed toward the head and the upper phase toward the tail. These hydrophilic solvent systems are characterized by high viscosity, low interfacial tension and relatively small density difference between the two phases.

The rest of the solvent systems, including hexane–methanol, ethyl acetate–acetic acid–water (4:1:4), chloroform–acetic acid–water (2:2:1), and n-butanol–water, possess physical properties intermediate between the above two extremes, i.e., moderate degrees of hydrophobicity in the non-aqueous phase and interfacial tension beween the two phases. Although, at the $\bar{\beta} = 0.5$–0.75 applied in the present studies, these solvent systems exhibit hydrodynamic trends similar to those of the hydrophobic solvent systems, at the lowered $\bar{\beta}$ values they would show the reversed hydrodynamic trend characteristic of the hydrophilic solvent systems.

In short, these retention diagrams obtained from the present Type J-L synchronous planetary motion closely resemble those of the Type J (Scheme IV) synchronous planetary motion[4,5], except that the whole retention diagrams are slightly shifted upward in the phase distribution format, as if β is reduced. Overall results obtained with the 1.6 mm I.D. coil indicate that the present system is capable of providing a satisfactory retention of the stationary phase for hydrophobic and intermediate solvent systems, except for the chloroform–acetic acid–water (2:2:1) system which requires a minimum $\bar{\beta}$ value slightly higher than 0.5. On the other hand, the hydrophilic solvent systems show low levels of retention even under a reduced flow-rate of 60 ml/h. As demonstrated in the previous studies on the Type J (Scheme IV) synchronous planetary motion[5], these solvent systems would improve retention at smaller $\bar{\beta}$ values of around 0.25.

Phase retention studies for the 0.85 mm I.D. columns have been carried out on the same set of nine solvent systems used for the retention studies on the 1.6 mm

I.D. columns. However, the runs were limited to one elution mode for each phase
distribution diagram by selecting the highest retention curve from the phase retention
diagram for the 1.6 mm I.D. column shown in Fig. 3, while for each elution mode
two different flow-rates were tested, *i.e.*, 24 and 60 ml/h for the hexane, ethyl acetate
and chloroform solvent systems, and 12 and 24 ml/h for the butanol solvent systems.

In Fig. 4 a set of phase distribution diagrams for the 0.85 mm I.D. columns
is displayed in the standard format with slightly modified symbolic designs for the
retention curves. As indicated in Table IB, solid lines are given for the head to tail
elution mode and broken lines for the tail to head elution mode, while for each
elution mode thick lines are for the lower flow-rates and thin lines for the higher
flow-rates. These phase distribution diagrams show unaltered general hydrodynamic
trends of the solvent systems with a substantial shift of all retention curves toward
the right, indicating the effects of solvent–wall interaction which is intensified in a
narrower opening of the column. Here, it is worthwhile to point out that the retention
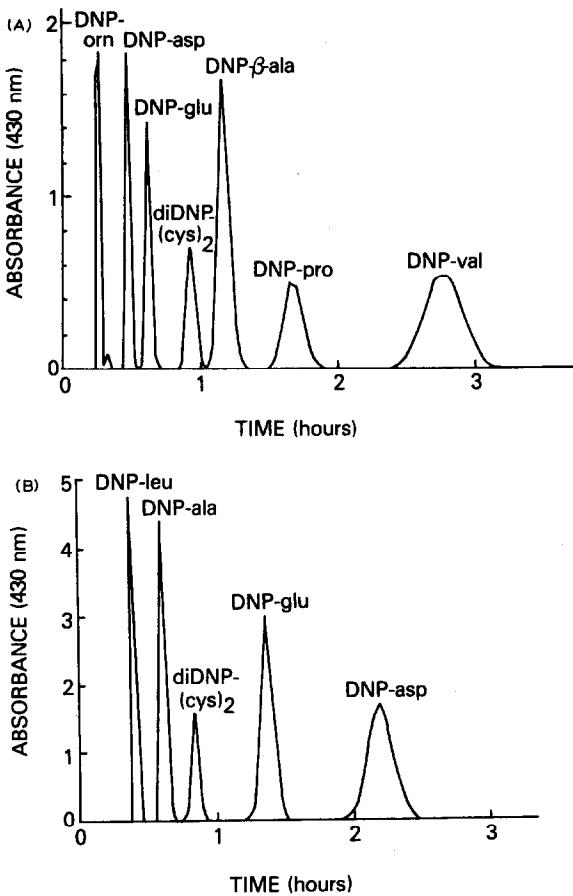curves for the chloroform–acetic acid–water (2:2:1) system at $\tilde{\beta} = 0.5$ are much

Fig. 5. Chromatograms of DNP-amino acids with chloroform–acetic acid–0.1 N hydrochloric acid (2:2:1)
obtained from the 1.6 mm I.D. multilayer coil: (A) upper phase mobile; (B) lower phase mobile.

improved even at the higher flow-rate of 60 ml/h, compared with those obtained from the 1.6 mm I.D. columns (see Fig. 3).

Overall results of the phase retention studies on the 0.85 mm I.D. columns clearly indicate the need for application of a stronger centrifugal force field to achieve higher and more stable stationary phase retention, which would be available at the plateau of the retention curve. This requirement may be fulfilled by increasing the speed and/or the radius of revolution.

Studies on partition efficiency

The partition capability of the present scheme was demonstrated on separations of DNP-amino acid samples with a two-phase solvent system composed of chloroform–acetic acid–0.1 N hydrochloric acid (2:2:1). These runs were performed with the semipreparative (1.6 mm I.D. and 190-ml capacity) and the analytical (0.85 mm I.D. and 55-ml capacity) multilayer coils under the suitable experimental conditions determined by the phase retention studies.

Fig. 5 shows chromatograms obtained with the 1.6 mm I.D. semipreparative multilayer coil at the flow-rate of 240 ml/h using both the upper (A) and the lower (B) phases as the mobile phase. In each chromatogram, all components were well resolved in symmetrical peaks and eluted out in a few hours. Partition efficiency was estimated for each peak according to the conventional gas chromatographic formula:

$$N = (4R/w)^2 \tag{1}$$

where N denotes the partition efficiency expressed in terms of the number of theoretical plates (T.P.); R is the retention time of the peak maximum; and w is the peak width expressed in the same time scale. The results gave 1200 T.P. for the 2nd peak (DNP-asp) and 400 T.P. for the 7th peak (DNP-val) in chromatogram A (upper phase mobile); and 1000 T.P. for the 2nd peak (DNP-ala) and 630 T.P. for the 5th peak (DNP-asp) in chromatogram B (lower phase mobile). The retention of the stationary phase measured after the separation was 54% (A) and 60% (B).

Fig. 6 similarly shows chromatograms obtained from the 0.85 mm I.D. analytical multilayer coil at a flow-rate of 24 ml/h using both the upper (A) and the lower (B) phases as the mobile phase. The partition efficiency similarly estimated from eqn. 1 ranged from 1150 T.P. for the 2nd peak (DNP-asp) to 890 T.P. for the 7th peak (DNP-val) in chromatogram A (upper phase mobile); and from 1600 T.P. for the 2nd peak (DNP-ala) to 1330 T.P. for the 5th peak (DNP-asp) in chromatogram B (lower phase mobile). The retention of the stationary phase measured after the separation was 14% (A) and 60% (B). The above low retention level in chromatogram A is considered to be the result of steady carryover of the stationary phase, which is a common complication often observed in the tail to head elution. Comparison between these two chromatograms reveals that the run with the lower phase mobile yields much higher peak resolution than the run with the upper phase mobile, apparently due to much greater retention of the stationary phase in the column. As described elsewhere[6], retention of the stationary phase produces a significant effect on peak resolution in CCC, especially for early eluting solute peaks. Therefore, for analytical-scale separations, it is desirable to provide higher and more stable retention of the stationary phase by adjusting the centrifugal conditions as discussed earlier.
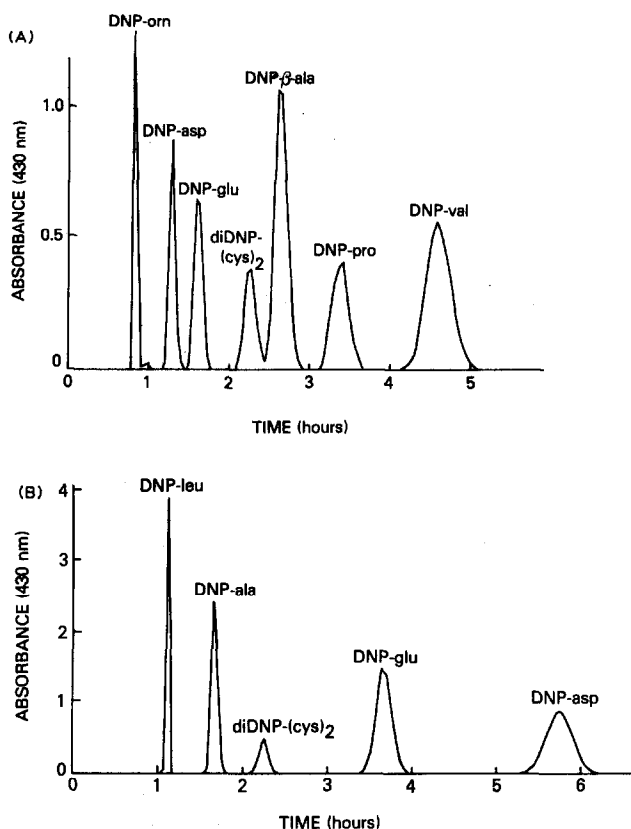
Fig. 6. Chromatograms of DNP-amino acids with chloroform–acetic acid–0.1 N hydrochloric acid (2:2:1) obtained from the 0.85 mm I.D. multilayer coil: (A) upper phase mobile; (B) lower phase mobile.

The chromatograms of DNP-amino acid samples obtained with both semipreparative and analytical multilayer coils clearly demonstrate the excellent partition capability of the present scheme, which is comparable to that of the existing high-speed CCC. The present studies should also be extended to the use of different types of coiled column, such as a toroidal coil, where the three-dimensional mixing effect may play a more critical role in improving the partition efficiency in CCC.

ACKNOWLEDGEMENT

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