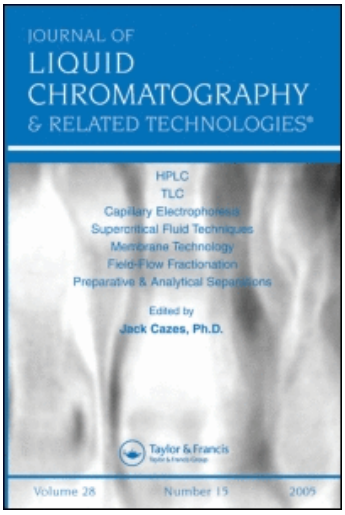

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Large-Scale Preparative Counter-Current Chromatography with a Coil Planet Centrifuge

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LARGE-SCALE PREPARATIVE COUNTER-CURRENT CHROMATOGRAPHY WITH A COIL PLANET CENTRIFUGE

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ABSTRACT

Development of the large-scale preparative countercurrent chromatographic schemes has been continued by increasing the diameter of the separation column. A 0.55 cm i.d. FEP tube was coaxially coiled around the holder (7.5 cm, 10 cm or 15 cm in diameter) of a horizontal flow-through coil planet centrifuge (15 cm revolutional radius). Performance of each column was evaluated on the separation of dinitrophenyl amino acid samples with a two-phase solvent system composed of chloroform, acetic acid, and 0.1N hydrochloric acid (2:2:1) by using both aqueous and nonaqueous phases as the mobile phase. Experiments with the short preliminary columns (114 ml capacity) revealed that the hydrodynamic distribution of the two solvent phases was sensitively affected by the helical diameter of the column. However, by choosing the proper elution mode of the mobile phase, satisfactory results were obtained with the helical diameters of 7.5 cm and 15 cm at a high flow rate of 500 ml/h under a moderate revolutional speed of 300 rpm. With the long coiled columns (750 ml capacity), the preparative capability of the present scheme was successfully demonstrated on separations of the 1g-quantity sample mixture under optimized operational conditions. Overall results indicated that the sample-loading capacity of the present scheme can be further increased by the use of longer and/or larger-diameter columns.

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INTRODUCTION

In the previous studies, the preparative capability of high-speed countercurrent chromatography (CCC) was demonstrated by the use of a 2.6 mm i.d. coil mounted on a compact table top model of the horizontal flow-through coil planet centrifuge (1). In the present series of studies, efforts have been mode to further increase the sample-loading capacity of the scheme by applying a larger diameter column of 0.55 cm i.d. on a bench top model of the coil planet centrifuge. By varying the diameter of the column holder and other operational conditions, the performance of the short preliminary columns was evaluated on separations of a standard set of dinitrophenyl (DNP) amino acids with a two-phase solvent system composed of chloroform, acetic acid, and 0.1N hydrochloric acid at a 2:2:1 volume ratio. The preparative-scale separations were performed with long columns under the optimum operational conditions determined by the preliminary studies.

PRINCIPLE

The present CCC method utilizes a complex hydrodynamic motion of the two immiscible solvent phases in a rotating coiled column (2). When a coil is rotated around its horizontally positioned axis, it exerts an Archimedean screw force on the column contents toward one end of the coil. This end is called the head and the other end is the tail. In a centrifugal force field induced by a particular type of planetary motion (Fig. 1), the two solvent phases are unilaterally distributed in the coil so that one phase (head phase), entirely occupies the head side and the other phase (tail phase), the tail side of the coil. This unilateral

hydrodynamic equilibrium condition is effectively utilized for performing CCC in the following two different ways: The coil filled with the head phase is eluted with the tail phase from the head toward the tail or the column filled with the tail phase is eluted with the head phase from the tail toward the head. In either case a large amount of the stationary phase is retained in the coil while the two phases are continuously mixed by rotation of the coil. Consequently, solutes locally introduced at the inlet of the column are subjected to an efficient partition process and separated according to their partition coefficients as in liquid chromatography.

The planetary motion which produced the desirable unilateral distribution of two solvent phases in a coil is illustrated in Fig. 1 where a planetary gear on the holder axis is coupled to an identical stationary sun gear placed on the central axis of the centrifuge. This gear arrangement effects the synchronous planetary motion of the holder, i.e., the rotation about its axis and revolution around the central axis of the centrifuge at the same angular velocity. This motion establishes unilateral hydrodynamic distribution of the two solvent phases in the coil coaxially mounted around the holder. Under this circumstance, either upper of lower phase can become the head phase depending on the operational conditions which include revolutional speed and radius, diameter of the holder, various physical properties of the solvent system, etc. However, recent studies (3) have shown that in tertiary two-phase solvent systems the hydrodynamic distribution mode of the two phases is most sensitively affected by a single parameter, $\beta = r/R$, where r is the holder radius and R, the revolutional radius as indicated in Fig. 1.

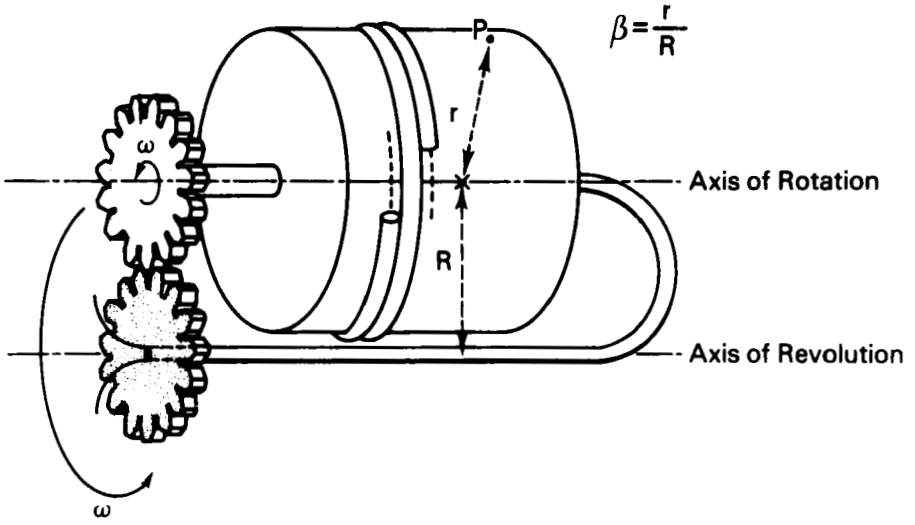


Figure 1. Synchronous planetary motion of the coil holder.

APPARATUS

The apparatus used in the present studies (Fig. 2) is a modified version of the original horizontal flow-through coil planet centrifuge free of rotary seals (4,5). Modifications mainly dealt with the column holder which was made removable from the rotary frame simply by loosening a pair of screws in each bearing block. The new holder is equipped with a pair of large flanges to support multiple layers of the coiled column in place.

The motor (Bodine Electric Co.) drives the rotary frame around the central stationary pipe by a pair of toothed pulleys coupled with a toothed belt. The rotary frame consists of a pair of aluminum plates to support a pair of holders symmetrically at 15 cm from the central axis of the centrifuge. Each holder is equipped with a planetary gear which is

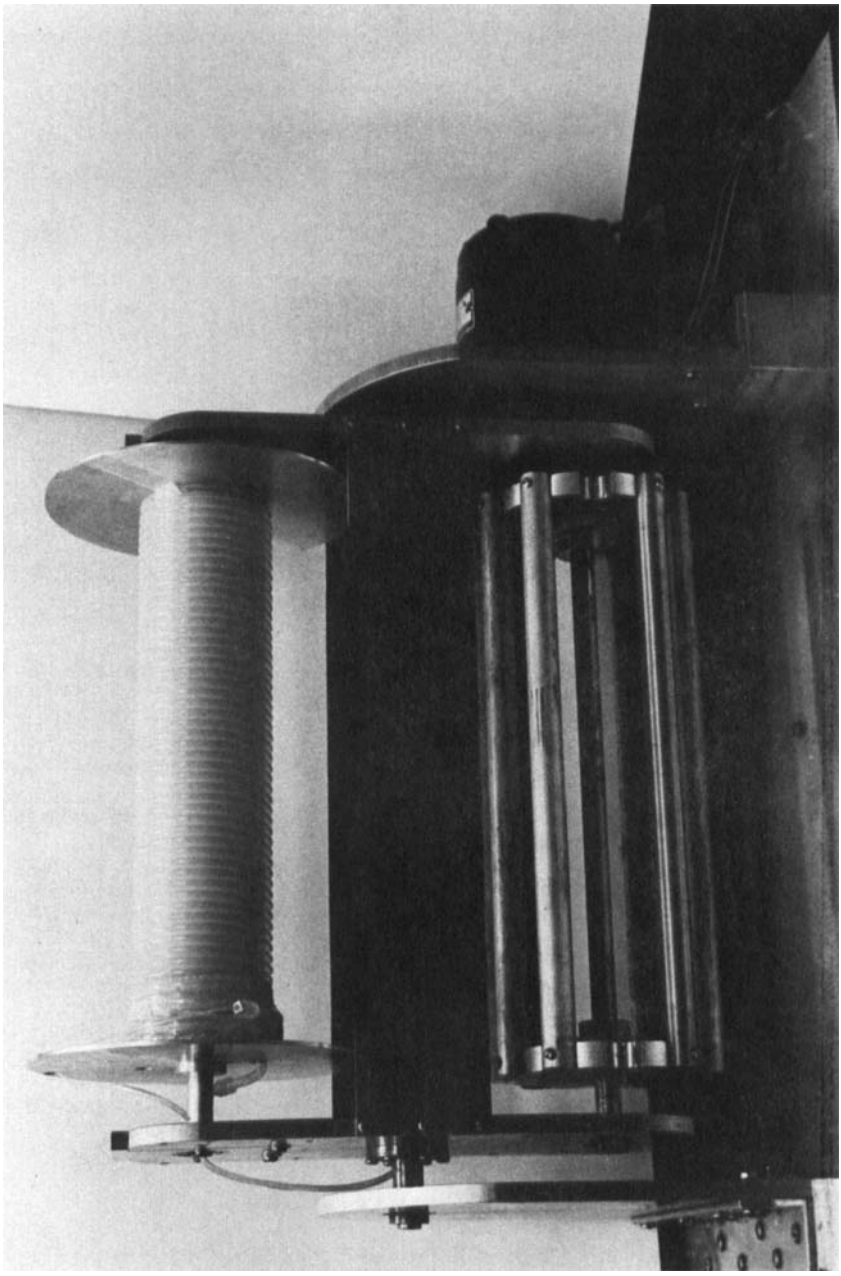


Figure 2. Modified Horizontal flow-through coil planet centrifuge.

coupled with an identical stationary sun gear rigidly mounted around the central stationary pipe. This gear arrangement produces the desirable synchronous planetary motion (Fig. 1) of each holder, i.e., rotation about its own axis and revolution around the central axis of the apparatus at the same angular velocity. The flanged holder holds the separation column while the other holder functions as a counterbalance.

Two types of the coiled columns were used in the present studies. Short columns used for the preliminary studies were made of a single piece of FEP tubing (Galtek) 430 cm long, 0.55 cm i.d., and 114 ml in capacity. The tubing was tightly wound around the holder core forming a single layer. The core of the holder measured 7.5 cm in diameter yielding a β value of 0.25. The β value was increased by arranging multiple plastic cylinders around the core of the holder over which the tubing was coiled. The use of the plastic cylinders of 1.9 cm and 3.8 cm o.d. yielded β values of 0.375 and 0.50, respectively. Long columns for the preparative separations were prepared from a single piece of similar tubing, 30 m long and 750 ml in capacity. Due to the length of the tubing, several layers were wound around the holder resulting in a range of β values, 0.25 - 0.30 without plastic cylinders and 0.50 - 0.55 with the plastic cylinders of 3.8 cm o.d. Flow tubes from the column were passed through the opening of the central stationary pipe to the outside of the centrifuge. The planetary motion of the apparatus prevents twisting of the flow tubes and thus eliminates the need for the conventional rotary-seal device and the associated problems of leakage and contamination.

Revolutional speed of the apparatus was adjustable from 0 to 400 rpm with a speed control unit (Bodine Electric Co.). For elution of the solvents, either a Beckman Accu Flo pump or a Milton Roy Minipump was

employed, depending upon the flow rates required. An LKB Uvicord S (280 nm) was used to monitor the effluent and an LKB fraction collector to collect fractions.

EXPERIMENTAL

Reagents

The following dinitrophenyl (DNP) amino acid samples were purchased from Sigma Chemical Co., Saint Louis, MO: N-2,4-DNP-L-aspartic acid (DNP-asp), N-2,4-DNP-DL-glutamic acid (DNP-glu), N,N-di-(2,4-DNP)-L-cystine (diDNP-$(cys)_2$), N-2,4-DNP-L-alanine (DNP-ala), and N-2,4-DNP-L-valine (DNP-val). Chloroform (glass-distilled chromatographic grade) was obtained from Burdick and Jackson Laboratories, Inc., Muskegon, MI, glacial acetic acid (reagent grade) from J. T. Baker Chemical Co., Phillipsburg, NJ, and 1N hydrochloric acid from Fisher Scientific Co., Fair Lawn, NJ.

Solvent System and Sample Solutions

The two-phase solvent system was prepared by mixing chloroform, acetic acid and 0.1N hydrochloric acid at a 2:2:1 volume ratio. Equilibration and degassing were accomplished in a 2 L separatory funnel at room temperature.

The sample solutions for the preliminary studies with the short columns were prepared by dissolving DNP-glu and DNP-ala in the upper aqueous phase to make a 0.5 g% concentration for each component. For preparative separations with the long columns, two different sample

solutions were prepared. For the first set of experiments (Fig. 4), 500 mg each of DNP-glu and DNP-ala were dissolved in 20 ml of a solvent mixture consisting of equal amounts of the upper and the lower phases. For the second set of experiments (Fig. 5), 250 mg each of DNP-asp, DNP-glu, DNP-ala, DNP-val, and 50 mg of diDNP-$(cys)_2$ were dissolved in 21 ml of the two-phase mixture in which 20 ml were charged into the column to make the net sample size of 1g.

Separation Procedure

In each separation the column was first filled with the stationary phase followed by injection of the sample mixture through the sample port. The mobile phase was then eluted through the column at the desired flow rate while the column was rotated at a given rpm. A series of experiments was performed by using both upper and lower phases as the mobile phase, each pumped at 120 ml/h and 500 ml/h under various rpm values of 50, 100, 200, 300 and 400. The effluent from the outlet of the column was continuously monitored via an LKB Uvicord S at 280 nm and fractionated with an LKB Ultrorac fraction collector. The collected fractions were further analyzed with a Beckman DU spectrophotometer at 430 nm. Following the sample separation the column contents were voided with nitrogen gas (60 psi) into a graduated cylinder to measure the volume of the stationary phase retained in the column.

Measurement of Partition Efficiency

The partition efficiency of the present CCC scheme was expressed in two different terms, i.e., theoretical plates and peak resolution. The

number of theoretical plates, N, was obtained according to the conventional gas chromatographic equation:

$$N = (4R/W)^2 \quad (1)$$

where R denotes the retention time of the peak maximum and W, the peak width provided both values are given in the same unit. The resolution between DNP-glu and DNP-ala peaks was computed by the following equation:

$$Ro = 2\Delta R/(W_1 + W_2) \quad (2)$$

where ΔR indicates the distance between the two peaks and W_1 and W_2, their peak widths, all expressed in the same unit.

RESULTS AND DISCUSSION

Preliminary Studies with Short Columns

A series of preliminary experiments has been performed to study the effects of the β values on the retention of the stationary phase and partition efficiency in the short columns.

The data for the stationary phase retention are summarized in Fig. 3 where a set of phase distribution diagrams are arranged according to the β values (0.25, 0.375, and 0.50) and the flow rates (120 ml/h and 500 ml/h) for the upper and the lower phases. In each diagram percentage stationary phase volume relative to the total column capacity is plotted against the applied revolutional speed in rpm. Two curves are drawn according to the elution mode of the mobile phase, i.e., the solid line for the head to tail elution mode and the broken line for the tail to head elution mode.

The retention profile of the stationary phase at β=0.25 (top row) shows a clearcut hydrodynamic trend that the lower phase is retained if

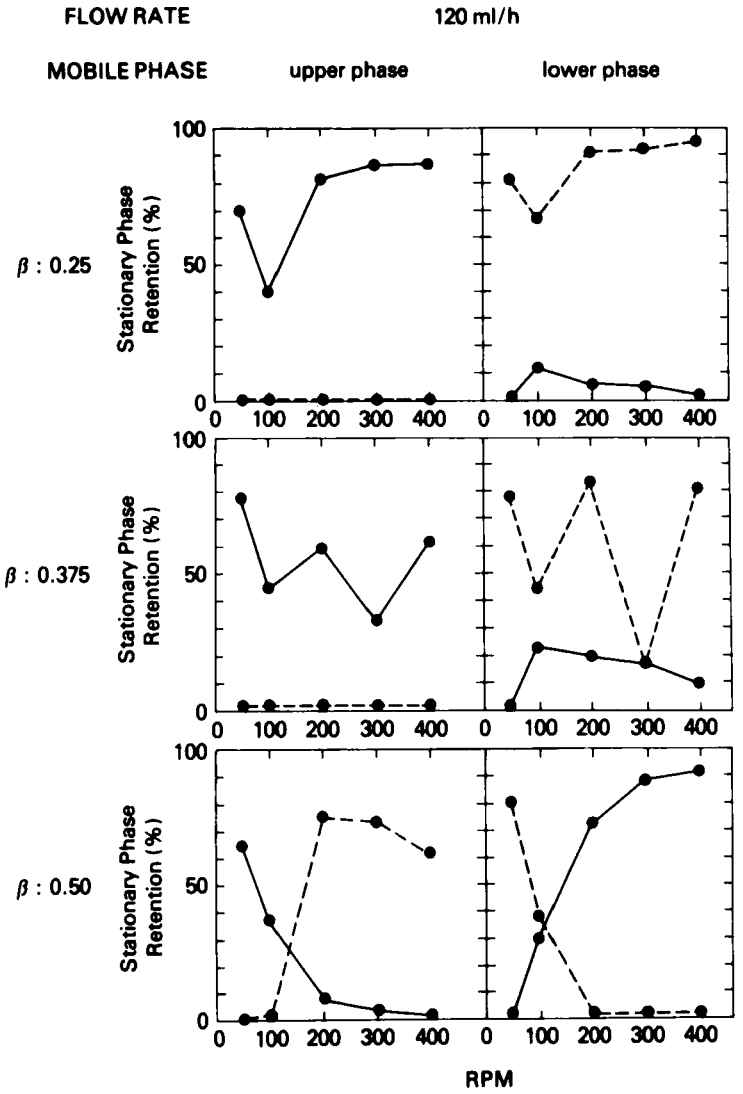


Figure 3. Phase distribution diagrams of chloroform/acetic acid/0.1N HCl (2:2:1) obtained with the short columns at three different β values.

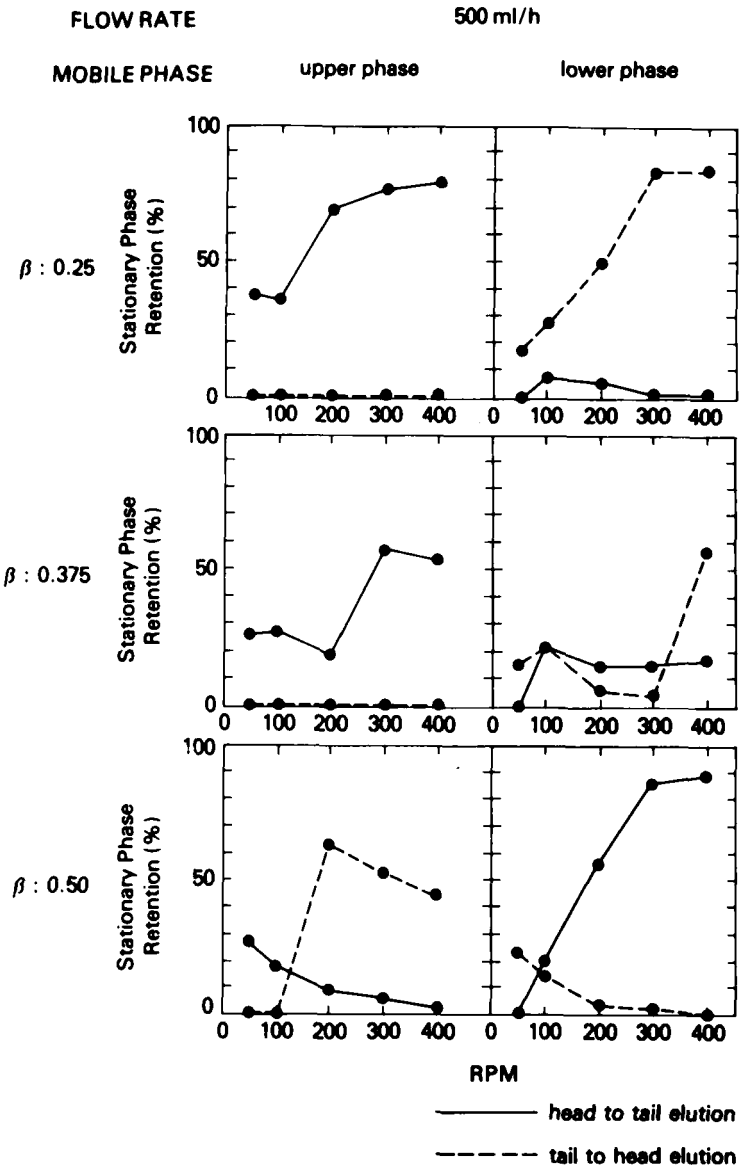


Figure 3 (continued)

the upper phase is eluted in the head to tail mode (solid line) and the upper phase is retained if the lower phase is eluted in the tail to head mode (broken line), regardless of the revolutional speed and flow rates within the applied ranges. This indicates that the two solvent phases are unilaterally distributed in the column, the lower phase on the head side and the upper phase on the tail side. When the β value is increased to 0.375 (middle row), the retention curves of both stationary phases become much lower with marked irregularity. At the large β value of 0.50 (bottom row), the relationship of the two curves is completely reversed at high revolutional speeds of over 200 rpm: the lower phase is retained in the tail to head elution mode (broken line) and the upper phase, in the head to tail elution mode (solid line). This retention profile indicates that the upper phase is now distributed to the head side and the lower phase to the tail side. The above findings are strongly supported by the recent hydrodynamic studies with a 1.6 mm i.d. coil in which the β values produced drastic effects on the unilateral distribution of tertiary solvent systems such as the one used in the present studies (3). Comparison between the data obtained with the two different flow rates shows that the higher flow rate of 500 ml/h gives a substantially lower retention of the stationary phase especially at the transitional condition of β=0.375.

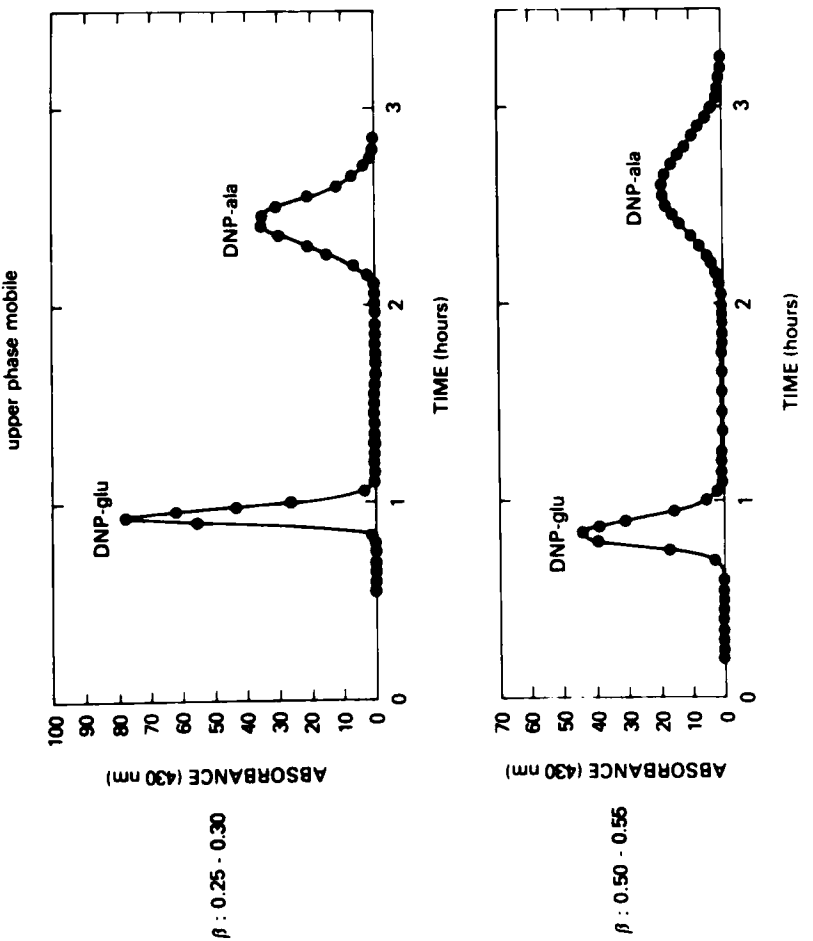
Partition efficiency attained with the short columns increased with the retention of the stationary phase reaching the maximum level at 300 to 400 rpm for all β values. Among those the intermediate β value of 0.375 yielded the lowest efficiency apparently due to the lowest retention of the stationary phase. The partition efficiencies produced at β = 0.25 greatly exceeded those at β = 0.5 in both theoretical plate number N and peak resolution Ro despite the similar retention volumes of the stationary phase (Table 1).

TABLE 1. Summary of Experimental Conditions and Results

Column	β	Sample	Mobile** Phase	% Retention	N*** DNP-glu	N*** DNP-ala	# Rs
Short	0.25		up	77.5	77.4	89.8	2.1
	0.25	DNP-glu & DNP-ala	lp	84.1	75.6	76.6	2.1
	0.50	total 5 mg	up	51.3	28.0	25.0	0.97
	0.50		lp	84.6	14.7	20.6	0.98
Long	0.25-0.30		up	65.3	495	357	4.4
	0.25-0.30	DNP-glu & DNP-ala	lp	83.7	337	410	4.7
	0.50-0.55	total 1 g	up	79.1	147	116	3.0
	0.50-0.55		lp	86.5	123	100	2.9
Long	0.25-0.30		up	61.2	792	353	4.4
	0.25-0.30	5 DNP amino acids*	lp	74.9	355	400	4.4
	0.50-0.55	total 1g	up	64.9	139	112	2.5
	0.50-0.55		lp	82.7	101	127	2.6

* DNP-asp, DNP-glu, di-DNP-$(cys)_2$, DNP-ala and DNP-val
** up: upper phase; lp: lower phase.
*** theoretical plate number
\# peak resolution between DNP-glu and DNP-ala

The above difference in partition efficiency between the two β values may be partially explained in the light of the findings obtained from the recent hydrodynamic studies (6). Stroboscopic observations on the motion of colored solvent phases in a spiral column (2.6 mm i.d. and β=0.5-0.9) have shown that in each spiral segment the two solvent phases are locally mixed to form a droplet zone which moves toward the head of the column at a rate equal to the revolutional speed of the centrifuge. Under ideal conditions each droplet zone may produce one theoretical plate. Since the length of each droplet zone nearly equals one fourth of each spiral turn, the internal segments with small β values can yield more theoretical plates per unit length of tubing than the external segments with large β values. Although this hypothesis appears to be consistent with the results of the present studies, the above



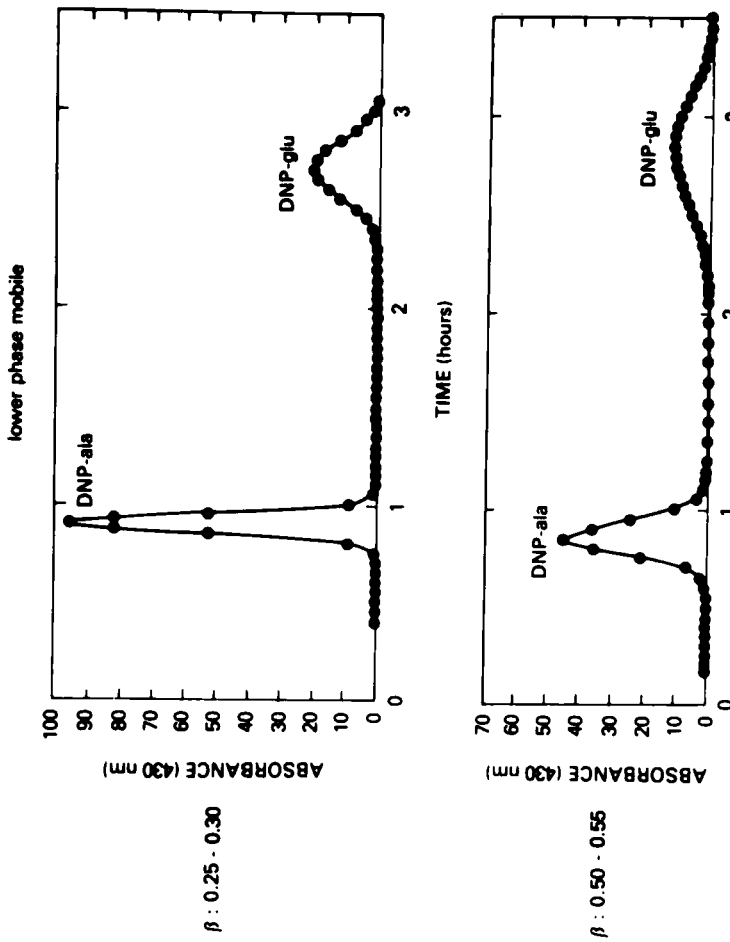


Figure 4. Preparative separations of DNP-glu and DNP-ala with the long columns at two different ranges of β values.

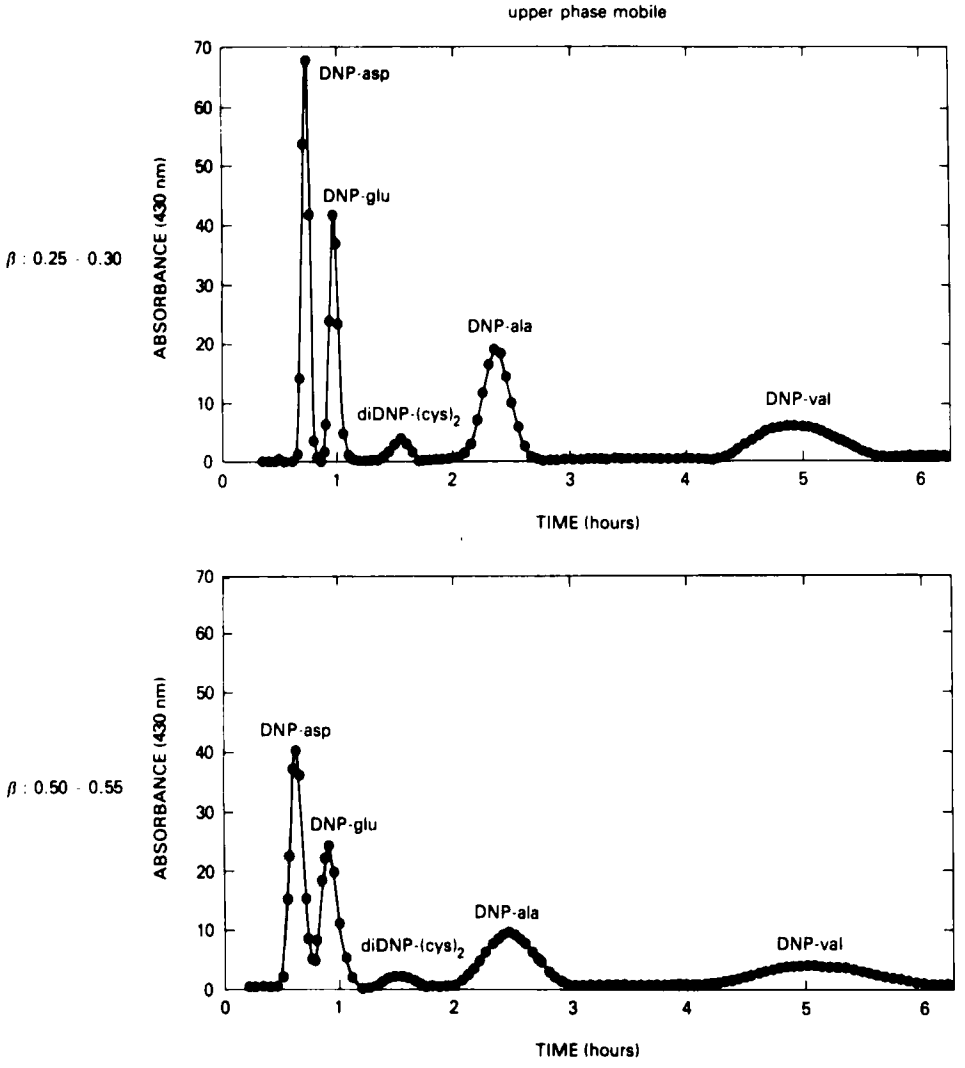
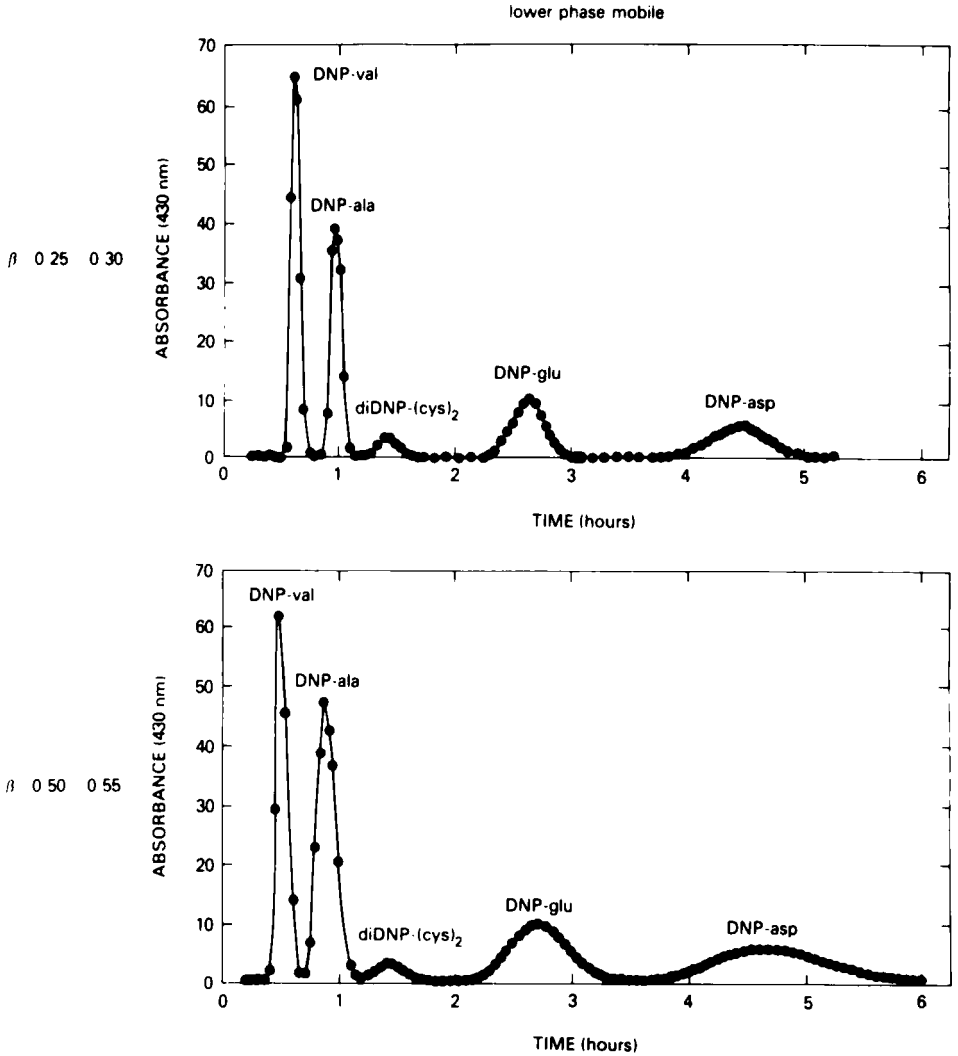


Figure 5. Preparative separations of five DNP amino acids with the long columns at two different ranges of β values.



Column 5.5 mm i.d. & 750 ml capacity; Revolution 300 rpm;
Flow rate 500 ml/h; Sample size 1 g

Figure 5 (continued)

experimental observation should be extended to cover a lower range of β values such as 0.25 where the hydrodynamic distribution mode of the two solvent phases is completely reversed as demonstrated in the present studies.

Preparative Separations with Long Columns

Preparative-scale separations have been performed with the long columns, 30 m long and 750 ml in capacity, under the optimum operational conditions determined by the preliminary experiments with the short columns. The two sets of standard sample mixture, each consisting of one-gram quantities of DNP amino acids, were used in these experiments (Table 1).

Fig. 4 shows a set of chromatograms of DNP-glu and DNP-ala at 300 rpm with the two different ranges of β values at 0.25 - 0.30 and 0.5 - 0.55 by using both the upper and the lower phases as the mobile phase. As expected from the preliminary separations obtained with the short columns, the runs at the small β value yielded substantially higher partition efficiencies in terms of both theoretical plate number N and peak resolution R_0 (Table 1). Fig. 5 similarly illustrates a set of chromatograms of five DNP amino acid under otherwise identical operational conditions. Here again separations obtained at the small range of β values produced substantially higher partition efficiencies than those at the large range of β values.

Overall results indicate that the present method quickly yields excellent separations of lg-quantity samples at a moderate revolutional speed. The results also suggest that the sample-loading capacity of the

present scheme can be further increased by many folds simply by applying a longer column. Large industrial-scale separations may also be feasible by the use of a larger-bore coiled column.

ACKNOWLEDGEMENT

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