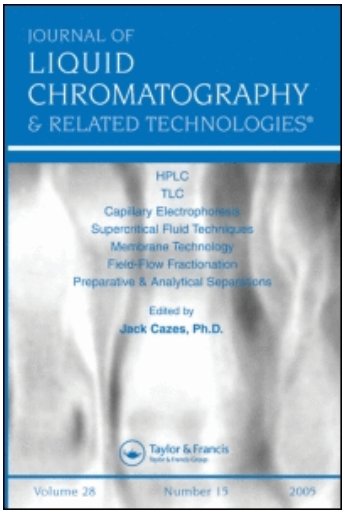

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Improved Scheme for Preparative Countercurrent Chromatography (CCC) with a Rotating Coil Assembly

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IMPROVED SCHEME FOR PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY (CCC) WITH A ROTATING COIL ASSEMBLY

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ABSTRACT

Efforts have been successfully made to improve a preparative CCC scheme utilizing a slowly rotating coil assembly by optimizing the orientation of the coiled column. By the aid of a standard set of test samples and a two-phase solvent system, the performance of the single coil was examined in both eccentric and coaxial orientations with respect to the axis of rotation. In the eccentric location 10 cm away from the rotation axis, changes in the skew angle and/or inclination of the apparatus failed to improve the separation significantly. On the other hand, the coils mounted coaxially around the rotation axis in various helical diameters all produced excellent peak resolution at critical rotational speeds due to a high level of stationary phase retention. This finding facilitated the development of a new separation column consisting of multiple layers of the coil coaxially arranged around the rotary support. The preparative capability of this multi-layer coil was demonstrated in the separations of lg-quantity samples with satisfactory results. The present scheme is amenable to be further scaled up for industrial applications.

INTRODUCTION

Countercurrent chromatography (CCC) has an advantage over liquid chromatography in that it eliminates complications arising from the use of solid supports (1). In the past preparative CCC has

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been performed with a slowly rotating coil assembly, which holds coiled columns in the eccentric positions around the horizontal axis of rotation (2,3). Recently, efforts have been made to increase the sample-loading capacity of the scheme by the use of larger-bore coils similarly arranged around the rotary shaft (4).

This paper describes the continued development of this preparative CCC scheme which involves changing the orientation of the coiled column. Experiments were performed to test both eccentric and coaxial locations of the coil on the rotary shaft. In the eccentric orientation of the coil, changing the column angle relative to the rotary shaft and/or tilting the device against the horizontal plane was found to give only slightly improved results. On the other hand, the same column mounted coaxially around the rotary shaft produced excellent peak resolution with an extremely high level of stationary phase retention. In this coaxially rotated coil, increasing the helical diameter from 3 cm to 20 cm showed little difference in peak resolution. This new finding led to the development of an efficient separation column which consists of multiple layers of coil mounted concentrically around the rotary shaft. The performance of these columns was evaluated in the separation of a standard set of DNP (dinitrophenyl) amino acid samples and a two-phase solvent system composed of chloroform, acetic acid and 0.1N hydrochloric acid at a 2:2:1 volume ratio.

PRINCIPLE

The principle of CCC with a slowly rotating coil has been described earlier (1-4). When a water-filled coil is held horizontal and slowly rotated around its own axis, any object either heavier or lighter than the water moves toward one end of the coil. This end is called the head and the other end, the tail of the coil. When such a coil contains two immiscible solvents, slow rotation soon establishes a hydrodynamic equilibrium between the two solvent phases in which the head side of the coil is occupied by nearly

equal amounts of the two phases and any excess of either phase is found at the tail end of the coil. Under this hydrodynamic equilibrium condition, the coil can be eluted with one of the phases through the head end while retaining the other phase stationary in the coil. Consequently, solutes locally introduced at the head of the coil are subjected to an efficient partition process between the mobile and stationary phases and are chromatographically separated according to their partition coefficients in the absence of solid supports. The eluate eluted through the tail end of the coil is continuously monitored for its absorbance and then fractionated as in liquid chromatography.

Peak resolution produced by this CCC scheme is greatly influenced by the volume of the stationary phase retained in the coil, i.e., the higher the retention level, the better the result. It has been observed that the retention of the stationary phase is quite sensitive to the orientation and rotational speed of the coil (5). In the coaxially rotated coil, slow rotation usually yields near 50% retention. Increasing the rotational speed of the coil radically changes the hydrodynamic equilibrium volume ratio of the two phases in the coil and, in some critical range, one of the phases almost entirely occupies the head end and the other phase, the tail end of the coil. This equilibrium condition permits a high level of stationary phase retention if the mobile phase is introduced in the proper direction (5). In the eccentric orientation of the coil, the centrifugal force field induced by the rotation tends to trap the heavier phase in the outer half and the lighter phase in the inner half of each helical turn resulting in a more or less even distribution of the two phases throughout the coil and, therefore, the retention of the stationary phase becomes rather insensitive to the rotational rate of the coil. Thus eccentrically rotated coils tend to retain the stationary phase no more than 50% of the column volume unless the axis is inclined against the horizontal plane (2,3). While hydrodynamic behavior of the solvents in the rotating coil are highly complex and difficult to predict, the optimum coil orientation is easily determined by a series of

experiments with a standard set of samples and a two-phase solvent system.

MATERIALS AND METHOD

Apparatus

Two types of rotary devices with comparable functions were employed. The first device was equipped with a rotary seal at each terminal of the rotary shaft to establish a flow-through system (2,3). In the present experiment a clamp rod was mounted on the rotary shaft to support a coiled column at an eccentric position 10 cm away from the axis of rotation. The coil was positioned at a desired skew angle while the rotary shaft was set at a desired incline against the horizontal plane (See Fig. 1, left). The second rotary device was equipped with a rotating-seal-free flow-through system similar to that reported earlier (4). In this device, the coil was coaxially mounted around the rotary shaft at various helical diameters over a spool-shaped support. In both rotary devices, rotational speed is continuously adjustable up to 400 rpm.

Coiled Column

Both glass and plastic coils were tested at the eccentric location. The glass coils (Kontes Scientific Glass Co., Vineland, NJ) consisted of 45 helical turns of 0.5 cm i.d., 3 cm helical diameter with a total capacity of about 90 ml. The plastic coil was prepared from a piece of 0.55 cm i.d., 420 cm long FEP (fluorinated ethylene propylene) tubing (Galtek Corp., Jonathan Ind. Ctr., Chaska, MN) by winding it on to a 2.5 cm o.d. aluminum pipe core to make about 45 helical turns with a total capacity of about 100 ml. The same plastic tubing was used to test the coaxial orientation on the second rotary device in which the tube was wound around the spool-shaped support to make a single layer coil with helical

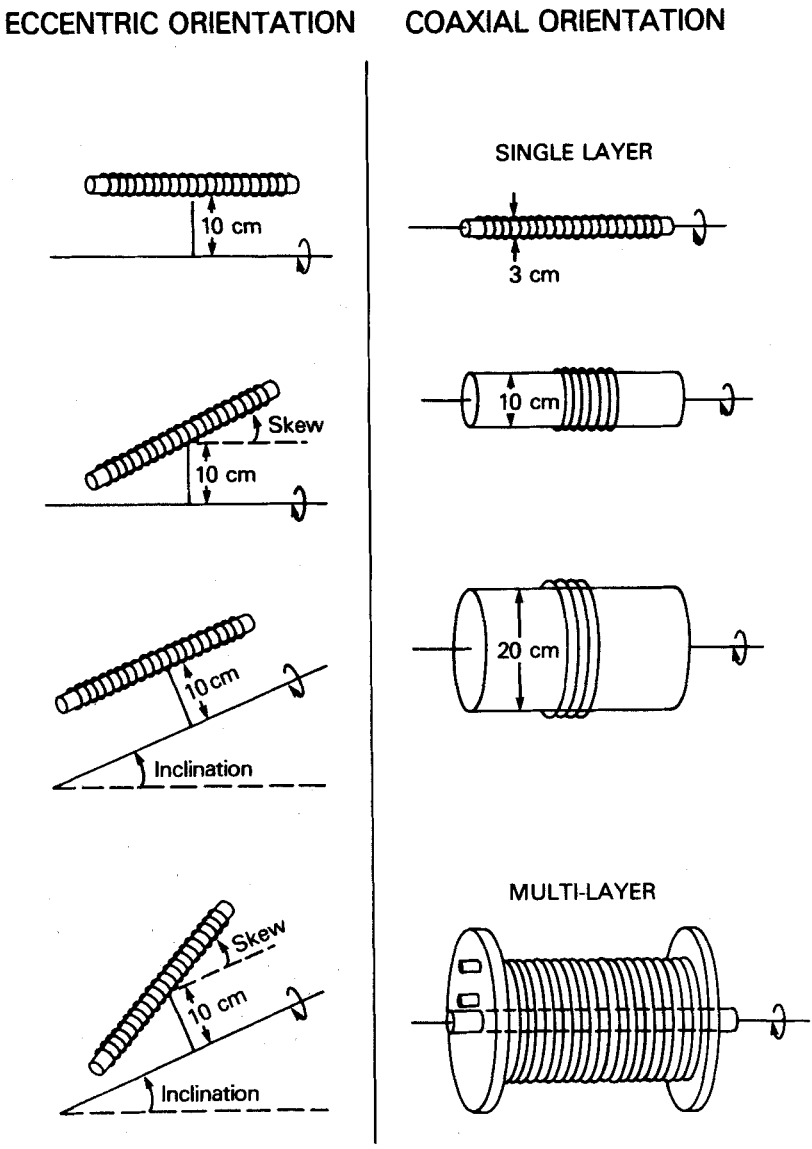

FIGURE 1. Various orientations and configurations of the coiled separation columns.

diameters of 3 cm, 10 cm and 20 cm (see Fig. 1, right top). For preparative separations, a multi-layer coil (Fig. 1, right bottom) was prepared from a 30 m long FEP tube of the same type by winding it coaxially over a 10 cm diameter, 25 cm wide spool support to make nearly 3 layers of the coil with a total capacity of about 750 ml. Each terminal of all these coiled columns was connected to 0.85 mm i.d. PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ) for continuous elution.

Solvent System and Sample Solution

The two-phase solvent system used in the present study consisted of chloroform (Burdick and Jackson Laboratories, Inc., NJ), glacial acetic acid and 0.1N hydrochloric acid (Fisher Scientific Co., Fairlawn, NJ) at a volume ratio of 2:2:1. The solvent mixture was equilibrated in a separatory funnel at room temperature and separated before use.

N-2,4-DNP-D,L-glutamic acid and N-2,4-DNP-L-alanine (Sigma Chemical Co., St. Louis, MO) were selected as test samples. For comparative studies with short columns, the sample solution was prepared by dissolving the DNP amino acid mixture in the upper aqueous phase to make the concentration of each component 0.5g%, and 0.5 ml was used for each separation. For preparative-scale separations with the multi-layer coil, the sample solution was prepared by dissolving 500 mg of each DNP amino acid for a total of 1g in 30 ml of the solvent, consisting of equal amounts of the upper and lower phases.

Measurement of Phase Distribution

The distribution of the two solvent phases in the rotating coil was studied with a 0.55 cm i.d. FEP coiled tube coaxially mounted on the rotary support in helical diameters of 3 cm, 10 cm and 20 cm.

In order to facilitate observation, the lower nonaqueous phase was colored with Sudan III. The column was first filled with equal volumes of the upper and lower phases and both inlet and outlet tubes were clamped. Then the apparatus was rotated at a given rate until the two solvent phases established the hydrodynamic equilibrium, in which one of the phases predominantly occupied the head side leaving the excess volume of the other phase at the tail side of the coil. Upon stopping the rotation, the number of helical turns, n, containing the predominant phase was noted. From the total number of helical turns, N, the percentage volume occupied by the predominant phase in the equilibrated coil was calculated from the expression, 50N/n. Alternatively, for a coil with large helical diameters, the length of the segment of each phase occupying in one helical turn was directly measured to obtain percentage figures for each phase. The above procedure was repeated without renewing the column contents while changing the rotational speed of the coil to obtain a series of measurements for each helical diameter of the coil.

Separation procedure

For comparative studies with short columns, the separations of the DNP amino acids were performed as follows: The column was first filled with the stationary phase. This was followed by injection of the 0.5 ml sample solution containing 5 mg of DNP amino acid mixture through the sample port which was located on the flow line between the outlet of the pump and the inlet of the coil. Then the coil was rotated at a given rate while the mobile phase was introduced through the coil at a rate of 120 ml/h with a Milton Roy Minipump or a Chromatronix Cheminert pump. Although the elution was usually performed from the head of the coil towards the tail, the reversed elution mode (tail to head) was also applied to the coils with the coaxial orientation. The eluate through the outlet of the coil was continuously monitored for absorbance at 280 nm with an LKB Uvicord

S and LKB recorder to obtain elution profiles of the samples. After the separation was completed, the apparatus was stopped and, by connecting the inlet of the coil to a pressured N_2 line (50 psi), the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the coil. During the process of filling the column with the solvent or emptying the column contents with N_2, the coil was slowly rotated in a reversed mode (tail to head) to eliminate trapped air bubbles or remaining solvent from the coil.

Preparative-scale separations were performed with a multi-layer coil mounted coaxially on the rotary support. The column was similarly filled with the stationary phase under the reversed mode of slow rotation to eliminate trapped air bubbles from the coil. After the filling process was completed, the coil was rotated at the optimum rate of 80 rpm in the desired direction, while the sample solution, 30 ml in volume containing lg DNP amino acid mixture as described above, was injected into the rotating coil through the sample port at a rate of 4 to 5 ml per minute. During the injection, the sample-loading syringe was kept substantially in the horizontal position so that both phases would be evenly introduced into the stream. Then the column was eluted with the mobile phase at a rate of 120 ml/h with a Milton Roy Minipump. Both the upper and the lower phases were used as the mobile phase at the identical operational conditions except that the upper phase was eluted in the normal mode (head to tail) and the lower phase in the reversed mode (tail to head). The eluates through the outlet of the coil were collected with an LKB fraction collector to obtain a 12-ml fraction in each test tube. A 20µl volume of each fraction was then mixed with 3 ml of methanol to measure the absorbance at 430 nm with a Beckman DU spectrophotometer. After the separation, the volume of the retained stationary phase was measured by emptying the column contents into a graduated cylinder by means of N_2 pressure and reversed slow rotation as described earlier.

RESULTS AND DISCUSSION

Eccentric Orientation of the Coil.

The performance of the short coils mounted 10 cm away from the axis of rotation was studied by varying both skew angle and inclination. The DNP amino acid separations obtained using glass and FEP coils are summarized in Fig. 2 where individual charts are arranged according to the applied operational conditions of coil orientation, choice of the stationary phase and the rotational speed.

In the first experimental group with the glass coil (Fig. 2A), the 0° skew, 0° inclination condition used in the previous studies (4) produced almost identical results (left column). These separations are considerably improved by changing either skew or inclination angle. The best peak resolutions are found in 0° skew, 15° inclination at 40 rpm for both nonaqueous and aqueous stationary phase groups (middle column). In the second experimental group with the FEP coiled tube (Fig. 2B), the best separations are observed at 40 rpm for the stationary nonaqueous phase and 60 rpm for the stationary aqueous phase, both under 0° skew and 0° inclination (left column). Application of either 15° skew or 15° inclination failed to give any appreciable improvement in the peak resolution. Although skew and inclination produce some significant improvement of the performance of the glass coil, the FEP coiled tube positioned at 0° skew and 0° inclination yields the best peak resolutions among all experimental groups.

Coaxial Orientation of the Coil

The FEP coiled tube mounted eccentrically for the above studies was uncoiled and rewound coaxially around the rotary shaft in three helical diameters of 3 cm, 10 cm and 20 cm to investigate the hydrodynamic behavior of the two solvent phases and the partition efficiency at various rotational speeds.

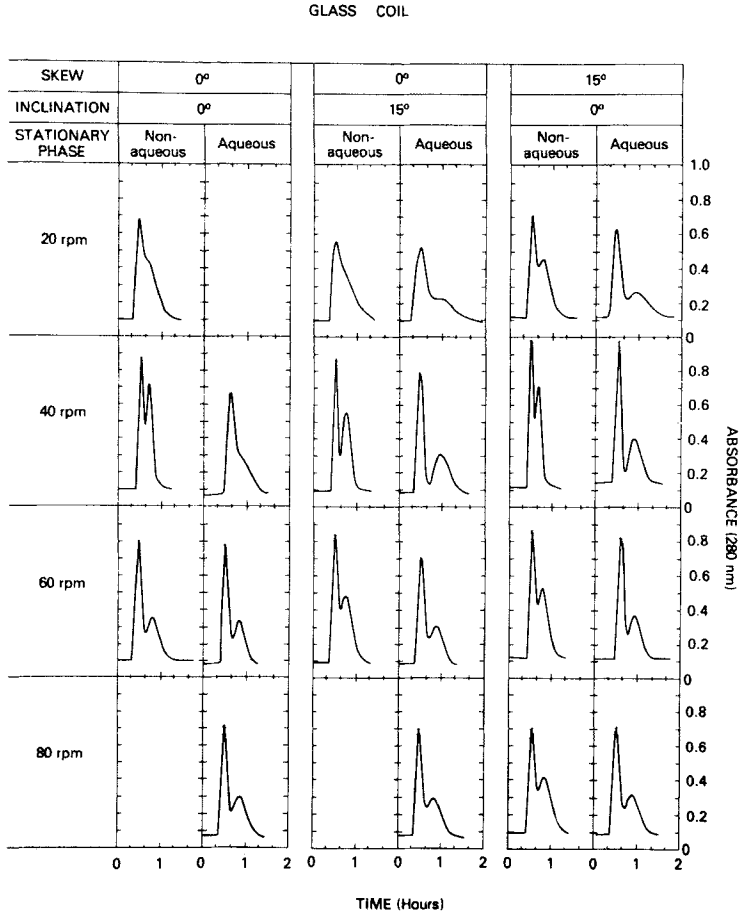

FIGURE 2. Effects of skew angle and inclination of the eccentrically rotated coil on the separations of DNP amino acids at various rotational speeds.

A. Glass Coil.

B. FEP Coil.

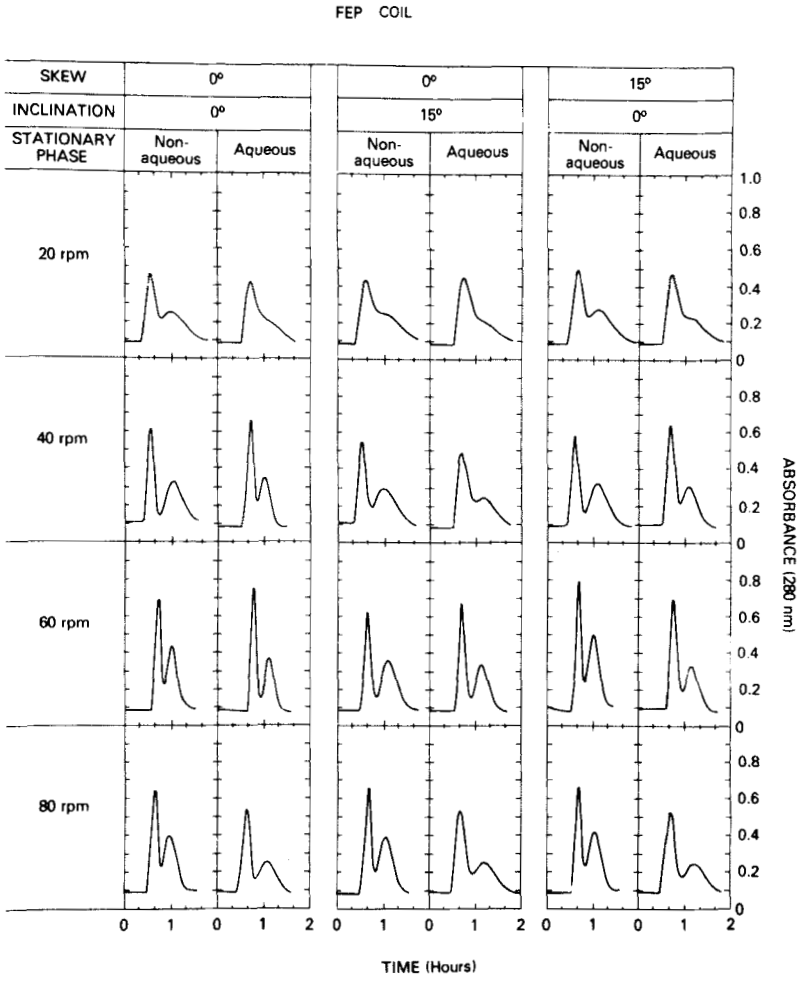

FIGURE 2B

The distribution of the two phases in the coaxially rotated coils measured at their hydrodynamic equilibrium is shown in Fig. 3. In each diagram, the volume percentage of each phase at the head side of the coil is plotted against the applied rotational speed. The three diagrams obtained from different helical diameters show common features characteristic of the coaxial orientation. In the

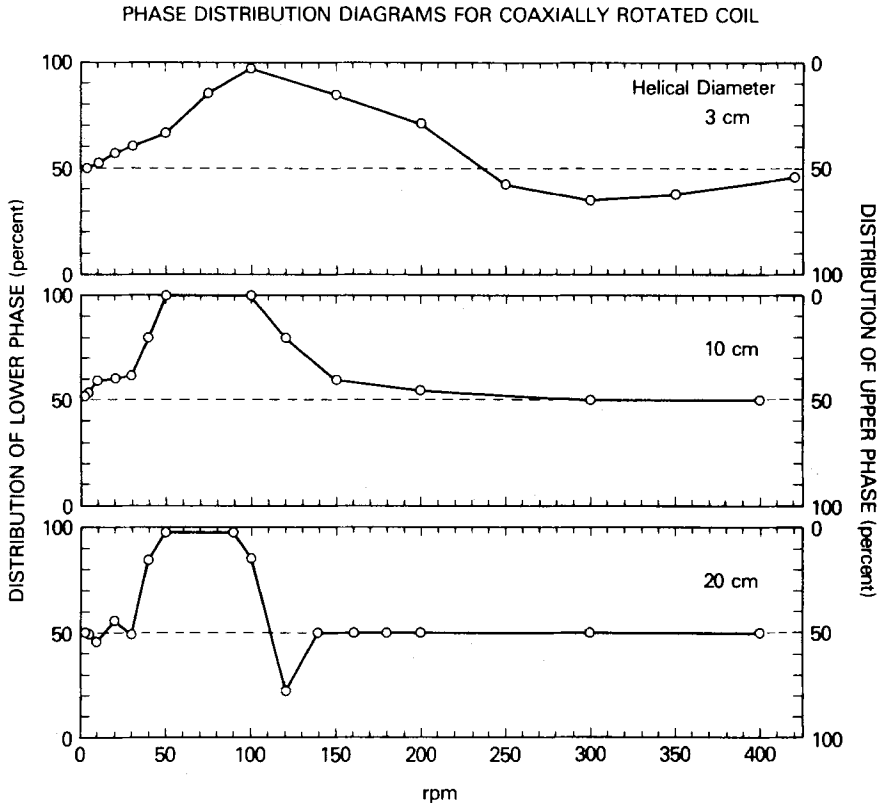

FIGURE 3. Phase distribution diagrams for coaxially rotated coils with three different helical diameters.

slow rotational speed between 0 and 30 rpm, the two phases distribute fairly evenly in the coil (stage I). When the rotational speed is increased, the lower nonaqueous phase tends to occupy more space on the head side of the coil and, at a critical speed between 60 and 100 rpm, the two phases are almost completely separated along the length of the coil, the lower phase occupying the head side and the upper phase, the tail side of the coil (stage II). After this critical range, the amount of lower phase on the head side tends to decrease rather sharply, crossing below the 50% line (stage III). A further increase in the rotational speed again yields an even

distribution of the two phases in the coil (stage IV). As the helical diameter increases all these stages tend to shift toward the lower rpm range apparently due to the enhanced centrifugal force field. For performing preparative CCC, stage II is considered to be of greater interest because the system permits retention of a large amount of the stationary phase for either phase under the proper mode of elution as described earlier (5). The uneven distribution of the two phases in the coil at the critical rpm permits an extremely high level of stationary phase retention when either the aqueous phase is eluted in the normal mode (head to tail) or the nonaqueous phase in the reversed mode (tail to head).

The partition efficiency of the coaxially rotated coils was measured by the set of DNP amino acid samples and the two-phase solvent system which were used in the studies on the eccentric orientation. The experimental results with the three different helical diameters are summarized in Fig. 4 in which individual charts are arranged according to various operational conditions. In addition to the normal elution mode (head to tail), the reversed elution mode (tail to head) was also applied for the nonaqueous mobile phase at the critical rpm range (stage II) to achieve a high level of stationary aqueous phase retention as is expected from the phase distribution diagrams shown in Fig. 3.

The overall results of these experiments with the coaxially rotated coils clearly indicate that the best peak resolutions are obtained at the critical rotational speed ranging between 60 rpm and 100 rpm, when either the aqueous phase was eluted in the normal mode (head to tail) or the nonaqueous phase in the reversed mode (tail to head). As predicted from the phase distribution diagrams in Fig. 3, the normal mode of elution with the stationary aqueous phase (middle column in each helical diameter in Fig. 4) gave unsatisfactory peak resolution due to a low level of stationary phase retention. Under the proper elution mode the resultant peak resolutions from the coaxially rotated coils are much higher than those from the eccentrically rotated coils shown in Fig. 2.

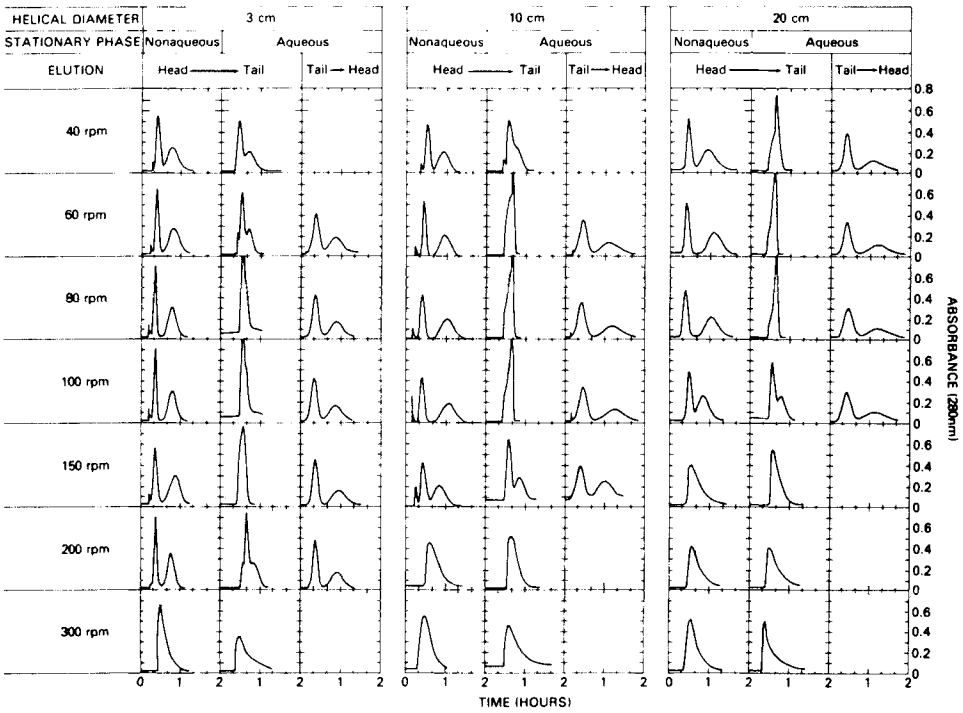

FIGURE 4. Effects of helical diameters of the coaxially rotated coil on the separations of the DNP amino acids at various rotational speeds.

Comparison of the results obtained from the three different helical diameters illustrated in Fig. 4 reveals the considerable shift of the optimal conditions toward the lower rpm range in the larger helical diameter coil, this being consistent with the finding from the phase distribution diagrams in Fig. 3. However, the satisfactory peak resolution observed in a wide range of rpm in each helical diameter suggests that the choice of a proper rotational speed such as 80 rpm would produce good separations in the coil with any helical diameter between 3 cm and 20 cm. In light of these experimental data with the coaxially rotated coils, a new column configuration called the multi-layer coil (Fig. 1, right bottom) has been made for large-scale preparative CCC.

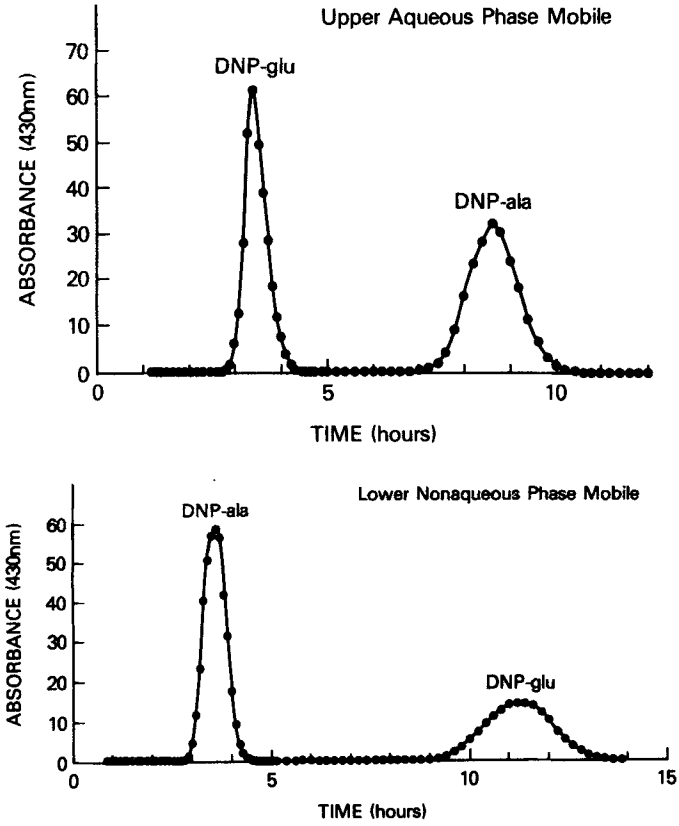

FIGURE 5. Chromatograms of DNP amino acids (1g quantity) obtained with the multi-layer coil.

A. Elution with the upper aqueous phase in the normal mode (head to tail).

B. Elution with the lower nonaqueous phase in the reversed mode (tail to head).

Preparative CCC with a Multi-Layer Coil

The preparative capability of the multi-layer coil has been demonstrated in the separations of 1g quantity of the DNP amino acid mixture under the optimum operational conditions determined by the preliminary studies on short coils as described above. Fig. 5 shows the chromatograms obtained with the multi-layer coil in which both the upper aqueous phase (A) and the lower nonaqueous phase (B) are used as the mobile phase in the suitable elution mode. The two DNP amino acids are well resolved and eluted out as symmetrical peaks. The retention of the stationary phase measured after the separation was 68% in A and 84% in B. The separations obtained with this multi-layer coil with a 750 ml capacity are much better than those from the eccentrically mounted glass coils with a similar internal diameter and having a total capacity of 900 ml (4).

The multi-layer coil has a number of advantages over the previous CCC schemes utilizing a slowly rotating coil assembly. Because of the extremely large volume of the retained stationary phase, both peak resolution and the sample-loading capacity of the column are much increased. The column is leak-free, easily prepared and relatively inexpensive. The compactness of the column reduces the size of the apparatus saving cost and space in the research laboratory. The present scheme is amenable to be scaled up for large-scale industrial applications.

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