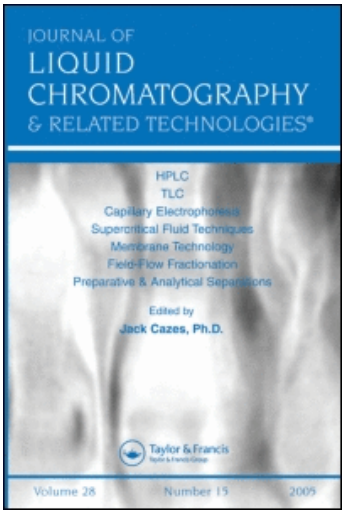

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Gram Quantity Separation of DNP (Dinitrophenyl) Amino Acids with Multi-Layer Coil Countercurrent Chromatography (CCC)

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GRAM QUANTITY SEPARATION OF DNP (DINITROPHENYL) AMINO ACIDS WITH MULTI-LAYER COIL COUNTERCURRENT CHROMATOGRAPHY (CCC)

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ABSTRACT

The efforts have been successfully made to extend the preparative capability of the high-speed CCC scheme with a multi-layer large capacity coiled column. The apparatus is a table top model of a horizontal flow-through coil planet centrifuge which produces a synchronous planetary motion of the column holder. The separation column was prepared from a single piece of 70 m long, 2.6 mm i.d., PTFE tubing coiled around the spool-shaped holder to form multiple layers of the coil with a total capacity of about 400 ml. The performance of the apparatus was assessed with a standard set of DNP amino acid samples and a two-phase solvent system composed of chloroform, acetic acid and 0.1N HCl (2:2:1). Preparative capability of the method was evaluated in terms of the retention level of the stationary phase and peak resolution for various sample size ranging from 0.05g to 2g. The effects of sample volume, sample concentration and the choice of the sample diluent on the separation were studied. The results indicated that both the retention level and the peak resolution tend to decrease with the increase of the sample volume applied at a given concentration. For separation of 1 gram quantity, best results were obtained by applying the sample dissolved in a small volume (10 ml) consisting of equal amounts of the two phases. Overall results indicate that the present scheme is capable of efficient separation for gram quantity of samples in a short period of time. The preparative capability may be further increased by the use of a larger-diameter and/or longer coil.

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INTRODUCTION

Countercurrent chromatography (CCC) has a great potential in performing preparative-scale separations (1). The method eliminates all complications arising from the use of solid supports and retains a large volume of the stationary phase in the free space in the column to provide a large sample-loading capacity. The preparative CCC schemes developed in the past, however, requires relatively long separation times ranging from an overnight to a few days for completing a sizable separation (2-5). Recently, we have developed a new CCC scheme which produces highly efficient separations in short periods of time (6,7). The scheme uses a separation column consisting of multiple layers of the coil around a spool-shaped holder which is subjected to a synchronous planetary motion by the use of a coil planet centrifuge. With the use of 1.6 mm i.d. tubing, the scheme has produced separation of various biological samples in the order of 100 mg quantities within 3 hours. Efforts have been successfully made to scale up the sample loading capacity of the present scheme by using larger-diameter tubing. This paper describes the results of 1-g quantity separations of dinitrophenyl (DNP) amino acid mixture with a 2.6 mm i.d. multi-layer coil. Effects of sample dose, sample volume and sample diluents on the retention of the stationary phase and the partition efficiency are studied to optimize the operational conditions.

PRINCIPLE

As described earlier (1), the mechanism of countercurrent chromatography (CCC) is based on the complex hydrodynamic motion of two immiscible solvent phases in a coiled tube. When a water-filled coil is held horizontal and rotated around its own axis, anything that is heavier than water (glass bead) or lighter than water (air bubble) will tend to move towards the head end of

the coil. This is in accordance with the Archimedean screw principle. When two immiscible solvents are introduced into the coil, a hydrodynamic equilibrium is established whereby a certain volume of each phase occupies each helical turn (equilibrium volume ratio) and any excess of either phase remains at the tail end of the coil. This hydrodynamic equilibrium behavior of the two solvent phases can be efficiently used for solute partitioning. The column is first filled entirely with the stationary phase and the mobile phase is then pumped through the head of the coil. As the mobile phase occupies the first helical turn, hydrodynamic equilibrium (above) is established, thus producing a large retention of the stationary phase in the first helical turn. This process repeats until the mobile phase reaches the tail end of the coil after which only the mobile phase is displaced.

The amount of stationary phase retained in the coil is dependent upon two factors. The first is the initial equilibrium volume ratio before the elution is started. This ratio determines the maximum attainable retention level of the stationary phase. When the mobile phase is introduced into the inlet of the coil some of the stationary phase is displaced thus altering the equilibrium ratio. The other factor that determines retention of the stationary phase is the returning rate of the stationary phase. The higher the rate of the returning stationary phase, the higher the percent of retention, but always within the maximal attainable equilibrium volume ratio. In order to achieve a satisfactory retention level of the stationary phase, a large initial equilibrium volume ratio must be obtained in addition to a high rate of flow of the returning stationary phase. In the above simple scheme described, the initial equilibrium volume ratio meets the criteria to obtain a high level of retention; however, the returning rate of the stationary phase is insufficient if a high elution rate is to be applied.

Recently modifications have been made to the above scheme so that both requirements are satisfied (8). In Figure 1 cylindrical holder coaxially mounted with a planetary gear is coupled to an

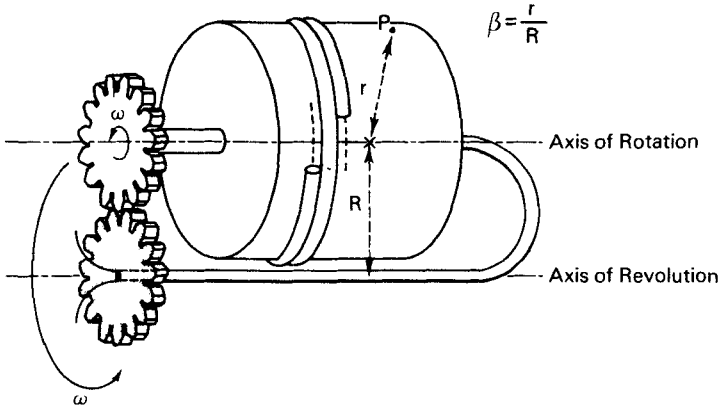

Figure 1. Synchronous planetary motion of the coil holder.

identical stationary sun gear (shaded) that is mounted along the central axis of the apparatus. This type of arrangement produces a beneficial planetary motion of the coil holder. The holder revolves around the central axis of the apparatus and simultaneously rotates about its own axis at the same angular velocity in the same direction. The holder always maintains its axis parallel to and at a distance R from the central axis of the apparatus.

This synchronous planetary motion produces a type of centrifugal force field that establishes a favorable hydrodynamic equilibrium that produces higher retention of the stationary phase. Experimental observations revealed that the magnitude and acting pattern of this centrifugal force field favor the heavier phase to remain in the peripheral layers of the coil (tail) and the lighter phase to remain in the internal layers of the coil (head). The centrifugal force field produced by this planetary motion is highly dependent upon the location of point P on the holder. Location of point P, expressed as β, is the ratio between the radii of rotation and revolution (r/R). When β is greater than 0.25, the centrifugal force vector is always directed outwardly from the coil (9). In addition to the beneficial hydrodynamic situation produced by this planetary motion, physical factors pertaining to the solvent system

also play a critical role in the retention of the stationary phase. These physical parameters include relative density, viscosity, and tube wall affinity.

When the upper phase is much lighter, has a greater tube wall affinity and is less viscous than the lower phase, then complete separation of the two phases is achieved along the entire length of the coil. The upper phase completely occupies the head of the coil while the lower phase occupies the tail side. In this situation retention of the stationary phase, be it the lower phase (tail to head elution) or the upper phase (head to tail elution), is maximized. However, if the upper phase is more viscous or has less tube-wall affinity than the lower phase, the above said separation of phases may not take place. Instead, a hydrodynamic equilibrium is established whereby the upper phase dominates the head side of the coil. In this situation the choice of which phase is to be the mobile phase is limited. Due to the hydrodynamic equilibrium a small portion of the stationary phase will remain in the head end of the coil. Therefore, if the upper phase is used as the mobile phase (tail to head elution) there would be a continuous carryover of the stationary phase (lower phase) from the head end since a small portion of the stationary phase always remains in the head side due to the equilibrium state. With continual loss of the stationary phase, solute resolution would drastically diminish. In this situation one is limited to the lower phase as the mobile phase since the tail end is completely occupied by the lower phase.

To solve the dilemma of limited mobile phase availability a recent modification has produced a situation where either phase can be used as the mobile phase with relatively the same percentage of stationary phase remaining in the column. The improved coil is made by winding a single piece of PTFE tubing onto a spool-shaped holder with a pair of large flanges (6,7). The tubing is wound around the holder in a continuous fashion in which multiple layers are formed. This multi-layer configuration produces a centrifugal force field gradient created from the internal layer of the coil (head end) toward the external layer of the coil (tail end). This gradient

forces the upper phase to move toward the head and the lower phase toward the tail. Due to this gradient the hydrodynamic equilibrium is altered in such a way that there is more complete separation of the two phases along the entire length of the coil. When there is complete separation of the phases, either phase can be used as the mobile phase without carryover of the stationary phase.

EXPERIMENTAL

Apparatus

The apparatus is a table top model of a horizontal flow-through coil planet centrifuge with a multi-layer coil as previously described (7). Figure 2 illustrates a cross-sectional view of our prototype. The motor drives a rotary frame about a stationary central shaft by means of a pair of toothed pulleys and a toothed belt. The rotary frame holds a freely rotating multi-layer coil column and a counterbalance equidistant from the central stationary shaft. The coil holder is coupled to the stationary shaft by means of a stationary sun gear, attached to the central shaft, and a planetary gear attached to the column holder. This arrangement produces the desired synchronous planetary motion, one rotation per one revolution in the same direction. Both holder and counterbalance are removable from the rotary frame by means of loosening a pair of screws. This easily facilitates changing coils with tubing of different i.d. The coil itself is comprised of a spool equipped with two large flanges, around which a single piece of 70 m long, 2.6 mm i.d. PTFE tubing is wound, thus producing a multi-layer arrangement. Each terminal of the coil is attached to an appropriate diameter flow tube. These flow tubes are passed through a side hole leading to the lumen of the central shaft of the column holder and emerge at the most proximal end of this shaft. The flow tubes are then passed through a side hole of the short coupling pipe that leads to the lumen of the central stationary shaft. The flow tubes are protected from metal contact

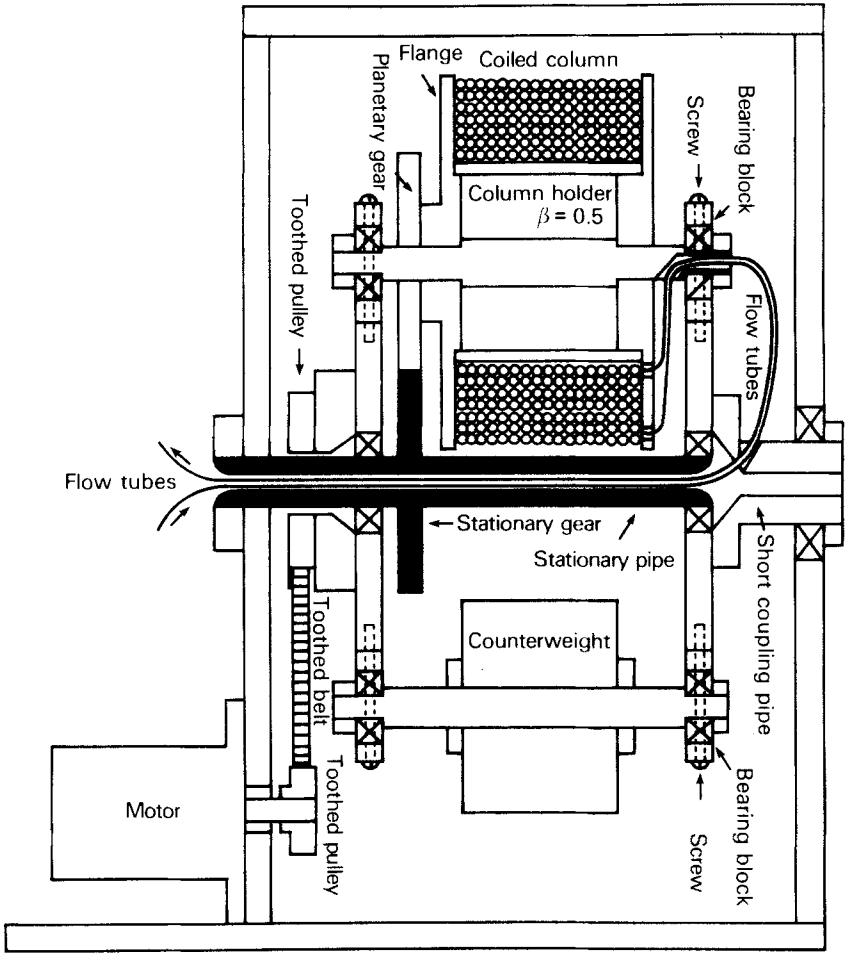

Figure 2. Cross-sectional view through the central axis of the apparatus.

by a piece of lubricated Tygon tubing along the entire length of the flow tubes to the point where they emerge from the lumen of the central stationary shaft. Revolutional speed (0-1000 rpm) is controlled with high accuracy and stability by the use of a speed control unit (Electro Craft or Bodine Electric Co.). The solvent is pumped through the column by means of a Beckman Accu Flow Pump or Milton Roy Mini Pump, while the effluent is monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes with an LKB fraction collector for further analysis.

The multi-layer coil is prepared from a single piece of PTFE tubing, 2.6 mm i.d. and 70 m long. The tubing is tightly wound onto a spool equipped with two large flanges, thus providing boundaries for which the multi-layer configuration can be obtained. To prevent movement of the multi-layer coil column with respect to the column holder, each layer of coil is attached to the flanges with a piece of fiberglass enforced tape across the width of the coil. To further enhance greater stability at higher revolutional speeds, the same fiberglass tape is wrapped (one continuous layer) around the circumference of the coil. Around this layer of tape is wound a single piece of wire in which the ends were anchored to each flange. Around the wire another single layer of tape is wrapped in effect, sandwiching the wire between the two layers of tape. This arrangement firmly anchors the multi-layer coil to the holder.

Reagents

The DNP amino acid samples used in this study include N-2,4-DNP-L-valine, N-2,4-DNP-L-alanine, N,N-di(2,4-DNP)-L-cystine, N-2,4-DNP-DL-glutamic acid, and N-2,4-DNP-L-aspartic acid and they were obtained from Sigma Chemical Co., Saint Louis, MO. Organic solvents used in the two-phase solvent system were mostly of chromatographic grade. Chloroform was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ and Burdick and Jackson Laboratories, Inc., Muskegon, MI, glacial acetic acid from MCN

Manufacturing Chemists, Inc., Cincinnati, Ohio, and Fisher Scientific Co., Fairlawn, NJ, and hydrochloric acid from Fisher Scientific Co., Fairlawn, NJ.

Preparation of Solvent System and Sample Solution

The two-phase solvent system was prepared by mixing chloroform, acetic acid and 0.1N hydrochloric acid at a 2:2:1 volume ratio. The mixture was equilibrated in a separatory funnel at room temperature and separated before use.

The sample solutions were prepared by dissolving a mixture of the 5 DNP amino acids in the upper and/or lower phase and stored in the dark at 4°C. For the first set of experiments where the sample concentration remained constant at 5g% (Figures 3 and 4), the sample solution was prepared by dissolving 1g each of DNP-valine, DNP-alanine, DNP-glutamic acid, DNP-aspartic acid, and 200 mg of diDNP-cystine (because of lower solubility) in 84 ml of the stationary phase. For the second set of experiments where the sample dose was fixed at about 1g for each run (Figure 5), the sample solution was prepared by dissolving 250 mg each of DNP-valine, DNP-alanine, DNP-glutamic acid, DNP-aspartic acid, and 50 mg of diDNP-cystine in 10 ml, 20 ml or 40 ml of the mobile and/or the stationary phase.

Separation Procedure

The column was first filled with the stationary phase followed by the introduction of the sample solution through the injection port. The mobile phase was then pumped through the column while the apparatus was run at a revolutional speed of 800 rpm. The eluate through the outlet of the column was continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes with an LKB fraction collector. Introduction of the sample solution and solvent phases is dependent upon which phase is chosen to be the mobile phase. When the mobile phase is the lower phase,

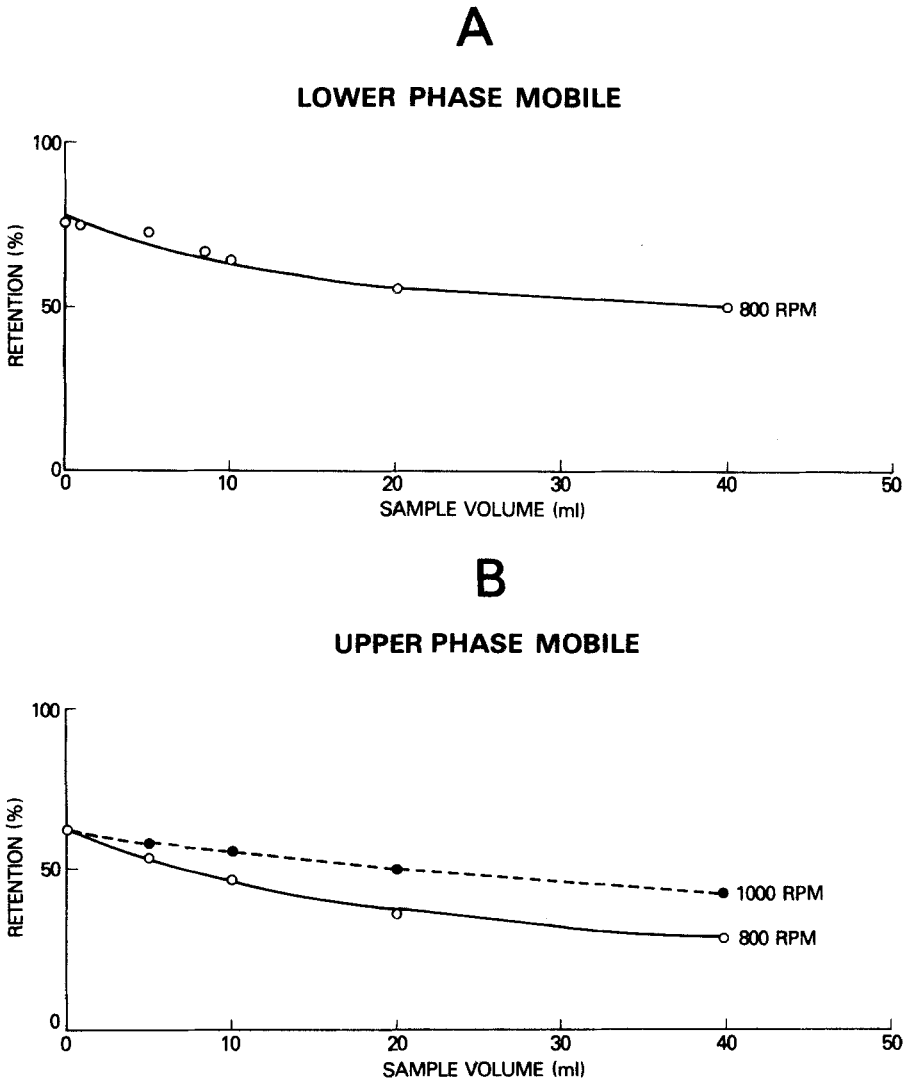

Figure 3. Effects of sample volume on retention level of the stationary phase.
(A) Lower Phase Mobile
(B) Upper Phase Mobile.

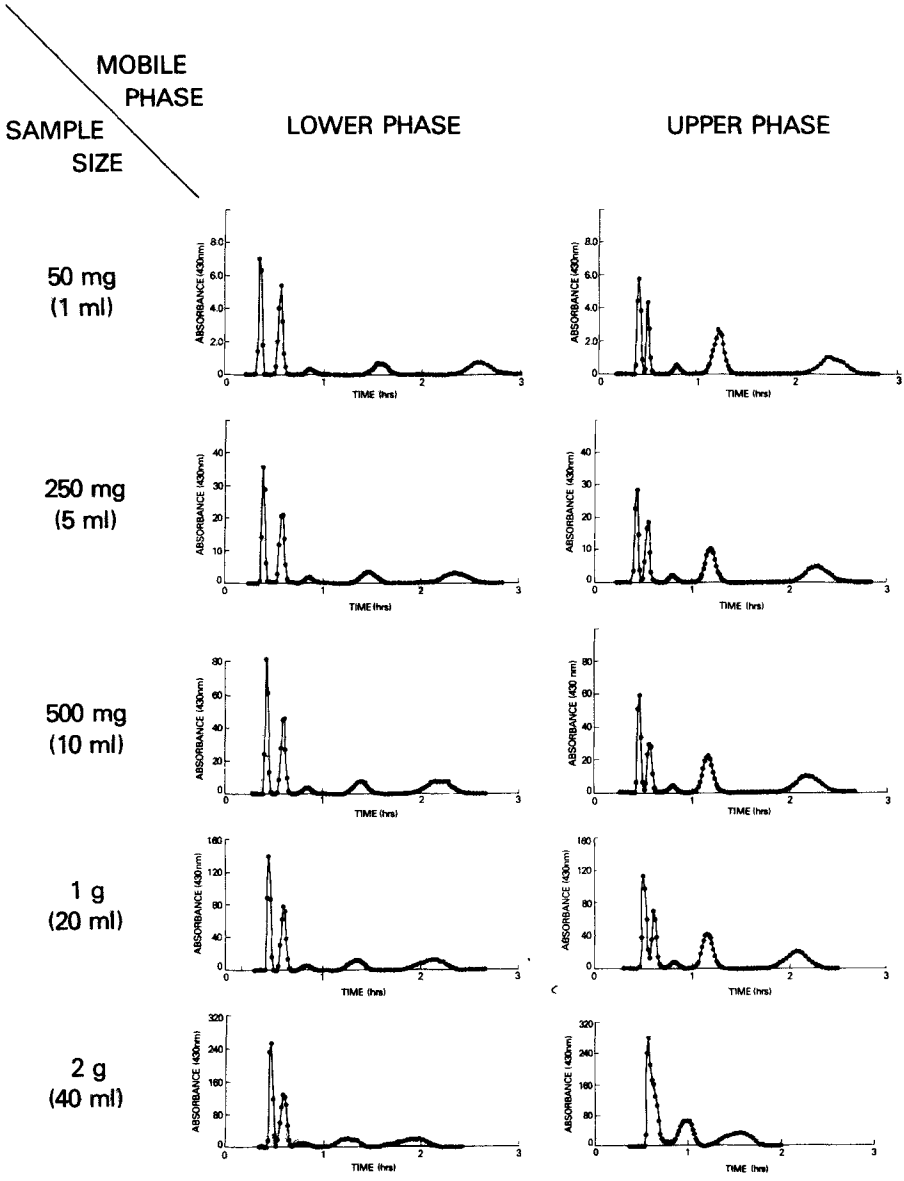

Figure 4. Effects of sample size on separation of a set of DNP amino acids.
Order of Elution:
Lower Phase Mobile (left column): DNP-valine, DNP-alanine, diDNP-cystine, DNP-glutamic acid, DNP-aspartic acid.
Upper Phase Mobile (right column): DNP-aspartic acid, DNP-glutamic acid, diDNP-cystine, DNP-alanine, DNP-valine.

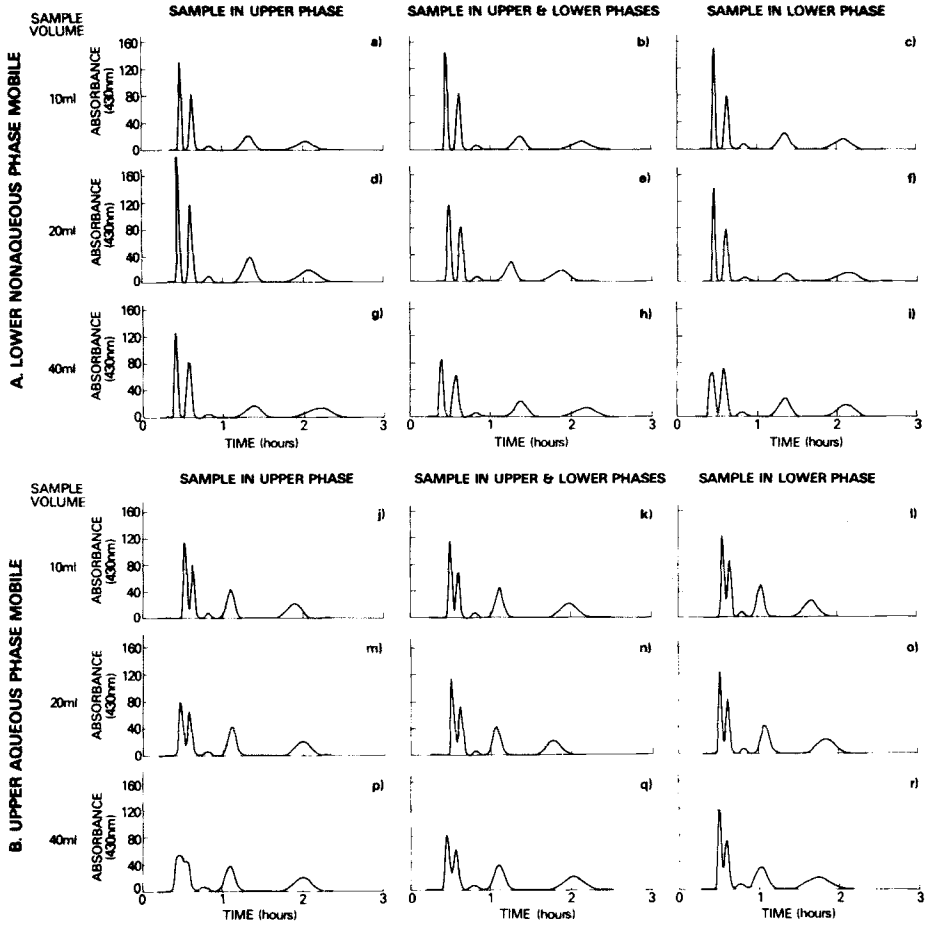

Figure 5. Effects of sample volume and sample diluent on separation of a set of DNP amino acids. Order of elution see Figure 4. The retention levels of the stationary phase for these separations are listed in TABLE II.

both sample solution and mobile phase are introduced at the head end of the coil (internal terminal). When the mobile phase is the upper phase, they are introduced through the tail terminal of the coil (external terminal). Upon completion of the separation, nitrogen gas at 100 psi was applied to the inlet of the column. Column contents were collected in a graduated cylinder and percent retention of the stationary phase remaining in the column was calculated. Aliquots of each fraction collected during the separation were mixed with 3 ml of methanol and analyzed the absorbance at 430 nm with a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Effects of the sample size on the retention of the stationary phase and partition efficiency have been investigated on separation of 5 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. Both lower nonaqueous and upper aqueous phases were used as the mobile phase at 500 ml/h under 800 rpm unless otherwise indicated. The sample was dissolved in the stationary phase at 5 g%.

Figure 3 shows effects of the sample dose on the retention of the stationary phase where the retention volume expressed in percentages relative to the total column capacity is plotted against the applied sample volume. The results clearly indicates a general trend that the retention of the stationary phase decreases with the increased sample size for both mobile phase groups. The results also show that the retention level of the nonaqueous phase (B) is substantially lower than that of the aqueous phase (A). However, this low retention level of the nonaqueous phase is improved by applying a higher revolutional speed at 1000 rpm as indicated by the dotted line (B).

Chromatograms obtained from these experiments are illustrated in Figure 4 where individual charts are arranged according to the sample dose and the mobile phase. Although the peak resolution gradually decreases with the increased sample dose, the integrity

TABLE I

Comparison of Stationary Phase Retention with Increased Sample Volume (Sample Concentration Constant)

Sample Volume (ml)	Sample Wt (mg)	UP (%)	LP (%)
1.0	50.0	73.3	57.9
5.0	250.0	70.3	53.8
10.0	500.0	60.8	46.6
20.0	1000.0	56.4	39.5
40.0	2000.0	51.3	28.1

TABLE II

Comparison of Stationary Phase Retention with Sample Dissolved in Increasing Volumes of UP, LP, ULP (Sample Dose Constant 1g)

A. Lower Nonaqueous Phase Mobile

Sample Solvent	UP	ULP	LP
10 ml	50.6	54.4	48.8
20 ml	53.8	48.7	56.4
40 ml	64.1	60.8	57.3

B. Upper Aqueous Phase Mobile

Sample Solvent	UP	ULP	LP
10 ml	39.2	44.2	33.2
20 ml	45.6	39.5	41.0
40 ml	46.4	46.8	40.0

UP = Upper Phase, LP = Lower Phase, and ULP = Upper and Lower Phases

of the individual peaks is well preserved in all charts except for the 2 g run with the mobile upper phase. This lowest peak resolution coincides with the lowest retention level of the stationary phase at 28% (See Figure 3B and TABLE I).

The above results strongly suggest that the retention level of the stationary phase plays a critical role in partition efficiency in the present method. Among a number of factors involved in the retention of the stationary phase, we consider the density difference between the two phases most important. The greater is the density difference, the higher retention level is expected. In the solvent system utilized in the present study, the specific gravity for the upper aqueous and the lower nonaqueous phases measured 1.11 and 1.34, respectively. Introduction of the sample mixture into the solvent system reduces not only the original density of each phase but also the density difference between the two phases. This explains the fact that the application of the large sample dose tends to lower the retention level especially when the sample is dissolved in the nonaqueous stationary phase. Alteration of other factors such as interfacial tension, viscosity, etc. may also contribute to the loss of the stationary phase. For example, lowered interfacial tension tends to produce emulsification of the phases which hinders countercurrent movement of the two phases and results in carryover of the stationary phase.

Effects of the sample volume and the choice of the sample diluents on the peak resolution were studied on separations of 1g quantity of the DNP amino acid mixture. The results are shown in Figure 5 and TABLE II where both the lower nonaqueous phase (A) and the upper aqueous phase (B) are used as the mobile phase.

These chromatograms clearly show that the choice of the sample diluent makes little difference in resolution for both early and late appearing peaks until the sample volume is increased to 40 ml. With a large sample volume a significant decrease in peak resolution is observed in early appearing peaks especially when the sample is dissolved entirely in the mobile phase (Figure 5, i and

p). The loss of peak resolution becomes minimized if the sample is dissolved in the stationary phase (Figure 5, g and r). Further observation reveals that chromatograms i and p (Figure 5) with the lowest resolution in the early peaks show the best resolved late peaks among all 40 ml sample groups. This peculiar elution profile of the early and late peaks can not be attributed to the loss of the stationary phase since all these separations yielded satisfactory retention levels as shown in TABLE II. Instead, this phenomenon may be clearly understood on the basis of the two-phase interaction within the sample compartment at the beginning of the partition process.

When the sample mixture is introduced with the stationary phase, the eluting mobile phase will pick up each individual component at a different rate according to the partition coefficient. The components which favor the partition in the mobile phase, hence producing the early appearing peaks, are quickly depleted from the sample compartment of the stationary phase and concentrated in a small volume of the mobile phase, resulting in a sharp sample band. Therefore, a large volume of sample can be injected into the column without causing significant peak broadening of early appearing peaks. Although the components favoring the partition in the stationary phase are not concentrated by the mobile phase, they are subjected to the partition process in the column for longer periods of time to produce broader but better resolved peaks. Therefore, the initial band width for these components becomes less significant for separation. The above effects become reversed when the sample mixture is introduced with a large volume of the mobile phase. In this case the components favoring the partition in the stationary phase, thus producing the late appearing peaks, are concentrated in the stationary phase at the beginning of the column, while other components tend to produce broader peaks affected by the sample volume. Because these early appearing peaks are eluted close together, the loss of resolution among those peaks becomes serious compared with the late eluted peaks. By dissolving the sample in equal volumes of the upper and

lower phases, resolution can be relatively maintained for both early and late appearing peaks. There is a decrease in resolution when the sample is dissolved in a large volume of the upper and lower phase mixture but not to the degree seen when the sample is dissolved in a large quantity of the mobile phase.

As discussed above, in large-scale separations the best results are usually attained by dissolving the sample mixture entirely in the stationary phase. However, in some instances the sample mixture contains a component or components having low solubility in the stationary phase and, therefore, an enormously large volume of the stationary phase is required to dissolve the sample. In this situation the addition of the mobile phase to the sample solution will give beneficial effects in reducing the sample volume and at the same time securing the formation of two phases in the sample compartment upon introduction into the column.

The overall results of the experiments described above indicate that the present scheme produces efficient separations for gram quantity of samples in a short period of time. Sample-loading capacity of the scheme may be increased by the use of a larger-diameter and/or longer coil. We believe that the present countercurrent chromatographic scheme will be useful in separation and purification of natural products and synthetic drugs.

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