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PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY WITH HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE

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SUMMARY

Capability of a new countercurrent chromatographic scheme is demonstrated on preparative-scale separation with two typical two-phase solvent systems. The scheme uses a helical column revolving around the horizontal axis of the centrifuge and rotating about its own axis at the same angular velocity. This planetary motion, while eliminating the need for the rotating seals, enables utilization of the gravitational and/or centrifugal force fields to perform a high-efficiency countercurrent chromatography with a variety of two-phase solvent systems.

INTRODUCTION

Countercurrent chromatography[1,2] by eliminating the use of solid supports has many ideal features for preparative-scale solute separations, such as good recovery, high purity, high reproducibility, and minimum denaturation of samples. However, its practical application has been limited by the time-consuming operation which often requires days to complete a separation. In droplet countercurrent chromatography, for example, the applicable flow-rate for a 1-butanol–acetic acid–water phase system is optimized at 4.3 ml/h which yields a separation equivalent to that in the countercurrent distribution method[3]. Recently, the method was substantially improved by a simple countercurrent chromatographic scheme consisting of a horizontal helical column slowly rotating in the gravitational field[4,5], allowing a flow-rate of 12 ml/h for a similar 1-butanol phase system. The new countercurrent chromatographic scheme to be described here permits a 60-ml/h flow-rate to separate a 10-ml sample solution with efficiencies as high as 1000 theoretical plates.

The horizontal flow-through coil planet centrifuge is a new development which efficiently utilizes both gravitational and centrifugal force fields to retain the stationary phase while providing constant mixing with the mobile phase flowing through the helical column. The principle of the scheme and the preliminary test results were briefly reported earlier[6]. This paper reviews the principle and the design of the appa-

ratus in detail and demonstrates the capability of the test system on preparative-scale separations of dinitrophenyl (DPN) amino acids and peptides using two typical two-phase solvent systems.

PRINCIPLE

The principle of the horizontal flow-through coil planet centrifuge is illustrated in Fig. 1. A horizontal helical column is connected to a mobile gear which rolls around

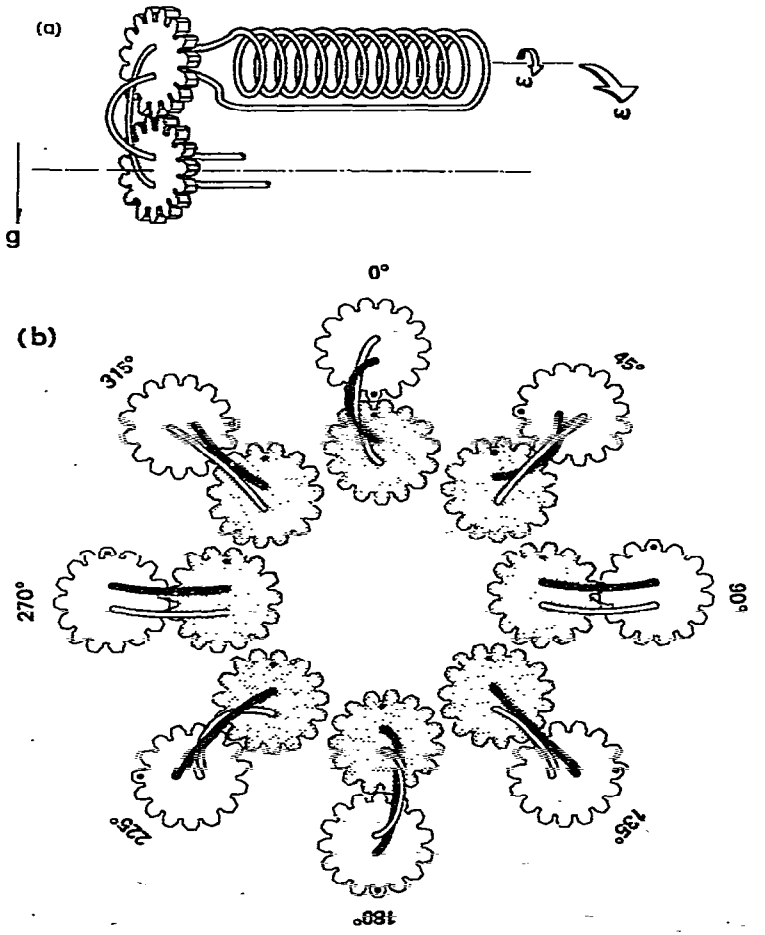


Fig. 1. Principle of the horizontal flow-through coil planet centrifuge. (a) The helical column is horizontally supported by a mobile gear that rolls around an identical stationary gear (shaded) mounted at the central axis of the centrifuge. Rolling motion of the mobile gear around the stationary gear produces a planetary motion of the helical column, a revolution about the central axis of the apparatus and rotation about its own axis at the same angular velocity as indicated by a pair of arrows. (b) successive positions of the black and white flow tubes during one complete revolution of the mobile gear. This planetary motion prevents twisting the flow tubes. Note that both tubes pass each other around 45° and 225°. A black dot on the mobile gear helps to visualize the motion of the helical column which rotates twice with respect to the gravitational field and once with respect to the centrifugal force field produced by revolution.

an identical stationary gear (shaded) fixed at the central axis of the centrifuge (Fig. 1a). Both feed and return flow tubes of the helical column are passed through the mobile gear and then tightly supported by the stationary gear, making loops as shown. In this arrangement, rolling motion of the mobile gear around the stationary gear produces a planetary motion to the helical column; the column revolves around the central axis of the apparatus and rotates about its own axis at the same angular velocity in the same direction as indicated by a pair of arrows. Consequently, during one complete revolution of the mobile gear, the helical column rotates twice with respect to the gravitational field and once with respect to the centrifugal force field introduced by revolution.

This planetary motion prevents twisting the flow tubes as they revolve with the mobile gear. Fig. 1b illustrates successive positions of the flow tubes (white and black) during one revolution of the mobile gear around the stationary gear. The black tube is pulled under the white tube at around 45° while the white tube similarly passes under the black tube at around 225°. The black dot on the mobile gear helps to visualize the planetary motion of the helical column connected to the mobile gear.

A simple mathematical analysis has been made of the motion of an arbitrary point on the helical column to study the resulting acceleration. Fig. 2a shows a coordinate system where two circles indicate positions of the helical column revolving around the central axis of point 0 and rotating about its own axis at the same angular velocity, ω. The arbitrary point on the helical column starts at $P_0(R + r, 0)$ and after time t locates at $P(x, y)$, where R denotes the radius of revolution and r, the radius of rotation of the helical column. Then the motion of the arbitrary point can be expressed as:

$$x = R\cos\theta + r\cos 2\theta \tag{1}$$

$$y = R\sin\theta + r\sin 2\theta \tag{2}$$

where $\theta = \omega t$.

From these equations, the orbit of the arbitrary point can be computed by eliminating the variable θ. The shape of the orbit greatly changes with β where $\beta = r/R$, *i.e.*, the ratio between the radii of rotation and revolution of the helical column. In Fig. 2b, three orbits are drawn corresponding to β values of 0.1, 0.5 and 1. For $\beta \leq 0.25$, the orbit is a single circular loop. As the β value increases, it changes into a heart shape and then forms a double loop which approaches a double circle with larger β values.

The acceleration acting on $P(x, y)$ is further computed from the second derivatives of eqns. 1 and 2. We obtain:

$$d^2x/dt^2 = -\omega^2(R\cos\theta + 4r\cos 2\theta) = -R\omega^2(\cos\theta + 4\beta\cos 2\theta) \tag{3}$$

$$d^2y/dt^2 = -\omega^2(R\sin\theta + 4r\sin 2\theta) = -R\omega^2(\sin\theta + 4\beta\sin 2\theta) \tag{4}$$

which gives the absolute total acceleration

$$a = \sqrt{(d^2x/dt^2)^2 + (d^2y/dt^2)^2} = R\omega^2\sqrt{1 + 16\beta^2 + 8\beta\cos\theta} \tag{5}$$

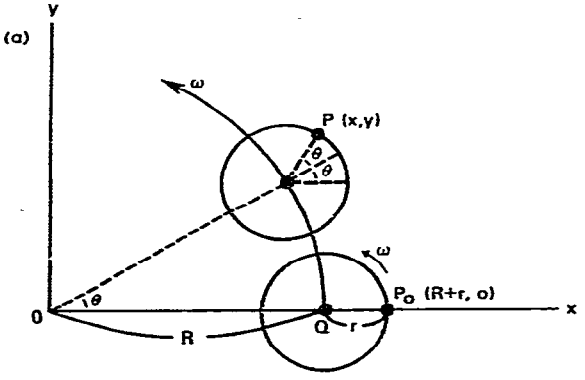
acting at an angle of $\tan^{-1}(\sin\theta + 4\beta\sin 2\theta)/(\cos\theta + 4\beta\cos 2\theta)$, provided $R \neq 0$.

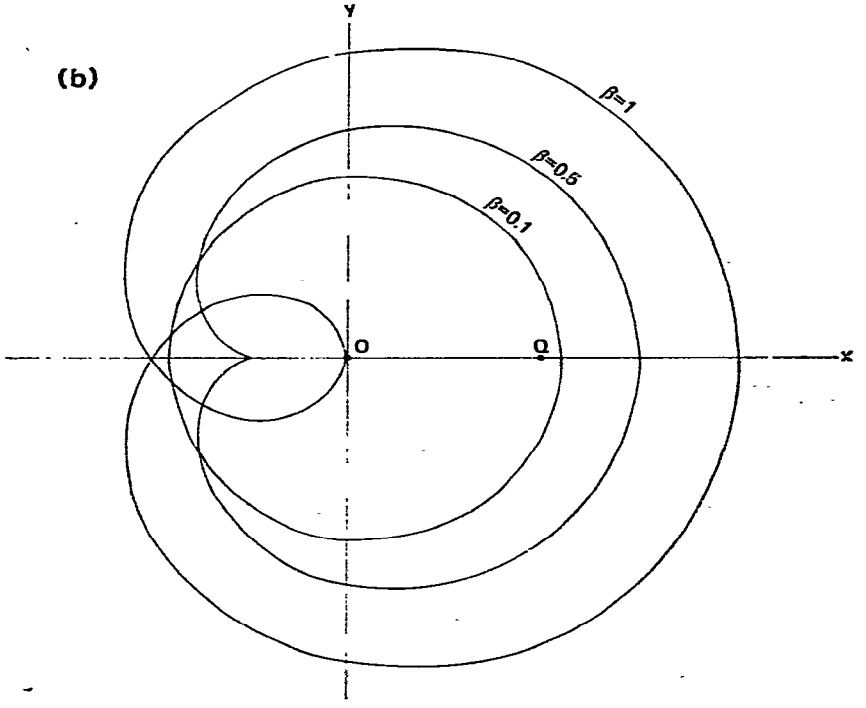
However, for the analysis of the motion of the two phases in the column, it is more meaningful to obtain the tangential acceleration α_t and the radial acceleration α_r with respect to the helical column. From eqns. 3 and 4, we get

$$\alpha_t = -(d^2x/dt^2)\sin 2\theta + (d^2y/dt^2)\cos 2\theta = R\omega^2 \sin\theta \tag{6}$$

$$\alpha_r = (d^2x/dt^2)\cos 2\theta + (d^2y/dt^2)\sin 2\theta = -R\omega^2(4\beta + \cos\theta) \tag{7}$$

As is clearly shown in eqns. 6 and 7 and Fig. 2c, α_t is independent of β whereas α_r is largely affected by β, $\beta > 0.25$ always giving a negative acceleration regardless of





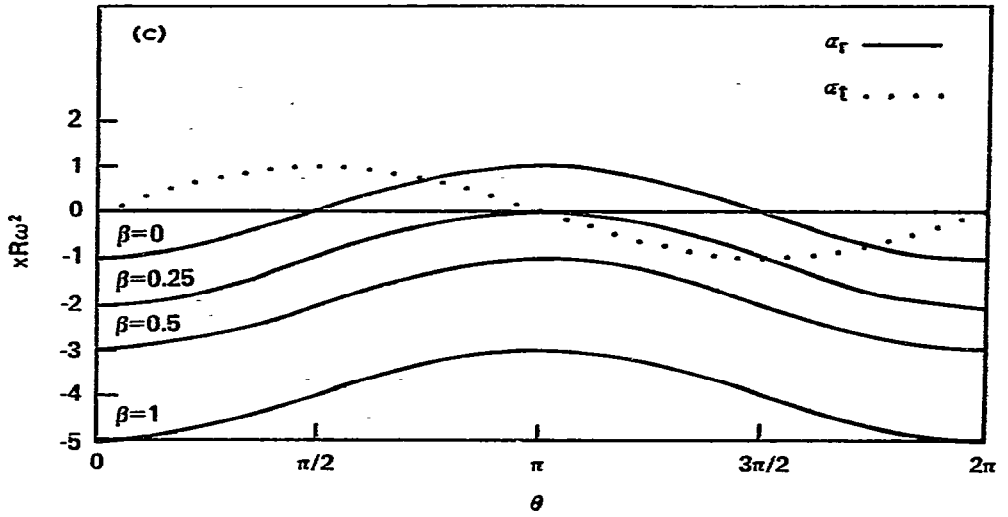


Fig. 2. Analysis of the planetary motion of the horizontal flow-through coil planet centrifuge. (a) The coordinate system for analysis. (b) Orbits of the arbitrary point on the helical column for β values of 0.1, 0.5, and 1, where β denotes the ratio between the revolutional radius and the rotational radius of the helical column. (c) Acceleration produced by the planetary motion during one complete revolution of the mobile gear. The tangential acceleration α_t shown by the dotted line is independent from β while the radial acceleration α_r is sensitive to the β values as shown by the four solid lines for $\beta = 0, 0.25, 0,5$, and 1.

θ values. This indicates that the large β values cause uneven phase distribution across the diameter of the column which may increase mass transfer resistance while small β values produce an additional phase mixing to contribute to the partition.

When the multiple helical columns are arranged around the column holder as shown in Fig. 3, the analysis of the acceleration field becomes more complex. However, the analysis also suggests an uneven phase distribution in the column with large β values. In the light of the results of the above analysis, we designed the test system to provide β values of less than 0.25.

Motion of the two immiscible liquids in a rotating helical column under an acceleration field has been described earlier[1,2,7,8]. The two-phase solvent system present in the column tends to distribute itself in such a way that each phase occupies nearly equal amounts in every turn of the helical column and any excess of either phase is accumulated at one end of the column. Once this dynamic equilibrium is reached, further rotation results in mixing of the two phases without changing the overall phase distribution throughout the column. Consequently, elution of either phase through the column in the proper direction results in an efficient partition process of solutes between the mobile and the stationary phases within each turn of the column.

EXPERIMENTAL

Apparatus

Fig. 3 shows a photograph of the test system used in this study. The rotary frame of the centrifuge consists of a pair of aluminum plates rigidly linked and driven

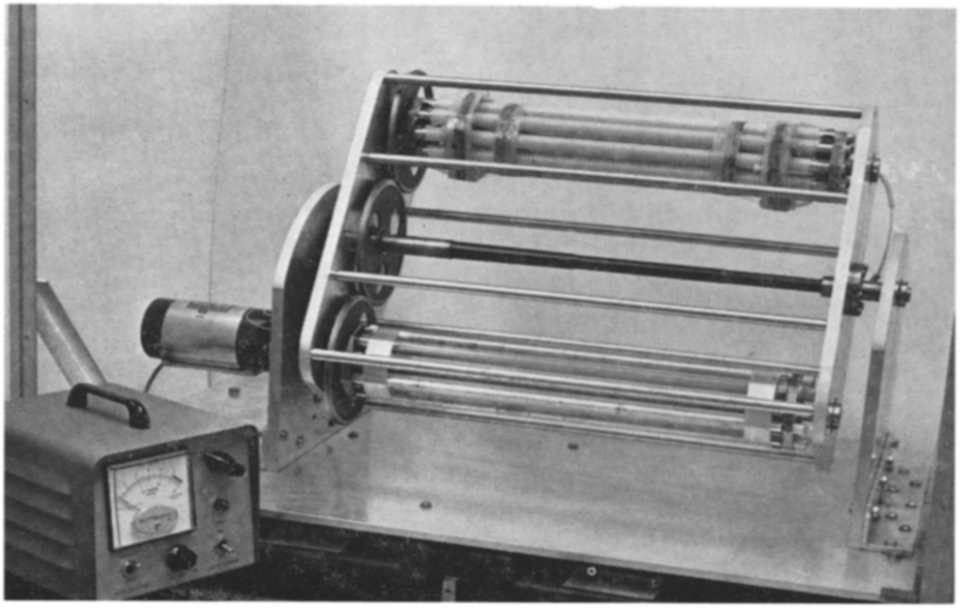


Fig. 3. Overall view of the test system.

by the motor (Electro-Craft Co.) around the hollow stationary shaft fixed at the central axis of the centrifuge. The frame holds a pair of rotary shafts, 54 cm long and 1.9 cm in diameter, symmetrically spaced a distance of 15 cm from the center of the apparatus. Each rotary shaft is equipped with a mobile gear which engages an identical stationary gear fixed on the stationary shaft. In order to secure the remote end (right) of the stationary shaft, a short coupling pipe is extended from the aluminum plate of the rotary frame and supported through a bearing by a standing support. The helical column can be mounted on either side of the rotary shaft and a counterweight is placed on the other side to obtain a proper balancing of the centrifuge. The revolutional speed of the test system is continuously adjustable from 0 to 300 rpm.

A Cheminert Metering Pump (Chromatronix) was used for eluting the column while an LKB Uvicord III was used for monitoring the eluate at 280 nm.

Column

PTFE tubing 2.6 mm I.D. with 0.4–0.5 mm wall thickness (Zeus, Raritan, N.J., U.S.A.) was mainly used for column preparation. The tube was wound onto an aluminum pipe, 48 cm long and 1.25 cm O.D. with 1 mm wall thickness, to make a short column or column unit. One column unit has approximately 100 helical turns with a total capacity of about 25 ml. A long column for preparative-scale separations was prepared by connecting 10 column units in series with PTFE tubes 0.45 mm I.D. and 15 cm in length. Application of these small tubes appeared to be necessary to prevent pulsative surging flow of the two-phase solvent system under a strong centrifugal force field which would cause unnecessary broadening of sample bands. The column was mounted as close as possible by using fuse holders symmetrically arranged around the rotary shaft to clamp the ends of each column unit. Then the column was secured in place by wrapping fiber glass tape in several places.

The flow tubes (0.85 mm I.D. PTFE) of the helical column were first passed through the center hole of the rotary shaft and then through the side-hole of the coupling pipe to lead into the opening of the stationary shaft. The moving portion of the flow tubes was lubricated with grease and protected with a piece of silicone rubber tube at each supporting point. These tubes, if properly protected, can maintain their integrity over several months of use.

Reagents

All reagents used in this work are of reagent grade. The two-phase system composed of chloroform–acetic acid–0.1 *N* HCl (2:2:1) was used for separation of DNP amino acids (Sigma, St. Louis, Mo., U.S.A.) and 1-butanol–acetic acid–water (4:1:5) for separation of peptides (Sigma). Each two-phase system was equilibrated at room temperature and separated before use. The sample solutions were prepared by dissolving the sample mixture in the stationary phase. They were stored in the dark at 4° before use.

Procedure

Preliminary tests were performed for each two-phase system to optimize revolutional speed and flow-rate with a single column unit while preparative-scale separations were carried out with a long column consisting of 10 column units under the optimized conditions.

In each separation, the column was first filled with the stationary phase and a sample solution was introduced through the feed tube followed by elution with the mobile phase at a given flow-rate while the apparatus was spun at a given rpm. The eluate was continuously monitored for absorbance at 280 nm to record the elution curves.

During separation of DNP amino acids the apparatus was covered with a black sheet to prevent decomposition of the sample upon exposure to the light.

RESULTS AND DISCUSSION

Preliminary experiments

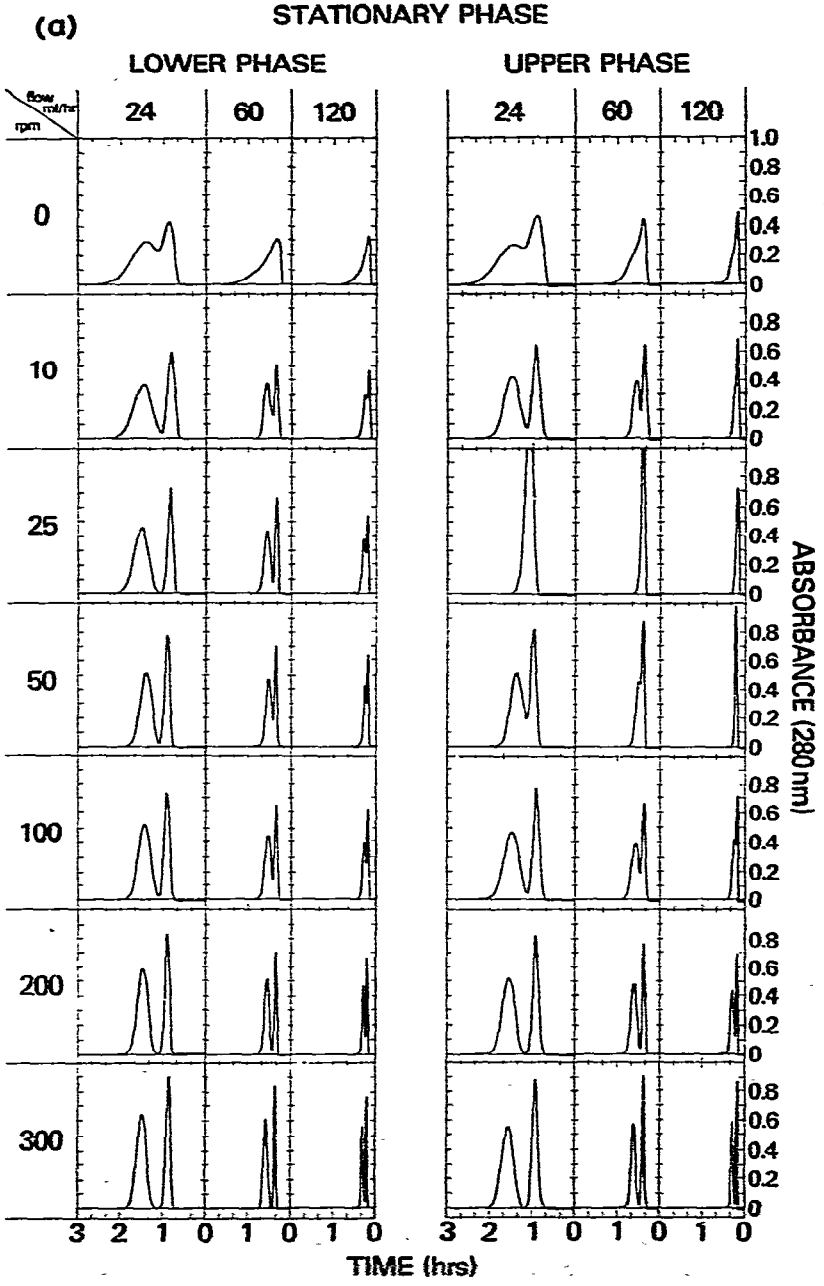
Using a single column unit the effects of revolutional speed and flow-rate on separation of two selected samples were studied in each two-phase system, the results being summarized in Fig. 4.

Fig. 4a shows separation of DNP-L-glutamic acid and DNP-L-alanine on the two-phase system composed of chloroform–acetic acid–0.1 *N* HCl (2:2:1). In each separation the partition efficiency is easily estimated by measuring the height of the trough between the two peaks. These diagrams clearly demonstrate the presence of two optimum revolutional speeds.

The efficiency sharply rises with revolution from 0 to 10 and 25 rpm where gravity plays a major role in partition as the centrifugal force produced by revolution is negligibly small. When the revolutional speed reaches a range of 25–50 rpm, the gravity fails to retain a satisfactory amount of the stationary phase against the flow and yet centrifugal force is not strong enough to compensate gravity. As a result, efficiency falls with the depletion of the retained stationary phase manifested by a decreased retention time of the second peak and a shortened time-lapse between the

two peaks at a given flow-rate. Further increase of revolutional speed up to 300 rpm results again in a sharp rise of efficiency where the centrifugal force plays a major role in partition.

Fig. 4b shows diagrams similarly obtained on separation of L-valyl-L-tyrosine and L-tryptophyl-L-tyrosine with the 1-butanol–acetic acid–water (4:1:5) phase



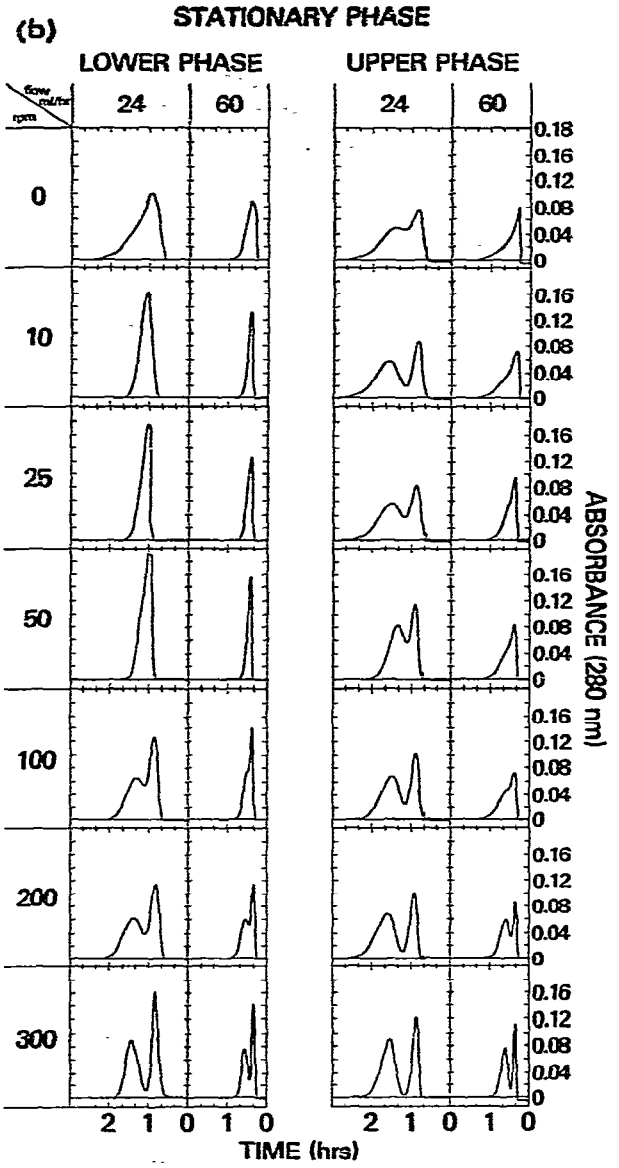


Fig. 4. Effects of revolutional speed and flow-rate on separation of two selected samples on typical two-phase solvent systems. In both a and b, two optimum revolutional speeds are present, one around 10 to 25 rpm and the other at 300 rpm. (a) Separation of DNP-L-glutamic acid in DNP-L-alanine on chloroform–acetic acid–0.1 *N* HCl (2:2:1) phase system (b) Separation of L-valyl-L-tyrosine and L-tryptophyl-L-tyrosine on 1-butanol–acetic acid–water (4:1:5) phase system.

system. In spite of contrasting physical properties of this 1-butanol phase system to the former chloroform phase system, presence of the two optimum revolutional speeds is evident — one around 10 rpm and the other at 300 rpm.

The unique capability of the present scheme among other countercurrent chro-

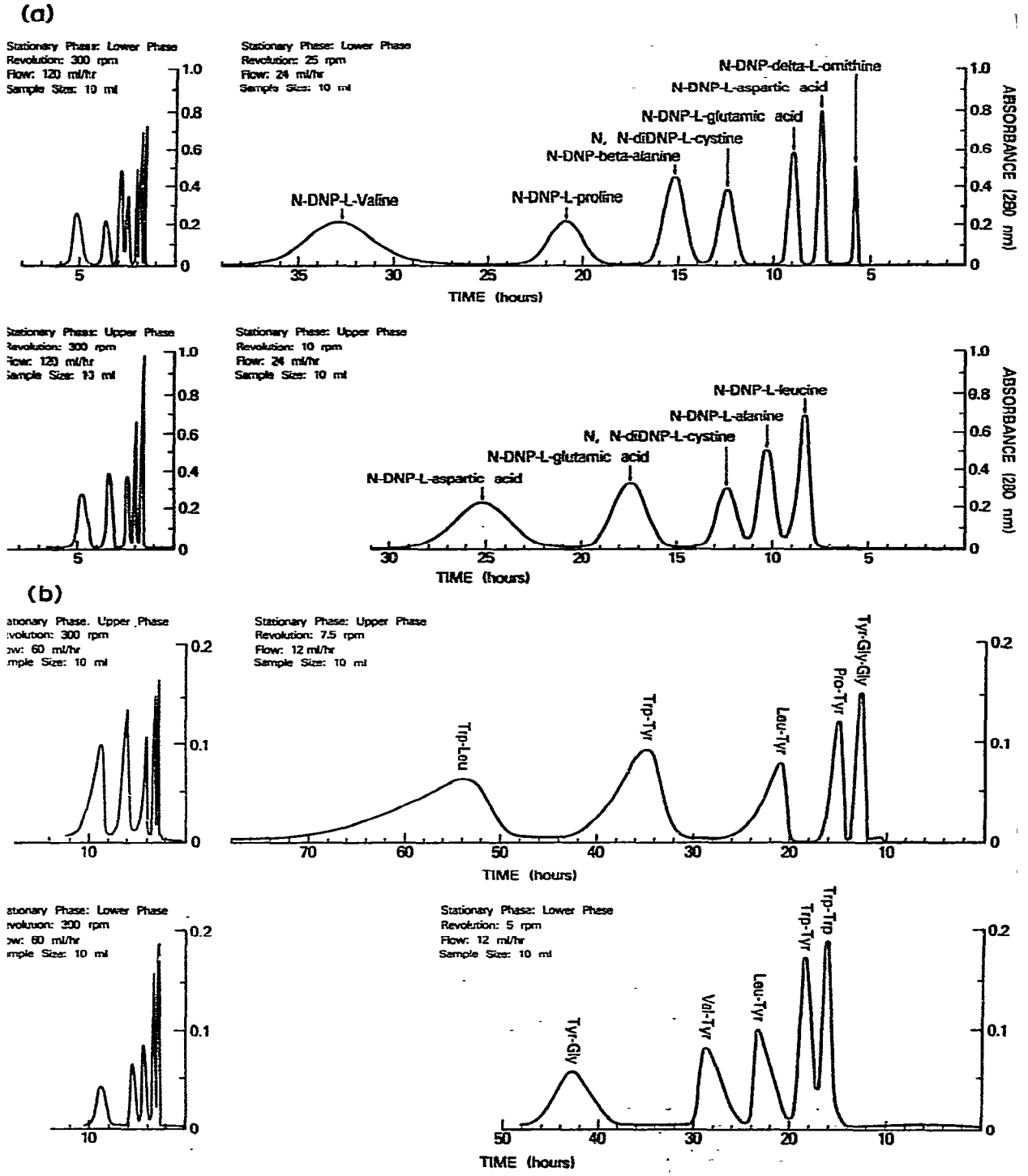


Fig. 5. Preparative-scale separations under the two optimum revolutional speeds. Application of a higher revolutional speed greatly decreases elution time without significantly affecting resolution. (a) Separation of a set of DNP amino acids on chloroform–acetic acid–0.1 *N* HCl (2:2:1) phase system. (b) Separation of a set of peptides on 1-butanol–acetic acid–water (4:1:5) phase system.

matographic systems derives from the fact that it has two optimum operational conditions, one at a slow revolutional speed in the gravitational field and the other at a high revolutional speed utilizing the centrifugal force field. When a large-bore preparative column is eluted with an extremely low interfacial tension phase system, a slow revolutional speed prevents emulsification of the phases which would cause carry-over of the stationary phase. For a small-bore analytical column a high revolutional speed can create a strong centrifugal force field that allows the two phases to counterflow through a narrow opening of the column without plug flow. Thus, the present scheme is capable of using a variety of two-phase solvent systems for both preparative- and analytical-scale separations.

Preparative-scale separations

Using a long column consisting of 10 column units, preparative-scale separations were performed under the optimum operational conditions predetermined by preliminary experiments.

Fig. 5a shows separations of a set of DNP amino acids on the chloroform phase system using 10 ml sample size in each separation. As indicated in Fig. 5 two chromatograms on the right were obtained by a slow revolutional speed using gravity and the other two chromatograms on the left by a high revolutional speed using the centrifugal force field. When efficiencies of separation are calculated according to the gas chromatographic formula, all separations show over 1000 theoretical plates in the early peaks which decrease to several hundred theoretical plates in the later peaks. Although a good resolution is achieved under the slow revolutional speed, similar results can be obtained under the high revolutional speed with a remarkably shorter elution time.

Fig. 5b shows separation of peptides on the 1-butanol phase system using 10 ml sample size in each separation. The efficiencies range from 1000 to a hundred theoretical plates in these separations. Here again application of high revolutional speed greatly decreases separation time without significantly affecting resolution.

Using typical two-phase solvent systems, we have demonstrated versatility of the horizontal flow-through coil planet centrifuge on preparative-scale separations. The sample size may be increased to grams by using a larger bore helical column by utilizing a slow revolutional speed to prevent emulsification of the solvent. For analytical separation, a high revolutional speed enables the use of a small-bore column to prevent plug flow as in the flow-through coil planet centrifuge previously reported[7,8]. When the apparatus is refined, it will perform both analytical- and preparative-scale separations with a variety of two-phase solvent systems to yield a high efficiency separation without complications arising from the use of solid supports.

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