*Journal of Chromatography*, 136 (1977) 189–198


CHROM. 9928

# PREPARATIVE COUNTERCURRENT CHROMATOGRAPHY WITH A SLOWLY ROTATING HELICAL TUBE

Y. ITO and R. L. BOWMAN

*National Heart, Lung, and Blood Institute, Laboratory of Technical Development, Bethesda, Md. 20014 (U.S.A.)*

(Received January 7th, 1977)

## SUMMARY

The capability of a simple countercurrent chromatographic scheme for obtaining high-resolution preparative-scale separations was demonstrated with the separations of a series of dinitrophenyl (DNP) amino acids and peptides. Basic studies on stationary phase retention and partition efficiency with a low-viscosity chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) phase system together with the results previously obtained with a viscous *n*-butanol system suggest a general applicability of low-interfacial tension phase systems by the present method.

## INTRODUCTION

A simple preparative countercurrent chromatographic scheme has been devised to extend the usefulness of previously described schemes[1,2]. We use a coiled tube rotating slowly in the gravitational field while the mobile phase countercurrents through the stationary phase trapped in each turn of the coil. The principle of the method and the preliminary test results using a *n*-butanol–acetic acid–water (4:1:5) phase system have been reported earlier[3]. Further investigations have demonstrated a broad applicability of low-interfacial tension two-phase solvent systems having various phase properties.

For the present studies, a two-phase system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) has been selected because of its contrasting phase properties to the *n*-butanol system previously examined. Basic studies on retention of the stationary phase and efficiency of separation are performed according to the procedure previously used for the *n*-butanol phase system in order to compare results obtained with these two solvent systems. Under the optimum operational conditions obtained for these phase systems, preparative-scale separations of a series of dinitrophenyl (DNP) amino acids and peptides are performed using a sample volume of 10 ml each.

## EXPERIMENTAL

### *Apparatus*

The test system used in the experiment is illustrated in Fig. 1. A separation column which consists of nine units of coiled PTFE tubes connected in series is mounted around the hollow rotary shaft equipped with a rotating seal at each end. The rotary shaft is driven by a motor (Electro-craft Co.) through a pair of toothed pulleys coupled with a toothed belt. The frame holding all these elements is made adjustable at a desired angle by tightening the center screw against the standing support. A Chromatronix metering pump is used to pump the solvent into the column through a bubble trap, a sample port and then the first rotating seal (right) and the eluate collected through the second rotating seal (left) is monitored with an LKB Uvicord III at 280 nm. Each rotating seal is fabricated with Kel-F block and a PTFE o-ring to prevent corrosion.

Each column unit is prepared by winding PTFE tubing (Zeus Industrial Products, Rariton, N.J., U.S.A.) 5 m long and 2.6 mm I.D. onto a lucite pipe of 50 cm long and 1.25 cm O.D. to make approximately 100 turns with a capacity of 25 ml.

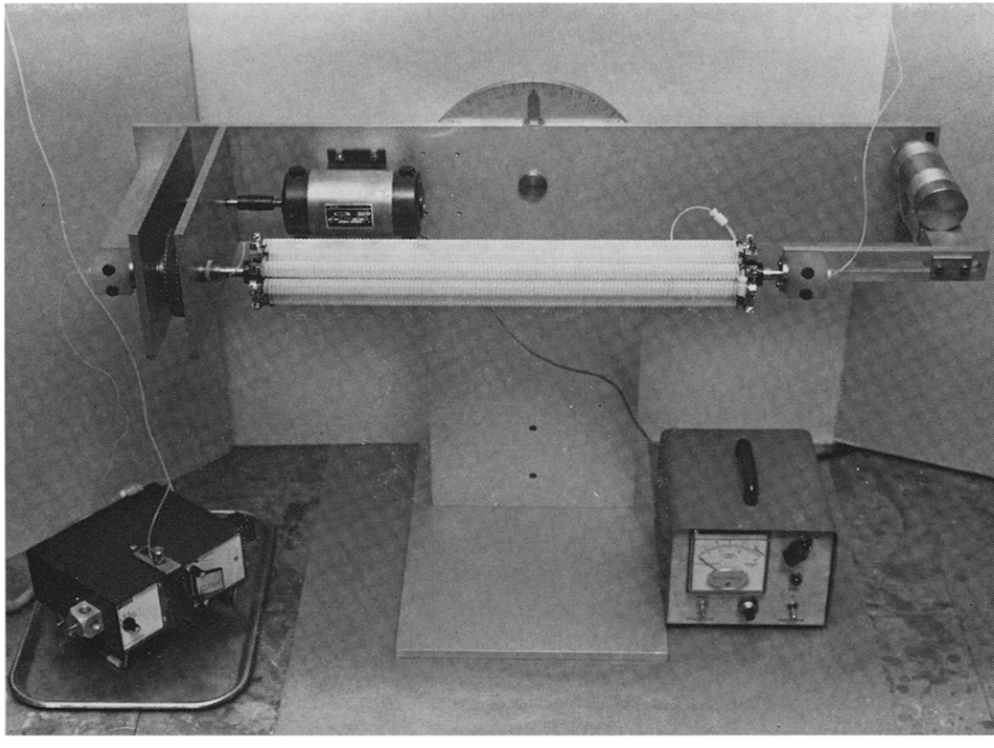

Fig. 1. Preparative countercurrent chromatograph. The apparatus consists of a helical column mounted around the rotary shaft equipped with a rotating seal at each end and driven by a motor through a pair of pulleys coupled with a toothed belt. The solvent is introduced into the column through the first rotating seal (right) and the eluate collected through the second rotating seal (left) is monitored with an LKB Uvicord III at 280 nm. The frame holding the rotary shaft is made adjustable to any desired angle.

The large-capacity preparative column as shown in Fig. 1 is made by connecting nine column units in series.

*Two-phase system preparation*

A two-phase solvent system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) is used. The phase mixture is equilibrated at room temperature and separated before use. A two-phase system composed of *n*-butanol–acetic acid–water at 4:1:5 volume ratio is similarly prepared for preparative separation of peptides.

*Phase retention studies*

Using a single-column unit, retention of the stationary phase is measured under various conditions with respect to column angle, rotational speed, and flow-rate. Both upper aqueous and lower organic phases are tested as a stationary phase. The phase distribution and behavior in the rotating coiled tube is conveniently studied if the stationary phase is colored with a dye which partitions almost entirely to the stationary phase. We used Sudan black B to color the lower phase and basic fuchsin for the upper phase. The column is first filled with the mobile phase and about 5 ml of the lightly colored stationary phase is introduced into the column which is held vertical so that the stationary phase is completely separated from the mobile phase. The length of the column occupied by the colored stationary phase is defined as A. When the column is set to the desired angle, rotated and eluted with the mobile phase at the desired rates, a dynamic equilibrium is reached and the length of the column now containing the colored stationary phase is defined as B. The retention percentage of the stationary phase ($R$) is given by $R = A/B \times 100$.

*Partition efficiency studies*

Two DNP amino acids (Sigma), DNP-L-glutamic acid (1.9) and DNP-L-alanine (0.56), are selected on the basis of their suitable partition coefficients indicated in the parentheses since these two mixtures give similar elution curves regardless of the choice of mobile phase. The sample mixture is dissolved into the upper aqueous phase at a concentration of 0.5 g% for each component and stored in dark at 4° before use. In each separation the column is filled with the stationary phase and 0.2 ml of sample solution is injected through the sample port followed by elution with the mobile phase under a given set of conditions of column angle, rotational speed, and flow-rate. Since DNP amino acids are decomposed by exposure to the light, the apparatus is covered with a black sheet during separation. The eluate is continuously monitored at 280 nm to record the elution curve. The partition efficiency in each separation can be evaluated from the relative height of the trough between the two peaks and/or by calculating the number of theoretical plates (T.P.) for each component if the two peaks are well resolved.

## RESULTS AND DISCUSSION

*Phase retention studies*

Retention of the stationary phase in the rotating coiled tube examined under various operative conditions is illustrated in Fig. 2. In each diagram the percentage

of stationary phase volume occupying the column space is plotted against the applied column angle, where 0° indicates the horizontal column position, + 90° and — 90° being the vertical column position with the inlet end upward and downward, respectively. Several curves drawn in each diagram show the effects of flow-rate. Ideal phase retention close to 50% is observed in a wide range of the column angle between 0° ± 60° in most cases.

Each curve exhibits a characteristic shape similar to those observed with the *n*-butanol phase system. An abrupt decrease or increase of phase retention around the horizontal column position is produced by the change of the flow pattern from the

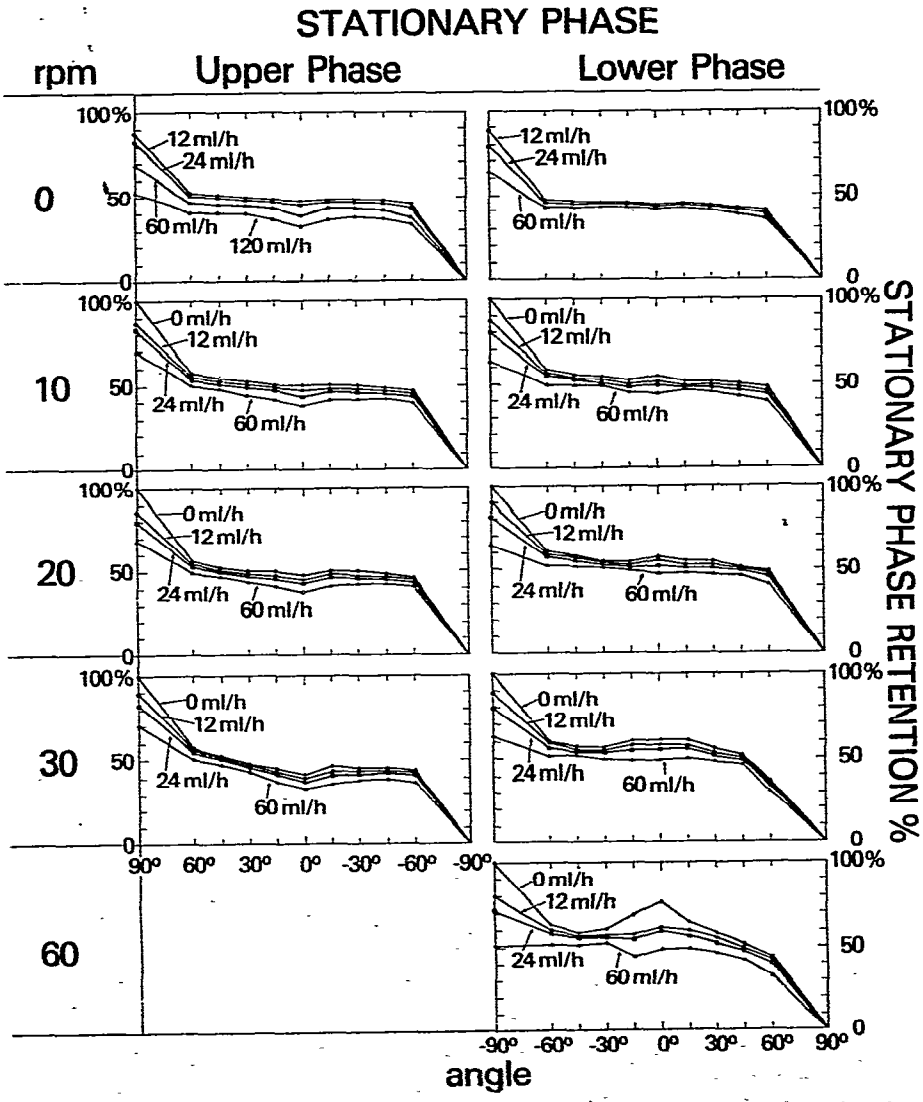


Fig. 2. Retention of the stationary phase in relation to the column angle, rotational speed and flow-rate. The column angle is expressed as 0° being horizontal.

laminary to the droplet. The size of the droplets decreases with the rotational speed and becomes much smaller than the internal diameter of the column at higher rotational speeds. These droplets, however, can be trapped in the coiled tube by gravity to maintain a satisfactory phase retention in the present phase system.

Comparative studies revealed that in both chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1) and *n*-butanol–acetic acid–water (4:1:5) phase systems the aqueous phase, having less affinity to the tube wall, tends to form droplets and, if

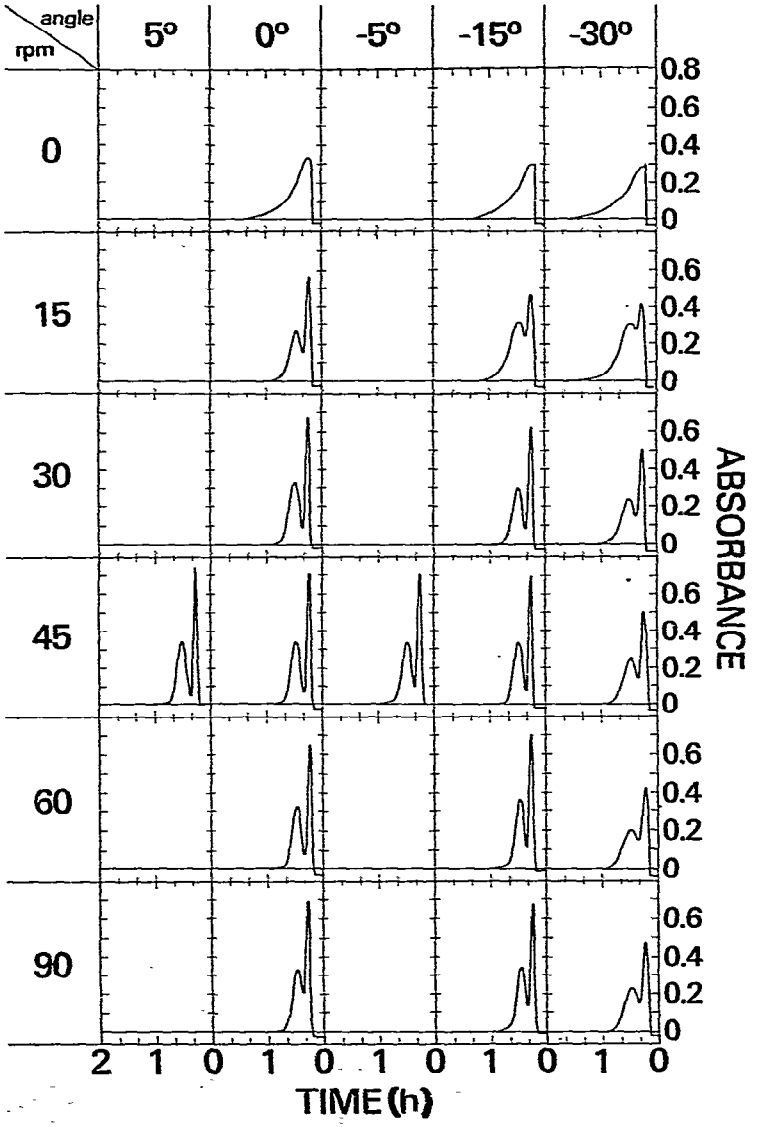


Fig. 3. Effects of column angle and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-glutamic acid; the second, DNP-L-alanine; mobile phase, upper phase; flow-rate, 60 ml/h.

used as the mobile phase, it allows higher rotational speeds or flow-rates for satisfactory phase retention than if used as the stationary phase. The present phase system, which has lower viscosity and a greater density difference between upper and lower phases, permits operations at higher rotational speeds and flow-rates.

Several other two-phase solvent systems have also been tested for phase retention. Satisfactory retention is found with extremely low interfacial tension phase systems such as ethylene glycol ethers–various salt solutions used for separation of proteins[4,5]. However, high interfacial tension phase systems including hexane–water and ethyl acetate–water produce a plug flow in the presently used column and it is necessary to increase the diameter of the column for successful operation.

*Partition efficiency studies*

Effects of the column angle and rotational speed on separation of two DNP amino acid samples are shown in Fig. 3, where the upper aqueous phase is used as the mobile phase at a flow-rate of 60 ml/h. The partition efficiency is conveniently estimated from the relative height of the trough between the two peaks. The best separation is obtained at 45 rpm with the column set close to the horizontal position. The efficiency rises sharply with the rotational speed from 0–30 rpm.

Using the horizontal column position, further experiments are performed to study the effects of flow-rate and rotational speed on partition efficiency. Fig. 4 shows the results obtained by using the upper aqueous phase as the mobile phase. The best resolution is seen at 60 rpm with a slow flow-rate of 12 ml/h, while the highest partition efficiency in terms of T.P./time is achieved at 45 rpm with a higher flow-rate of 60 ml/h. Fig. 5 shows similarly the results obtained by using the lower organic phase as the mobile phase. Here, the best resolution is seen at 20 rpm with a flow-rate of 24 ml/h where the T.P./time figure shows little improvement with a higher flow-rate of 60 ml/h.

Comparing the above results with those previously obtained with the *n*-butanol phase system, it may be concluded that the aqueous phase, if used as the mobile phase, allows a higher rotational speed and flow-rate to yield a higher partition efficiency regardless of the viscosity of the organic phase. The higher partition efficiency achieved by the present phase system may be attributed to the lower phase viscosity and the greater density difference between the upper and the lower phases.

*Preparative countercurrent chromatograms*

Preparative-scale separation is carried out with the column consisting of nine column units connected in series which has approximately 1000 turns and 240 ml in capacity. In each separation, the sample mixture is dissolved in the stationary phase and a sample volume of 10 ml is applied.

Fig. 6 shows three chromatograms of DNP amino acids on the present phase system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). The top and middle chromatograms are obtained by using the upper aqueous phase as the mobile phase under optimum operative conditions of 45 rpm at flow-rates of 60 ml/h and 24 ml/h, respectively. The bottom chromatogram is obtained by using the lower organic phase as mobile phase at 20 rpm and a flow-rate of 24 ml/h.

Fig. 7 shows preparative chromatograms of peptides on the phase system com-

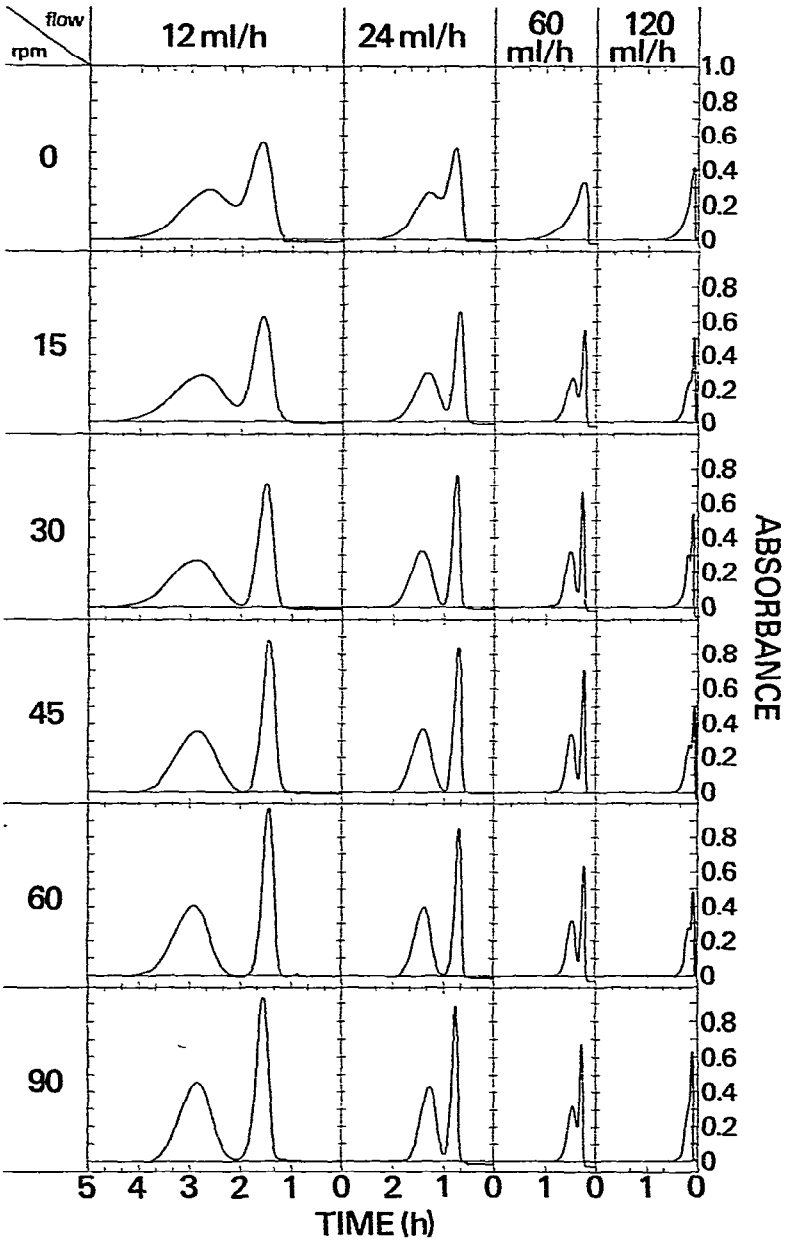


Fig. 4. Effects of flow-rate and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-glutamic acid; the second, DNP-L-alanine; mobile phase, upper phase; column angle, 0°.

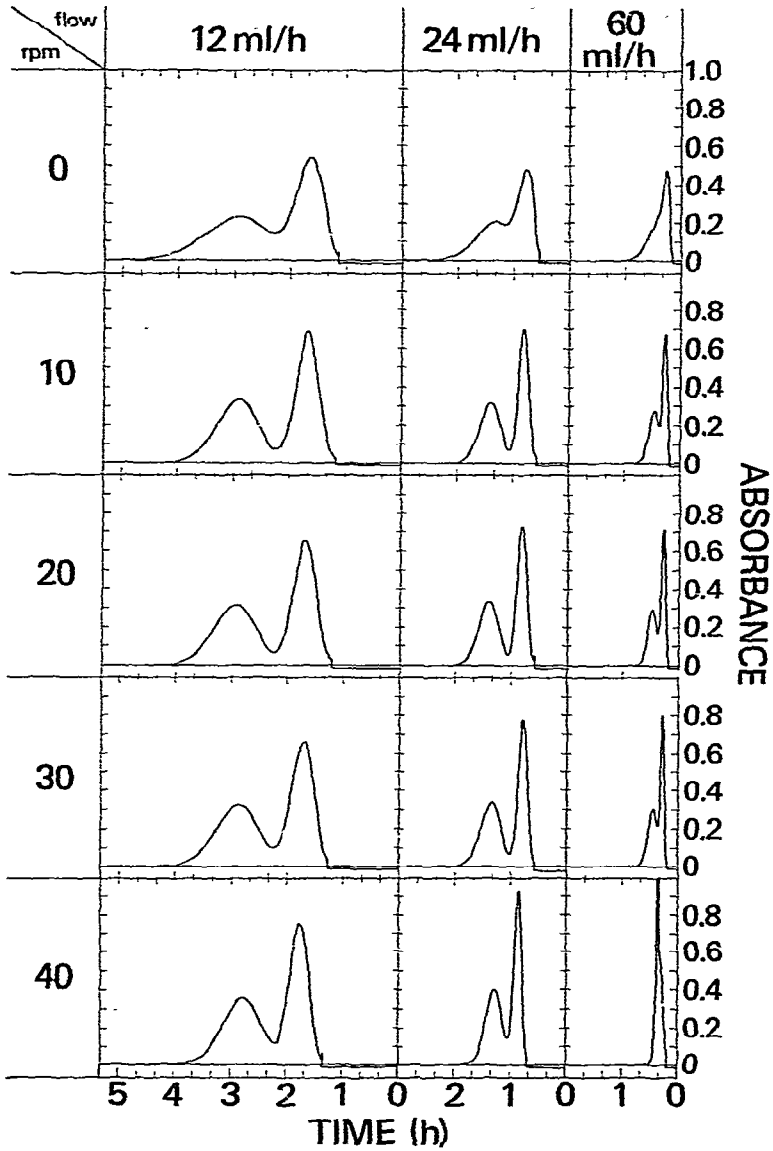


Fig. 5. Effects of flow-rate and rotational speed on the separation of DNP amino acids. The first peak is DNP-L-alanine; the second, DNP-L-glutamic acid; mobile phase, lower phase; column angle, 0°.

posed of *n*-butanol–acetic acid–water (4:1:5) with the same preparative column. The top chromatogram is obtained by using the lower aqueous phase as a mobile phase at 15 rpm and 12 ml/h of flow-rate while the bottom is obtained by using the upper organic phase as a mobile phase at 10 rpm and 12 ml/h.

These preparative separations were produced on the two typical phase systems

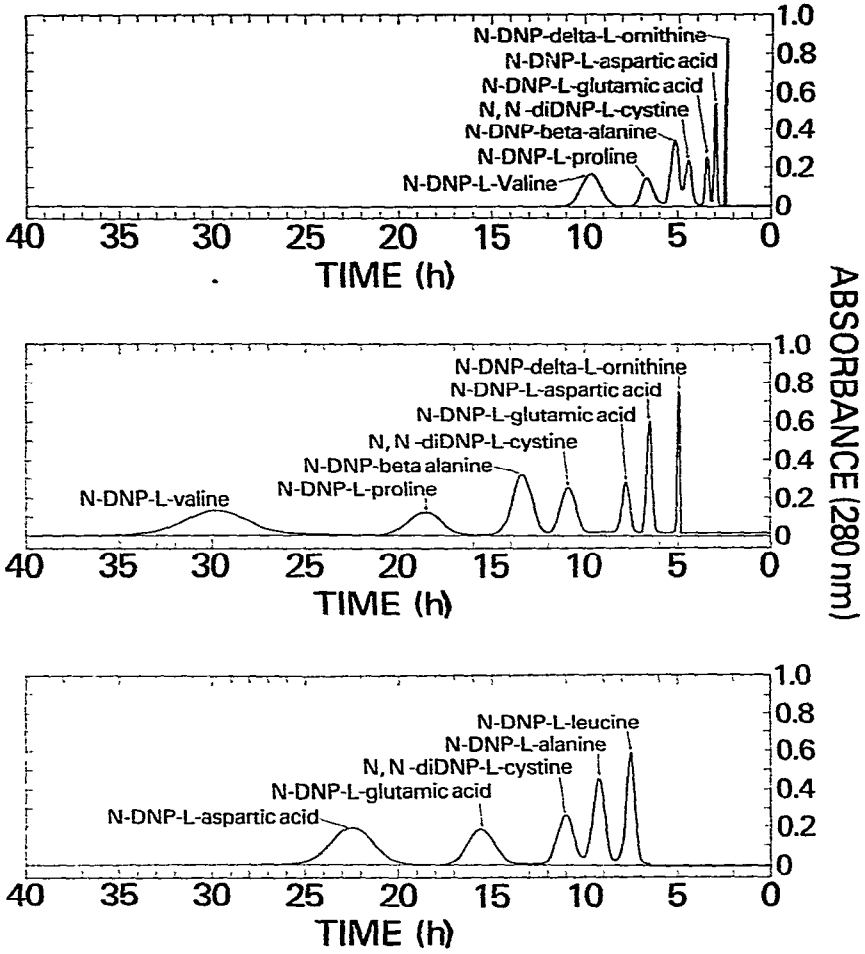


Fig. 6. Preparative countercurrent chromatograms of DNP amino acids on chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Top: mobile phase, upper phase; flow-rate, 60 ml/h; rotation, 45 rpm; column angle, 0°. Middle: mobile phase, upper phase; flow-rate, 24 ml/h; rotation, 45 rpm; column angle, 0°. Bottom: mobile phase, lower phase; flow-rate, 24 ml/h; rotation, 20 rpm; column angle, 0°.

which differ greatly in viscosity and phase density difference. We think that other low interfacial tension phase systems used for extraction or partition chromatography may be applied successfully by choosing the above operational conditions with minor modifications. The present countercurrent chromatographic scheme allows 10-ml sample charge to yield a high partition efficiency ranging from one thousand to several hundred theoretical plates where either aqueous or organic phase can be used as the mobile phase.

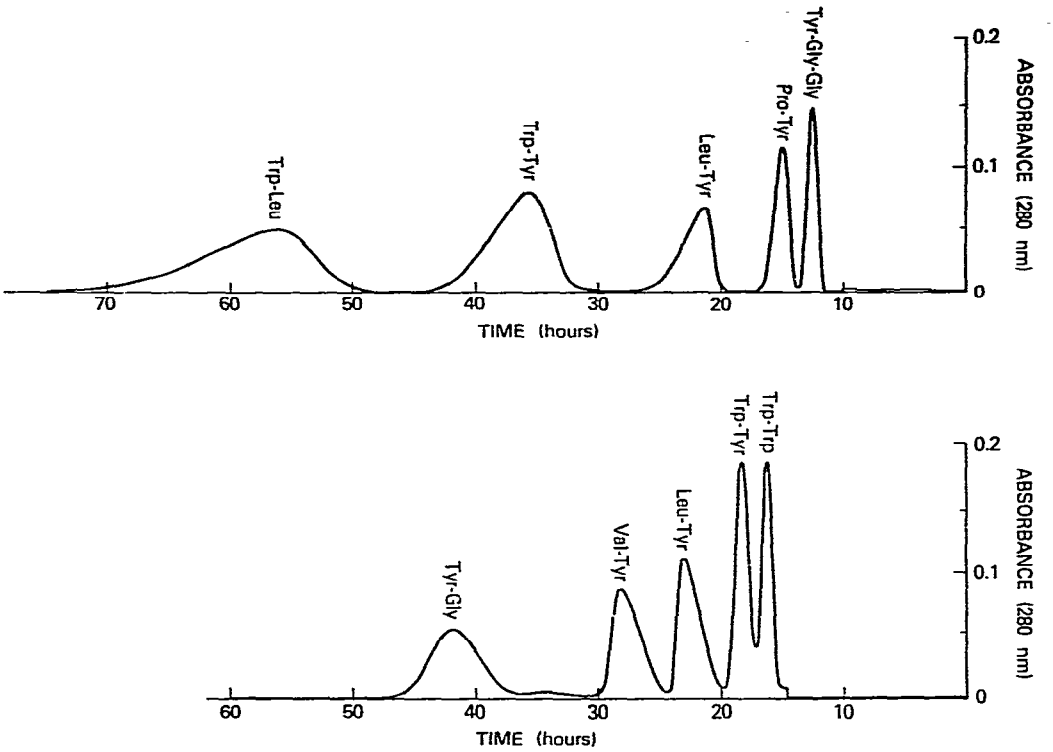


Fig. 7. Preparative countercurrent chromatograms of peptides on *n*-butanol–acetic acid–water (4:1:5). Top: mobile phase, lower phase; flow-rate, 12 ml/h; rotation, 15 rpm; column angle, 0°. Bottom: mobile phase, upper phase; flow-rate, 12 ml/h; rotation, 10 rpm; column angle, 0°.

## REFERENCES

1 Y. Ito and R. L. Bowman, *Anal. Chem.*, 43 (1971) 69A.
2 Y. Ito, R. E. Hurst, R. L. Bowman and E. K. Achtor, *Sep. Purif. Methods*, 3 (1974) 133.
3 Y. Ito and R. L. Bowman, *Anal. Biochem.*, in press.
4 A. J. P. Martin and R. R. Porter, *Biochem. J.*, 49 (1951) 215.
5 R. R. Porter, *Biochem. J.*, 53 (1953) 320.