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CROSS-AXIS SYNCHRONOUS FLOW-THROUGH COIL PLANET CENTRIFUGE FOR LARGE-SCALE PREPARATIVE COUNTER-CURRENT CHROMATOGRAPHY

II*. STUDIES ON PARTITION EFFICIENCY IN SHORT COILS AND PREPARATIVE SEPARATIONS WITH MULTILAYER COILS

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SUMMARY

A series of experiments was conducted to evaluate the partition efficiency of the cross-axis synchronous flow-through coil planet centrifuge. The preliminary studies indicated that short coils (5 m) mounted on 15- and 25-cm diameter holders in either a central or a lateral location produce a high partition efficiency of over 100 theoretical plates. Under the optimum operating conditions, the preparative capability of the countercurrent chromatograph was demonstrated on gram-scale separations of DNP-amino acids and dipeptides in a pair of multilayer coils connected in series with a total capacity of 1600 ml.

INTRODUCTION

Successful separation in counter-current chromatography (CCC) mainly depends on two parameters, retention of the stationary phase in the separation column and partition efficiency[1]. Studies on stationary phase retention described in Part I have shown that the present CCC scheme yields satisfactory levels of retention for various two-phase solvent systems and that the retention can be improved by choosing lateral coil positions on the holder. This second series of experiments was performed to investigate the partition efficiency of the coiled column using the operating conditions which have produced significant levels of stationary phase retention.

This paper describes the results of partition efficiency studies using two sets of standard test samples and two-phase solvent systems: DNP (dinitrophenyl) amino acid separation in chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1) and dipeptide separation in *n*-butanol–acetic acid–water (4:1:5). Preliminary studies were performed

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with single-layer coils (5 m) mounted on a set of holders with various hub diameters each at central and lateral positions as in the previous studies on retention of the stationary phase (Part I). The preparative capability of the method was demonstrated by the separation of gram amounts of samples in a pair of long multilayer coils connected in series (total volume of 1600 ml) and mounted symmetrically on both sides of the rotary frame of the centrifuge.

EXPERIMENTAL

Apparatus

The design of the apparatus was described in detail in Part I. This second prototype cross-axis synchronous flow-through coil planet centrifuge has a 20-cm revolutional radius and can be rotated at up to 500 rpm, which produces about 56 *g* on the axis of the holder.

Separation columns

Two different types of coiled columns were used for partition efficiency studies: single-layer short coils for preliminary or model studies and long multilayer coils for preparative separations.

Short coils were each prepared from 5 m of 2.6 mm I.D. PTFE tubing of approximately 28-ml capacity by winding it onto the holder hub (5-, 15- or 25-cm diameter), making a single layer coil. The number of helical turns varied according to the hub diameters: 6 turns for 25-cm hub diameter, 10 turns for 15-cm hub diameter and 30 turns for 5-cm hub diameter. As in Part I, the coil was mounted at two different locations on each holder, at the center ($l = 0$ cm) and 10 cm to the left of the center ($l = -10$ cm). At the side of the 5-cm diameter holder there was not enough space, so 5 m of tubing was wound in a double layer. Each coil was securely fixed on the holder hub with several pieces of fiber-glass reinforced adhesive tape. Each end of the coil was connected to a 1-m long flow tube of 0.85 mm I.D., using short pieces of intermediate-size PTFE tubing. These flow tubes were led to the outside of the centrifuge as described in Part I.

The multilayer coil was prepared from a long piece of 2.6 mm I.D. PTFE tubing by winding it onto a spool-shaped holder (15-cm hub diameter), making multiple coiled layers between a pair of flanges spaced 5 cm apart. The column consisted of 14 layers of coil with a total capacity of about 800 ml. The β value varied from 0.375 at the internal terminal to 0.625 at the external terminal. A pair of similar multilayer coils was symmetrically mounted, one on each side of the rotary frame, in either the central or lateral location on the holder shafts. These two columns were connected in series with a flow tube in such a way that the external terminal of the first column joins the internal terminal of the second column. In this way perfect balancing of the centrifuge system is ensured and the two columns are always subjected to an ideal elution mode. Fig. 1 shows the apparatus equipped with a pair of multilayer coils in the central position of the holder shafts. The columns can be shifted along the holder shaft to optimize the hydrodynamic conditions.

Reagents

Solvents such as chloroform and *n*-butanol were of glass-distilled chromato-

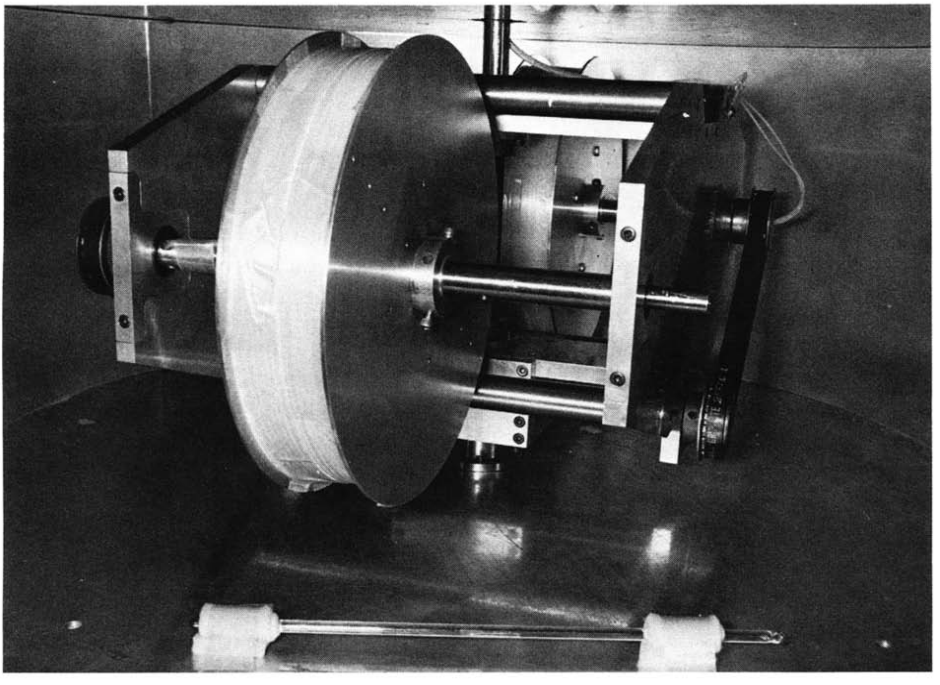

Fig. 1. The cross-axis synchronous flow-through coil planet centrifuge with 20-cm revolutional radius equipped with a pair of large multilayer coil separation columns.

graphic grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and others, acetic acid and *n*-butanol for preparative separations, were of analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). Hydrochloric acid (1 *M*) and all test samples including L-valyl–L-tyrosine (Val–Tyr), L-tryptophyl–L-tyrosine (Trp–Tyr), N-2,4-dinitrophenyl-DL-glutamic acid (DNP-Glu) and N-2,4-dinitrophenyl-L-alanine (DNP-Ala) were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of two-phase solvent systems and sample solutions

Two types of solvent systems were prepared: chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1) for DNP-amino acid separations and *n*-butanol–acetic acid–water (4:1:5) for dipeptide separations. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature by repeated vigorous shaking and degassing several times. The samples were also dissolved in these solutions.

Sample solutions for DNP-amino acid separations were prepared as follows. For the preliminary studies with short coils, equal amounts of DNP-Glu and DNP-Ala were dissolved in the upper aqueous phase to make the concentration of each component 0.5% (w/v), and 0.2 ml of the sample solution was loaded in each experiment. For large-scale preparative chromatography in the multilayer coils, 2 or 5 g of the DNP-amino acids were dissolved in equal volumes of the upper and the lower phases to bring the total volume to 50 or 100 ml, respectively.

For the preliminary separations in short coils, the peptides were dissolved in the

lower aqueous phase to make concentrations of Val–Tyr and Trp–Tyr 1 and 0.3% (w/v), respectively, and 0.2 ml of this solution was used for each run. For the preparative separations in the multilayer coils, 1 g of Val–Tyr and 0.3 g of Trp–Tyr were dissolved in 100 ml of the solvent system.

For concentrated sample solutions used in preparative separations, the settling times of the two solvent phases were measured to ensure satisfactory retention of the stationary phase in the column[2,3]. A 2-ml volume of each phase of a sample solution was delivered into a 5-ml capacity graduated glass cylinder which was then sealed with a glass stopper. For each test, the cylinder was gently inverted five times to mix the contents and then placed in the upright position to measure the time required for the solvent mixture to form two distinct layers. The test was repeated several times to obtain the mean value.

Preliminary separations with short coils

As described in Part I, preliminary studies were performed with short coils mounted at the center ($l = 0$) or to one side ($l = -10$ cm) on three different holders (5-, 15- and 25-cm hub diameters).

For each separation, the coil was first entirely filled with the stationary phase followed by injection of the sample solution (0.2 ml) through the sample port. Then, the apparatus was run at a uniform revolutional speed while the mobile phase was eluted through the coil at a flow-rate of 120 ml/h. The effluent from the outlet of the coil was monitored continuously with an LKB Uvicord S instrument at 278 nm and fractionated into test-tubes with an LKB fraction collector. Each fraction containing 1 ml was diluted with 2 ml of methanol and the absorbance was determined at 430 nm (DNP-amino acids) or 280 nm (dipeptides) in a Zeiss PM6 spectrophotometer. The absorbance is plotted in Figs. 2, 3, 5, 6, etc. The experiments were performed by applying revolutional speeds of 200, 300, 400 and 500 rpm using both the upper and the lower phases as the mobile phase. After each separation, the volume of the stationary phase retained in the coil was measured by collecting the column contents in a 50-ml graduated cylinder after connecting the coil inlet to a pressurized nitrogen line under slow rotation of the coil in the tail-to-head elution mode.

At the central coil position ($l = 0$ cm), the above separations were performed for each mobile phase in a single elution mode of either head-to-tail or tail-to-head elution, whichever yielded the higher retention of the stationary phase as determined in the studies of Part I. With the lateral coil position ($l = -10$ cm), where the retention of the stationary phase is affected by both the planetary motion and head–tail elution mode, separations were performed by selecting the suitable elution mode for each planetary motion (P_I and P_{II}) for each choice of the mobile phase.

Preparative separations with multilayer coils

Using the optimal experimental conditions determined by preliminary separations, preparative-scale CCC separations were performed with a pair of multilayer coils symmetrically mounted one on each side of the centrifuge rotor at either the central or the lateral location on the holder shafts.

The preparative separations were carried out as follows. The entire column (a pair of multilayer coils connected in series) was first filled with the stationary phase. This was followed by injection of the sample solution through the sample port. Then,

the centrifuge was run at 400 or 450 rpm in the desired direction (P_I or P_{II}) while the mobile phase was eluted through the column at a uniform flow-rate of 120 ml/h in the appropriate head–tail elution mode. The effluent from the outlet of the column was monitored continuously with an LKB Uvicord S instrument at 278 nm and collected in 15-ml fractions (7.5 min) in an LKB fraction collector. An aliquot of each fraction (20–50 μl) was mixed with 3 ml of methanol and the absorbance was determined at 430 nm for the DNP-amino acids and 280 nm for the dipeptides in a Zeiss PM6 spectrophotometer.

After each separation was completed, the retention of the stationary phase was measured by collecting the column contents by pushing with pressurized nitrogen as described above.

Determination of partition efficiency

In both preliminary and preparative separations, the partition efficiency was measured in terms of theoretical plates from the elution profile of the sample peaks using the conventional gas chromatographic equation

$$N = (4R/w)^2 \tag{1}$$

where N is the partition efficiency expressed as the number of theoretical plates (T.P.), R the retention volume or time of the peak maximum, and w the peak width measured in the same unit as R.

The partition efficiency was also evaluated by the degree of resolution between the two peaks using the equation

$$R_\sigma = 2(R_2 - R_1)/(w_2 + w_1) \tag{2}$$

where R_σ is the peak resolution expressed in 4σ units (σ is the standard deviation in a normal distribution), R_1 and R_2 the retention volumes or times of the first and second peaks, respectively, and w_1 and w_2 the peak widths for the first and second peaks, respectively, expressed in the same unit as retention R[4].

RESULTS AND DISCUSSION

Partition efficiency studies with short coils

Fig. 2 shows three sets of chromatograms of DNP-amino acids (DNP-Glu and DNP-Ala) obtained with short coils mounted on the holders with hub diameters of 5 cm (top), 15 cm (middle) and 25 cm (bottom), each in the central and lateral positions on the holder. These separations were obtained under the optimal operating conditions, which were determined by preliminary studies on stationary phase retention as described in Part I. In each set of diagrams, the chromatographic charts are arranged according to the applied revolutional speeds (rows) and elution mode (columns). The three left-hand columns were obtained with the upper aqueous phase mobile and the three right-hand columns with the lower non-aqueous phase mobile. For each mobile phase group, the results shown in the left column were obtained with the central coil position (l = 0 cm) and those in the middle and right columns were

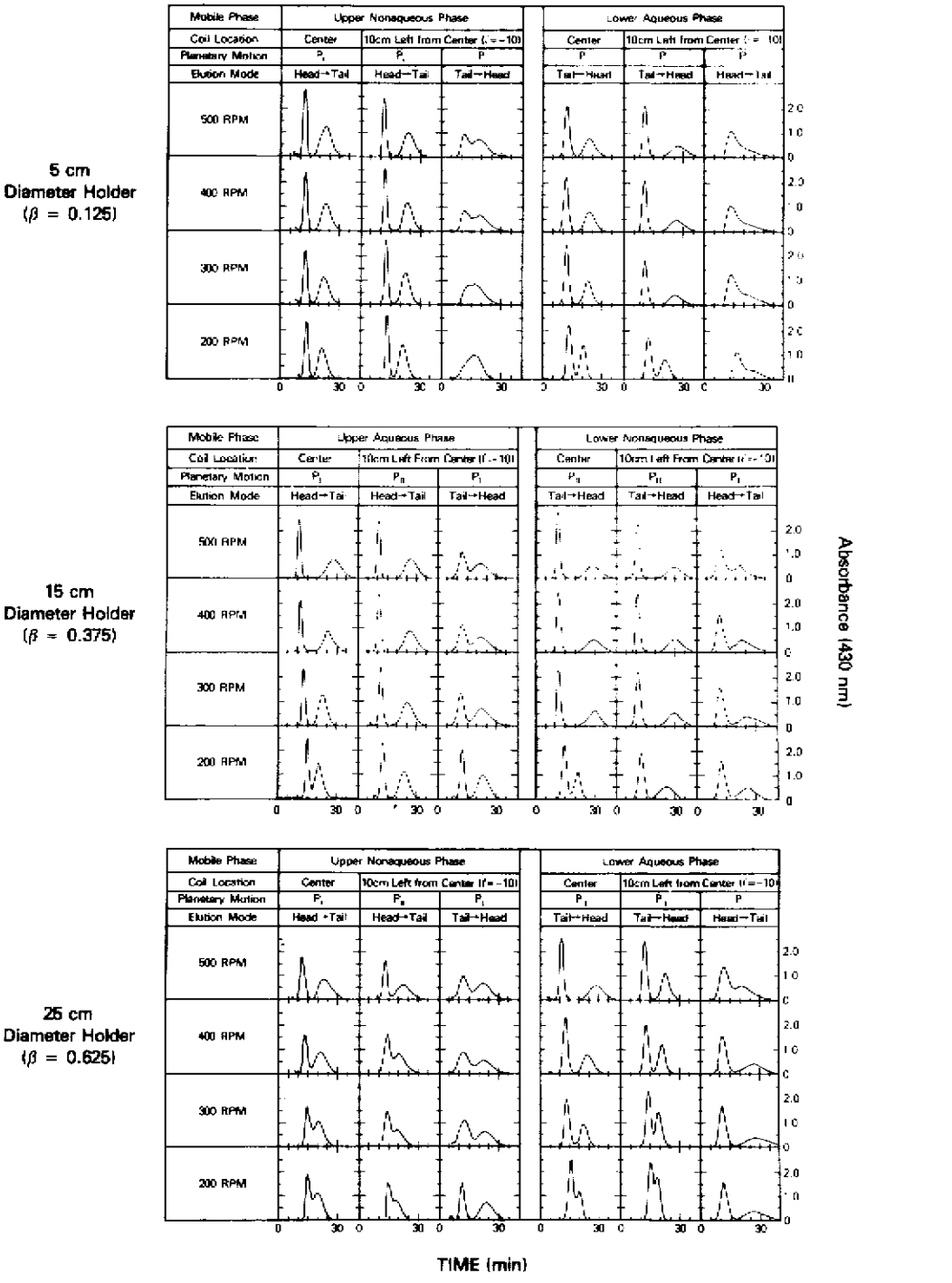

Fig. 2. Results of DNP-amino acid separations with short coils. Solvent system, chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); sample size, 2 mg; flow-rate, 120 ml/h.

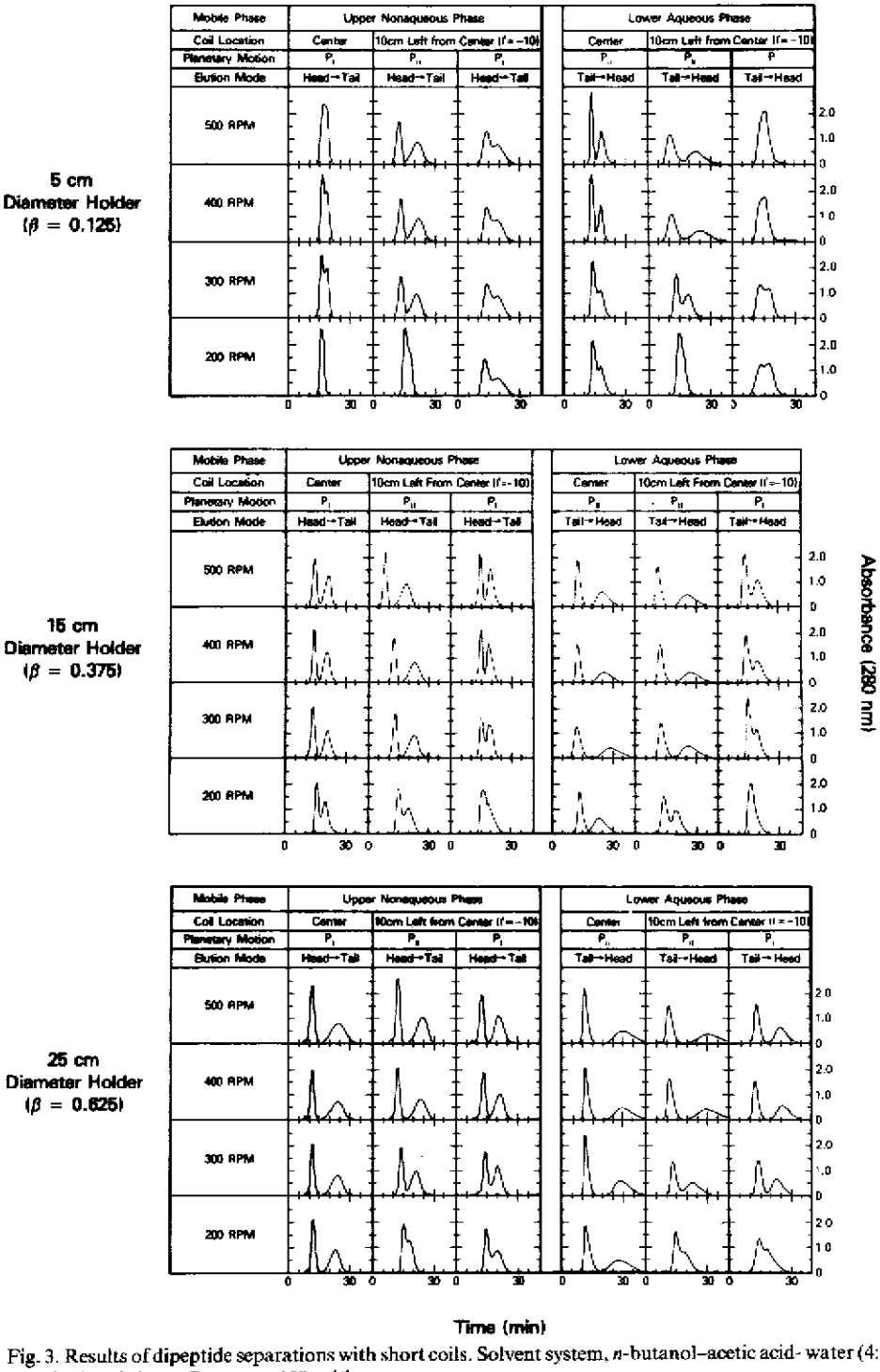

Fig. 3. Results of dipeptide separations with short coils. Solvent system, *n*-butanol–acetic acid- water (4:1:5); sample size, 2.6 mg; flow-rate, 120 ml/h.

obtained with the laterally positioned coil ($l = -10$ cm), each using the indicated planetary motion and head–tail elution mode.

In Fig. 2 (top) (5-cm hub diameter, $\beta = 0.125$) a good peak resolution is observed in both the central and lateral positions with head-to-tail elution of the upper phase or tail-to-head elution of the lower phase. Chromatograms obtained with the reversed elution mode in the lateral coil position (P_I planetary motion) show poor peak resolution despite the fair degree of stationary phase retention of over 40%. With one exception (right column, lower phase mobile), separations are substantially improved with an increased revolutional speed of up to 400–500 rpm where, with both mobile phase groups, the lateral coil position gives a slightly higher peak resolution than the central coil position.

All the separations shown in the middle panel of Fig. 2 are considerably improved with the coil mounted on the 15-cm hub diameter ($\beta = 0.375$), which coincides with a higher retention of the stationary phase [see the phase distribution diagrams for chloroform–acetic acid–water (2:2:1) in Figs. 3 and 4 in Part I]. In both central and lateral positions with the head-to-tail elution of the upper phase or the tail-to-head elution of the lower phase, two peaks were completely resolved at revolutional speeds of 300 rpm or greater. Chromatograms obtained with the lateral coil position using the reversed elution mode (P_I planetary motion), which gives poor peak resolution at $\beta = 0.125$, yield fair degrees of peak resolution at the higher β value, especially at lower rpm. With the larger hub diameter of 25 cm ($\beta = 0.625$), the peak resolutions of most chromatograms obtained with the head-to-tail elution mode (the left and the middle columns in each mobile phase group) are again decreased, apparently owing to depletion of the stationary phase retained in the coil.

Fig. 3 similarly shows separations of dipeptides (Val–Tyr and Trp–Tyr) using *n*-butanol–acetic acid–water (4:1:5) with short coils mounted on a set of holders with 5-cm (top), 15-cm (middle) and 25-cm (bottom) hub diameters at the central and lateral positions, with the same format as in Fig. 2. Because of the unique hydrodynamic distribution characteristic of this hydrophilic (or polar) solvent system, head-to-tail elution of the upper phase and tail-to-head elution of the lower phase were applied throughout the experiment. In the central coil position, the small β value of 0.125 (5-cm hub diameter) gives a poor peak resolution, apparently owing to the lower retention of the stationary phase, whereas the large β value of 0.625 (25-cm hub diameter) yields excellent peak resolution at revolutional speeds ranging from 300 to 500 rpm. In the lateral coil position, the two modes of planetary motion (P_I and P_{II}) produced different results. Chromatograms obtained under planetary motion P_{II} (middle column in each mobile phase group) show an excellent peak resolution, which substantially exceeds that obtained at the central coil position, whereas chromatograms obtained under planetary motion P_I (right column in each mobile phase group) show much lower peak resolution. These results are closely correlated with the retention level of the stationary phase (see Figs. 3 and 4 in Part I): the greater the retention of the stationary phase, the higher is the peak resolution. This finding strongly suggests that various other two-phase solvent systems, including the hydrophobic hexane and ethyl acetate systems and the hydrophilic *sec.*-butanol system, will produce much more efficient separations in the lateral coil position than in the central position (compare Fig. 3 with Fig. 4 in Part I).

Fig. 4 illustrates the relationship between the percentage retention of the

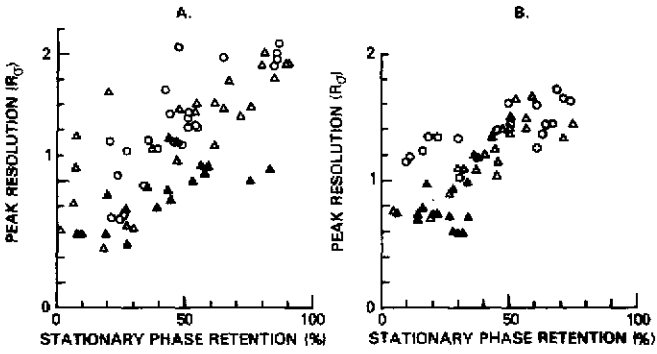

Fig. 4. Correlation between peak resolution and stationary phase retention. (A) DNP-amino acid separation; (B) dipeptide separation. ○, Central coil position; △, lateral coil position (P_{II}); ▲, lateral coil position (P_I).

stationary phase and peak resolution R_s for (A) DNP-amino acid separations and (B) dipeptide separations. In both diagrams, circles indicate the data obtained with the central coil position (left columns in each mobile phase group in Fig. 2) and open and solid triangles the data obtained with the lateral coil position under the planetary motions P_{II} and P_I, respectively (middle and right columns in each mobile phase group in Fig. 2). All these groups show a significant positive correlation ($r = 0.70$–0.86) between the stationary phase retention and the resulting peak resolution. These figures may be an underestimation as unresolved peaks due to extensive loss of the stationary phase are all eliminated from the data.

Similar studies on the relationship between the stationary phase retention and partition efficiency (theoretical plates) revealed various degrees of negative correlation ranging from -0.7 to -0.22. The above results clearly indicate that increased stationary phase retention in CCC will improve the peak resolution, while the number of theoretical plates may be a useful parameter for evaluating the performance of the preparative CCC systems only if the retention of the stationary phase remains in a normal range (over 50% of the total column capacity).

Partition efficiencies (mean T.P. values of two peaks) of DNP-amino acid separations obtained from the short coils under the optimal experimental conditions of over 50% stationary phase retention are about 100 T.P. or 5 cm/T.P. (one T.P. is produced by a 5-cm length of coil), which is comparable to the performance of the first prototype X-axis CPC with a 10-cm revolutional radius as reported previously[5]. The above partition efficiency doubles those produced by the existing high-speed CCC apparatus[6] while the time required for the separation in the present method is also proportionally increased owing to the slower flow-rate necessary for the elution of the mobile phase to achieve a satisfactory level of stationary phase retention.

Preparative separations with multilayer coils

In order to demonstrate the preparative capability of the present system, gram-scale separations were performed on each set of test samples in a pair of multilayer coils connected in series (1600 ml capacity) using the optimal operational conditions determined in the preliminary studies with the short coils.

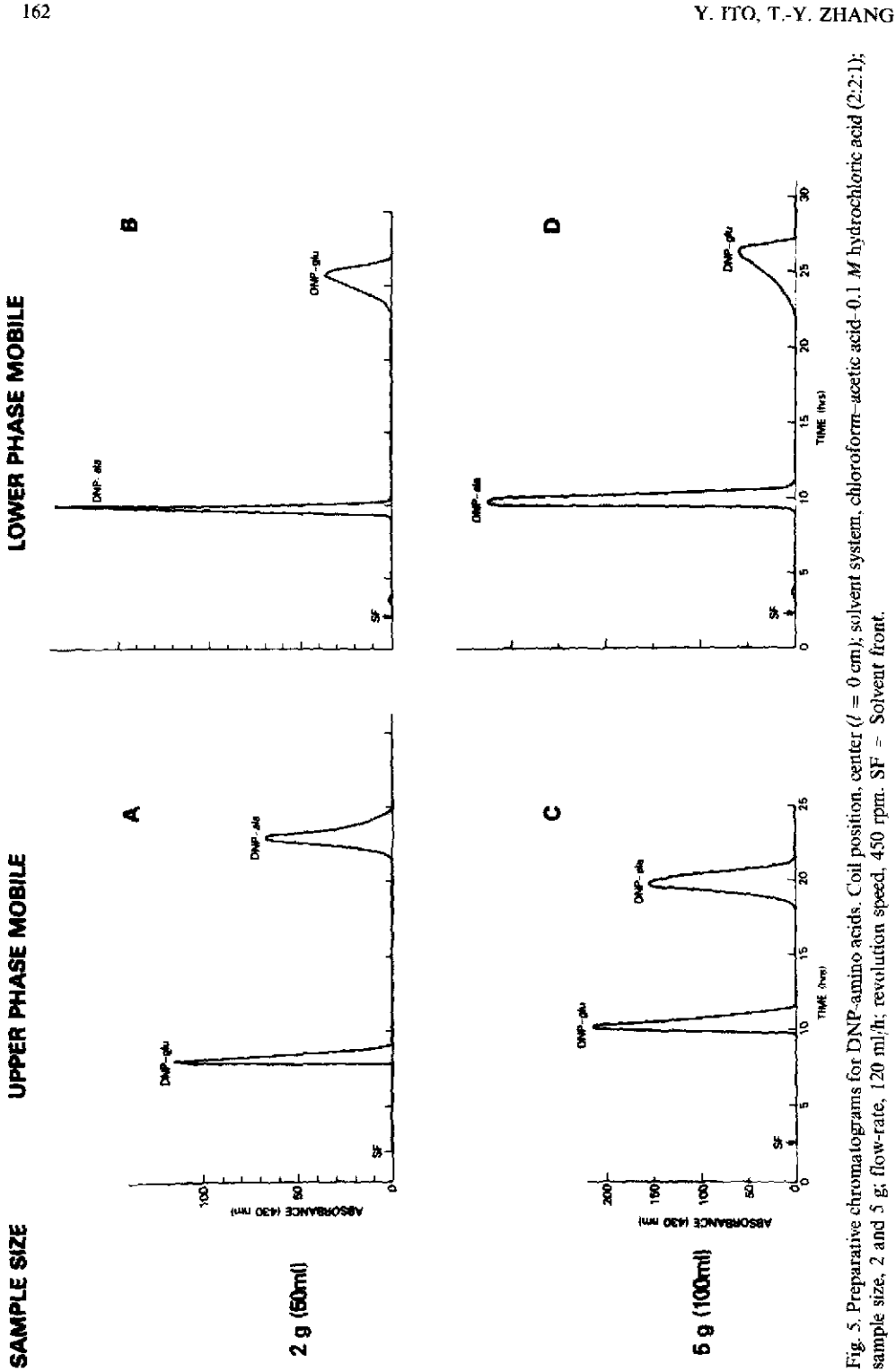

Fig. 5. Preparative chromatograms for DNP-amino acids. Coil position, center (l = 0 cm); solvent system, chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1); sample size, 2 and 5 g; flow-rate, 120 ml/h; revolution speed, 450 rpm. SF = Solvent front.

Fig. 5 shows preparative separations of the DNP-amino acid mixture in the central column position. Four chromatograms are arranged according to the sample size and the choice of the mobile phase as indicated. Samples of 2 and 5 g were separated at a flow-rate of 2 ml/min at 450 rpm using either the upper or lower phase as the mobile phase. In all chromatograms, DNP-Glu and DNP-Ala peaks are well resolved and eluted in 22–27 h. Owing to the non-linear isotherm produced by high sample concentrations, all peaks display various degrees of skewness, which is enhanced with the 5-g sample size especially for DNP-Glu with the lower phase mobile (Fig. 5, bottom right). The partition efficiency measured from the DNP-Ala peaks ranges from 2000–2700 T.P. for the 2-g sample to 1000–1250 T.P. for the 5-g sample. The above T.P.s are substantially lower than the estimated value of 5000 T.P. calculated from the results obtained on the short coils, apparently owing to the increased sample size. Separations of DNP-amino acids obtained with the lateral column position produced similar chromatograms.

Preparative separations of the dipeptide mixture (containing 1 g of Val- Tyr and 0.3 g of Trp–Tyr) were difficult, mainly for the following reasons. The high concentration of the dipeptide sample in the *n*-butanol solvent system raised the phase viscosity, as manifested by an increase in the settling time from 39 to 58 s, which in turn reduced the retention of the stationary phase. Further, a high sample concentration increased the non-linear isotherm, particularly for Trp–Tyr, which produced markedly skewed peaks with a considerable shift of the peak maximum. Under these circumstances, multilayer coils in the central column position failed to resolve two peaks when the upper phase was used as the mobile phase. However, chromatography in the lateral column position produced complete separation of the two peaks regardless of the choice of the mobile phase.

Fig. 6 shows chromatograms of the dipeptides obtained with the lateral column position by eluting with (A) the upper and (B) the lower phase mobile. The non-linear isotherm of Trp- Tyr produced skewed peaks deviating toward the Val–Tyr peaks. Compared with the DNP-amino acid separations shown in Fig. 5, these dipeptide

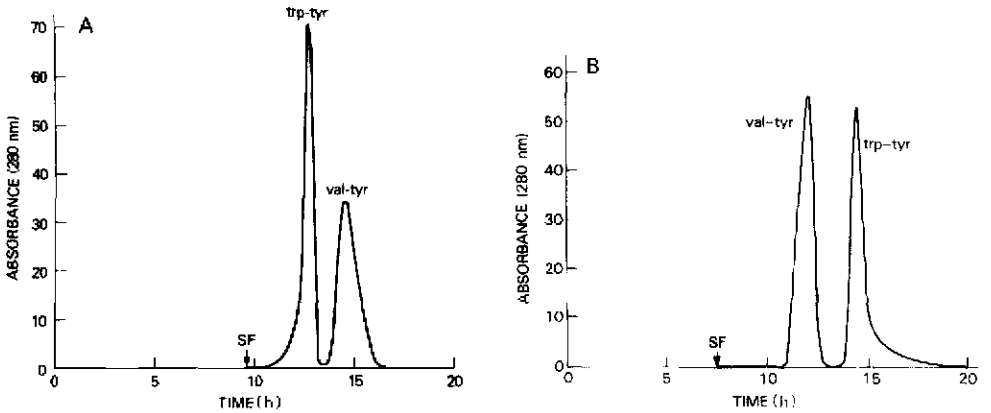

Fig. 6. Preparative chromatograms of dipeptides. Coil position, lateral ($l = -10$ cm); solvent system, *n*-butanol–acetic acid–water (4:1:5); sample size, 1.3 g; flow-rate, 120 ml/h; revolution speed, 400 rpm. (A) Upper phase mobile; (B) lower phase mobile. SF = Solvent front.

separations show a much lower peak resolution, which resulted from extensive loss of the stationary phase from the column. The retention of the stationary phase in these dipeptide separations was 17% for the upper phase mobile and 26% for the lower phase mobile. Various problems associated with the use of hydrophilic solvent systems with low interfacial tension and high viscosity may be further alleviated by optimizing the location of the separation column. The results of these studies indicate that the retention of the hydrophilic butanol systems can be improved by increasing the β value and/or the distance of lateral deviation ($\pm l$) of the multilayer coil.

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