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# IMPROVED HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPH WITH THREE MULTILAYER COILS CONNECTED IN SERIES

## I. DESIGN OF THE APPARATUS AND PERFORMANCE OF SEMIPREPARATIVE COLUMNS IN 2,4-DINITROPHENYL AMINO ACID SEPARATION

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SUMMARY

A compact desktop model of a high-speed counter-current chromatograph holds three identical multilayer coils in the symmetrical positions around the rotary frame to maintain perfect balance of the centrifuge system without the use of a counterweight. These multilayer coils are connected in series to make up a total capacity of 400 ml while the unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal. The high performance of the present system was successfully demonstrated in separations of 10–250 mg of 2,4-dinitrophenyl amino acid mixtures in a two-phase solvent system composed of chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v).

## INTRODUCTION

High-speed counter-current chromatography (HSCCC) is the most advanced form of the CCC technology which is characterized by rapid and efficient separation on both analytical and preparative scales[1]. As in other centrifugal CCC systems, the partition efficiency of HSCCC can be improved by using a greater length of the coild column. The original HSCCC apparatus is equipped with a column holder on one side of the rotor and the counterweight on the opposite side of the rotor to balance the centrifuge system[1–3]. The use of a single column holder not only limits the column

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capacity, hence the partition efficiency, but also necessitates careful adjustment of the counterweight mass according to the density of the solvent system applied for separation. Recently, efforts were successfully made to solve these problems by mounting two identical multilayer coils symmetrically, one on each side of the rotary frame, and connecting these columns in series to double the column capacity[4].

The present paper introduces a novel design of the apparatus which holds three multilayer coils symmetrically around the rotary frame. These columns are interconnected in series on the rotary frame to triple the column capacity. The unique mechanical design of the present centrifuge system permits single passage of flow tubes; the flow tube enters from one side and leaves from the other side of the centrifuge without twisting. Performance of the apparatus was evaluated by separations of 2,4-dinitrophenyl (DNP) amino acid samples in a set of three semipreparative multilayer coils.

## APPARATUS

The design principle of the present apparatus is illustrated in Fig. 1 where three cylindrical column holders are symmetrically arranged around the centrifuge axis. Each holder undergoes an identical synchronous planetary motion: revolution around the centrifuge axis and rotation about its own axis at the same angular velocity in the same direction as indicated by arrows. These holders are connected in series with flow tubes in the following manner: the inflow tube enters the centrifuge system from the right along the centrifuge axis and is bent to enter the first holder (bottom) on the left side. The connecting tubing running between the first and the second holders leaves the first holder on the right side and is arched around to the left side of the centrifuge and via another bend reaches the second holder (middle) on the left side. The connecting tubing from the second holder similarly reaches the third holder (top) on the left side. The return flow tube from the third holder leaves the holder on the right side and is arched reaching the left side of the centrifuge where it exits the centrifuge system along the central axis of the centrifuge.

In order to prevent twisting the flow tubes, the horizontal portions of the flow tubes on the rotary frame should be counter-rotated synchronously with the rotation of the holder as indicated by arrows. If these requirements are fulfilled, the system

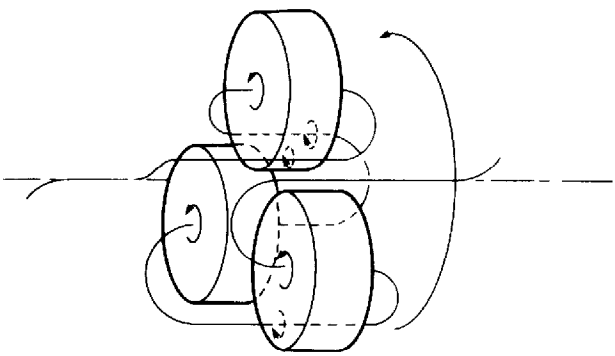

Fig. 1. Orientation and motion of column holders and flow tubes in the present apparatus.

permits flow in and out through the rotating columns without the use of a rotary seal which would become a source of leakage and contamination.

The planetary motion of the column holders and the counter-rotation of the flow tubes above described can be accomplished by the use of a set of 10 identical gears, as shown in Fig. 2, where one stationary sun gear (S) is held on the central axis of the centrifuge and surrounded by 9 planetary gears, *i.e.*, 3 double gears (C) each mounted on the column holder shaft and 3 single gears (T) each affixed to the tube holder shaft. The left half of each double gear interlocks to the stationary sun gear and the right half, to the single planetary gear, while the sun gear is entirely free from the single planetary gears. With this gear arrangement, rotation of the rotary frame around the stationary sun gear causes synchronous rotation of the double gears on the rotary frame and this motion is further conveyed to each single planetary gear to counter-rotate the tube holder shaft holding the flow tube. The number of gears required may be reduced to 8 or 7 but this will sacrifice the symmetry of the gear arrangement in the present design.

Fig. 3 shows a cross-sectional view of the apparatus through the central axis of the centrifuge with a plane across the center of the first column holder. For simplicity, the figure shows a column holder (bottom) and a tube holder shaft (top) on the rotary frame while all other structures behind the scene (including the second column holder, a tube holder shaft, and links) are omitted from the diagram. The motor (right side behind the centrifuge case, as indicated by an arrow) drives the rotary frame around the central stationary pipe via a pair of toothed pulleys coupled with a toothed belt. The rotary frame consists of a pair of aluminum discs rigidly bridged together with multiple links and holds three sets of column holders and tube holder shafts symmetrically at 7.5 cm from the central axis of the centrifuge all through sealed ball bearings. Each column holder is equipped with two identical plastic gears put together on the holder shaft. The first gear on the left side is coupled with the identical stationary sun gear mounted on the central axis of the centrifuge around the stationary pipe. This gear arrangement produces a desired planetary motion of the column

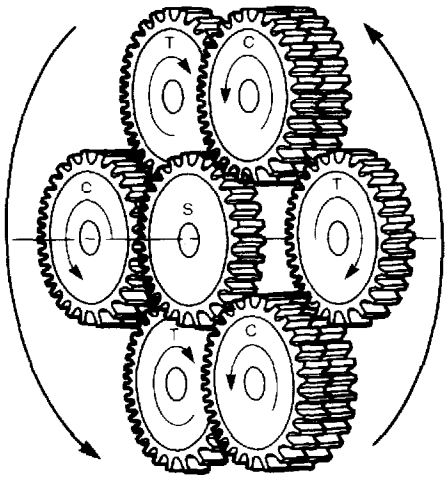


Fig. 2. Gear arrangement of the present apparatus.

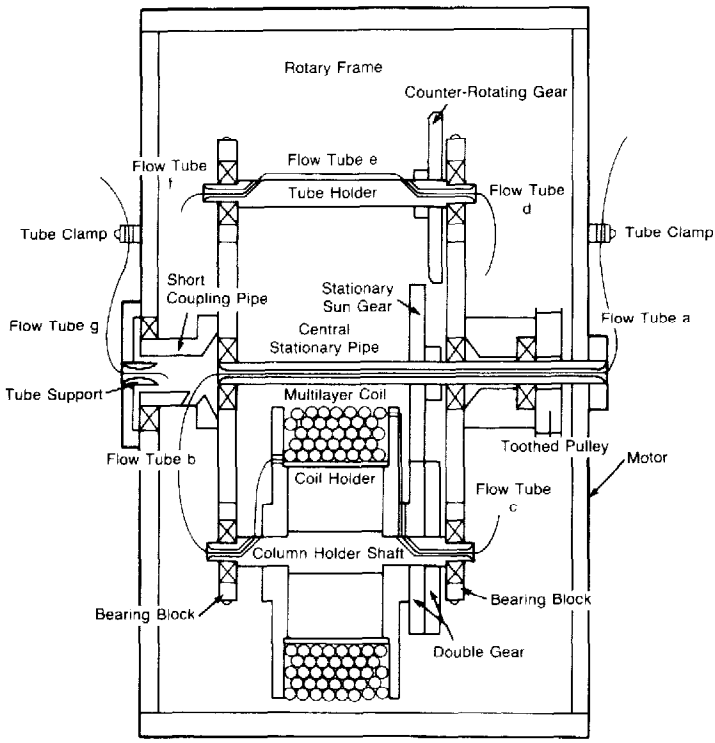


Fig. 3. Cross-sectional view of the apparatus.

holder: revolution around the central axis of the centrifuge and rotation about its own axis at the same angular velocity in the same direction. The second gear on the right side of the column holder shaft is engaged with the identical gear mounted on the tube holder shaft to produce synchronous counter-rotation of the flow tubes, thus fulfilling all the requirements described earlier (see Figs. 1 and 2).

In order to facilitate preparation of multilayer coils, the column holders are designed to be easily removed from the rotary frame by loosening a pair of screws on each bearing block. Each separation column was made by winding a single piece of 1.6 mm I.D. standard wall (0.4 mm thick) PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) directly onto the holder hub of 7.5 cm diameter, forming nine layers of coils between a pair of flanges spaced 5 cm apart. The total capacity of each column measures approximately 135 ml. The $\beta$ value of the multilayer coil measures 0.5 at the internal terminal to 0.75 at the external terminal. Here, $\beta$ is given by the ratio of the rotational radius (distance from the holder axis to the coil) to the revolutional radius (distance between the holder axis and the central axis of the centrifuge) and represents an important parameter to govern hydrodynamic distribution of the two solvent phases in the rotating coil[5]. In order to prevent dislocation of the multilayer coil on the holder, the whole column and flanges were covered by heat shrunk vinyl wrapping.

The present centrifuge system requires flexible narrow-bore flow tubes which

can maintain their integrity under repetitive flexing. Each terminal of the multilayer coil was connected to a flow tube of 0.85 mm I.D. and 0.46 mm wall thickness in the following manner: about 1 cm length of the flow tube was inserted into the coil terminal and the junction was covered with several turns of copper wire of about 0.7 mm diameter. The application of this copper wire is to limit heat expansion of the junction, thus forcing the overlapping two tube walls to contact under high pressure. Then, heat was locally applied with a heat gun until the whole junction becomes transparent and fused together. After cooling, the copper wire was removed from the junction. The fused joint thus formed usually can hold the pressure up to several hundred p.s.i. If space is available, the connection can also be made with commercial adaptors on the outside of the flanges.

Interconnection of three multilayer coils were made with flow tubes in such a manner that the external terminal of the first column joins the internal terminal of the second column and, similarly, the external terminal of the second column joins the internal terminal of the third column. In this way all multilayer coils are subjected to the identical elution modes.

The arrangement of the tubing is partially indicated in Fig. 3 (tube a–g); the whole passage is schematically shown in Fig. 1. The inflow tube enters the centrifuge from the right side through the opening of the central stationary pipe, and it passes the side hole of the short coupling pipe at the left end of the rotary frame where it forms an arch to reach the first column holder. Interconnection flow tube between the first and the second columns leaves the first column holder from the right side and, after forming an arch, it runs along the first tube holder towards the left across the rotary frame. On the left side of the rotary frame, the flow tube again forms an arch to reach the second column holder through the center hole on the holder shaft. The interconnection flow tube between the second and the third columns similarly runs through the second tube holder shaft. The outflow tube from the third column leaves the holder from the right side and, after passing through the third tube holder shaft, it reaches the left side of the rotary frame where it enters another side hole of the short coupling pipe to reach the stationary tube support projecting from the left wall of the centrifuge.

These flow tubes on the rotary frame were secured onto each tube holder with a pair of nylon ties while inflow and outflow tubes were each firmly held onto the centrifuge wall with a silicone-rubber-padded clamp. These flow tubes were lubricated with silicone grease and protected with a sheath of tygon tubing where supported or secured to prevent direct contact with metal parts. With this precaution, the flow tubes can maintain their integrity for many months of operation.

The apparatus can be operated up to the maximum revolutional speed of 1500 rpm with a speed control unit (Bodine Electric Co., Chicago, IL, U.S.A.). In the present studies, a Beckman Accu-Flo pump was used to pump the solvents, an LKB Uvicord S (2.5 mm light path) and a six-channel recorder to monitor the absorbance at 275 nm, and an LKB fraction collector to obtain fractions.

## EXPERIMENTAL

### *Reagents*

Chloroform was of glass distilled chromatographic grade containing 1% etha-

nol preservative (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) while glacial acetic acid (J. T. Baker, Phillipsburg, NJ, U.S.A.) and 1 *M* hydrochloric acid (Fisher Scientific, Pittsburgh, PA, U.S.A.) were both reagent grade. All DNP amino acids were reagent grade (Sigma, St. Louis, MO, U.S.A.) and include N-DNP-L-aspartic acid (DNP-asp), N-DNP-DL-glutamic acid (DNP-glu), N,N-diDNP-L-cystine [diDNP-$(cys)_2$], N-DNP-L-alanine (DNP-ala), and N-DNP-L-valine (DNP-val).

*Preparation of two-phase solvent system and sample solutions*

A two-phase solvent system used in the present study consisted of chloroform, glacial acetic acid and 0.1 *M* hydrochloric acid at a volume ratio of 2:2:1. The solvent mixture was thoroughly equilibrated in a separatory funnel by repeating vigorous shaking and degassing several times and the two solvent phases separated shortly before being applied to the column.

Two sample solutions were prepared, one for the runs with the lower non-aqueous phase used as the mobile phase and the other for the runs with the upper aqueous phase mobile. In the experiment with the lower phase mobile, a sample mixture consisting of 100 mg of DNP-val, 100 mg of DNP-ala, 20 mg of diDNP-$(cys)_2$, and 200 mg of DNP-glu was dissolved in the upper stationary phase for a final volume of 42 ml at a concentration of 1% (w/v). Similarly, in the experiment with the upper phase mobile, a mixture of DNP-asp, DNP-glu, diDNP-$(cys)_2$, and DNP-ala at a weight ratio of 5:5:1:10 was dissolved in the lower stationary phase at a concentration of 1% (w/v). In both series of experiments, various sample volumes of 1 ml (10 mg), 5 ml (50 mg), and 25 ml (250 mg) were charged to the column.

*Separation procedures*

The separations were performed according to the standard procedure used for HSCCC as follows: in each experiment, the entire column (including the dead space in the flow tubes) was filled with the stationary phase. This was followed by injection of the sample solution through the sample port. Then, the centrifuge was rotated at 1250 rpm while the mobile phase was pumped into the column at a flow-rate of 4 ml/min in the proper elution mode, *i.e.*, from the head toward the tail for the lower phase and from the tail toward the head for the upper phase. The effluent from the outlet of the column was continuously monitored with a Uvicord S at 275 nm and fractionated with a fraction collector to obtain 3 ml fractions. After all peaks were eluted from the column, the centrifuge run was terminated, and the column inlet was connected to a pressured $N_2$ line (100 p.s.i.) to collect the column contents into a graduated cylinder to measure the volume of the stationary phase retained in the column. During the collection of the column contents, the column was rotated at about 200 rpm in the tail-to-head elution mode to accelerate the process. The column was then washed by pumping with methanol and water (each about 100 ml in volume) while slowly rotating the column in the tail-to-head elution mode. Finally, the column-coil was flushed and dried with $N_2$ prior to the next experiment.

*Analysis of fractions*

An aliquot of each fraction containing the colored sample was diluted with a known amount of methanol and the absorbance was determined at 430 nm with a Zeiss PM6 spectrophotomcter.

## RESULTS AND DISCUSSION

The capability of the present apparatus was examined in separation of DNP amino acid samples in a two-phase solvent system composed of chloroform, acetic acid, and 0.1 *M* hydrochloric acid at a 2:2:1 volume ratio using both the upper and the lower phases as the mobile phase. All separations were performed under the optimum experimental condition using a flow-rate of 4 ml/min and a revolutional

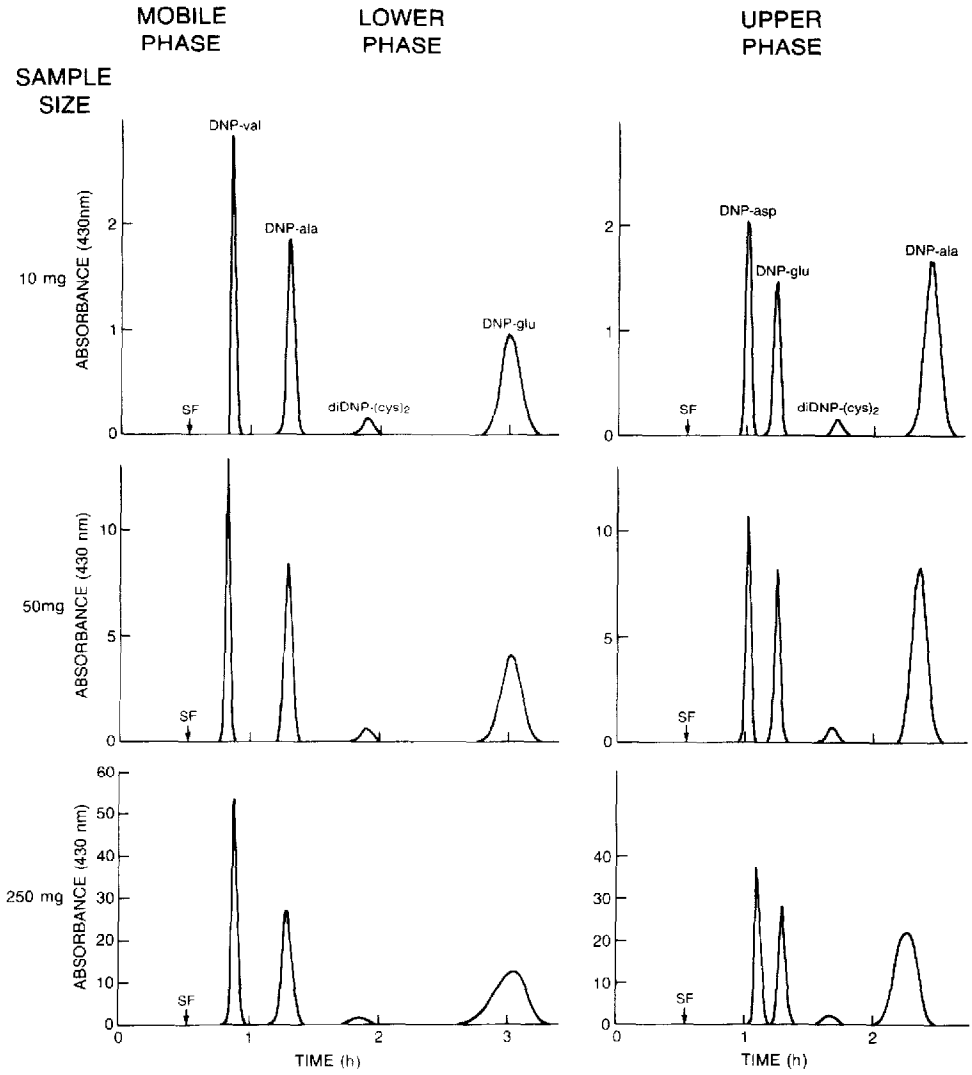


Fig. 4. Chromatograms of DNP amino acids obtained from the present apparatus. Experimental conditions are as follows: Two-phase solvent system: chloroform–acetic acid–0.1 *M* hydrochloric acid (2:2:1, v/v/v); elution mode: head-to-tail for the lower phase of the mobile phase (left column) and tail to head for the upper phase (right column); flow-rate: 240 ml/h; revolution: 1250 rpm.

speed of 1250 rpm. The results are summarized in Fig. 4 where a set of chromatograms is arranged according to the choice of the mobile phase and the applied sample size. In all chromatograms, four components are completely separated and eluted within 4 h.

Partition efficiency of these srparations can be estimated according to the conventional gas chromatographic equation

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

where $N$ is the partition efficiency expressed in terms of theoretical plates (TP); $R$ is the retention time or volume of the peak maximum; and $W$ is the peak width expressed by the same unit as $R$.

The results show that the highest partition efficiencies are found in the 10-mg sample group where the average value for the lower phase of the mobile phase is 2100 TP and that for the upper phase is 2400 TP. These figures are so far the highest among the results obtained with the existing HSCCC instruments. As the sample size is increased to 250 mg, the average partition efficiencies decrease to 1100 TP for the lower phase of the mobile phase and 1400 TP for the upper phase. Using these TP values, the partition efficiencies of the present system can be expressed in various ways such as the length of tubing per TP (cm/TP), TP number produced by each helical turn of the column (TP/turn), and the time required to yield one TP (s/TP) as listed in Table I.

The multilayer coils of the present apparatus, consisting of about 600 helical turns of a 200-m length of tubing, can produce one TP in every 10 cm of the tubing or each helical turn yields 3–4 TP which is comparable to other HSCCC schemes. These results clearly indicate that the use of multilayer coils connected in a series on the rotary frame yields the partition efficiency increased in proportion to the tubing length or total number of helical turns as demonstrated in the previous studies with a pair of multilayer coils[4]. The degree of speed in the separation can be expressed by the time required to produce one TP which is computed by dividing the retention time of the solvent front by the experimental TP value. The present column produces a high partition rate of one second/TP, while the classical counter-current distribution appa-

TABLE I

SUMMARY OF EXPERIMENTAL RESULTS

| *Exp.*[a] *No.* | *Mobile phase* | *Sample size (mg)* | *Partition efficiency (TP)* | | | | | *cm/TP* | *TP/turn* | *s/TP* | *Retention (%)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *P-1*[b] | *P-2* | *P-3* | *P-4* | *Mean* | | | | |
| 1 | LP | 10 | 2300 | 2200 | 2300 | 1800 | 2125 | 9.4 | 3.5 | 0.9 | 62.6 |
| 2 | LP | 50 | 2000 | 1900 | 1800 | 1400 | 1775 | 11.2 | 3.0 | 1.0 | 62.5 |
| 3 | LP | 250 | 1600 | 1200 | 1000 | 700 | 1125 | 17.5 | 1.9 | 2.0 | 63.5 |
| 4 | UP | 10 | 2800 | 2800 | 2300 | 1800 | 2425 | 8.2 | 4.0 | 0.9 | 56.1 |
| 5 | UP | 50 | 2700 | 2500 | 2300 | 1700 | 2300 | 8.6 | 3.9 | 1.0 | 51.6 |
| 6 | UP | 250 | 1800 | 1900 | 1100 | 800 | 1400 | 14.2 | 2.3 | 1.7 | 44.5 |

[a] See Fig. 4 for experimental conditions.
[b] Peak. For identity of peaks see Fig. 4.

ratus generally requires a few minutes for each transfer which corresponds to one plate, and the droplet CCC developed 10–20 years ago produces one TP in every 45 seconds[6].

One important characteristic feature of HSCCC is its unique ability to produce a highly efficient separation under a relatively low column pressure compared with other CCC schemes. All DNP amino acid separations described above were obtained at the maximum back pressure of 80 p.s.i. for the head-to-tail elution and 30 p.s.i. for the tail-to-head elution as measured with a pressure gauge mounted at the outlet of the pump. Being a rotary-seal-free flow-through mechanism, the present system can safely function against several hundred p.s.i., and the critical pressure which causes leakage may be substantially increased by the use of thick-wall separation columns. Therefore, the partition efficiency of the present apparatus can be further increased several times without a risk of leakage simply by increasing the width of the column holder and/or applying multilayer coils with a smaller I.D. tubing.

The overall results of our studies indicated that the present apparatus can separate hundred milligram quantities of samples at a high partition efficiency in several hours of elution. The results also suggest that small quantities of material can be isolated from the bulk of impurities with a high recovery rate as demonstrated in the separation of diDNP-cystine from the rest of the components. Preliminary applications of the present apparatus to various natural products will be described in Part II.

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