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A NEW CENTRIFUGAL COUNTER-CURRENT CHROMATOGRAPH AND ITS APPLICATION

WATARU MURAYAMA*, TETSUYA KOBAYASHI, YASUTAKA KOSUGE, HIDEKI YANO*, YOSHIAKI NUNOGAKI and KANICHI NUNOGAKI
Sanki Engineering, Ltd., 2-16-10 Imazato, Nagaokakyo, Kyoto (Japan)

SUMMARY

A centrifugal counter-current chromatograph with a rotary seal joint has been developed. The capability of the apparatus was demonstrated on separations of a set of dinitrophenyl amino acids and application examples of the method to the separation of some biological materials are presented.

INTRODUCTION

Counter-current chromatography (CCC) is defined as liquid–liquid chromatography without solid support and it has many advantages in the separation and purification of a wide variety of compounds. The classical counter-current distribution method using Craig's apparatus is very complicated to operate and types of continuous-flow counter-current chromatography such as droplet counter-current chromatography (DCCC)[1] and coil planet centrifugation (CPC)[2–6] have been developed.

We have developed another kind of centrifugal partition chromatography which employs a rotary seal joint (CCCC–RJ). The apparatus is of simple construction and is suitable for the fractionation of materials on a preparative scale in short reaction times.

The key point of the apparatus is the construction of the rotary seal joint which enables the solvents to be pumped into the rotating separation columns in the centrifuge at a continuous high pressure.

Partition efficiency of CCCC–RJ has been tested using some 2,4-dinitrophenyl (DNP) amino acids as test samples, and some examples of its application in the separation of some biological materials are reported.

APPARATUS

The principle of the separation in CCCC-RJ is similar to that for DCCC and is shown in Fig. 1. The separation columns, arranged on the rotor of a centrifuge with

* Present address; Central Research Laboratories, Unichica Co. Ltd., 23 Kozakura, Uji, Kyoto, Japan.

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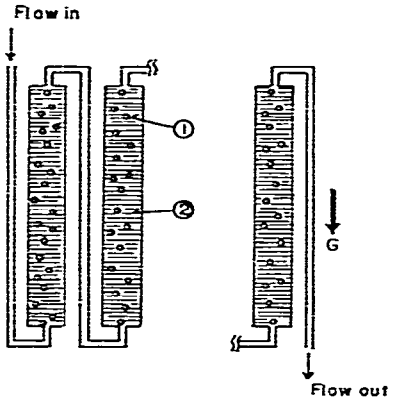


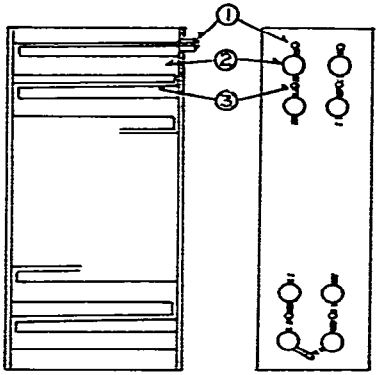
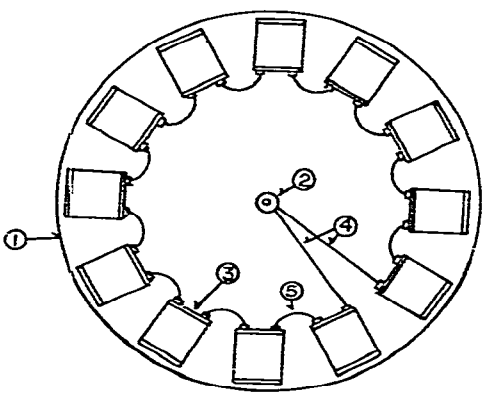


Fig. 1. Separation columns: 1 = mobile phase; 2 = stationary phase.

Fig. 2. Separation column cartridge: 1 = connectors for solvent inlet and outlet; 2 = separation columns; 3 = connecting tubes.

their longitudinal axes parallel to the direction of the centrifugal force, are connected to each other by fine tubes. The separation columns are filled with stationary liquid phase (lower phase in the case shown in Fig. 1) prior to the experiment. While the rotor of the centrifuge is in motion, the mobile liquid phase (upper phase in this case) is pumped into the separation columns and bubbles up through the stationary phase in the columns by the action of centrifugal force.

The separation columns and connecting tubes are actually arranged in the form of column cartridges (Fig. 2). The polytrifluoroethylene resin block (150 × 40 × 40 mm) has drilled in it fifty holes for the separation columns (40 × 3 mm)



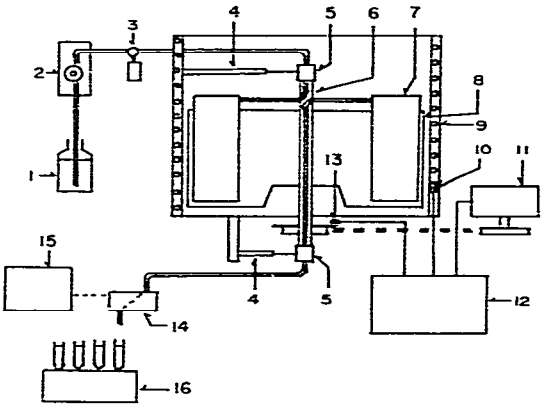


Fig. 3. Rotor of the centrifugal partition chromatograph: 1 = rotor of the centrifuge; 2 = rotary seal joint; 3 = column cartridge; 4 = connecting tubes between rotary seal joint and column cartridges; 5 = connecting tubes between column cartridges.

Fig. 4. Centrifugal partition chromatograph system: 1 = Solvent reservoir; 2 = high-pressure constant flow pump; 3 = sample injector; 4 = rotary seal joint stopper; 5 = rotary seal joint; 6 = rotor axis; 7 = column cartridge; 8 = rotor; 9 = heater and cooler; 10 = temperature-control sensor; 11 = motor; 12 = temperature and rotational-speed control unit; 13 = rotational-speed control sensor; 14 = monitor; 15 = recorder; 16 = fraction collector.

alternating with fifty holes for the connecting tubes (1 mm diameter). The holes are connected by fine troughs on both sides of the block, and are sealed by polytetrafluoroethylene sheets which are pressed tightly on both sides by metal plates and screws. The inlet and outlet for the solvents are attached to one end of the column cartridge. The inner volume for each column cartridge is *ca.* 15 ml.

Twelve column cartridges are arranged around the rotor of the centrifuge and are connected to each other by fine tubes as shown in Fig. 3. Solvents are pumped into the column cartridges through the rotary seal joint attached at the upper end of the rotor axis of the centrifuge, and collected through the rotary seal joint at the lower end. A clockwise flow of the mobile phase corresponds to the cases where a heavier phase of the two-phase solvent system is used as the stationary phase, and a counter-clockwise flow corresponds to the opposite case.

Fig. 4 shows the complete chromatographic system of the chromatograph; a photograph of the system is shown in Fig. 5.

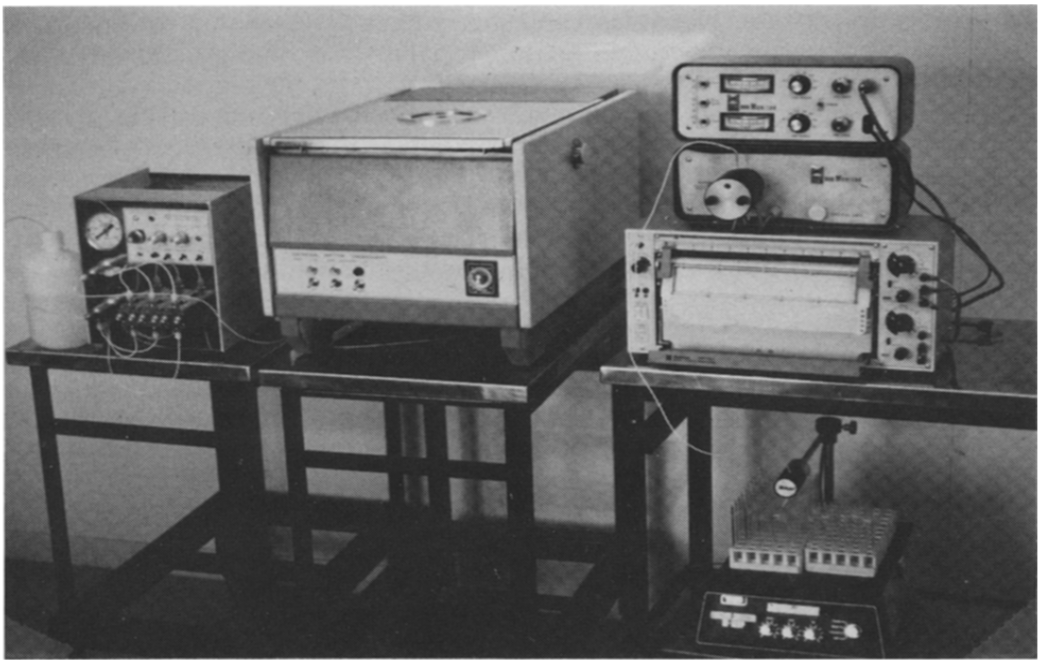


Fig. 5. Photograph of the centrifugal counter-current chromatograph system.

The rotary seal joint consists of two discs, one of which is fixed and the other which rotates with the axis of the centrifuge. The two discs are pressed against each other by a spring so that they provide a leak-free seal under a high-pressure pumping of the solvents into the separation column cartridges. The faces of the two discs, one of which is made from graphite and the other from ceramics, are highly polished. This rotary seal joint enables the apparatus to be operated continuously under high pressures for long periods of time.

TABLE I

RESOLUTION OF DNP-AMINO ACIDS

Flow-rate (ml/min)	*Resolution ($R_{s_{i,j}}$)*		
	$R_{s_{1,2}}$	$R_{s_{2,3}}$	$R_{s_{3,4}}$
4.0	2.8	2.0	2.5
1.9	3.8	2.3	2.6
1.2	4.1	2.3	3.1
0.50	4.1	2.2	2.9
0.28	4.2	2.3	–

EXPERIMENTAL

The solvents used for the preparation of the two phase partitioning solutions are of reagent grade. DNP-amino acids were purchased from Sigma (St. Louis, MO, U.S.A.), fatty acids from Tokyo Kasei and sugars from Nakarai (Kyoto, Japan). A crude extract of *Bepleurum falcatum* (Mishima Saiko) was obtained from Kotaro Shoten (Osaka, Japan).

The DNP-amino acids were detected by a flow-cell monitor at 350 nm. For the experiments involving separation of sugars or saponins the eluates were collected in 1–5 ml fractions using a fraction collector; fractions were coloured by sulphuric acid–phenol to measure the absorbance at 490 nm. The purities of the saponin fraction were checked by thin-layer chromatography (TLC) on silica gel. Fatty acids were detected gravimetrically and were checked by gas chromatography.

RESULTS AND DISCUSSION

Resolution of the centrifugal counter-current chromatograph was tested by

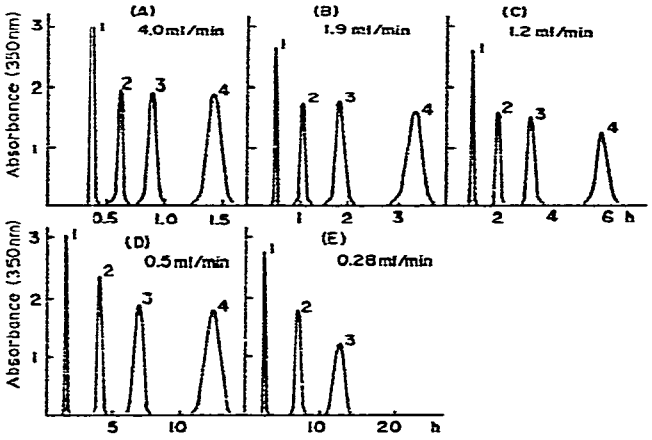
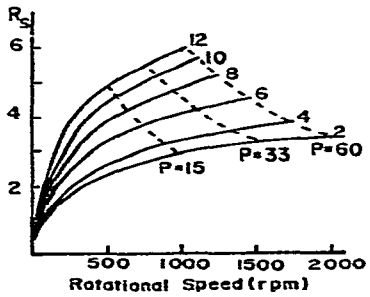


Fig. 6. Separation of DNP-amino acids. Solvent system: chloroform–0.1 *M* hydrochloric acid–acetic acid (2:1:2). Stationary phase: lower. Peaks: 1 = N^{α}-DNP-L-Ornithine HCl, 4.0 mg, $K = 50$; 2 = DNP-L-Threonine, 4.0 mg, $K = 2.0$; 3 = N,N′-di-DNP-L-Cystine, 8.0 mg, $K = 0.92$; 4 = DNP-L-Proline, 12.0 mg, $K = 0.54$. Rotational speed, 700 rpm.



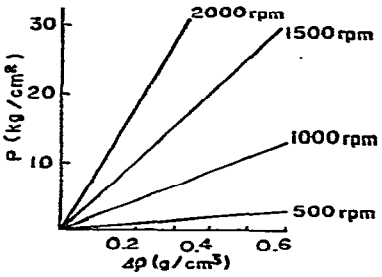


Fig. 7. Rotational speed *vs.* resolution (R_s). The numbers denoted at solid lines indicate the numbers of column cartridges used, and the values denoted at dotted lines indicate the pumping pressure.

Fig. 8. Density difference between upper and lower phase *vs.* pumping pressure.

separation of mixed DNP-amino acids (N^{δ}-DNP-L-ornithine, N,N'-di-DNP-L-cystine, DNP-L-threonine and DNP-L-proline). The solvent system reported by Tanimura *et al.*[1] was used. The rotational speed of the centrifuge was 700 rpm and the flow-rate of the mobile phase varied from 0.28 to 4.0 ml/min. The resultant chromatogram is shown in Fig. 6. Resolution (R_s) between two adjacent peaks, defined by eqn. 1, were calculated from the chromatogram and are shown in Table I.

$$R_s = \frac{L_{ij}}{\frac{1}{2}(W_i + W_j)} \tag{1}$$

where L_{ij} = peak to peak distance between component i and component j, W_i = peak width of component i, and W_j = peak width of component j.

No significant changes were observed in resolution when the flow-rate of the mobile phase was increased to 1.9 ml/min, and slight decreases in R_s values was observed when the flow-rate was raised to 4.0 ml/min.

These results indicate that the "true" flow-rate of the bubbles of the mobile phase through the stationary phase is determined by the strength of the applied centrifugal force. As long as the pumping rate (the apparent flow-rate) does not exceed the "true" flow-rate, it does not effect the resolution of the peaks. This means that the separation can be completed in a short time provided a sufficient centrifugal force is applied.

Resolution between DNP-L-threonine and N,N'-di-DNP-L-cystine were measured at a constant pumping rate by varying the speed of rotation of the centrifuge and the numbers of column cartridges used (Fig. 7). The results show that a high rotational speed gives better peak resolution.

The pumping pressure necessary for injecting the mobile phase into the separation columns during the centrifuge run is proportional to the differences in density between the upper and lower phases of the partitioning solvent system, and to the strength of the centrifugal force applied. The relationship between the density difference ($\Delta\rho$) of the two phases in the solvent system and the pumping pressure is shown in Fig. 8. For density differences of 0.10–0.25 the solvent system used was *n*-butanol–ethanol–water and for differences of 0.25–0.60 chloroform–methanol–water

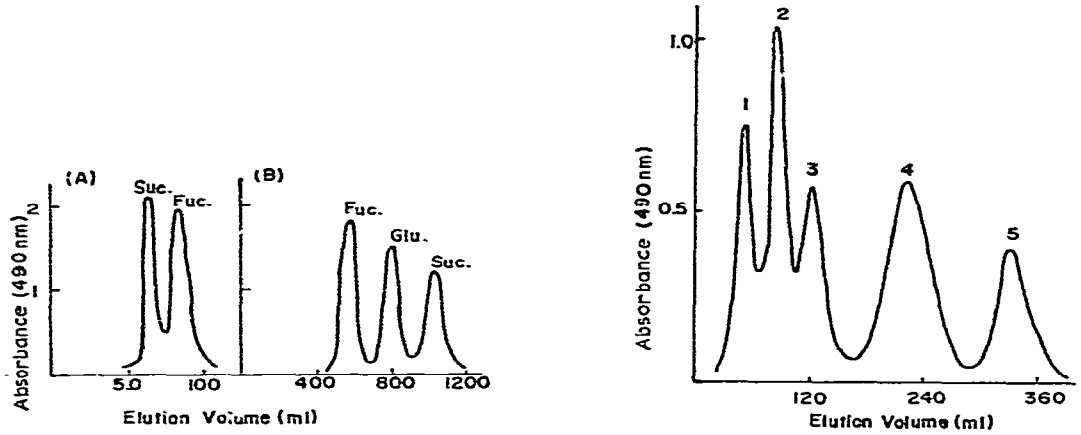


Fig. 9. Separation of sugars. Solvent system: *n*-butanol–ethanol–water (10:2.5:10). Sample: 100 mg of each sugar. A, Stationary phase = upper, flow-rate = 1.0 ml/min. B, Stationary phase = lower, flow-rate = 4.0 ml/min. Rotational speed, 1500 rpm.

Fig. 10. Separation of saponins from extracts of *Bepleurum falcatum* (Mishima Saiko). Solvent system: chloroform–methanol–water–benzene–ethyl acetate (45:60:40:2:3). Stationary phase = lower. Sample = 20 mg. Flow-rate = 2.0 ml/min. Rotational speed = 1000 rpm.

was used. Because of instrumental limitations, all the experiments were carried out at a rotational speed of the centrifuge at which the pumping pressure did not exceed 60 kg/cm^2. It is important to select the appropriate rotational speed for the solvent systems employed when using the CCCC–RJ system.

The separation of a mixture of sugars was performed using an *n*-butanol–ethanol–water (10:2.5:10) solvent system (Fig. 9). Sugars have a tendency to partition into the aqueous layer of a solvent mixture. When the aqueous phase was used as the mobile phase, two components, sucrose and fucose, were separated, while in the opposite case three components, fucose, glucose and sucrose, were resolved.

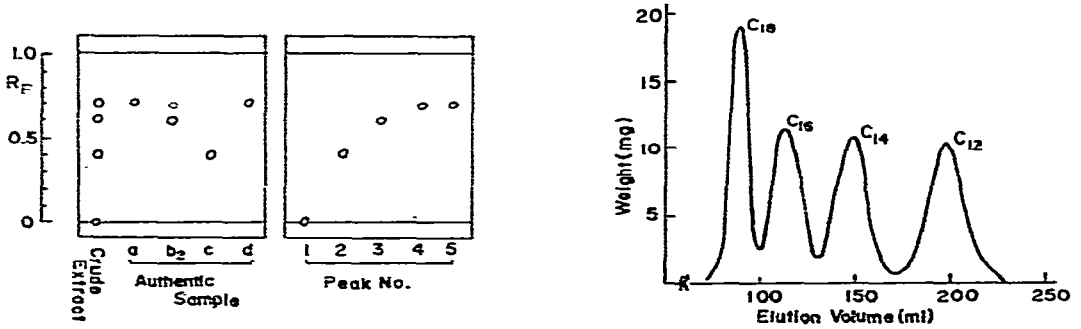


Fig. 11. TLC of peak fractions in Fig. 10. Elution: chloroform–methanol–water = (30:10:1). Detection: Sulphuric acid.

Fig. 12. Separation of saturated fatty acids. Solvent system: *n*-heptane–methanol–acetic acid (1:1:1). Sample: C_{18}, stearic acid; C_{16}, palmitic acid; C_{14}, myristic acid; C_{12}, lauric acid. Flow-rate = 1.0 ml/min. Rotational speed = 1000 rpm.

Separation of saponins from *Bepleurum falcatum* (Mishima saiko) have been reported by Ogiwara *et al.*[7] using DCCC. A crude extract from the root of Mishima Saiko, which was known to contain Saiko saponins a, b_2, c and d, was fractionated using CCCC–RJ with the same solvent system as reported. Four saponin components were completely resolved within 3 h (Fig. 10) and were identified by TLC (Fig. 11).

Separation of saturated fatty acids was also demonstrated using a non-aqueous two-phase solvent (*n*-heptane–acetic acid–methanol, 1:1:1) (Fig. 12).

It has been reported that in DCCC there are limitations on the solvent systems that can form stable droplets during the experiments[8]. In CCCC–RJ separations have been achieved when sufficient amounts of the stationary phase solution (usually 70–80% of the column volume) were retained in the column cartridges while the mobile phase solution was continuously eluted through the columns. This state of dynamic equilibrium could be achieved by selection of suitable speeds for rotation of the centrifuge and for pumping; we have encountered few cases which do not have a solvent system suitable for the present method.

Centrifugal counter-current chromatography may have many uses in the fields of normal- or reversed-phase partition chromatography. Improvements of the apparatus are now in progress and results of its application will be published later.

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