Journal of Chromatography, 479 (1989) 53–60
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 654

MULTILAYER COIL PLANET CENTRIFUGE FOR ANALYTICAL HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

HISAO OKA[a], FUMIE OKA and YOICHIRO ITO*

Laboratory of Technical Development, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 5D-12, Bethesda, MD 20892 (U.S.A.)

(First received March 20th, 1989, revised manuscript received May 30th, 1989)

SUMMARY

A compact portable model of a high-speed counter-current chromatograph enables efficient analytical separations of microgram sample quantities within 10 min. A series of preliminary experiments was conducted to study retention of the stationary phase of various two-phase solvent systems in short coils with different helical diameters. Under the optimal experimental conditions, analytical capability of the apparatus was successfully demonstrated in separation of flavonoids from a crude sea buckthorn ethanol extract in a multilayer coil with a total capacity of 8 ml.

INTRODUCTION

Counter-current chromatography (CCC) has many desirable features for performing analytical separations because it eliminates various complications such as sample loss, tailing of solute peaks, contamination, etc., all arising from the use of solid supports[1]. Nevertheless, the method has been used almost exclusively for preparative purposes mainly due to its long separation times required. Advent of high-speed CCC in early 1980s, however, remarkably improved the CCC technology to shorten the separation time from days to a few hours[1–3]. Recent development of an analytical coil planet centrifuge with a 5-cm revolution radius further reduced the separation time, thus providing a powerful tool for analyses of various natural products[4].

This paper introduces a new coil planet centrifuge with a 2.5-cm revolution radius which can be operated at the maximum speed of 4000 rpm. Performance of the apparatus was evaluated in terms of retention of the stationary phase of several two-phase solvent systems, and the analytical capability of the method was demonstrated in separation of microgram quantities of flavonoids from a crude ethanol extract of sea buckthorn (*Hippophae rhamnoides*) using a two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v/v).

[a] Visiting Scientist from Aichi Prefectural Institute of Public Health, Nagoya, Japan.

0021-9673/89/$03.50

APPARATUS

Fig. 1 shows a photograph of our prototype apparatus. The motor (Electro-Craft Corp., Hopkins, MN, U.S.A.) directly drives the rotary frame around the central stationary pipe of the centrifuge. The rotary frame consists of a pair of aluminum plates rigidly bridged together with multiple links and holds a column holder and a counterweight holder in the symmetrical positions at a distance of 2.5 cm from the central axis of the centrifuge. The column holder is equipped with a plastic planetary gear which interlocks to an identical stationary sun gear mounted around the central stationary pipe. This gear arrangement produces the desired planetary motion of the column holder, *i.e.*, rotation about its own axis and revolution around the central axis of the centrifuge at the same angular velocity in the same directions. This particular type of planetary motion not only permits high retention of the stationary phase in the rotating column but also prevents twisting the flow tubes, thus eliminating the need for the conventional rotary seal device which would produce leakage and contamination[2,3]. Both column and counterweight holders can be removed from the rotary frame by loosening a pair of screws on each bearing block.

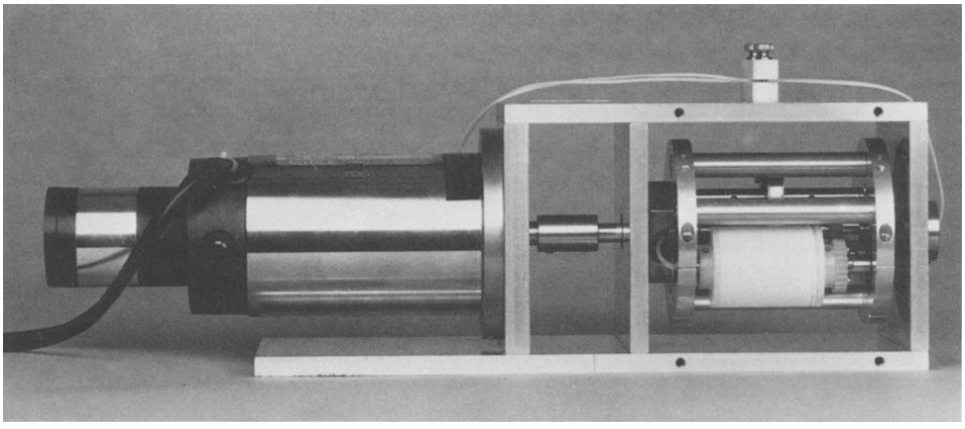

Fig. 1. Photograph of the apparatus.

Two types of coiled columns were prepared: short coils for preliminary studies on stationary phase retention and a multilayer coil for analytical separations, all from 0.85 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.). The short coils were mounted on three interchangeable column holders measuring 1.4, 2.5 and 3.75 cm in hub diameters. Each coil was made by winding a 3.5–4 m length of the tubing directly onto the holder hub making tight helical turns between a pair of flanges spaced 5 cm apart. On the smallest holder hub of 1.4-cm diameter, the coil was made in double layers, while a single layer coil was formed on the other two holders. Each column had a total capacity of about 2 ml. The multilayer coil was prepared by winding approximately a 15-m length of the tubing onto the holder with 2.5-cm hub diameter making four coiled layers with a total capacity of about 8 ml.

Each terminal of these coils was connected to a PTFE flow tube of 0.45 mm I.D. and 0.5-mm wall thickness in the following manner: approximately a 1-cm length of the flow tube was inserted into the column terminal, and a piece of copper wire with 0.7-mm diameter was wound over the junction to limit heat expansion of the tubing. Then, heat was applied with a heat gun until the whole joint became transparent and fused together. The joint thus formed generally holds a pressure up to several hundred p.s.i. A pair of flow tubes from the coiled column first passes through the center hole of the column holder shaft, then forms an arch to reach the opening of the yoke mounted on the left end of the rotary frame and finally enters the opening of the central stationary pipe to exit the centrifuge. At the top of the centrifuge, these flow tubes are tightly supported with a silicone-rubber-padded clamp. These flow tubes were lubricated with silicone grease and protected with a sheath of Tygon tubing at each supported portion to prevent direct contact with metal parts. If these cautions are followed, the flow tubes can maintain their integrity for many months of operation.

EXPERIMENTAL

Reagents

Organic solvents used for preparation of the two-phase solvent systems include *n*-hexane, ethyl acetate, methanol, chloroform, acetic acid and *n*-butanol. Among those, *n*-hexane, acetic acid and *n*-butanol were of reagent grade and obtained from Polyscience (Warrington, PA, U.S.A.), Mallinckrodt (Paris, KY, U.S.A) and Tedia Company (Fairfield, OH, U.S.A.), respectively. All other solvents were glass-distilled chromatographic grade and purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Dried powder of sea buckthorn ethanol extract used as the test sample was obtained from China by the courtesy of Professor Tian You Zhang at Beijing Institute of New Technology Application, Beijing, China.

Preparation of two-phase solvent systems and sample solution

The following six volatile two-phase solvent systems were prepared for preliminary studies on stationary phase retention in short coils: *n*-hexane–water, *n*-hexane–ethyl acetate–methanol–water (1:1:1:1), chloroform–water, chloroform–acetic acid–water (2:2:1), *n*-butanol–water, *n*-butanol–acetic acid–water (4:1:5). The two-phase solvent system composed of chloroform–methanol–water (4:3:2) was prepared for the analytical separation of the sea buckthorn ethanol extract with the multilayer coil. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature by repeated shaking and degassing by opening the stopcock and two phases separated before use.

The sample solution for the analytical separation was prepared by dissolving 30 mg of the sea buckthorn extract dried powder in 1 ml of the lower non-aqueous phase of the above solvent system used for separation.

Procedures for preliminary studies on stationary phase retention in short coils

For each experiment, the short coil was first entirely filled with the stationary phase. Then, the apparatus was run at the desired revolutional speed while the mobile phase was continuously eluted through the coil at a flow-rate of 60 ml/h. In order to

provide a uniform flow, a 20-ml capacity glass syringe was driven by a multi-speed transmission syringe driver (Harvard Apparatus, Millis, MA, U.S.A.). The effluent from the outlet of the column was collected into a 5-ml capacity graduated cylinder. After 3 ml were eluted, the results were recorded by simply observing the amount of upper and lower phases comprising the total volume of the effluent. Then, the apparatus was stopped and the column flushed by allowing nitrogen gas to push out the contents. The experiments were performed by varying the experimental conditions such as revolutional speed (2000, 2500, 3000, 3500, 4000 rpm), elution modes (head to tail or tail to head) and the use of upper and lower phases as the mobile phase. The head-tail elution modes were changed by altering the direction of the planetary motion.

Construction of phase distribution diagram

From the volume of the stationary phase eluted from the column, V_s, the percentage retention of the stationary phase relative to the total column capacity was computed according to the expression $100(V_c + V_f - V_s)/V_c$, where V_c and V_f are the total column capacity and the dead space volume in the flow tubes, respectively. Using these retention data obtained from the suitable elution mode, a set of phase distribution diagrams was prepared by plotting the percentage retention of the stationary phase against the applied revolution speed in rpm.

Procedure for analytical separation of flavonoids from sea buckthorn ethanol extract with the multilayer coil

The column was first entirely filled with the upper aqueous stationary phase. Then, the coil planet centrifuge was rotated at the optimum speed of 3500 rpm, while the mobile phase was pumped into the head of the column at a flow-rate of 120 ml/h by a Shimadzu Model 6A HPLC pump (Shimadzu Corporation, Kyoto, Japan). After a steady state hydrodynamic equilibrium was reached, 4 μl of the sample solution containing 120 μg of the sample were injected into the head of the coil with a microsyringe via a Rheodyne Model 7152 syringe loading sample injector. Effluent from the outlet of the column was continuously monitored by the absorbance at 254 nm using a Shimadzu Model SPD-6A detector equipped with a preparative flow cell to record the elution curve with a Pharmacia 482 recorder (Pharmacia, Uppsala, Sweden).

The lower non-aqueous phase used as the mobile phase tends to produce microdroplets upon a slight fall of the ambient temperature. When this phenomenon occurs in the detector cell, the recording of the elution curve is disturbed by an intensive noise. In order to prevent this detrimental effect, the effluent from the column was first passed through a narrow PTFE tube (3 m × 0.45 mm I.D.) which was immersed into a water-bath heated at 40°C before entering the detector. A similar narrow tube was applied at the outlet of the detector to prevent sudden pressure drop which would generate gas bubbles from the mobile phase.

RESULTS AND DISCUSSION

The results of the preliminary studies on stationary phase retention in short coils are summarized in Fig. 2 where a set of phase distribution diagrams are arranged

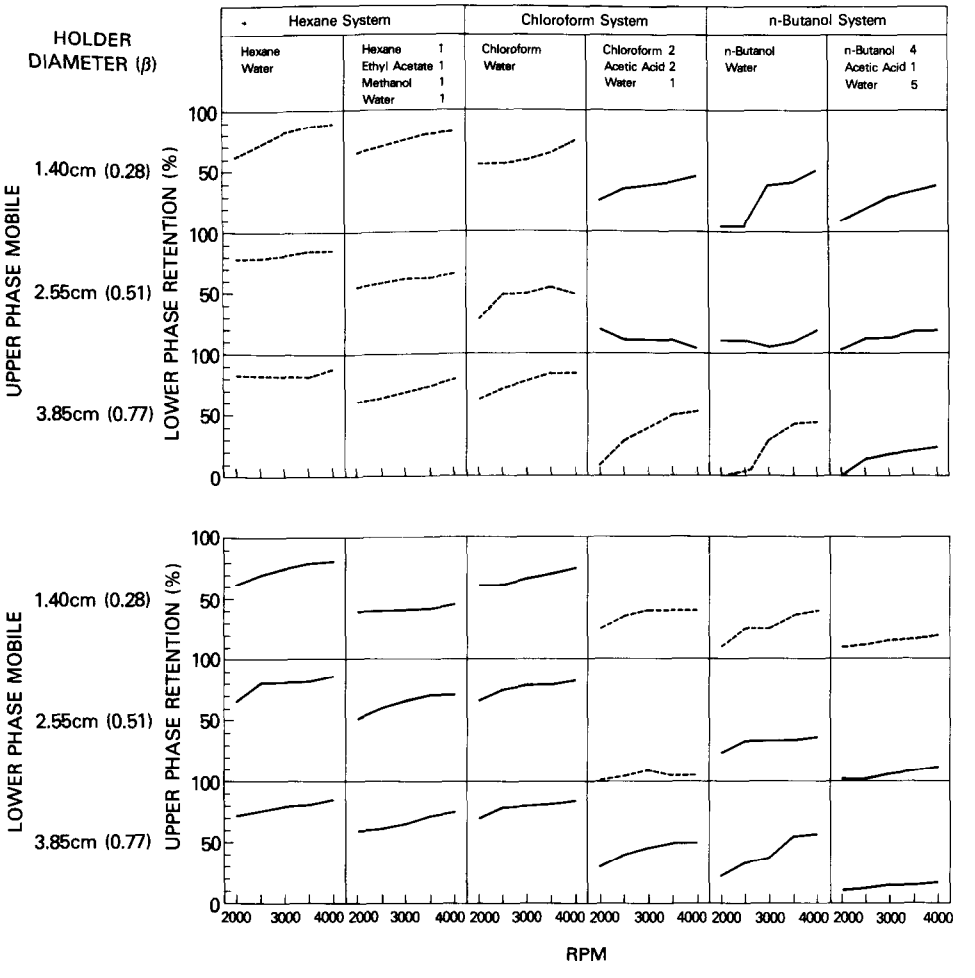

Fig. 2. Phase distribution diagrams of six volatile two-phase solvent systems obtained from short coils mounted on the present apparatus. ——, head to tail elution; – – –, tail to head elution. Flow-rate: 60 ml/h.

according to the applied two-phase solvent systems and the choice of the mobile phase.

Each column contains retention curves obtained from the same solvent system indicated at the top. These columns are placed from left to right in the order of hydrophobicity of the major organic solvent, *i.e.*, *n*-hexane, chloroform and *n*-butanol. In each solvent group, the left column shows the binary solvent system with a high interfacial tension, while the right column shows ternary or quaternary solvent system containing one or two additional solvents to moderate the interfacial tension.

These diagrams are also divided into upper and lower panels according to the choice of the mobile phase as indicated in the left margin: the upper panel was obtained by the upper phase mobile and the lower panel by the lower phase mobile. Each mobile phase group consists of three rows each obtained from a different helical

diameter or β value of the coil as indicated on the left. Here, parameter β indicates the ratio of the rotation radius (distance from the holder axis to the coil) to the revolution radius (distance from the holder axis to the central axis of the centrifuge). As stated elsewhere[5], the β value plays an important role in retention of the stationary phase in the rotating coil. Each diagram contains a single retention curve determined at five different rpms: the solid line indicates the head to tail elution mode and the broken line, the tail to head elution mode.

In all phase distribution diagrams obtained from the *n*-hexane systems, excellent retention of the lower phase is obtained by the tail to head elution of the upper phase (the upper panel) or by the head to tail elution of the lower phase (the lower panel). This phase distribution pattern indicates an ideal hydrodynamic trend that the upper phase distributes toward the head and the lower phase toward the tail, regardless of the applied β value. The above results clearly indicate that these hexane solvent systems can be retained in a multilayer coil with the full range of β values from 0.28 to 0.77.

In the chloroform solvent systems with moderate hydrophobicity, the binary system shows retention curves similar to those observed in the *n*-hexane solvent systems. However, the chloroform–acetic acid–water system displays a quite different phase distribution pattern which is largely affected by β values. In this ternary chloroform system, the largest β value of 0.77 produces satisfactory retention of around 50% at 4000 rpm as shown by the broken line with the upper phase mobile (the third row in the upper panel) or the solid line with the lower phase mobile (the third row in the lower panel) according to the typical hydrodynamic trend similar to that in the binary chloroform system. However, at the smaller β values of 0.51 to 0.28, the hydrodynamic trend of the ternary chloroform system is completely reversed as indicated by the solid lines in the upper phase mobile (first and second rows in the upper panel) and the broken lines in the lower phase mobile (first and second rows in the lower panel). At the transitional β value of 0.51, the retention level becomes lowest in both mobile phase groups. The large difference in retention between these two chloroform systems may be caused by an addition of a large proportion of acetic acid which lowered the interfacial tension between the two phases.

The hydrophilic *n*-butanol solvent systems show somewhat similar retention behavior to the ternary chloroform system. The ternary *n*-butanol system shows substantially lower retention level due to its reduced interfacial tension by the addition of acetic acid.

The above results provide useful information for the design of the multilayer coil with respect to the solvent systems to be used for the separation. A large capacity multilayer coil with full β values ranging from 0.28 to 0.77 can be used for various hexane systems and some chloroform systems containing a small amount of a modifier. On the other hand, the multilayer coil with the β value around 0.77 can yield satisfactory retention to all solvent systems except for the ternary *n*-butanol system, while the column capacity is limited to several milliliters. In the light of the above experimental results, a multilayer coil with the β value ranging from 0.51 to 0.77 was selected for performing analytical separations.

Using a multilayer coil consisting of four coiled layers with a total capacity of about 8 ml, analytical capability of the present apparatus was evaluated in separation of flavonoids in the crude ethanol extract of sea buckthorn with a two-phase solvent

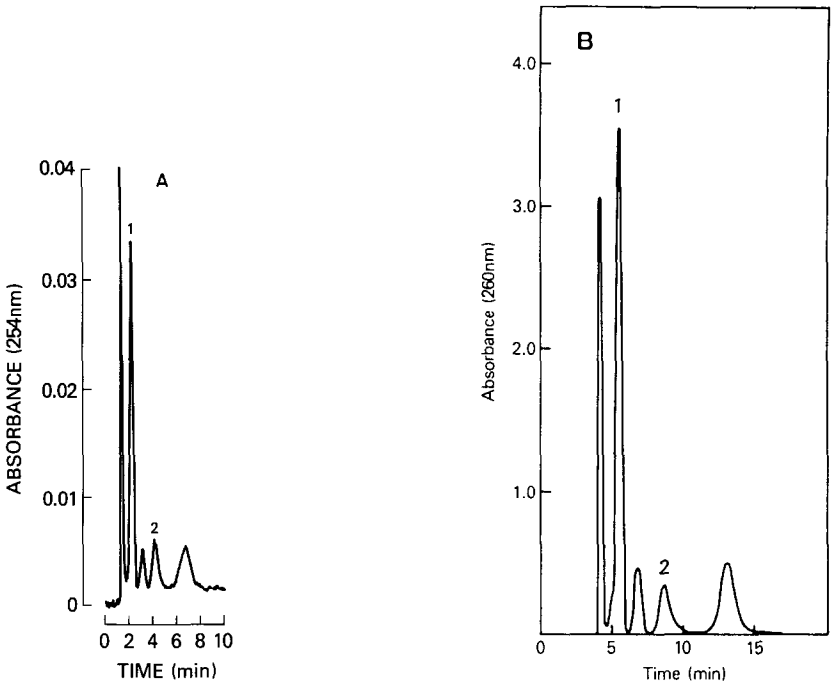

Fig. 3. Chromatograms of flavonoids in the crude sea buckthorn ethanol extract by the present apparatus (A) and by the existing analytical CCC centrifuge (B). The experimental conditions were as follows: (A) Coil planet centrifuge with 2.5-cm revolution radius; column, multilayer coil, 0.85 mm I.D. and 8-ml capacity with β = 0.51–0.77; solvent system, chloroform–methanol–water (4:3:2, v/v/v); mobile phase, lower phase; flow-rate, 2 ml/min; sample size, 120 μg; revolution, 3500 rpm. (B) Coil planet centrifuge with 6.35-cm revolution radius; column, multilayer coil, 0.85 mm I.D. and 43 ml capacity with β = 0.4–0.75; solvent system and mobile phase as in (A); flow-rate, 5 ml/min; sample size, 3 mg; revolution, 1800 rpm.

system composed of chloroform-methanol-water (4:3:2, v/v/v). Separation was performed with the lower non-aqueous mobile phase in the head to tail elution mode at a flow-rate of 2 ml/min under a coil rotation of 3500 rpm. Fig. 3A shows a chromatogram of flavonoids in a 120-μg quantity of the crude extract obtained by the present method by on-line UV monitoring of the effluent at 254 nm. Five peaks, including isorhamnetin (1) and quercetin (2) were well resolved and eluted within 8 min.

Fig. 3B shows a similar chromatogram obtained by Zhang *et al.*[6] which represents the most efficient analytical CCC separations ever achieved in the past. In an analytical column with a 40-ml total capacity, mounted on a high-speed CCC centrifuge with a 6.35-cm revolution radius, a 3-mg quantity of the same sample was separated at a flow-rate of 5 ml/min at 1800 rpm of coil rotation under otherwise identical conditions. In this experiment, on-line UV monitoring of the effluent produced an intensive noise in recording due to a thermolabile nature of the chloroform mobile phase and, therefore, the elution curve was manually drawn following the spectrophotometric analysis of each fraction.

The present apparatus further improved the analytical capability of CCC by reducing the sample size by less than 1/20 and shortening the separation time without

significantly affecting the peak resolution. In addition, the method permits on-line UV monitoring of the effluent by a minor modification of the conventional LC detection system as described elsewhere[7].

ACKNOWLEDGEMENTS

We are deeply indebted to Biomedical Engineering and Instrumentation Branch, National Institutes of Health, Bethesda, MD, U.S.A., for engineering and fabrication of the instrument.

REFERENCES

1 Y. Ito, in N. B. Mandava and Y. Ito (Editors), *Countercurrent Chromatography, Theory and Practice*, Marcel Dekker, New York, 1988, Ch. 3, pp. 79–442.
2 Y. Ito, *CRC Crit. Rev. Anal. Chem.*, 17 (1986) 65.
3 Y. Ito, J. Sandlin and W. G. Bowers, *J. Chromatogr.*, 244 (1982) 247.
4 Y. Ito and Y. W. Lee, *J. Chromatogr.*, 391 (1987) 290.
5 Y. Ito, *J. Chromatogr.*, 301 (1984) 387.
6 T. Y. Zhang, R. Xiao, Z. Y. Xiao, L. K. Pannell and Y. Ito, *J. Chromatogr.*, 445 (1988) 199.
7 H. Oka and Y. Ito, *J. Chromatogr.*, 475 (1989) 229.