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Note

Analytical high-speed counter-current chromatography with a coil planet centrifuge

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In the past, the analytical capability of counter-current chromatography (CCC) has been demonstrated in separations of dinitrophenyl amino acids in a long narrow-bore coiled column. In order to sustain the stationary phase in the column against a plug flow, the separations were performed at a low flow-rate under a strong centrifugal force field. Although high partition efficiencies of many thousand theoretical plates have been achieved, the method required long elution times ranging between 10 and 30 h[1,2]. Recently, the development of high-speed CCC has remarkably shortened separation times of semi-preparative-scale separations by the use of a multilayer coil coaxially mounted on the holder[3,4]. However, the application of an analytical column of 0.85 mm I.D. to the standard coil planet centrifuge with a 10-cm revolutional radius has resulted in steady carryover of the stationary phase, apparently due to the increased solvent–wall interaction in a narrow-bore tube[5]. Analytical-scale separations may be effected by decreasing the revolutional radius and weight of the column holder so that the system permits application of higher revolutional speeds which would promote counter-current flow of the two solvent phases through a small-diameter tube.

The present paper introduces a new coil planet centrifuge with a 5-cm revolutional radius which is ideal for performing analytical-scale separations with a narrow-bore multilayer coil. Using typical two-phase solvent systems, retention of the stationary phase was studied on single-layer coils of 0.85 mm I.D. tubing mounted on the holders with two different hub diameters of 5 and 7.5 cm. Efficient analytical-scale separations were demonstrated on separations of indole plant hormones with a multilayer coil at a high revolutional speed of 2000 rpm.

EXPERIMENTAL

Apparatus

An overall view of the apparatus is shown in Fig. 1. The motor (Bodine Electric Co., Chicago, IL, U.S.A.) directly drives the rotary frame around the central axis of

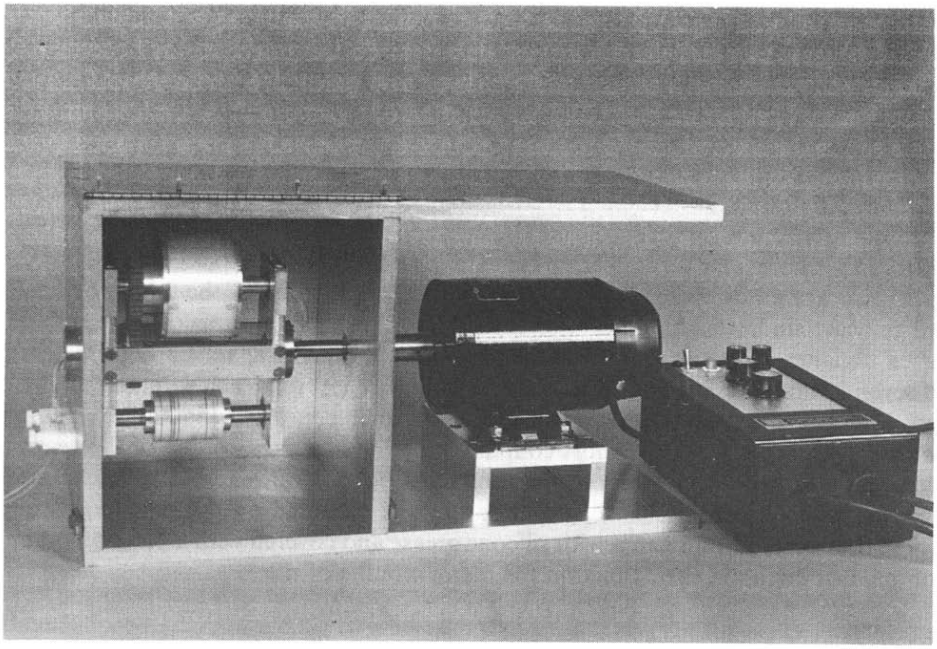

Fig. 1. Photograph of the apparatus.

the centrifuge. The rotary frame consists of a pair of aluminum plates rigidly bridged by links and holds a column holder (top) and the counterweight holder (bottom) in the symmetrical positions at 5 cm from the central axis of the centrifuge. The holder shaft is equipped with a plastic planetary gear which is coupled to an identical stationary sun gear rigidly mounted on the central axis of the centrifuge. This gear coupling produces a desired synchronous planetary motion of the column holder: The holder revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity. As described elsewhere[2], this particular type of planetary motion permits the flow tubes to rotate around the central axis of the centrifuge without twisting, thus facilitating continuous elution of the mobile phase through the rotating column. The revolutional speed of the centrifuge is continuously adjustable up to 2000 rpm with a speed control unit shown on the right. Both column holder and counterweight holder can be removed from the rotary frame simply by loosening a pair of screws in each bearing block, hence facilitating the column preparation and determination of the counterweight mass required for balancing centrifuge system.

Two types of coiled columns were prepared each from a 0.85 mm I.D. polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.): single-layer coils for phase retention studies and a multilayer coil for analytical separations. The single-layer coil was made from a 5-m length of tubing by winding it tightly around the holder hub, forming a single layer with a uniform helical diameter. Two different hub diameters were selected for the present studies, 5 cm ($\beta = 0.5$) and 7.5 cm ($\beta = 0.75$), where β is the ratio between the helical radius (r) and the revolutional radius (R): $\beta = r/R$. As described elsewhere[4], β is an important param-

eter to determine the orientation of the coiled column on the holder. The multilayer coil was prepared by winding a long piece of tubing onto the holder with a 5-cm hub diameter, making multiple coiled layers. The β value varied from 0.5 at the internal terminal to 0.8 at the external terminal. The total column capacity of the multilayer coil measured approximately 38 ml.

Each coiled column was equipped with a pair of flow tubes measuring 0.5 mm I.D. and 0.5 mm wall thickness (Pierce, Rockford, IL, U.S.A.). The junction between the flow tube and the column terminal was made with a short piece of PTFE tubing of 1.7 mm I.D. as an adaptor. After the connection was made, a piece of copper wire was open wound over the adaptor tubing (to limit expansion of the tubing by heat) which was then heated by a heat gun until the whole junction turns transparent and fused together. This simple procedure provided a tight seal against a high pressure of several hundred p.s.i. Both feed and return flow tubes from each column were first led through the center hole of the holder shaft, and then by making a loop passed through the side-hole of the short coupling pipe to enter the opening of the central stationary pipe. At the exit from the centrifuge (left) the flow tubes were clamped between silicone rubber sheets by a tube support mounted on the centrifuge wall. These flow tubes, if lubricated with silicone grease and covered with a piece of Tygon tubing, can maintain their function for many months of use.

Reagents

Organic solvents used for preparation of the two-phase solvent systems, including *n*-hexane, ethyl acetate and methanol, are glass-distilled chromatographic grade and purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The two-phase solvent system was prepared by thoroughly equilibrating the solvent mixture in a separatory funnel at room temperature.

Indole plant hormones including indole-3-acetamide, indole-3-acetic acid, indole-3-butyric acid, and indole-3-acetonitrile, were obtained from Sigma (St. Louis, MO, U.S.A.).

Procedure for phase retention studies

Retention studies were performed mainly with a typical two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1). Experiments were carried out according to the procedure previously described[6].

In each measurement the column was first entirely filled with the stationary phase followed by rotation of the column set at a desired speed. After the revolutional speed reached the uniform rate, the mobile phase was pumped into the column at the desired rate. The effluent from the outlet of the column was collected into a 5-ml capacity graduated glass cylinder to measure the volume of the stationary phase eluted from the column. The centrifuge run was continued until the total elution volume reached 5 ml or over 1.5 times the volume of the column capacity. The measurement was repeated by applying various operational conditions, such as mobile phases (upper and lower phases), revolutional speeds (500, 1000, 1500 and 2000 rpm), flow rates (12, 24, 60 and 120 ml/h), etc.

Retention of the stationary phase was calculated from the eluted stationary phase volume (V_s), total column capacity (V_c), and the free space in the flow tubes (V_f), and expressed in terms of percentage retention to the total column capacity according to the expression $100(V_c + V_f - V_s)/V_c$.

Procedure for analytical separations

Analytical capability of the present scheme was demonstrated with the multilayer coil on separations of indole plant hormones. Experiments were performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water with a modified volume ratio of 3:7:5:5 which provided suitable partition coefficients for a set of samples.

In each separation the column was first entirely filled with the upper non-aqueous stationary phase. This was followed by injection of the sample solution (0.8 ml phase mixture containing each component 250 to 750 μg) through the sample port. Then, the column was rotated at 2000 rpm while the mobile phase was introduced into the column at a flow-rate of 60 ml/h. The effluent from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and fractionated with an LKB fraction collector. Each fraction was mixed with 2.5 ml of methanol and the absorbance was measured at 280 nm with a Zeiss spectrophotometer.

RESULTS AND DISCUSSION

Phase retention studies

The results of the retention experiments on the *n*-hexane–ethyl acetate–methanol–water (1:1:1:1) are summarized in Fig. 2 where percentage retention of the

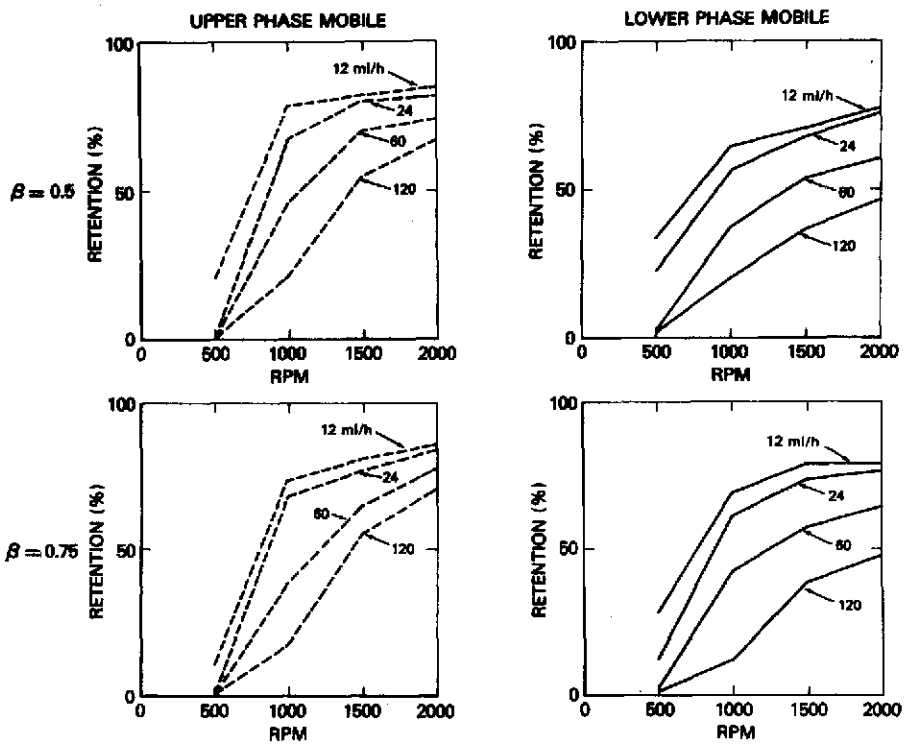

Fig. 2. Phase retention diagrams of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1).

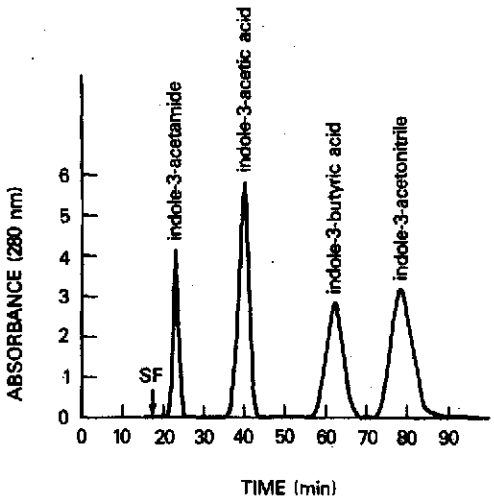

Fig. 3. Analytical chromatogram of indole plant hormones. SF = Solvent front.

stationary phase relative to the total column capacity is plotted against the applied revolutional speeds. Both the upper non-aqueous phase (left) and the lower aqueous phase (right) were used as the mobile phase, each at two different β values of 0.5 (top) and 0.75 (bottom). Several lines drawn in each diagram show the effects of flow-rates on retention. The solid lines indicate the head to tail elution mode and the broken lines, the tail to head elution mode. In general, retention of 50% is considered to be satisfactory and over 70% is ideal.

All four phase retention diagrams show somewhat similar profile of retention curves which exhibit steep rise from 500 to 1000 rpm and then gradually reach near plateau at 2000 rpm in low-flow-rate groups. Overall results clearly indicates that the present scheme permits satisfactory retention at 2000 rpm for a broad range of flow-rates from 12 to 60 ml/h regardless of the choice of the mobile phase and the applied β values.

The retention profile of the present solvent system also indicates that the two solvent phases establish a unilateral hydrodynamic equilibrium in the rotating coil where the upper phase tends to distribute toward the head and the lower phase toward the tail. Excellent retention with the similar hydrodynamic trend was also observed in chloroform phase systems while hydrophilic butanol phase systems revealed low retention with transitional to reversed hydrodynamic trends at the presently applied β values.

Analytical separations

Fig. 3 illustrates a typical chromatogram of indole plant hormones on a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). The lower aqueous phase was used as the mobile phase at a flow-rate of 60 ml/h under a maximum revolutional speed of 2000 rpm.

All components were well resolved in symmetrical peaks and eluted within 90 min. The partition efficiencies calculated from the conventional gas chromatographic

formula range from 1000 to 1300 theoretical plates. Retention of the stationary phase was near 50% of the total column capacity. The lower retention compared with the data shown in the phase retention diagram (Fig. 2) is apparently due to the modified volume ratio between *n*-hexane and ethyl acetate.

The above preliminary result clearly demonstrates the potential capability of the present apparatus in performing analytical-scale separations. Similar solvent systems have been successfully applied to separations of various biological samples such as antibiotics[7], steroids[8], *s*-triazine herbicides[9], etc. Recent studies have further indicated that the present scheme permits application of chloroform solvent systems which are extremely useful for separation and purification of natural products[10]. Being a support-free chromatography, the analytical high-speed CCC enables rapid and efficient separations of microgram quantities of materials without adsorptive loss or deactivation caused by the solid supports. Future development will be interfacing the system to a mass spectrometer with a thermospray method.

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