Journal of Chromatography, 454 (1988) 382–386
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 845

Note

New high-speed counter-current chromatograph equipped with a pair of separation columns connected in series

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(First received May 30th, 1988; revised manuscript received July 28th, 1988)

Recent advent of high-speed counter-current chromatography (HSCCC) has radically improved performance of counter-current chromatography in both separation time and partition efficiency[1–3]. The existing HSCCC centrifuge, however, has common problems in balancing the centrifuge system where a separation column is mounted on one side and a counterweight on the opposite side of the rotor. Because two-phase solvent systems applied to the separation column have a wide range in density, the weight of the counterbalance should be adjusted according to the solvent density and also to the volume of the stationary phase retained in the column. Failure to achieve proper balancing would produce excessive vibration of the centrifuge which often results in detrimental loss of the stationary phase from the column.

The present device conveniently eliminates the above problem by mounting a pair of identical multilayer coils symmetrically, one on each side of the rotary frame of the centrifuge. This provides an additional advantage of increased sample loading capacity for peforming preparative-scale separations.

PRINCIPLE AND DESIGN OF THE APPARATUS

The principle of the present device is diagrammatically illustrated in Fig. 1A where two identical columns are symmetrically arranged around the central axis of the centrifuge system. Each column undergoes synchronous planetary motion in such a way that it revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity (ω) as indicated by arrows. These two columns are equipped with a pair of flow tubes; both feed and return flow tubes enter the centrifuge system from the left side along the central axis and by forming an arch reach the right side of the first column. These flow tubes then exit the first column in the opposite side and again form an arch to return to the right side of the centrifuge through a straight path parallel to the centrifuge axis. On the right side of the centrifuge, they form a similar arch to reach the second column. With the above flow-tube arrangement, synchronous planetary motion of the column can maintain

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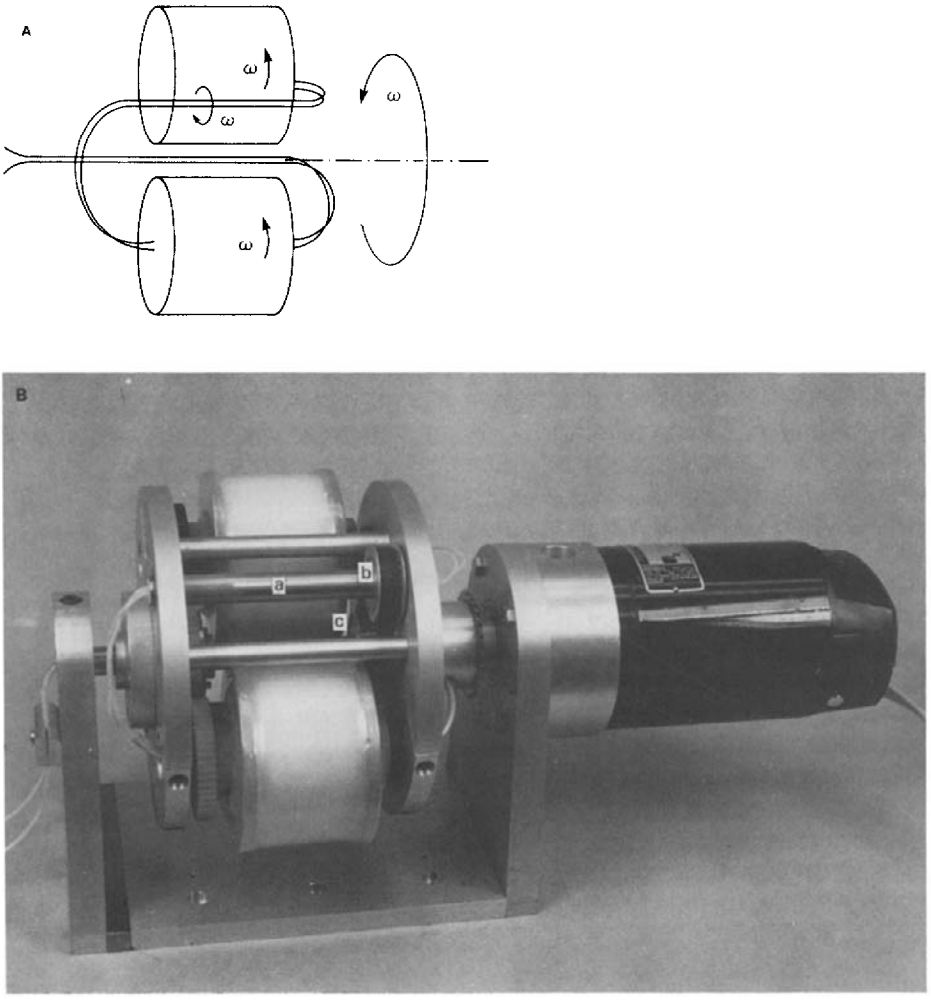

Fig. 1. (A) Design principle of the apparatus. (B) Photograph of the apparatus. Coupling the toothed pulley (b) on the rotary pipe (a) with a toothed belt to the identical stationary pulley (c) mounted on the central stationary shaft produces counter-rotation of the rotary pipe (a) to unwind the twist of the flow tubes caused by planetary motion of the column holders.

the integrity of the flow tubes without twisting, provided that the portion of the tubes connecting beween the two columns is counter-rotated ($-\omega$), either actively or passively, as indicated by the arrow.

Fig. 1B shows a photograph of our prototype based on the design principle described above. The motor drives the rotary frame around the central axis of the centrifuge. The rotary frame consists of a pair of aluminum discs rigidly bridged with multiple links and holds a pair of column holders in the symmetrical positions at a distance of 6.35 cm from the central axis of the centrifuge. Each column holder is equipped with a plastic planetary gear which interlocks with the identical stationary sun gear mounted around the central stationary shaft of the apparatus. This gear

arrangement produces the desired planetary motion of the column holder, *i.e.*, revolution around the central axis of the centrifuge and rotation about its own axis at the same angular velocity as in the existing HSCCC centrifuge.

The rotary frame of the present apparatus also holds a pair of identical rotary pipes, each equipped with a toothed pulley adjacent to the bearing, one rotary pipe (a) being used as a tube support and the other as a dummy to balance the centrifuge system. The pulley (b) on the rotary pipe (a) is coupled with a toothed belt to an identical stationary pulley (c) mounted around the central stationary shaft. This pulley coupling produces counter-rotation of the rotary pipe (a) to unwind the twist of the flow tubes caused by the planetary motion of the column holders. Consequently, the present design of the apparatus fulfills the mechanical requirements illustrated in Fig. 1A.

Both column holders can be removed from the rotary frame by loosening a pair of screws on each bearing block. Each separation column was prepared from a single piece of PTFE (polytetrafluoroethylene) tubing of 0.85 mm I.D. (Zeus Industrial Products, Raritan, NJ, U.S.A.) by winding it directly onto the holder hub and making multiple coiled layers with a capacity of about 45 ml. The β value (ratio of the rotational radius to the revolutional radius) of the multilayer coil ranged from 0.4 at the internal terminal to 0.75 at the external terminal. The layout of the flow tubes is schematically illustrated in Fig. 1A and summarized as follows: second column holder (upper-rear)–rotary pipe (a)–first column holder (lower-front) (one flow tube is connected to the column while the other bypasses it)–central stationary shaft-exit from the centrifuge system (clamped). As mentioned earlier, these flow tubes are free from twisting as the column holders undergo a synchronous planetary motion as indicated in Fig. 1A.

The apparatus can be operated up to the maximum speed of 2000 rpm with a Bodine speed controller. A Milton Roy metering pump was used to deliver the solvent while an LKB Uvicord S was used to monitor the absorbance of the effluent.

EXPERIMENTAL

All organic solvents, *n*-hexane, ethyl acetate and methanol, were glass-distilled chromatographic grade and purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). A two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 3:7:5:5 was used in the present study. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and two phases separated shortly before use.

Test samples of indole-3-acetamide, indole-3-acetic acid, and indole-3-butyric acid were obtained from Sigma (St. Louis, MO, U.S.A.). The sample solution was prepared by dissolving a mixture containing 1 mg of each component in 1 ml of the above solvent system consisting of equal volumes of the two phases. The partition coefficient ($K = C_m/C_s$) for each component in this solvent system was 3.83 for indole-3-acetamide, 1.01 for indole-3-acetic acid, and 0.57 for indole-3-butyric acid.

The separation was initiated by filling the entire column with the upper non-aqueous phase followed by injection of sample solution through the sample port. Then the apparatus was rotated at 1600 rpm while the lower aqueous phase was introduced into the internal head terminal of the first column at a flow-rate of 1 ml/min. The effluent from the outlet of the second column was continuously mon-

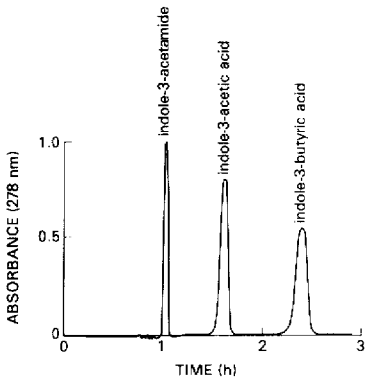

Fig. 2. Chromatogram of indole auxins obtained by the present method. Experimental conditions: solvent system: *n*-hexane–ethyl acetate–methanol–water (3:7:5:5); mobile phase: lower aqueous phase; elution mode: head to tail; flow-rate: 1 ml/min; sample size: 3 mg; revolution: 1600 rpm.

itored by the absorbance at 278 nm. After all peaks were eluted from the column, the apparatus was stopped and the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column.

RESULTS AND DISCUSSION

Potential capability of the present apparatus was demonstrated by separation of indole plant hormones with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 3:7:5:5. Fig. 2 shows a UV trace of the chromatographic run where three components were well resolved in 3 h. From this chromatogram, partition efficiency was calculated according to the conventional gas chromatographic formula, $N = (4R/W)^2$, where N is the partition efficiency expressed in terms of theoretical plate number (TP); R, the retention time of the peak maximum; and W, the peak width expressed in the same unit as R. The results revealed high efficiencies ranging from 3000 TP (first peak) to 2400 TP (third peak), which double those obtained with the similar HSCCC centrifuge equipped with a single separation column[4]. The solvent front of the mobile phase emerged in 48 min (48 ml of elution), and retention of the stationary phase was 45%. The maximum pressure measured at the outlet of the pump was 105 p.s.i.

The above results clearly demonstrate high performance of the present HSCCC system. The symmetrical arrangement of paired identical separation columns on the centrifuge rotor ensures perfect balancing of the centrifuge system, regardless of the density of the applied solvents, once the separation column is equilibrated with the mobile phase*. This eliminates the necessity of tedious counterweight adjustment to

* During the pre-equilibrium period, the mobile phase gradually displaces the stationary phase starting from the first column to cause transient unbalance of the centrifuge system. This problem is common in all the centrifugal CCC systems. In the present semianalytical column with 45 ml capacity (90 ml total), a chloroform–acetic acid–water (2:2:1) system (density difference between the two phases is 0.24 g/cm^3) will cause, at a 70% retention level, the maximum unbalance of $0.24 \times 45 \times 0.3 = 3.2$ (g). In a larger column this unbalance will be increased proportionally to the column capacity. When a chloroform–water binary system (0.5 g/cm^3 in density difference) is applied, the increased density difference may be largely offset by the increase in stationary phase retention.

meet the density of the solvent system in the existing HSCCC system. Because of minimum vibration of the centrifuge system, the present system enables stable retention of the stationary phase in the rotating coil to yield higher peak resolution. Doubled column capacity in the present system further provides an important advantage for performing preparative-scale separations by increasing both the sample loading capacity and partition efficiency. We believe that the present new centrifuge design will greatly facilitate the use of the HSCCC technology in the near future.

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