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HIGH-SPEED PREPARATIVE COUNTER-CURRENT CHROMATOGRAPHY WITH A COIL PLANET CENTRIFUGE

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SUMMARY

A compact table-top model of a flow-through coil planet centrifuge produces an efficient chromatographic separation of solutes on a preparative scale within 2–5 h. The use of a multi-layer coiled column promotes retention of the stationary phase with a high flow-rate of the mobile phase and permits almost universal application of conventional two-phase solvent systems. The capability of the counter-current chromatography scheme has been demonstrated on separations of various biological samples such as dinitrophenyl amino acids, dipeptides, gramicidins, auxins, purines and pyrimidines.

INTRODUCTION

The performance of preparative counter-current chromatography (CCC) schemes depends on the amount of stationary phase retained in the column, which determines both the resolving power of the solute peaks and the sample loading capacity. Various CCC schemes developed in the past[1] were usually not capable of yielding retention of the stationary phase of more than 50% of the total column space. This maximum retention level tends to fall sharply with higher flow-rates of the mobile phase, resulting in loss of peak resolution. Consequently, the flow-rate has become one of the major limiting factors in CCC and the methods require relatively long separation times, ranging from overnight to several days for completion of a sizable separation.

In recent years, successful efforts have been made to develop a CCC scheme that performs efficient extractions with a high feed rate of the sample solution[2]. In this scheme a multi-layer coiled column (a coiled tube wound on a spool) is used. It is ideal for performing preparative CCC, as demonstrated on a preliminary separation of dinitrophenyl (DNP) amino acids with a conventional two-phase solvent system[3].

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This paper describes the results of subsequent work, which has revealed the great versatility of the scheme in allowing almost universal application of conventional two-phase solvent systems. Because a high flow-rate of the mobile phase is used, together with the excellent retention of the stationary phase, the method can produce an efficient chromatographic separation on a preparative scale within a few hours.

PRINCIPLE

As briefly described earlier[1-8], the present method utilizes a complex hydrodynamic motion of the two immiscible solvent phases in a rotating coiled tube. Let us consider a simple model which consists of a coil coaxially mounted around a rotary shaft held in a horizontal position. When the coil is filled with water and slowly rotated around its own axis, any object, either heavier (glass bead) or lighter (air bubble) than water, present in the coil tends to move towards one end of the coil. This end is called the head and the other end the tail of the coil. When the coil is filled with two immiscible solvent phases, rotation sooner or later establishes a hydrodynamic equilibrium between the two solvent phases, where the two phases are distributed in each helical turn at a given volume ratio (equilibrium volume ratio) and any excess of either phase remains at the tail of the coil.

This hydrodynamic equilibrium can be efficiently utilized for performing CCC. When the coil is eluted with one of the phases through the head end, the hydrodynamic equilibrium tends to maintain the original equilibrium volume ratio of the two phases in the coil and thereby a certain volume of the other phase is permanently retained in the coil while the two phases are undergoing vigorous agitation with rotation of the coil. Consequently, the solutes introduced locally at the inlet of the coil are subjected to an efficient partition process between the two phases and chromatographically separated according to their partition coefficients in the absence of solid supports.

In this CCC scheme, the volume of the stationary phase retained in the coil is mostly determined by the following two factors. One is the equilibrium volume ratio of the two phases before the elution is started, and this determines the maximum attainable retention level of the stationary phase. When the mobile phase is introduced into the coil, it displaces a part of the stationary phase to alter the equilibrium volume ratio where the rate of the mobile phase towards the outlet of the coil is just balanced by the rate of the stationary phase returning toward the inlet of the coil. This returning rate of the stationary phase is the other important factor which determines the actual retention level of the stationary phase at a given flow-rate of the mobile phase. The higher the relative rate of the stationary phase against the flowing mobile phase, the greater is the volume of the stationary phase retained in the coil but always within the maximum level determined by the initial equilibrium volume ratio of the two phases. In order to achieve a satisfactory retention level of the stationary phase against a high flow-rate of the mobile phase, the CCC scheme should produce not only a large initial equilibrium volume ratio of the stationary to the mobile phase, but also a high flow-rate of the stationary phase towards the inlet of the coil against the flowing mobile phase. Although the simple rotary coil device described above may give a desired equilibrium volume ratio to the stationary phase at the optimum rotational speed of the coil, the scheme fails to produce a sufficiently high flow-rate of the stationary phase under a unit gravitational field.

Recently, it has been found that the above requirements are well satisfied by subjecting a coil to a particular type of synchronous planetary motion[2] produced by the centrifuge scheme schematically illustrated in Fig. 1. A large cylindrical coil holder coaxially holds a planetary gear which is coupled to an identical stationary sun gear (shaded) mounted on the central axis of the centrifuge. This gear arrangement produces a synchronous planetary motion to the coil holder. The holder revolves around the central axis of the apparatus and simultaneously rotates about its own axis at the same angular velocity in the same direction. In doing so, the holder always maintains its axis parallel to and at a distance R from the central axis of the apparatus. The coil is prepared by winding a piece of flexible tubing around the holder having a radius r as shown in Fig. 1.

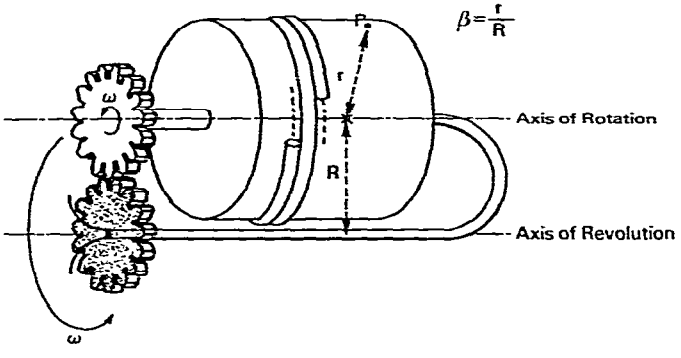

Fig. 1. Synchronous planetary motion of the coil holder.

The centrifugal force field produced by this type of planetary motion has been analyzed earlier[4,5]. The results show that the centrifugal force field varies greatly with the location of point P on the holder, which is conveniently expressed as $\beta = r/R$, *i.e.*, the ratio between the radii of rotation and revolution. When β is greater than 0.25, the centrifugal force vector is always directed outwards from the inside of the holder while it fluctuates periodically in both magnitude and direction during each revolutional cycle.

A series of preliminary experiments have been performed in order to study the effects of such centrifugal force fields on the motion of two immiscible solvents in the coil[2]. Observations made with our prototype on various types of two-phase solvent systems revealed that this type of centrifugal force field establishes a favorable hydrodynamic equilibrium in the coil in such a way that the upper phase always dominates on the head side of the coil. With a given pair of solvent phases, the application of a higher revolutional speed on a large helical diameter coil increases both the equilibrium volume ratio and the flow-rate of each phase through the coil to produce more favorable conditions for the retention of the stationary phase.

Among various physical properties of the solvent system, relative density, viscosity and tube-wall affinity of the two phases seem to play the greatest role in retention of the stationary phase. When the upper phase is much lighter, less viscous and of higher wall affinity than the lower phase, the two phases are quickly and completely separated along the length of the coil. The upper phase entirely oc-

cupies the head side and the lower phase the tail side of the coil. Under these circumstances, an excellent retention of the stationary phase is accomplished by introducing either the lower phase through the head of the coil, or the upper phase through the tail of the coil, after filling the coil with the other phase as the stationary phase. The solvent pairs which provide this ideal performance include (if a PTFE tube is used as the column) a number of useful extraction media such as hexane, diethyl ether, ethyl acetate, toluene, methyl ethyl ketone and benzene, mixed with an aqueous solution. Various salts can be added to adjust the pH and ionic strength of the aqueous phase to obtain suitable partition coefficients of solutes for separation[2].

When the upper phase has a higher viscosity and/or a lower wall affinity than the lower phase, the present scheme usually fails to achieve the complete separation of the two phases along the length of the coil and, instead, produces a hydrodynamic equilibrium of the two phases with the upper phase dominating in volume on the head side of the coil. In this instance, the choice of the mobile phase is limited to the lower phase which can be introduced at the head of the coil initially filled with the stationary upper phase. Introduction of the upper phase through the tail of the coil containing the stationary lower phase would result in steady carryover of the stationary phase through the head where a small amount of the lower phase is always present under this hydrodynamic equilibrium condition. Several commonly used solvent systems such as *n*-butanol, *sec*.-butanol, chloroform and ethylene dichloride, each mixed with an aqueous solution, are included in this group.

However, the lack of versatility in the choice of the mobile phase in the present scheme is greatly improved if the column is made by winding a single piece of tubing on to a spool-shaped holder to make multiple layers of the coil or multi-layer coiled column, as illustrated in Fig. 2. The holder with the multi-layer coiled column is always rotated in one direction so that the internal terminal of the column becomes the head and the external terminal the tail. Because of the spiral configuration of the multi-layer coiled column, a gradient of the centrifugal force field is created from the internal layer of the coil towards the external layer of the coil. This grad ent forces the upper phase to move towards the head and the lower phase towards the tail. As a result, the hydrodynamic equilibrium of the solvent systems is altered in a more favorable way so that the two phases are completely separated along the length of the column and, therefore, either phase becomes usable as the mobile phase without carryover of the stationary phase.

APPARATUS

Fig. 2 illustrates the design of the coil planet centrifuge with a multi-layer coiled column which shows a substantial improvement over the previous model[3]. The motor drives the rotary frame around the central stationary pipe (shaded) by means of a pair of toothed pulleys and a toothed belt. The frame holds a coil holder (top) and a counterweight (bottom) symmetrically at a distance of 10 cm away from the central axis of the centrifuge. The coil holder is equipped with a planetary gear which is coupled with an identical stationary sun gear (shaded) mounted around the central stationary pipe. This gear arrangement produces the desirable synchronous planetary motion of the holder. The holder revolves around the central axis of the apparatus and synchronously rotates about its own axis in the same direction. For mechanical

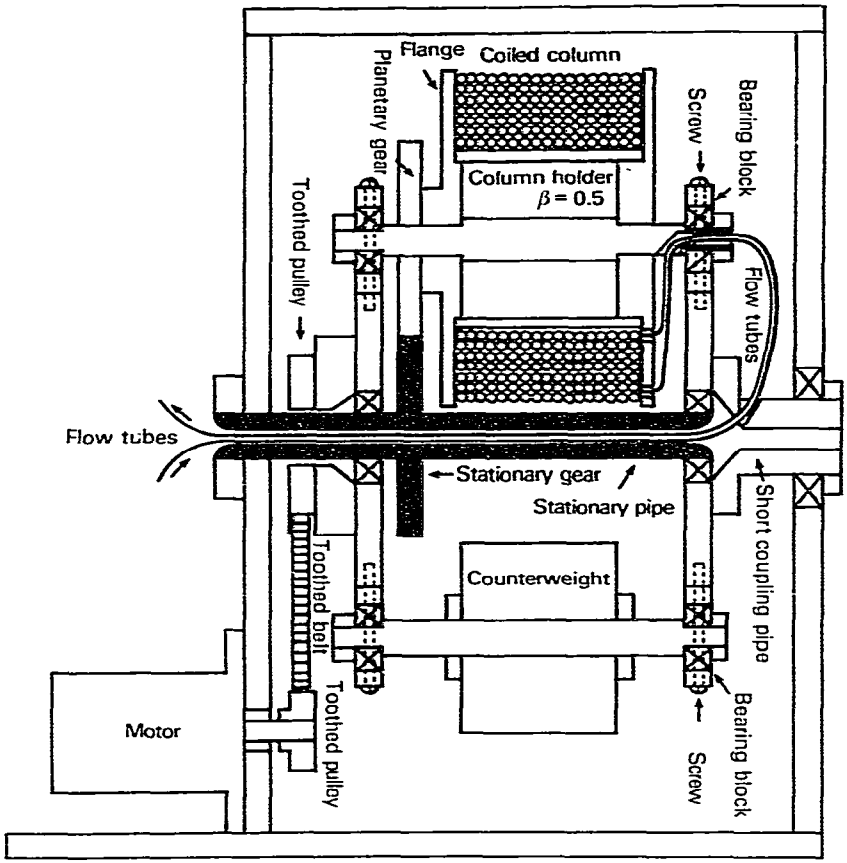

Fig. 2. Cross-sectional view through the central axis of the apparatus.

stability the free end of the rotary frame (right) is coaxially connected to a short coupling pipe, the other end of which is supported by the stationary wall member of the centrifuge through a ball bearing. Both the coil holder and the counterweight are made removable from the rotary frame by loosening a pair of screws, which greatly facilitates the preparation of the coiled column. The multi-layer coiled column is prepared by winding a long piece of PTFE tubing tightly around the coil holder equipped with a pair of large flanges to make multiple layers of the coil up to the rim of the flanges. Each terminal of the column is connected to a flow tube of the proper diameter. The pair of flow tubes are first led through the center-hole of the column holder shaft and then passed through a side-hole of the short coupling pipe to enter the opening of the central stationary pipe. These tubes are lubricated with grease and protected with a piece of Tygon tubing at each supported end to prevent direct contact with metal parts.

The revolutional speed of the apparatus is continuously regulated up to 1000 rpm with a speed control unit (Electro Craft or Bodine Electric Co.) at a high accuracy and stability. The apparatus is a compact table-top model measuring 17 × 17 × 17 in. The solvent is pumped with a Milton-Roy Mini-Pump and the effluent is

continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test-tubes with an LKB fraction collector for further analysis.

EXPERIMENTAL

Preparation of the separation columns

The multi-layer coiled column was prepared from a single piece of PTFE tubing, 1.6 mm I.D. and 130 m long (Zeus Industrial Products, Raritan, NJ, U.S.A.) by winding it tightly onto the coil holder equipped with a pair of large flanges. The total capacity of the column was approximately 285 ml. To prevent dislocation of the column from the holder, each layer of the coil was taped to the flanges by applying a piece of fiber-glass reinforced adhesive tape across the width of the coil. The same tape was also used to wrap the entire column. Each terminal of the column was directly connected to a flow tube of 0.85 mm I.D. which was inserted and then fused by applying heat with a heat gun. The column was then filled with water to measure both the total capacity and weight. These figures were used to determine the proper weight to be applied on the counterweight shaft, a minor adjustment being made according to the density of the solvent system employed.

Preparation of two-phase solvent systems and sample solutions

The organic solvents used were mostly of chromatographic grade. Chloroform, methanol, ethyl acetate and *n*-butanol were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), acetic acid from Fisher Scientific (Fairlawn, N.J., U.S.A.) and benzene from J. T. Baker (Phillipsburg, NJ, U.S.A.). Each two-phase solvent system was equilibrated in a separatory funnel at room temperature and separated before use.

All chemicals used as samples including dinitrophenyl (DNP)-amino acids, oligopeptides, auxins, purines and pyrimidines were purchased from Sigma (Saint Louis, MO, U.S.A.), except for the gramicidin mixture (Penick 641 N0F4), which was obtained by courtesy of the late Dr. Erhard Gross. The sample solutions were prepared by dissolving a mixture of samples in the upper and/or the lower phase of the respective solvent system and stored in the dark at 4°C.

Separation procedure

The separation was usually performed as follows: The column is first filled with the stationary phase and the sample solution is introduced into the inlet of the column. Then the mobile phase is pumped into the column while the apparatus is run at a revolutional speed of 800 rpm. The eluate through the outlet of the column is continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test-tubes with an LKB fraction collector. When the mobile phase is the lower phase, both sample solution and the mobile phase are introduced at the head or the internal terminal of the multi-layer coiled column. When the mobile phase is the upper phase, the elution is performed through the tail or the external terminal of the column. In the latter instance, combination of a slow flow-rate and a high revolutional speed may produce a negative pressure at the inlet of the column, resulting in increase in the flow-rate. This complication, if it occurs, is easily corrected by placing a piece of small-bore PTFE tubing, typically 0.3 mm I.D. and 50 cm long, at the outlet of the

column to produce a positive back-pressure to the pump. After the separation is completed, the apparatus is stopped and the column contents are emptied into a graduated cylinder by applying nitrogen through the inlet of the column at 100 p.s.i. This allows the measurement of the stationary phase volume retained in the column.

In the gradient elution, an LKB Ultrograd gradient mixer is placed at the inlet of the pump to produce a linear gradient between the starting and the ending media. The column is first entirely filled with the stationary phase of the starting medium and the sample mixture dissolved in the starting medium is injected into the inlet of the column. Then the mobile phase is introduced into the column through the gradient mixer while the apparatus is run at 800 rpm. After the gradient is completed, the elution is continued with the ending medium until all solute peaks are eluted from the column.

Analysis of the fractions

In addition to recording the elution profile of the solute peaks with the UV monitor, collected fractions were also analyzed by mixing aliquots of each fraction with a given volume of methanol or water and measuring the absorbance at the optimum wavelength with a Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Fig. 3 shows typical chromatograms of seven DNP-amino acids obtained with a two-phase solvent system composed of chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). We used the separation of DNP-amino acids as a reference separation to compare various CCC methods. The upper aqueous phase was used as the mobile phase at 240 ml/h. Separations were performed on sample mixtures of (A) 10 mg and (B) 360 mg. In both chromatograms all components were well resolved and eluted in 4 h. The results indicate that both the retention level of the stationary phase and the resolution of solute peaks are not substantially altered by the sample size within this range.

Fig. 4 shows similar chromatograms of five DNP-amino acids obtained by using the lower non-aqueous phase as the mobile phase. Separations were performed at a high flow-rate of 420 ml/h for sample mixtures of (A) 30 mg and (B) 650 mg. In both separations all DNP-amino acids were resolved and eluted in 2 h. However, in this instance, comparison between the two chromatograms clearly shows the effects of the sample size on both the retention of the stationary phase and the peak resolution. In chromatogram B, where a large sample dose of 650 mg was applied, the peak resolution became greatly decreased, largely owing to the decreased retention level of the stationary phase evidenced by the longer retention time of the first peak and the shortened time lapse between the peaks. The effects of the sample size on the retention level of the stationary phase are extremely complex phenomena which involve changes in a number of physical parameters of the solvent system such as interfacial tension, viscosity, density, volume ratio and composition of the two phases.

Fig. 5 shows a separation of a gramicidin mixture on a two-phase solvent system composed of chloroform–benzene–methanol–water (15:15:23:7). The upper aqueous phase was used as the mobile phase at a relatively slow flow-rate of 120 ml/h. An amount of 100 mg of gramicidins was separated into three major components (A,

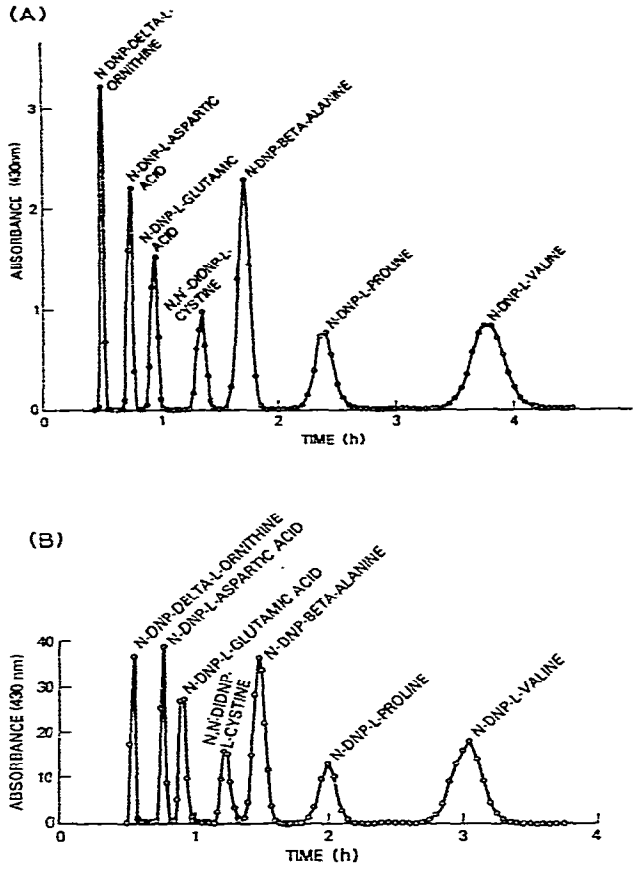

Fig. 3. Chromatograms of DNP-amino acids obtained with tail–head elution. Solvent system: chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Mobile phase: upper aqueous phase. Flow-rate: 240 ml/h. Revolution: 800 rpm. (A) Sample dose (volume), 10 mg (1 ml); retention of the stationary phase, 58%; column pressure, 40 p.s.i. (B) Sample dose (volume), 360 mg (10 ml); retention of the stationary phase, 51%; column pressure, 40 p.s.i.

B and C), each being partially resolved into the valine and isoleucine analogs. The relatively poor resolution of the gramicidin C analogs in this chromatogram would be much improved if the lower non-aqueous phase is used as the mobile phase.

Fig. 6 shows chromatograms of purines and pyrimidines on a two-phase solvent system composed of *n*-butanol–1 *M* potassium phosphate (pH 6.5) (1:1). Separations were performed by using both (A) the lower aqueous phase and (B) the upper non-aqueous phase as the mobile phase at a flow-rate of 240 ml/h. Although the upper *n*-butanol phase has a higher viscosity than the lower aqueous phase, these separations demonstrate that either phase can be chosen as the mobile phase to obtain similar peak resolutions.

Fig. 7 shows a chromatogram of indole plant hormones on a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). The separation was carried out at a flow-rate of 240 ml/h using the lower aqueous phase as the

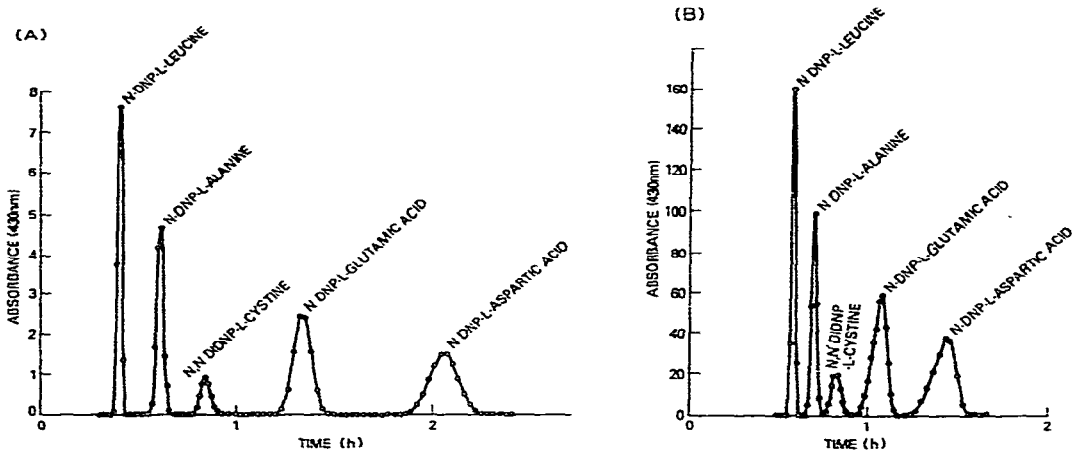

Fig. 4. Chromatograms of DNP-amino acids obtained with head–tail elution. Solvent system: chloroform–acetic acid–0.1 *N* hydrochloric acid (2:2:1). Mobile phase: lower non-aqueous phase. Flow-rate: 420 ml/h. Revolution: 800 rpm. (A) Sample dose (volume); 30 mg (1 ml); retention of the stationary phase, 51%, column pressure, 80 p.s.i. (B) Sample dose (volume), 650 mg (10 ml); retention of the stationary phase, 30%; column pressure, 50 p.s.i.

mobile phase. This solvent system has ideal physical properties for the present CCC method in that the upper phase is less viscous and has a higher affinity than the lower phase to the column wall. In addition, the partition coefficients of the solutes are conveniently adjusted by changing the volume ratio between *n*-hexane and ethyl acetate in the solvent system.

In order to demonstrate versatility of the present CCC scheme, a gradient elution was performed to separate a set of dipeptides using an *n*-butanol–aqueous sol-

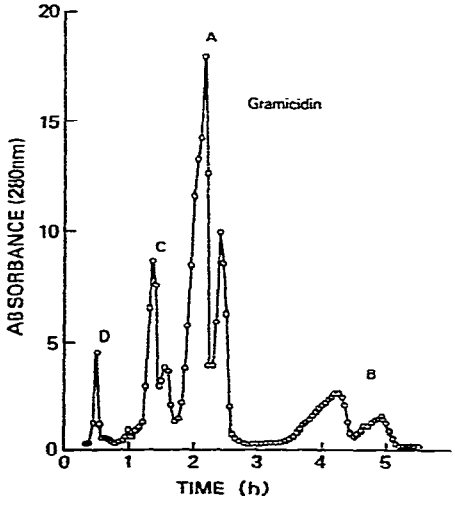

Fig. 5. Chromatogram of gramicidins. Sample dose (volume): 100 mg (5 ml). Solvent system: chloroform–benzene–methanol–water (15:15:23:7). Mobile phase: upper aqueous phase. Flow-rate: 120 ml/h. Revolution: 800 rpm. Column pressure: 25 p.s.i.

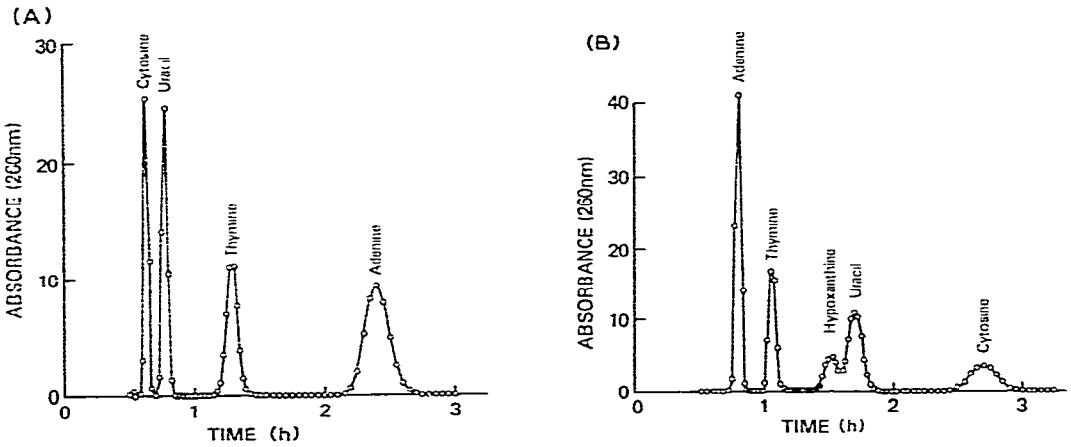

Fig. 6. Chromatograms of purines and pyrimidines. Solvent system: *n*-butanol–1 *M* potassium phosphate (pH 6.5) (1:1). Flow-rate: 240 ml/h. Revolution: 800 rpm. (A) Sample dose (volume), 20 mg (5 ml); mobile phase, lower aqueous phase; retention of the stationary phase, 58%; column pressure, 100 p.s.i. (B) Sample dose (volume), 25 mg (5 ml); mobile phase, upper non-aqueous phase; retention of the stationary phase, 37%; column pressure, 30 p.s.i. (small-bore tubing was inserted after the column outlet to produce a positive pressure).

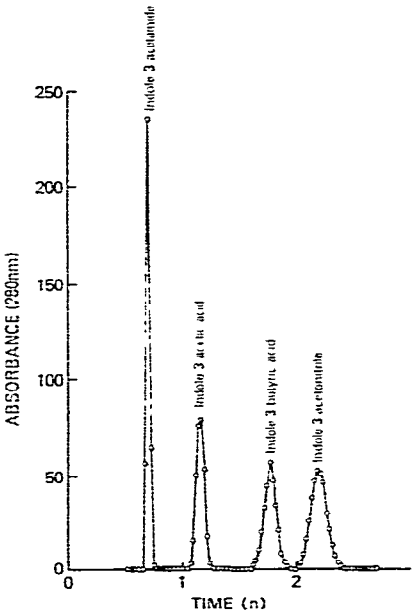

Fig. 7. Chromatogram of indole plant hormones. Sample dose (volume): 200 mg (5 ml). Solvent system: *n*-hexane–ethyl acetate–methanol–water (3:7:5:5). Mobile phase: lower aqueous phase. Flow-rate: 240 ml/h. Revolution: 800 rpm. Retention of the stationary phase: 51%.

vent system. A linear gradient of dichloroacetic acid was applied in a decreasing concentration on the base of *n*-butanol–0.1 *M* ammonium formate (1:1) by eluting with the lower aqueous phase at a flow-rate of 214 ml/h. Fig. 8A shows the chromatogram obtained by applying a gradient of 2 h duration where all components were resolved and eluted in 3 h. The third peak is an unknown impurity which can be completely resolved by doubling the duration of the gradient application, as shown in Fig. 8B.

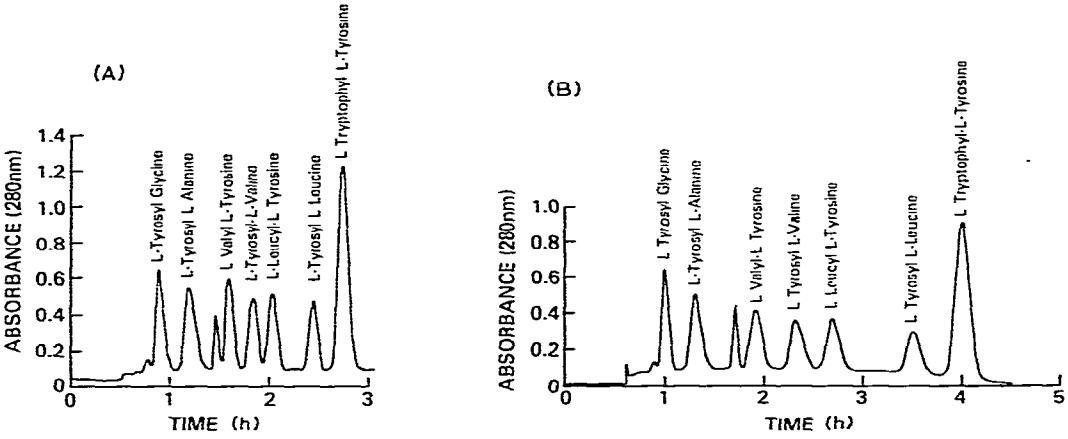

Fig. 8. Chromatograms of dipeptides by gradient elution. Sample dose (volume): 70 mg (5 ml). Starting medium: *n*-butanol–dichloroacetic acid–0.1 *M* ammonium formate (1:0.01:1). Ending medium: *n*-butanol–0.1 *M* ammonium formate (1:1). Mobile phase: lower aqueous phase. Flow-rate: 214 ml/h. Revolution: 800 rpm. Retention of the stationary phase: 55%. Duration of gradient: (A) 2 h; (B) 4 h.

The capability of the present scheme to perform preparative-scale CCC has been demonstrated on separations of a variety of biochemical samples. Compared with the countercurrent distribution method (CCD), the present method yields a higher partition efficiency in much shorter periods of time. In contrast to liquid chromatography, the method uses no solid support and, therefore, eliminates various complications caused by the adsorption effects. Thus, sample loss and denaturation, contamination and tailing of the solute peaks are minimized.

Among the existing CCC schemes, the present scheme has the greatest capacity for the retention of the stationary phase against a high flow-rate of the mobile phase. This fact enables one to perform efficient separations in short periods of time ranging from 2 to 5 h, instead of overnight or several days as required for other preparative CCC schemes. The low column pressure, ranging 20 to 100 p.s.i., in the present scheme suggests that the efficiency can be further improved by the use of a longer column and/or a higher flow-rate at an increased revolutional speed. The method also permits almost universal application of the conventional two-phase solvent systems where either phase can be used as the mobile phase.

The apparatus is a compact table-top model (17 × 17 × 17 in.) which can be fabricated in a small machine shop. The present model is equipped with an interchangeable column holder which is extremely versatile because the same apparatus

can be used for analytical separations[5] and continuous extraction[2] with respective types of separation columns.

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